DETECTION OF GENETIC VARIABILITY AMONG *Staphylococcus aureus* ISOLATES FROM VARIOUS CLINICAL AND NON-CLINICAL ANIMAL AND HUMAN SETTINGS IN RELATION TO SOME VIRULENCE FACTORS

पशु एवं मनुष्य के नैदानिक और गैर नैदानिक परिस्थितियों से वियोजित *स्टेफायलोकॉकस ऑरियस* में कुछ डाह कारकों के संबंध में आनुवांशिक विभिन्नता का पता लगाना

SANDEEP KUMAR SHARMA
M.V.Sc.

THESIS

DOCTOR OF PHILOSOPHY

(Veterinary Microbiology)

।पशुधनं नित्यं सर्वलोकोपकारकम्।

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Department of Veterinary Microbiology and Biotechnology
College of Veterinary and Animal Science, Bikaner
Rajasthan University of Veterinary and Animal Sciences
Bikaner – 334001

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Place: Bikaner
Date: **(Sandeep Kumar Sharma)**

ABBREVIATIONS

AP-PCR	=	Arbitrarily Primed PCR
agr	=	Accessory gene regulator system
bp	=	Base pair
BME	=	β-mercaptoethanol
*Coa*NSA	=	Coagulase Negative *S. aureus*
CP	=	Capsular Polysaccharide
CTAB	=	Cetyltrimethylammonium bromide
DEB	=	DNA extraction buffer
EDTA	=	Ethylene diamine tetraacetic acid
h	=	Hour/hours
lbs	=	Pounds
mcg	=	Microgram
min	=	Minutes
MLEE	=	Multilocus Enzyme Electrophoresis
MRSA	=	Methicillin Resistant *S. aureus*
MSSA	=	Methicillin Sensitive *S. aureus*
ng	=	Nanogram
nm	=	Nanometre
OD	=	Optical density
PAGE	=	Polyacrylmide Gel Electrophoresis
PBS	=	Phosphate buffer saline
PCR	=	Polymerase Chain Reaction
PFGE	=	Pulse Field Gel Electrophoresis

RAPD	=	Randomly amplified polymorphic DNA
REs	=	Restriction Endonuclease
rep-PCR	=	repetitive element sequence-based PCR
RFLP	=	Restriction Fragment Length Polymorphism
rRNA	=	Ribosomal Ribonucleic Acid
SDS	=	Sodium dodecyl sulphate
TBE	=	Tris borate EDTA
TE	=	Tris EDTA
Tris	=	Hydroxymethyl amino methane
TSS	=	Toxic Shock Syndrome
UV	=	Ultraviolet

Rajasthan University of Veterinary and Animal Sciences, Bikaner
College of Veterinary and Animal Science, Bikaner

CERTIFICATE-I

Date…....……

This is to certify that **Mr. Sandeep Kumar Sharma** had successfully completed the **comprehensive examination** held on 18-11-13, 19-11-13, 20-11-13 and 23-12-13 as required under the regulations for **Ph.D. degree.**

(S.K.Kashyap)
Head
Department of Veterinary Microbiology and Biotechnology,
College of Veterinary and Animal Science,
Bikaner-334001

Rajasthan University of Veterinary and Animal Sciences, Bikaner
College of Veterinary and Animal Science, Bikaner

CERTIFICATE-II

Date.....……

This is to certify that this thesis entitled **"Detection of genetic variability among *Staphylococcus aureus* isolates from various clinical and non-clinical animal and human settings in relation to some virulence factors**" submitted for the degree of Doctor of Philosophy in the subject of Veterinary Microbiology embodies bonafide research work carried out by **Mr. Sandeep Kumar Sharma** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on ……..

(S.K. Kashyap)
Head
Department of Veterinary Microbiology
and Biotechnology
College of Veterinary and Animal Science,
Bikaner-334001

(A.K. Kataria)
(Major Advisor)

(Tribhuwan Sharma)
Dean

College of Veterinary and Animal Science
Bikaner

Rajasthan University of Veterinary and Animal Sciences, Bikaner
College of Veterinary and Animal Science, Bikaner

CERTIFICATE-III

Date…....…….

This is to certify that the thesis entitled **"Detection of genetic variability among *Staphylococcus aureus* isolates from various clinical and non-clinical animal and human settings in relation to some virulence factors"** submitted by **Mr. Sandeep Kumar Sharma** to Rajasthan University of Veterinary and Animal Sciences, Bikaner, in partial fulfillment of requirements for the degree of Doctor of Philosophy in the subject of Veterinary Microbiology after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination on his thesis has been found satisfactory; we therefore recommend that the thesis be approved.

(A.K. Kataria)
Major advisor

(S.C. Mehta)
Advisor

(S.K. Kashyap)
Advisor

(B.N. Shringi)
Advisor

(H. Dadhich)
Advisor

(N. Kataria)
Dean, PGS Nominee

External Examiner

(S.K. Kashyap)
Head
Department of Veterinary Microbiology and Biotechnology
College of Veterinary and Animal Science, Bikaner

DEAN
Post Graduate Studies
RAJUVAS, Bikaner
Rajasthan University of Veterinary and Animal Sciences,
Bikaner
College of Veterinary and Animal Science, Bikaner

CERTIFICATE – IV

Date……

This is to certify that **Mr. Sandeep Kumar Sharma** of the Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Bikaner has made all corrections/modifications in the thesis entitled **"Detection of genetic variability among *Staphylococcus aureus* isolates from various clinical and non-clinical animal and human settings in relation to some virulence factors"** which were suggested by the external examiner and the advisory committee in the oral examination held on……The final copies of the thesis duly bound and corrected were submitted on…....., are enclosed herewith for approval.

(A.K. Kataria)
Major Advisor

Enclosed are one original and two copies of bound thesis. Forwarded to the Dean, Post Graduate Studies, RAJUVAS, Bikaner through the Dean, College of Veterinary and Animal Science, Bikaner.

(S.K. Kashyap)
Head

Department of Veterinary Microbiology and Biotechnology,

College of Veterinary and Animal Science,
Bikaner

Dean
College of Veterinary and Animal Science
Bikaner

Dean,
Post Graduate Studies
RAJUVAS, Bikaner

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1. INTRODUCTION

Staphylococcus aureus is a spherical gram positive, non-motile, non-spore forming, facultative anaerobic firmicute bacterium of family *Staphylococcaceae.* It commonly resides in the nasal passages, skin and mucous membranes of the animal and human. *Staphylococcus aureus* is the most important pathogen of various clinical and sub clinical infections in animals and human. It is the most notorious for causing bacteremia in human with high morbidity and mortality as compared to other organisms. In humans, it may also cause serious infections, particularly in persons debilitated by chronic illness, traumatic injury, burns and immunosuppression. These infections include pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, boils, furuncles, styes, impetigo, toxic shock syndrome and meningitis, and are often associated with hospitalized patients rather than healthy individuals in the worldwide communities (Carter *et al.*, 1990). This organism also known to cause variety of suppurative infections, septicemia and toxinoses in domestic animals and birds such as mastitis, udder impetigo, endometritis, pyoderma, cystits, dermatitis, botryomycosis of mammary gland, arthritis, scirrhous cord, bumble foot and tick pyaemia (Naber, 2009).

Staphylococcus aureus is unique with many typical phenotypic characteristics as rigid cell due to high content of teichoic acid helping in tolerance to drying, high salt and sucrose concentration enabling it to grow at a temperature range of 15 to 45^0C and at sodium chloride concentrations as high as 15%. They grow in grape bunches clusters, pairs and occasionally in short chains that grow by aerobic respiration or by fermentation that yields principally lactic acid. However, the organism *S. aureus* was named so because of production of golden coloured colonies but production of variable pigments *viz*. pale yellow, mustard and white by this organism has also been reported (Jonsson and Wadstrom, 1993; Khichar, 2011). The organism also shows variations in fermentation reactions, coagulase activity, haemolytic patterns, DNase activity, thermostable nuclease phenomena, slime production and biofilm formation (Yadav *et al.*, 2015a). Likewise, variations in other phenotypic properties have also been reported by many workers.

Because of its phenotypic variations molecular methods for its identification are preferred and overall DNA-based techniques have been found most effective in typing the isolates (Tenover *et al*., 1994). The molecular typing approaches have been reported to be of great advantages in identifying and monitoring the local and international spread of its strains (Diep *et al.*, 2003). The identification of strain is also important to confirm the epidemiological relationships among them (Aarestrup *et al*., 1995). Various PCR based detection systems were developed for *S. aureus* identification but were not found sufficiently reliable to detect all strains of *S. aureus.* Later, Straub *et al*. (1999) developed a PCR system that relied on one primer pair targeted against 23S rRNA-based species-specific probe, allowing specific detection of all strains of species. The target sequence 23S rRNA was chosen because it fulfilled the requirement of phylogenetic marker with highest reliability. This method is now being used extensively by many workers throughout the world for genotypic identification and confirmation of *S. aureus* from various clinical and subclinical infections (Salasia *et al*., 2004; Upadhyay *et al*., 2010a; Khichar *et al.*, 2012; Yadav *et al.*, 2015a).

Staphylococcus aureus strains were reported with variations in their genome so it is necessary to differentiate epidemic, nonepidemic and comparison of *S. aureus* strains from human and animal staphylococcal infections. At present, several molecular typing systems are available to monitor *S. aureus* infections and to establish their epidemiological relationship. The ideal system for the typing of *S. aureus* strains should be easy, rapid, reliable, highly discriminatory, and reproducible. Amplification of repeat DNA sequences found in prokaryotic chromosomes, seemingly random distribution of such repeats allows complementary oligonucleotide primers to generate DNA fragment patterns that are specific for individual strains. his general methodology is referred to as repetitive element sequence-based PCR or rep-PCR. The rep-PCR was proven to be a highly discriminating and rapid screening molecular typing method to classify variety of staphylococcal infections (del Vecchio *et al.*, 1995; van der Zee *et al.*, 1999; Reinoso *et al.*, 2007).

Staphylococcus aureus produces a variety of extracellular, intracellular and cell wall associated virulence factors *viz.* adherence factors, antiphagocytic factor (capsule), exoenzymes, immune evasion factor, plasminogen activator factor (staphylokinase) and toxin secretory system. These factors are involved in the organism's pathogenesis, invading the host, diseases causation and evasion of host defense system by facilitating attachment, colonization, cell-cell interactions, immune evasion and tissue damage (O'Riordan and Lee, 2004; Sindhu *et al*., 2008; Momtaz *et al*., 2010). It is also proved that there is certain relationship between severity of infections and the virulence factors possessed / produced by the organism (Akineden *et al*., 2001).

Bacterial chromosomal DNA, bacteriophage DNA, plasmids, or transposons may encode expression of above virulence factors. These factors may also be influenced by bacterial cell density and host health status or many environmental factors such as pH, oxygen and carbon dioxide. In *S. aureus,* functions of virulence factors are regulated through various regulatory systems. Those regulatory systems can be divided into two broad categories: two-component signal transduction systems and global transcriptional regulators (Cheung and Zhang 2002; Cheung *et al.,* 2004). The best-studied two-component regulatory system is the accessory gene regulator system (*agr*) (Gilot *et al.*, 2002). The *agr* locus regulates more than 70 genes including 23 virulence genes (Ziebandt *et al.,* 2004). It contains two divergent promoters, P2 (RNA II) and P3 (RNA III). The P2 promoter drives transcription of an auto-inducing signal transduction module which is composed of four genes, *agr*BDCA and these genes can be further classified into *agr* groups (*agr*I, *agr*II, *agr*III and *agr*IV). The P3 promoter activation produces a regulatory RNA called RNAIII (TRAP-Target of RNA III-Activating Protein governed by *trap* gene). This is the effector molecule of the system encoded outside of *agr* locus. It is also observed that TRAP has role in virulence and up regulation of the *agr* locus to produce RNA II (Gilot *et al.,* 2002).

The *agr* system is a quorum-sensing system, a signaling system for communication of bacteria to support adherence, colonization, growth and survivability of bacteria with adequate virulence. It has been also reported that *agr* system also up-regulates the transcription of certain extracellular toxins, biofilm formation and antibiotic resistance so it is necessary to study the variations in *agr* groups and *trap* gene (Keller and Surette 2006; Boles and Horswill, 2008).

Adherence is considered a critical first step in the pathogenesis of any pathogen. *Staphylococcus aureus* synthesizes two broad categories of adhesins namely microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and secreted expanded-repertoire adhesive molecules (SERAMs) (Clarke and Foster 2006). MSCRAMMs are covalently bound to the peptidoglycan and function in helping bacteria adhere to different host extracellular matrices such as collagen, fibrinogen and fibronectin. This group of adhesins include clumping factor (*Clf*A and *Clf*B) with other collagen and fibronectin-binding proteins. Clumping factors are thought to play a significant role in pathogenesis of wound, foreign body infections and bovine mastitis (Stephan *et al*., 2001). It has been shown that *clf*A mutant is less virulent than the wild type isogenic strain (Foster and Hook, 1998). Since, antibodies against *clfA* enhanced the protection against infection provided by capsular polysaccharides thus it has been also reported as potential vaccine candidate (Tuchscherr *et al*., 2008).

The second group of adhesins (SERAMs) is bound to the cell wall with inter-cellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface and altering T cell function (Chavakis *et al.,* 2005). These inter-cellular adhesion molecule-1 (ICAM-1)/ *ica* operon govern capability of *S. aureus* to attach the surface of the biomaterial and consequently to form a mucoidbiofilm. Biofilms are complex bacterial populations that are surface-attached and enclosed in a polysaccharide matrix, composed of poly-N-acetylglucosamine (PNAG). The term biofilm and slime are often used interchangeably (Ammendolia *et al.,* 1999; Arciola *et al.,* 2001; Fitzpatrick *et al.,* 2005). Among the *ica* genes, the *icaA* gene encodes N-acetylglucosaminyl transferase, the enzyme involved in the synthesis of N-acetylglucosamine oligomers from UDP-N-acetylglucosamine (Arciola *et al.,* 2001). Further, *icaD* has been reported to play a critical role in the maximal expression of N-acetylglucosaminyl

transferase, leading to the phenotypic expression of the capsular polysaccharide (Gerke *et al.,* 1998). Thus, it is necessary to study adherence properties of *S. aureus* through clumping factor (*clfA* and *clfB*) and *ica* genes.

Phagocytosis is an important process of host defense system but bacterial pathogen especially *S. aureus* has developed many strategies to escape from phagocytosis. Capsular polysaccharides (CP) produced by *S. aureus* are not only considered to be important virulence factors as antiphagocytic but also important factors in the survival of bacteria in neutrophils during pathogenesis of staphylococcal infections. Of the 11 CP types, majority of the human and animal staphylococci produces CP5 and CP8. Through in-vitro experiments, it has been recorded that mice immunized with antibodies to CP5 or CP8 had significantly reduced tissue bacterial burden four days after intra-mammary challenge with encapsulated *S. aureus* strains. Similarly, other studies have also shown that antibodies to capsular polysaccharides have some protective efficacies for preventing infections in experimental animal (Fatton *et al*., 1996; Tuchscherr *et al*., 2008). The variable occurrence of *cap*5K and *cap*8K genes has been reported from different geographical regions while they did not observe any polymorphism in *cap*5K and *cap*8K genes (Sordelli *et al*., 2000; Salasia *et al*., 2004; Upadhyay *et al*., 2010a; Khichar and Kataria, 2014). Since the *cap* genes are highly conserved and the capsular polysaccharides have got limited antigenic specificity thus the capsular polysaccharides CP5 and CP8 offer promise as target antigens for a vaccine to prevent staphylococcal infections (O'Riordan and Lee, 2004).

Staphylococcus aureus secrete various exoenzymes such as staphylocoagulase, aureolysin, V8 protease, hyaluronate lyase, lipase and staphopain which are implicated in pathogenesis of disease. Out of these enzymes, staphylocoagulase and aureolysin are important virulent exoenzyme to trigger staphylococcal infections. Coagulase is a collagen binding protein encoded by *coa* gene which is extracellular protein, binds to prothrombin to form a complex called staphylothrombin. The protease activity of the thrombin complex is activated, resulting in the conversion of fibrinogen to fibrin. This is suggested to lead to dissemination of staphylococci into deeper and more remote tissues (Guggenberger *et al*., 2012). Coagulase is produced by almost all clinical isolates of *S. aureus* thus classification based on *coa* gene has been considered to be an simple and accurate method as molecular typing (Goh *et al.,* 1992; da Silva and da Silva, 2005). Since *coa* gene has distinct molecular polymorphism patterns thus it is an important tool for epidemiological investigation of *S. aureus* from varied geographical regions and source of infections (Sanjiv *et al.,* 2008; Upadhyay *et al.,* 2010b; Yadav *et al.,* 2015b).

The aureolysin (*Aur*), member of the thermolysin family, is an extracellular and zinc dependent metalloprotease. This enzyme promote the detachment of bacterial cells from colonized sites and facilitate the spread of infection in the host system and destroys host defense molecules via cleaving the plasma proteinase inhibitors, a 1-antichymotrypsin, a 1-proteinase inhibitor and by inactivation of antimicrobial peptides. It may also affect the stimulation of T and B lymphocytes by polyclonal activators and display inhibitory activity against immunoglobulin production by lymphocytes (Sabat *et al.,* 2000).

The metalloprotease aureolysin encoded by *aur* gene, cloned and sequenced analysis of this gene revealed two allelic forms (types I and II). Many researchers demonstrated that *aur* gene occurred in two allelic forms was strongly conserved among human and animal isolates of *S. aureus* and suggested that this protease may have important housekeeping functions and also have important role in virulence of *S. aureus* (Sabat *et al.,* 2000; Takeuchi *et al.,* 2002; Zdzalik *et al.,* 2012).

Staphylococcus aureus has several proteins that can have a profound suppression impact on the innate and adaptive immune system, staphylococcal protein A (*SpaA*) encoded by *spa* gene is one of them. The gene encoding protein A (*spa*) is composed of some functionally distinct regions: IgG Fc binding region (*spa-IgG*) and X region (*spa-X*) at C terminus, a sequence required for cell wall attachment and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes (Gao and Stewart, 2004). The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains (Frenay *et al.,* 1994; Kuzma *et al.,* 2005). It has also been proved that more than seven repeats in the X-region tended to be epidemic or

more virulent while the presence of less than seven repeats is a non-epidemic or less virulent *S. aureus* strain (Frenay *et al.,* 1994; van Belkum *et al.,* 1995; Khichar *et al.,* 2012; Yadav *et al.,* 2015c).

DNA sequence analysis of the staphylococcal protein A gene variable repeat region (*spa* typing) provides a rapid and accurate method to discriminate *S. aureus* isolates from those deemed epidemiologically unrelated. It also accurately reflects variation occurring throughout the entire chromosome, enabling its use in global population studies and as a simple and rapid way of assigning strains to phylogenetic lineages among *S aureus* (Koreen *et al.,* 2004). *Staphylococcus aureus* has also other immune evasion specific proteins such as staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein of *S. aureus* (CHIPS) and staphylokinase (SAK). Staphylococcal complement inhibitor is a C3 convertase inhibitor, which blocks the formation of C3b on the surface of the bacterium inhibiting complement activation and governed by *scn* gene. Chemotaxis inhibitory protein is a *chp* gene governing, chemotaxis inhibitory protein of *S. aureus* that inhibits activation of neutrophils (Bien *et al.,* 2011).

Staphylokinase is a 16-kd extracellular protein expressed by most of the strains of *S. aureus of* human origin. It forms complex with human plasminogen, promoting its activation to plasmin. Staphylokinase protein governed by *sak* gene, mediates proteolytic activity in *S. aureus* infected skin, facilitates local spreading, increases tissue damage of skin and reduces bacterial clearance by the host (Kwiecinski *et al.,* 2010). The *sak* gene has a highly conserved sequence with an important function in immune evasion, such as complement inhibitory factors and enterotoxins. Staphylokinase was shown to enhance the breaching of tissue barriers *in vitro* (Peetermans *et al.,* 2014). *Staphylococcus aureus* strains showed host specificity with more presence of *sak*, *chp* and *scn* genes in human strains while strains from veterinary sources commonly lack these genes (Sung *et al.,* 2008; Stastkova *et al.,* 2011).

Staphylococcus aureus produces several different exoproteins, including exotoxins such as haemolysins (α, β, γ, δ), enterotoxins, superantigens (toxic shock syndrome toxin-1/ TSST-1) and leukocidins, etc. as important virulence determinants (Dinges *et al.,* 2000). Haemolysins are cytolytic pore forming toxins to a variety of host cells that not only lyse cells, but also alter the host immune response such as inducing caspase dependent and caspase-independent apoptosis (Bownik and Siwicki, 2008). Alpha-haemolysin (*hla* gene), the best characterized pore-forming exotoxin, is a heptameric protein of 34 kd monomer size. It forms pores in the cell membranes and causes lysis of erythrocytes, mast cells and thrombocytes of different animal species, particularly rabbit (Haslinger *et al.,* 2003).

Beta-haemolysin (encoded by *hlb* gene) is less well investigated than other staphylococcal haemolysins and exotoxins, probably because it has rarely been found to be produced by human and animal isolates of *S. aureus* and its role in pathogenesis is not yet clear. However, interestingly majority of isolates of *S. aureus* associated with bovine mastitis and chronic skin infections are beta-haemolytic (Anderson, 1983). Beta-haemolysin is a 39kd protein exotoxin enzyme, having Mg^{++} dependent sphingomyelinase C activity to degrade sphingomyelin in cell membranes of erythrocytes, leukocytes, neurons and other tissue cells (Gow and Robinson, 1969; Bernheimer *et al.,* 1974).

Delta haemolysin (encoded *hld* gene) is a 26 amino-acid peptide capable of causing membrane damage in a variety of mammalian cells. Delta haemolysin produced by most of *S. aureus* strains with wide range of cytotoxic effects and active against erythrocytes of many animal species and acted synergistically with staphylococcal beta-haemolysin against sheep erythrocytes (Peng *et al.,* 1988).

Superantigens of *S. aureus* are a class of protein toxins that can cause nonspecific T-lymphocyte activation and massive cytokine release. These include several enterotoxins (SEs), toxic shock toxin-1 (TSST-1), and exfoliative toxins (ETs). Toxic shock toxin (*tst* gene) is a pyrogenic toxin superantigen (PTSAgs) first described by Todd *et al.* (1978) causing severe acute illness in young children which is characterized by fever, rash, hypotension, multiple-organ-system dysfunction,

lethal shock, and desquamation in the host (Fraser and Proft 2008). Toxic shock syndrome toxin (TSST-1) may also act as superantigen for cells of the bovine immune system. The super antigenic toxins seem to induce immunosuppression in dairy animals that promote the persistence of bacteria in cattle and contribute to chronic mastitis (Farahmand-Azar *et al.,* 2013).

Staphylococcus aureus is naturally susceptible to virtually every antibiotic that has ever been developed but it is very notorious also, for its ability to become resistant to various antibiotics. It acquired antimicrobial resistance because of multifactorial reasons that include the widespread, huge and inappropriate use of antimicrobials, the extensive use of these agents as growth enhancers in animal feed and with the increase in regional and international travel, the relative ease with which antimicrobial-resistant bacteria cross geographic barriers (Lowy, 2003). As rapidly as new antibiotics are introduced, staphylococci have developed many efficient mechanisms to neutralize them. Resistance mechanisms of staphylococci include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic (penicillin-binding protein 2a of methicillin-resistant *S. aureus* and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (vancomycin and daptomycin) and efflux pumps (fluoroquinolones and tetracycline). Complex genetic arrays (staphylococcal chromosomal cassette *mec* elements or the *van*A operon) have been acquired by *S. aureus* through horizontal gene transfer, while resistance to other antibiotics, including some of the most recent ones (e.g., fluoroquinolones, linezolid and daptomycin) have developed through spontaneous chromosomal and plasmid mutations and antibiotic positive selection (Lowy, 2003; Pantosti *et al.,* 2007).

In the series of resistance development, *S. aureus* showed resistance towards penicillin by the production of a specific enzyme called penicillinase (β-lactamase) encodedby a *blaZ* gene often located in plasmid. This is an extracellular enzyme hydrolyzing the β-lactam ring of β-lactam antibiotics (penicillin) (Lowy, 2003). Only after two years of this, first case of MRSA (methicillin resistant *S. aureus*) was reported. MRSA strains acquired resistance by PBP2a (penicillin binding protein 2a) through blocking the proteins responsible for the construction and maintenance of the bacterial cell wall. PBP2a protein is encoded by *mecA* gene, which is the hallmark of MRSA. As opposed to the penicillinase gene, *mecA* does not reside on a plasmid but on the chromosome, embedded in a large mobile genetic element called *staphylococcal chromosome cassette mec* or *SCCmec*. The presence of PBP2a means MRSA is not only resistant to methicillin but also to all β-lactam antibiotics, including synthetic penicillins, cephalosporins and carbapenems (Pantosti and Venditti, 2009).

Vancomycin was identified as first line of drug for treatment of MRSA thus unfortunately use of vancomycin was dramatically increased not only for *S. aureus* infections but also for other infections which resulted in emergence of vancomycin resistance among bacterial population. Vancomycin resistant *S. aureus* (VRSA) strains acquire resistance by conjugal transfer of the vanA operon (*vanA* gene) from other bacterial population also (Loomba *et al.,* 2010). Establishment of MRSA and the emergence of VRSA has great concern because these are not only resistant to methicillin but also to vancomycin, monobactams and cephalosporins through production of ESBL (Extended-spectrum beta-lactamases). Antibiotic resistance, the overuse of antibiotics increased healthcare costs and sepsis-related deaths warrants exploration of the phenotypic and genotypic variations of resistance among *S. aureus* strains obtained from human and animal infections.

Looking towards the importance of *S. aureus* in causation of various ailments in man and animals, their confirmatory diagnosis, variations in the strains in regards to virulence factors such as adherence factors, antiphagocytic factor (capsule), exoenzymes, immune evasion factor, plasminogen activator factor (staphylokinase) and toxin secretory system and antimicrobial resistance shown towards various antibiotics, the present study was undertaken with the following objectives:

i. Isolation and identification of *Staphylococcus aureus* from various clinical and non-clinical settings and their genotypic confirmation.

ii. Phenotypic characterization of obtained isolates for coagulase production, slime production, haemolytic properties and toxin assays.

iii. Antibiogram study of *S. aureus* isolates including screening for Methicillin-Resistant *S. aureus* (MRSA), Vancomycin-Resistant *S. aureus* (VRSA), beta lactamase and Extended Spectrum Beta-Lactamase (ESBL) producers.

iv. To study genotypic variations among *S. aureus* isolates in relation to following virulence factors.

a) Adherence (*clfA, clfB, icaA, icaD, agr* and *trap* gene)
b) Antiphagocytosis (*cap5k* and *cap8k* gene)
c) Exoenzymes (*coa* and *aur* gene)
d) Immune evasion (*spa, chp* and *scn*)
e) Toxins (*hla, hlb, hld* and *tst* gene)

2. REVIEW OF LITERATURE

Staphylococcus aureus was first discovered by Alexander Ogoston in late 1870's from post-operative wound sepsis. In 1883, it was named *Staphylococcus* (the clusters of grape-like organisms), after the greek word '*Staphyle*' meaning bunch of grapes and '*coccus*' meaning granules. The species name '*aureus*' originated from the Latin word *'aurum'* which refers to the golden colour produced by the organism when grown on solid media (Howard and Kloos, 1987).

Staphylococcus aureus of *Staphylococcaceae* family forms a large yellow colony on rich medium. The cell wall is rigid due to high contents of teichoic acid and confers tolerance to drying, high salt and sucrose concentration. *Staphylococcus aureus* is often coagulase positive and haemolytic on blood agar. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative. *Staphylococcus aureus* can grow at a temperature range of 15 to 45^{0}C and at sodium chloride concentrations as high as 15% (Jonsson and Wadstrom, 1993).

Infectious diseases are a leading cause of morbidity and mortality in human and domestic animals, thereby incurring loss of life and production. Although, *S. aureus* is present as commensal on the skin and nasopharynx of the human and animal body, this bacterium is one of the most prevalent and contagious pathogen among the various causative agents of infectious diseases (Kumar *et al.*, 2011). It is commonly associated with various ailments *viz.* superficial skin infections, pneumonia, mastitis, meningitis, urinary tract infections, corneal infections, arthritis, osteomyelitis, food poisoning, toxic shock syndrome, scalded skin syndrome, endocarditis and toxinosis (Barg and Harris, 1997; Lowy, 2003; Herron-Olson *et al.*, 2007). *Staphylococcus aureus* causes pyogenic abscesses by infecting breached skin and mucous membrane by trauma or surgery. Since *S. aureus* secretes various toxins and exoenzyems, it is associated with septicemia through the lymphatic channels or blood (Projan and Novick, 1997). In Human, it causes toxic shock syndrome characterized by fever, rash, diarrhoea and inability to maintain proper haemostasis. In severe cases, the disease may progress further involving multiple organs, desquamation of skin over the entire body and death in some instances (Todd *et al.*, 1978). In swine, it

causes osteomyelitis and meningitis. In canine, it is associated with pyoderma, otitis externa and urinary tract infections. In equine, it causes suppurative lesions, abscesses and arthritis. In bovines and caprine, it is an important pathogen causing subclinical and clinical mastitis (Quinn *et al.*, 2000).

Mastitis is an inflammation of mammary gland tissue, which is caused by number of bacterial pathogens but *S. aureus* is the most important and the most common etiological agent. It causes chronic and deep infection in mammary glands, which is very difficult to treat (Hassan *et al.*, 2010). Mastitis is responsible for reduced milk production, decline in milk quality, and increased labour cost (Beck *et al.*, 1992). *Staphylococcus aureus* is not only recognized as most important mastitis associated pathogen which cause huge economic losses to the dairy industry worldwide but also a serious public health threat due to its ability to cause various diseases and to acquire antimicrobial resistance (Taverna *et al.*, 2007; Kavitha *et al.*, 2009).

The ability of this organism to cause diseases is thought to depend in part on three major activities: i) colonization of mucosal and skin surfaces with concomitant surface immune evasion, ii) production of cytolysins that target large numbers of host cells locally for additional immune evasion and also nutrient acquisition, and iii) production of super-antigens that become systemic and induce body-wide immune evasion (Lowy, 1998; McCormick *et al.*, 2001). Development of multiple drug resistant strains and emergence of new pathogens due to indiscriminate use of antimicrobials over long periods is a constant threat and danger of increasing spread of infectious diseases of zoonotic importance and endemic diseases are still a global concern (Mandell *et al.*, 1995; Kuhn, 2002).

Thus, the present work was undertaken with the objectives of isolation and phenotypic characterization of *S. aureus* isolates from various clinical and non-clinical settings *viz.* mastitic milk of cattle, buffalo and goat, clinical samples from human, camel, dog, sheep, horse and pig and meat samples in terms of cultural characteristics, biochemical properties, coagulase production, haemolytic properties, assay of beta and delta-toxins, slime production, antibiotic resistance pattern with beta-lactamase, extended-spectrum beta-lactamases (ESBL), MRSA and VRSA strains and associated genes, genotypic confirmation based on species specific 23S rRNA gene and molecular typing of *S. aureus* isolates with *rep* PCR. Genetic differentiation of isolates on the basis of some virulence factors *viz.* adherence factors, antiphagocytic factor (capsule), exoenzymes, immune evasion factor, plasminogen activator factor (staphylokinase) and toxin secretory system associated genes.

The literature available on *S. aureus* related to above cited parameters have been reviewed under following heads and subheads:

1. ***Staphylococcus aureus* isolation and genotypic confirmation**
2. **Phenotypic characterization of *S. aureus*:**
   a. Cultural and biochemical properties: (Pigmentation, Mannitol fermentation, catalase and oxidase production).
   b. Coagulase production
   c. Slime production
   d. Haemolytic properties and Toxin assays
   e. Antibiotic resistance pattern and MIC determination
   f. Beta-lactamase (*blaZ* gene), ESBL, MRSA (*mecA* gene) and VRSA activity
3. **Molecular typing of *S. aureus* with Repetitive sequence-based PCR *(rep* PCR)**
4. **Genotypic characterization of *S. aureus* in relation to following virulence associated genes:**
   a. Adherence (*clfA, clfB, icaA, icaD, agr* and *trap* gene)

b. Antiphagocytosis (*cap5K* and *cap8K* gene)
c. Exoenzymes (*coa* and *aur* gene)
d. Immune evasion (*spa-X, spa-IgG, chp* and *scn* gene)
e. Plasminogen activator/ Staphylokinase (*sak* gene)
f. Toxins (*hla, hlb, hld* and *tst* gene)

1. *Staphylococcus aureus* isolation and genotypic confirmation:

Brakstad *et al*. (1992) developed a PCR assay specific for *S. aureus* based on *nuc* gene coding for the staphylococcal thermonuclease and was found suitable for diagnostic purposes.

Tenover *et al*. (1994) also used traditional (antibiogram, bacteriophage, biotyping, immunoblotting) and molecular typing methods (insertion sequence typing, multilocus enzyme electrophoresis, restriction analysis of plasmid DNA, pulse field or field inversion gel electrophoresis, restriction analysis of PCR amplified coagulase gene sequences, RFLP typing and ribotyping) for 59 *S. aureus* isolates at eight different institutions. In their studies they recorded that overall, the DNA based technique and immune-blotting were most effective but no single technique was superior to other.

van Belkum *et al*. (1995) carried out a multicenter evaluation of Arbitrarily primed-polymerase chain reaction (AP-PCR) in 7 different laboratories for typing of *S. aureus* strains with a standardized protocol using template DNA isolated in a single institution, and a common set of three primers with different resolving powers in order to study reproducibility and discriminatory ability. Though difference in resolution was obtained in different laboratories, but that did not hamper the epidemiological correct clustering of related strains. They found AP-PCR well suited for genetic analysis and monitoring of nosocomial spreading of staphylococci.

Pentucek *et al*. (1996) used PFGE with genomic DNA of 95 *S. aureus* subsp. *aureus* strains of different origins by using *Sma*I. They recorded 30-100% levels of similarity, which was considered characteristic of this species. Within this range of similarity values *S. aureus* intra-species groups were identified whose levels of similarity ranged from 65 to 100%. They also found 44 kb *Sma*I fragment in all *S. aureus subsp. aureus* characteristic of the species *S. aureus.*

The method of ribotyping with specific-primers targeting 23S rRNA gene was developed for *S. aureus* by Straub *et al.* (1999) where a PCR product of 1250-bp size was found to be species-specific for this organism. This method achieved a high degree of sensitivity and unambiguity as required for the detection of contaminants in food starter preparations. The method permitted detection of *Staphylococcus aureus* in preparations of meat starter cultures containing *Staphylococcus carnosus* either alone or in combination with lactobacilli, pediococci, and/or Kocuria varians. Detection limits were sufficiently low to show within 12 h the presence of 10(0) CFU of *S. aureus* in starter preparations containing 10(10) CFU of *S. carnosus*.

Diep *et al*. (2003) developed a low cost strain-typing technique based on restriction fragment analysis of seven loci used in multi locus sequence typing (MLST) for the strain characterization of *S. aureus.* In this technique sequence variation in the MLST house keeping gene loci were detected by restriction fragment analysis rather than sequencing.

From bovine sub-clinical mastitis 35 *S. aureus* isolates were identified by Salasia *et al*. (2004) through amplification of 23S rRNA gene specific to *S. aureus* wherein they obtained a species specific amplicon of 1250bp. Presently this method of identification is being used by many of the laboratories.

Mork *et al*. (2005) carried out PFGE of 905 epidemiologically unrelated *S. aureus* isolates from bovine, ovine and caprine mastitis recovered from 588 herds in 12 counties in Norway. Their results suggested that a small number of closely related genotypes are responsible for a great proportion of *S. aureus* mastitis cases and these genotypes exhibited little or no host preference among these species.

The investigation of 40 *S. aureus* isolated from milk samples of clinical and subclinical mastitic cows from 40 different farms revealed that *S. aureus* were uniformly positive for the gene segment encoding a species specific part of the 23S rRNA (El-Sayed *et al.,* 2006).

Sequences for 23S rRNA gene enabled evaluation of the specificity of 39 previous and four newly designed primers specific for bacteria. This study of Hunt *et al.* (2006) incorporated data from large-scale sequencing efforts to develop new and evaluate existing bacterium-specific 23S rRNA PCR amplification primers.

Sanjiv *et al.* (2008) carried out genotypic confirmation of *S. aureus* from bovine mastitis using PCR techniques and obtained amplified product of 1250bp.

Milk samples were collected from 98 cows with subclinical mastitis. A total of 65 coagulase-positive *Staphylococcus* isolates were identified by phenotypical assays. Further, among the 65 staphylococcal isolates, 21 were identified as *Staphylococcus aureus* by PCR amplification of the 23S rDNA fragment species specific to *S. aureus* (Coelho *et al.,* 2009).

Out of the total 60 mastitic milk samples collected by Kumar (2009), 47 were from clinical cases and 13 were from sub clinical cases of buffalo mastitic milk. All these were confirmed by three different primers, Sta I (F) and Sta II (R) primers, Sau 327 (F) and Sau 1645 (R) and Staur 4(F) and Staur 6(R), sourced from Forsman *et al.* (1997), Riffon *et al.* (2001) and Straub *et al.* (1999) respectively. Out of the 36 mastitic milk samples that reacted with Sta I & Sta II primers, 21 samples yielded two PCR products of 190bp and 200bp, and 15 samples yielded three PCR products of 190bp, 200bp and 300bp of low intensity. But when all these 36 mastitic samples were further tested for their reactivity in PCR with *S. aureus* specific primer sets Sau 327 and Sau 1645 and Staur 4 and Staur 6, only 21 samples reacted positively. The remaining 15 samples didn't react with either of the *S. aureus* specific primer sets, even though they were found positive for staphylococcus species in PCR.

All the 43 *S. aureus* isolates of the bovine mastitc milk were identified by the PCR method using specific oligonucleotide primer encoding for 23S rRNA of *S. aureus* which yielded an amplification product of 1250bp (Bhanderi *et al*., 2009).

Molecular assessment of *S. aureus* from milk and dairy workers was carried out by Abeer *et al.* (2010) using multiplex PCR assay including 16S rRNA which revealed a successful amplified product in 49.61% and 82.6% of tested samples, respectively.

A total of 100 milk samples were collected from 41 indigenous cattle, 18 H. F cross cattle and 41 Marwari goats with mastitis for isolation of *S. aureus. Staphylococcus aureus* was isolated in 10 samples from indigenous cattle (24.39%) and in 10 samples from cross-bred H.F cattle (55.55%) with overall recovery of *S. aureus* from cattle to the tune of 33.89%. In Marwari goats, *Staphylococcus aureus* was isolated in 10 samples (24.39%) (Upadhyay *et al.,* 2010b).

In the study of Momtaz *et al.* (2010), 86 isolates (23.88%) out of 360 samples of clinical and subclinical bovine mastitis were confirmed as *S. aureus*, the existence of 1250bp fragment in samples showed positive PCR assay with species-specific 23srRNA gene fragment.

Proietti *et al.* (2010) confirmed a total of 170 *S. aureus* isolates from 1257 quarter milk samples collected from 320 cows suffering from subclinical mastitis. The isolates were genotypically identified by targeting species-specific gene encoding the 23S rRNA. Ariyanti *et al.* (2011) isolated 11 *S. aureus* isolates from foods of animal origin from various cities and all were confirmed with species-specific primers of 23S rRNA gene.

Thirteen *S. aureus* isolates obtained from 47 mastitc milk samples of H-F cross cattle and 15 isolates were recovered from 25 mastitc milk samples of Rathi cattle were identified with species specific primers sets on the basis of 23S rRNA gene segment. The samples were collected from various localities of Bikaner region (Khichar *et al.*, 2012).

Suleiman *et al.* (2012) reported 102 *S. aureus* from 339 quarter milk samples of 136 cows. The species of the 20 strains were confirmed by PCR amplification using *S. aureus* specie-specific primers derived from the 23S rDNA and epidemiological relationships of the strains were studied by macro-restriction analysis of their chromosomal DNA using pulse field gel electrophoresis (PFGE). Among the 20 *S. aureus* strains identified, PFGE revealed an identical DNA pattern for 18 strains while two strains differed in two bands.

Out of total 22 pus samples collected from skin infections and chronic wound of camels in and around the Bikaner city 15 isolates of *S. aureus* were obtained and confirmed with species specific primers based PCR for 23S rRNA gene (Rathore and Kataria, 2012).

Yang *et al.* (2012) identified 39 *S. aureus* isolates from bovine clinical mastitis by conventional methods and by PCR technology. The oligonucleotide primers targeted to species-specific parts of the gene encoding 23 S rRNA produced amplicons of 1250bp in all the isolates.

Fifteen isolates (10.6%) were identified as *S. aureus* from studied 141 samples collected from animal and human clinical infection. The isolates were confirmed as *S. aureus* with species-specific primers on the basis of amplification of the extracellular thermostable nuclease (*nuc*) gene (Abd El-Hamid and Bendary, 2013).

Nathawat *et al.* (2013) determined the prevalence of *S. aureus* in mastitic goats. From a total of 71 mastitic milk samples 27 isolates were obtained with a recovery rate of 38.03%. All the isolates were genotypically confirmed on the basis of 23S rRNA ribotyping.

Sharma *et al.* (2013) studied 46 nasal discharge samples from pneumonic camels (*Camelus dromedarius*) and obtained 15 isolates with 32.6% recovery of this organism. All the 15 isolates obtained and identified were genotypically confirmed on the basis of 16S-23S rRNA spacer region with a species-specific amplicon of 420bp.

Castelani *et al.* (2013) carried out molecular typing of mastitis causing *S. aureus* isolated from heifers and cows. Strains were typified through pulsed-field gel electrophoresis (PFGE) and grouped according to patterns of restriction enzyme *Sma*I.

For the purpose of identification of *S. aureus* strains from mastitic milk samples, species specific primer pair Staur4 (5'-ACG GAGTTACAAAGGACGAC-3') and Staur6 (5′-AGCTCAGCCTTAACGAGTAC-3′) was used for the partial amplification of the 23S rRNA gene by Vazquez *et al.* (2013) and found that the positive *S. aureus* were confirmed with an amplicon size of 1270bp.

Yadav *et al.* (2015a) isolated and confirmed 32 (16 of each cattle and buffalo) *S. aureus* isolates from 89 mastitic milk samples (41 were from cattle and 48 were from buffalo) on the basis of specific-primers targeting 23S rRNA gene.

2. Phenotypic characterization of *Staphylococcus aureus*:

A. Cultural and biochemical properties:

The most of the strains of *S. aureus* isolated from skin lesion of camels produced golden yellow or white colonies on milk salt agar and nutrient agar (Arzo, 1973).

In a study Cousins *et al*. (1980) found that 72% of the *S. aureus* isolates recovered from cows with suspected mastitis metabolized mannitol aerobically.

Garcia *et al.* (1980) observed that neither mannitol fermentation nor production of other enzymes appeared to be a specific property of bovine *Staphylococcus aureus* strains and also revealed that a total of 43 of the 46 strains of *S. aureus* fermented glucose anaerobically but three strains were weak producers of acid (pH 5.8 to 6.0).

Mannitol fermentation was favored as a primary and important criterion in identification of the *S. aureus* (Pennell *et al.*, 1984). A total of 314 clinical and non-clinical isolates of the genus *Staphylococcus* were tested for coagulase production and glucose and mannitol fermentation by Adekeye (1984).

Chatterjee *et al.* (1990) analyzed coagulase positive *S. aureus* isolates from cattle and found 48.8%, 12.4% and 31.4% produced golden yellow, yellow and white colonies, respectively.

Quinn *et al*. (2000) reported difference in colony pigmentation of *S. aureus* from cattle, human and other domestic animals.

The cultural, biochemical and hemolytic properties were used by Annemuller *et al*. (1999) to identify 25 *S. aureus* isolates from bovine subclinical mastitis at five locations in one region of Germany.

Ajuwape and Aregbesola (2001) reported that all the 108 isolates of *S. aureus* from nasal swab samples of rabbits were mannitol fermenter within 8-24 h.

In a study on phenotypic properties of *S. aureus* isolated from bovine sub-clinical mastitis Salasia *et al*. (2004) detected three types of pigments (orange, yellow and pale yellow) produced by *S. aureus*. Similarly, Qureshi and Kataria (2012) also reported a variation in the colony pigmentation of *S. aureus* from skin wounds in camel as golden yellow, yellow and white colonies.

Staphyloxanthin is membrane bound carotenoid responsible for golden yellow pigmentation, which was studied by Clauditz *et al.* (2006). In their study, they found that staphyloxanthin scavenges free radicals and probably protect lipid and DNA in the cell. They suggested that staphyloxanthin can be regarded as a biological antioxidant against hydrogen peroxide and hydroxyl (OH^-) radical and might be used as therapeutic radical scavenger to combat oxidative stress.

Mannitol fermentation was recorded with all the 23 *S. aureus* from mastitic milk samples obtained from buffaloes and cows by Arshad *et al.* (2006). Singh (2006), Sanjiv (2006) and Upadhyay (2009) reported that 100% isolates were mannitol fermenter obtained from cattle subclinical mastitis, cattle clinical mastitis and from mastitis of cattle and goats, respectively.

In a study by Islam *et al.* (2007), 28 coagulase positive *S. aureus* isolates were found to be produce three distinct types of colony pigments on nutrient agar *i.e.* golden yellow (46.4%), yellow (39.3%) and white (14.3%).

Sanjiv *et al.* (2008) characterized 21 isolates of bovine mastitis milk origin, of which 20 produced round, smooth, and glistening colonies with golden yellow pigmentation on nutrient agar whereas one colony was white in colour.

El-Jakee *et al.* (2008) characterized 78 isolates of *S. aureus* obtained from 409 samples of human and animal sources and found that 100% isolates were mannitol fermenter. In colony characteristics, they reported that five isolates were white, 23 creamy and 50 were golden yellow pigmented. Upadhyay (2009) characterized *S. aureus* isolates from cattle (20) and goat (10) suffering from clinical mastitis and found all the isolates to produce smooth, round colonies with golden yellow pigmentation.

Biochemical characterization of *S. aureus* strains isolated from pus samples of human samples was carried out by Chakraborty *et al*. (2011) in which they found all the isolates to ferment mannitol.

Makwana *et al.* (2012) concluded that sensitivity of mannitol fermentation test is more as compared to coagulase test for identification of *S. aureus*.

Qureshi and Kataria (2012) characterized 40 *S. aureus* from camel wound samples and recorded 15 isolates to produce golden yellow, 24 to produce yellow and one to produce white colony.

The yellow to orange colour of *S. aureus* is one of the classical criteria for identification of the species. However, the pigmentation of *S. aureus* is not a very stable character (Sun *et al.,* 2012). They observed that *S. aureus* in bio-film pattern was nearly colourless and *S. aureus* grown in anaerobic and aerobic patterns was colourless and yellow, respectively.

The applicability of mannitol salt agar was tested for 69 and 47 phenotypically different colonies of *S. aureus* obtained from bulk milk and teat apices by Visscher *et al.* (2013). They recovery of separate colonies of CNS was much more convenient on MSA compared to a non-selective blood agar.

Sharma *et al*. (2013) reported 100% mannitol fermentative, *S. aureus* (15) isolates obtained from 46 nasal swabs from pneumonic camels.

B. Coagulase production

The plasma of several animals were examined for suitability in coagulase test by Orth *et al.* (1971). The coagulase-reacting factor (CRF) activities of the different sera had following relative concentrations of CRF: human > pig > rabbit > horse > bovine, chicken, and lamb. Human, pig, and rabbit sera had adequate amounts of CRF for coagulase testing.

A tandem coagulase/thermonuclease agar plate method for the identification of *S. aureus* was developed by Boothby *et al.* (1979) wherein rabbit plasma was used in detecting the coagulase reaction on solid agar plates. The *S. aureus* colonies were transferred to coagulase test agar, the plates were incubated at 37°C for 18 h, and the coagulase reaction was recorded. The plates were then heated at 65°C for 2.5 h, overlaid with toluidine blue-metachromatic diffusion agar, and re-incubated at 37°C for 3 h, and the thermonuclease reaction was recorded. Their experiments demonstrated 100% agreement between the tandem agar plate method and standard coagulase and thermonuclease tests. They further emphasized that tandem agar plate method was rapid and convenient approach contributing to the identification of *S. aureus.*

Of the 354 isolates tested by Pennell *et al.* (1984), 156 were identified as *S. aureus* by the three agglutination tests and by the 4h and 24 h tube coagulase tests. Discrepancies between agglutination and tube coagulase identifications were resolved by use of the thermonuclease, mannitol fermentation and slide coagulase tests.

Jasper *et al*. (1985) isolated *S. aureus* from cows and found that 1-2% of the organisms did not produce tube coagulase.

Adesiyun and Shehu (1985) evaluated effect of sources of *S. aureus* and plasmas, concentration of plasmas, temperature and duration of incubation on coagulase test results. With *S. aureus* strains of food origin, the value of plasmas in order of superiority was Human and Rabbit > Pig > Donkey > Chicken > Cattle > Duck > Goat > Dog. Regardless of the source of staphylococci horse plasma was found unsuitable in coagulase test as it clotted spontaneously. The temperature and duration of incubation and type of anticoagulant had no significant effect on coagulase test results.

The slide coagulase and tube coagulase using rabbit plasma correctly identified 115 (97.5%) and 117 (99.1%), respectively, of the *S. aureus* isolates. The tube was incubated in a water bath at 37°C and examined after 4 and 24 h and formation of clot at either reading was recorded as positive (Berke and Tilton, 1986). Previously, biochemical, immunological, tube coagulase, and thermostable nuclease methods had shown variable sensitivity and specificity, but even though tube coagulase method was considered as the specific traditional methods for differentiating *S. aureus* from coagulase-negative staphylococci (Speers *et al.*, 1998)

Qureshi *et al.* (2002) in a study on coagulase types of 30 *S. aureus* isolated from wounds and abscesses in dromedary camel found that isolates coagulated the plasma from rabbit, human, buffalo, horse, cattle, goat, camel and sheep in decreasing order of superiority.

Boerlin *et al.* (2003) stated that the coagulase test was not sensitive after 4 h, and acceptable results were obtained only after 24 h of incubation. The high specificity and sensitivity of the coagulase test have made it a standard method for the identification of *S. aureus* in milk.

Coagulase production was recorded in all strains of *S. aureus* by Hookey *et al*. (1998). However, El-Jakee *et al.* (2008) studied 78 isolates of *S. aureus* obtained from 409 samples of human and animal sources and reported that the production of coagulases and thermonucleases were not unique features of *S. aureus* but were shared by *S. intermedius* and *S. hyicus*.

Sanjiv *et al*. (2008) recorded one isolate which did not produce coagulase in tube test. In an investigation by Upadhyay (2009) all the isolates from cattle and goat were found to produce coagulase enzyme detected against plasmas from cattle and human.

In one study Kateete *et al*. (2010) reported that sensitivity and specificity of the tube coagulase test with human plasma was markedly improved when MSA and DNase were introduced as a tri-combination test for routine identification of *S. aureus*.

Rubin *et al.* (2010) compared the utility of dog and rabbit plasma in the coagulase test for *S. aureus* isolated from canine (28), bovine (29), and human (30) hosts. Their study revealed that coagulation times were significantly faster for dog (2.38 hr) than rabbit (3.19 hr) plasma. When coagulation times were compared by isolate origin, no significant differences were found for rabbit plasma, whereas bovine isolates clotted dog plasma significantly faster (1.86 h) than canine (2.79 h) or human (2.38 h) isolates.

The prevalence of coagulase production was recorded highest by Singh *et al.* (2011) in Murrah buffalo milk isolates (90.7%) followed by isolates of Karan Fries cattle milk and Sahiwal cattle milk i.e. 88.3% and 78.3%.

Coagulase is a collagen binding protein encoded by *coa* gene which had been demonstrated to be directly related with bovine mastitis (Momtaz *et al*., 2011). In a study on the comparison of sensitivity of mannitol fermentation test with coagulase test for diagnosis of *S. aureus*, Makwana *et al*. (2012) reported that among 100 staphylococcal isolates, the mannitol fermentation test reaction was seen in all 100 isolates while positive tube coagulase test was seen in 94 isolates. They further concluded that sensitivity of mannitol fermentation test was more as compared to tube coagulase test and the mannitol fermentation can be used for diagnosis of *S. aureus* along with coagulase test to improve the sensitivity of coagulase test. They reported that the sensitivity of human plasma was 94%.

Sharma *et al*. (2013) reported 15 (100%) coagulase producing *S. aureus* isolates obtained from 46 nasal swabs from pneumonic camels. In a comparison of plasmas for coagulation reaction, human plasma was found superior than cattle plasma.

Abd El-Hamid and Bendary (2015) isolated 46 staphylococci from 141 human origin samples. Among these 46 isolates, 26 were coagulase positive *S. aureus* and 20 were identified as coagulase negative staphylococci.

Yadav *et al.* (2015a) also found 32 (100%) coagulase positive *S. aureus* from 89 mastitic milk sample of both cattle and buffalo. Strongest coagulation reaction was recorded for human plasma followed by pig, rabbit, horse, bovine, chicken, and lamb in decreasing order.

Khichar and Kataria (2015) characterized 28 *S. aureus* isolates obtained from 59 mastitis samples of Holstein-Friesian crossbred and Rathi cattle. All of the isolates produced coagulase and the overall strongest coagulation reaction in regards to early onset and firmness of clot was recorded with plasma from rabbit followed by plasma from buffalo, cattle, camel, human, goat, sheep, dog, horse, chicken and pig in decreasing order.

C. Slime (Biofilm) production

Freeman *et al*. (1989) studied two different methods of slime production i.e. Christensen method and Congo red method. The results showed that there was complete agreement between methods in 107 out of a total of 124 test strains. The Congo red method was rapid, sensitive, and reproducible and had the advantage that colonies remained viable on the medium.

Thirty-two of the 35 isolates of *S. aureus* from bovine mastitis produced slime on Congo red agar by showing black color colonies (Vasudevan *et al.*, 2003). Slime production of *S. aureus* from raw milk samples was investigated by Citak *et al.* (2003) using Congo red agar (CRA) method. Slime production was positive in 5.1% of *S. aureus.*

In one study Turkyilmaz and Kaya (2006) found 77.80% of the 180 strains of coagulase positive *S. aureus* to show positive slime production activity while 44.4% coagulase negative *S. aureus* showed slime production on CRA plate which produced black colonies with dry crystalline consistency.

There is increasing evidence that *S. aureus* can form biofilms in the udder of cows suffering from mastitis. Biofilms impair the action of both the host immune system and antimicrobial agents and allow the persistence of the infection (Melchior *et al*., 2006).

Yazdani *et al*. (2006) evaluated the biofilm forming capacity of *S. aureus* isolated from wound infections phenotypically by slime production assay on Congo red agar (CRA) and by microtiter plate assay.

Gundogan *et al.* (2006) investigated slime production by *S. aureus* using Congo red agar method wherein they recorded slime production in 52.7% of *S. aureus.* Arslan and Ozkarde (2007) identified 129 *S. aureus* from several clinical specimens by the API Staph System (Biomerieux). Slime production was detected both by the conventional Christensen's method as well as by the Congo red agar method. Seventy-two strains of staphylococci isolates (38.5%) were found to be slime producers by Christensen's test tube method whereas 58 strains (31%) were slime positive with Congo red agar method.

The evaluation of modified Congo red agar (MCRA) based on the phenotypic characteristics of 100 methicillin resistant *S. aureus* (MRSA) isolated from different clinical samples was done by Mariana *et al.* (2009), in which MCRA was optimized to get strong black pigment in all colonies of MRSA isolates with constant pigmentation at 48 h incubation and then for 2-4 days at room temperature. A black color interpreted as positive biofilm producing strains in contrast with red colonies which interpreted as negative biofilm producing.

Some of the *S. aureus* strains exhibit the ability of producing a viscous extracellular polysaccharides layer (Slime) which promotes bacterial adhesion on to mammary epithelial cells and protects bacteria from opsonization and phagocytosis this extracellular layer of polysaccharides is considered a virulence factor. Dhanawade *et al.* (2010) investigated biofilm production by *S. aureus* by employing different methods. Of the 102 strains tested, 48.03% were detected as biofilm producers by Congo red agar method, 36.27% by tube method and 29.41% by tissue culture plate method.

The slime production by 46 *S. aureus* strains isolated from auricular infection was assessed on CRA plates by Zmantar *et al.* (2010) who found 26 strains (56.5%) to be slime producers developing almost black or very black colonies while other 20 strains were non-producers developing red or bordeaux colonies.

Khan *et al.* (2011) evaluated biofilm formation by *S. aureus* and its role in treatment of MRSA infections. Out of 262 clinical strains tested, 38 (14.51%) were found to be strong biofilm producers, 132 (50.38%) as moderate biofilm producers and 92 (35.11%) strains as non-producers by tissue culture plate method. Biofilm production was high in methicillin resistant strains as compared to methicillin sensitive strains of *S. aureus*.

Singh *et al.* (2011) observed slime production in 346 isolates of *S. aureus* from Sahiwal (107), Karan fries cattle (128) and Murrah buffalo (111) suffering from intramammary infections. They recorded 65.4%, 83.6% and 81.4% slime producer isolates in Sahiwal, Karan fries cattle and Murrah buffalo respectively.

The ability of biofilm formation by *S. aureus* isolates was tested using two phenotypic methods, congo red plate assay and spectrophotometric microtitre plate assay. Out of these, microtitre plate assay was found to be more efficient. Most of the isolates, 76/132 (57.6 %) were classified as biofilm producers depending on the value of absorbance in the microtitre plate test (Szweda *et al.*, 2012).

Congo red agar assay (CRA), semi quantitative adherence assay and microbial adhesion to solvent (MATS) test were used by Bekir *et al.* (2012) to study qualitative and quantitative biofilm formation by *S. aureus* strains. Twenty one strains (51.2%) were slime producers on CRA and 19 strains were slime producer on polystyrene plates.

Growth properties of *S. aureus* in biofilm formed on polystyrene plate was studied by Sun *et al.* (2012). They recorded that *S. aureus* cell in biofilm was nearly colorless because it produced less staphyloxanthin. The cells existed in a poorly acidic environment. Further, they found that the organic acid accumulated by *S. aureus* in biofilm growth pattern included acetate, isobutyrate, isovalirate, lactate and butyrate especially acetate and lactate.

Melo *et al.* (2013) carried out congo red agar for slime production in 94 *S. aureus* strains obtained from cows with subclinical mastitis from two farms in the state of Sao Paulo wherein they recorded 85% of the isolates to produce slime.

A total of 40 *S. aureus* were isolated from raw calf minced meat, chicken drumsticks, raw milk, ice cream and white cheese samples by Gundogan *et al.* (2013) wherein they recorded a high proportion of isolates forming slime (80%) and biofilm (70%).

Ozpinar and Gumussoy (2013) studied 61 *S. aureus* isolates and found 37 (60.65%) isolates were biofilm producers and Ebrahimi *et al.* (2014) also reported 41 (83.7%) of isolates were biofilm producers from 61 human wound infections.

Yadav *et al.* (2015a) studied 32 *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis and found that 31 (96.87%) isolates were slime producer with characteristic black color colonies on congo red agar.

Fabres-Klein *et al.* (2015) studied 54 *S. aureus* strains obtained from cows with subclinical mastitis. Slime was detected using the Congo Red agar (CRA) test in 35.18% (19/54) of the strains; however, 87.04% (47/54) of the strains were considered biofilm-positive based on crystal violet staining.

Barbieri *et al.* (2015) studied breast peri-implant infections causing 13 *S. aureus* isolates. All were producing biofilm in microtitre plate assays and Congo Red Agar (CRA) plate method with characteristic brown-black colonies while Castelani *et al.* (2015) studied 110 *S. aureus* isolates from mammary gland secretions of heifers and cows with mastitis and only 55.5% of all isolates produced a biofilm on Congo red agar.

Two hundred fifteen *S. aureus* strains were collected from human and dairy cow's infections and approximately 70% of the isolates produced biofilm. Among these, 59.3% were producers of weakly adherent biofilms while 34.8% and 5.8% produced moderate and strong biofilms, respectively (Khoramian *et al.*, 2015).

D. Haemolytic properties and Toxin assay

Production of haemolysins is another important phenotypic property of *S. aureus* isolates. Different workers have examined *S. aureus* isolated from variety of sources and found variations in the production of haemolysin which is greatly affected by the environmental conditions. *Staphylococcus aureus* produces three types of haemolysins namely α, β and δ designated in order of their discovery (Elek and Levy, 1950).

The delta haemolysin had an injurious effect on wide variety of cell cultures and leucocytes (Jackson and Little, 1957). The delta haemolysin produced by *S. aureus* was identified as a polypeptide consisting of five identical subunits each weighing $1x10^4$ (Kantor *et al*., 1972).

The beta toxin is a protein with a molecular weight ranging from $1.2x10^4$ to $5.9x10^4$ (Chesbro and Kucic, 1971) and is a "Hot-cold" haemolysin (Wiseman, 1975).

In a study by Garcia *et al.* (1980) only four of the 57 cultures failed to produce any of the hemolysins. Delta-hemolysin (δ) was produced by 47 strains, beta-hemolysin (β) by 45 strains and alpha-hemolysin (α) was produced by 30 strains. The hemolysin patterns found most frequently were α-β-δ (25 strains), β-δ (14 strains) and δ (6 strains).

Two hundred and three isolates were confirmed as *S. aureus* from cow milk by Jasper e*t al.* (1985) and the results of the tests for α- or α-β toxin, tube coagulase and hemolytic toxin tests were each positive for 99% of the isolates tested.

The bovine mammary isolates of *S. aureus* were examined by Kenny *et al*. (1992) for production of α-hemolysin and they found 94% of the isolates positive by an assay which consisted of a dot immune-blotting technique employing bacterial culture supernatant and mouse monoclonal antibody specific for α-hemolysin.

Fifty eight *S. aureus* strains isolated by Matsunaga *et al.* (1993) from bovine mastitic milk were examined for their virulence associated factors. The positive rates of the total isolates for various haemolysin were as follows: alpha-haemolysin (74.1%), beta-haemolysin (65.5%) and delta-haemolysin (12.1%).

Beta-toxin had property of damaging bovine mammary secretary epithelial cells by increasing the damaging effect of α- toxin, increased adherence of *S. aureus* to mammary epithelial cell and proliferation of *S. aureus* (Cifrian *et al*., 1996).

Bedidi-Madani *et al.* (1998) studied coagulase negative strains of *S. aureus* from goat milk and recorded haemolysis on blood agar plate as α-haemolysin, β-haemolysin and δ-haemolysin by 56.9%, 75.1% and 76.3% isolates, respectively.

The phenotypic expression of haemolysins and the presence of genes encoding alpha- and beta-haemolysin were determined in 105 *S. aureus* isolates from bovine mastitis by Aarestrup *et al.* (1999). Alpha-haemolysin was expressed phenotypically by 39 (37%) of the bovine isolates and β-haemolysin was expressed by 76 (72%). Phenotypic expression of β-haemolysin was shown by 75% of isolates which carried the gene encoding β-haemolysin.

A total of 319 *S. aureus* isolates obtained from 15 dairy herds in Israel were studied for their haemolytic properties on blood agar by Younis *et al.* (2000). They recorded majority (62.7%) of the isolates to be non-haemolytic, α- and β- haemolysis was shown by 23.2%, β- haemolysis by 12.5% and α- haemolysis by only 1.6% of the isolates.

Salasia *et al.* (2004) subjected 35 *S. aureus* isolates to haemolysin production test by cultivation on sheep blood agar plates. Complete haemolysis was observed for 7 isolates, partial haemolysis for 10 isolates, α/β haemolysins for seven isolates and δ- haemolysin for one isolate whereas 10 of the isolates were recorded as non-haemolytic.

Forty six *S. aureus* of camel origin were characterized by Solanki (2004) wherein he demonstrated that 21 isolates showed complete haemolysis, 13 isolates exhibited incomplete haemolysis and 12 isolates did not show any haemolysis on blood agar and none of the isolates showed both types of haemolysis. On toxin titration, it was recorded that 33.33% isolates were β-toxin producer, 26.66% produced both α- and β-, 23.33% produced all the three toxins, 6.66% produced δ- toxin, 3.33% produced α- only, 3.33% produced combination of β- and δ- toxin whereas 3.33% did not produce any toxin.

Out of the total 228 *S. aureus* strains from goat mastitic milk studied by da Silva *et al.* (2005), 80% were detected to produce haemolytic activity of which 76.7% produced alpha-hemolysin, 74.4% beta-hemolysin and 83.7% produced delta-hemolysin, either alone or in combination.

Out of 28 coagulase positive *S. aureus* from cattle 25 (86.3%) produced partial haemolysis, indicating their ability to produce β-haemolysin toxin (Islam *et al.*, 2007). El-Jakee, *et al.* (2008) studied 78 isolates of *S. aureus* obtained from 409 samples of human and animal origin and found 70 isolates to be haemolytic and eight non haemolytic.

In a study by Sanjiv *et al.* (2008) of the 21 isolates 20 were revealed to produce both α- and β-haemolysin and one isolate to produce β-toxin only. The β-toxin producing isolates did not give coagulation reaction in tube test and was further confirmed as coagulase gene deficient strain.

Coelho *et al.* (2009) studied 21 *S. aureus* of bovine mastitic milk origin for the haemolytic properties wherein they found that only nine isolates were haemolytic with seven of them producing both complete and partial haemolysis and two producing only partial haemolysis.

In an investigation Ebrahimi and Taheri, (2009) recorded hemolytic activity, out of eight *S. aureus* isolates where five strains produced α/β/δ and three produced α/β hemolysins.

All the 122 *S. aureus* isolates from various cow dairy products showed haemolysis on blood agar plates. Out of which, 62% of the isolates showed β-haemolysis, 36% showed double haemolysis (α+β), and only two cow isolates showed complete haemolysis (Morandi *et al.*, 2009).

Out of the 82 *S.aureus* isolates from cases of acute clinical mastitis in cattle, Capurro *et al.* (2010) found that α, β or α haemolysins was found in 66% or 34% of the isolates, respectively.

Twenty one of the isolates (14 from cattle, seven from goats) were streaked on 5% sheep bold agar and all isolates produced complete haemolysis, three produced partial haemolysis (two from cattle and one from goat), and six isolates (four from cattle and two from goats) produced both haemolysis (Upadhyay and Kataria, 2010).

Of the 129 isolates complete haemolysis was exhibited by 56 (43.4%), partial by 43 (33.11%) whereas 29 (22.48%) isolates did not show any haemolysis (Wang *et al*., 2011).

In a study Salasia *et al.* (2011) recorded types of haemolysis by *S. aureus* on the sheep blood agar plate and observed complete hemolysis by two isolates (18.18%), partial haemolysis by three isolates (27.27%) and no hemolysis by six isolates (54.55%).

Of the 60 *S. aureus* isolates from bovine mastitic milk, 33 were found haemolytic of which 20, 11 and two isolates showed complete, partial and both type haemolysis, respectively (Hussain *et al.*, 2012).

Bautista-Trujillo *et al.* (2012) observed that the use of culture media combined with coagulase activity and haemolysis as secondary tests improved accuracy of the identification of the *S. aureus* from bovine mastitis.

Staphylococcus aureus spreads on the surface of soft agar, and this phenomenon is termed as "colony spreading" activity. Omae *et al.* (2012) purified hemolysin (hld-toxin), a major protein secreted by *S. aureus*, as a compound that inhibits colony spreading. The *hld*-disrupted mutant's culture lowers the colony spreading activity by 30 folds. Further, it had higher colony-spreading ability than the parent strain. These results suggested that *S. aureus* negatively regulates colony spreading by secreting hemolysin.

Qureshi and Kataria (2012) obtained 40 *S. aureus* isolates from skin wound infections of camel and reported that 16 isolates showed complete haemolysis, 11 showed incomplete and 13 exhibited both types of haemolysis on sheep blood agar.

Graber *et al.*, (2013) reported that all clinical isolates *S. aureus* showing complete, incomplete or double hemolysis while non-hemolytic strains of *S. aureus* showed very low prevalence (0–2%) and suggested that might be good indicator of virulence.

The identification of ADAM10 as a proteinaceous receptor for the alpha toxin provides a greater appreciation and opportunity to more extensively probe and understand the role of α-toxin in modulation of the complex interaction of *S. aureus* with its human host (Berube and Wardenburg, 2013).

Gundogan *et al.* (2013) observed that out of 40 *S. aureus* isolates from different sources such as raw calf meat, raw milk, ice cream and white cheese samples only 16 (40%) possessed haemolytic activity.

Sharma *et al*. (2013) reported all the 15 isolates obtained from 46 nasal swabs from pneumonic camels showed hemolysis on sheep blood agar. Among these, only 12 isolates were partial haemolysis of which four isolates later showed hot-cold lysis whereas three isolated were complete haemolysis.

Ebrahimi *et al.* (2014) studied 40 isolates (87%) of *S. aureus* from 61 human wound infections and reported that, 20 (40.8%), 20 (40.8%), 6 (12.25) and 6 (12.25%) of *S. aureus* isolates were positives for α, β, ∂ and combined αβ-hemolysin productions, respectively.

Helal *et al.* (2015) isolated 72 *S. aureus* from examined 100 samples of human and animal (poultry, cow and sheep) source. All isolates were found to be hemolytic with presence of *hlg* gene.

Khichar and Kataria (2015) recorded that of the 28 *S. aureus* isolates from cattle mastitis, 15 produced incomplete haemolysis which turned into complete haemolysis when the plates were further incubated at 4^0C overnight (Hot-cold lysis) and 13 showed complete zone surrounded by incomplete zone of haemolysis.

Yadav *et al.* (2015d) investigated 32 *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis for haemolytic reactions on sheep blood agar which revealed five (15.62%) isolates to show complete haemolysis, 20 (62.50%) to show partial haemolysis, four (12.50%) isolates to show both complete and partial and three (9.37%) isolates did not show any haemolysis.

Quantitative assays

A high tendency of production of β-toxin with a titre of 1:640 to 1:1280 was recorded by Arzo (1973) followed by α-toxin, whereas the production of δ-toxin was found negligible.

Mittal (1997) characterized *S. aureus* isolates from milk samples of cows and goats showing clinical signs of mastitis for quantitative properties of α-, β- and δ-toxins. In his studies he recorded that the titres of α-, β- and δ-haemolysins ranged from less than 1:3 to 1:1280, 1:8 to 1:600 and 1:2 to 1:8, respectively. In a study Qureshi *et al.* (2002) found that all *S. aureus* isolates from camel produced α-, β- and δ-toxin either in pure or in combinations and the titre of α-, β- and δ-toxin ranged between 1:20 to 1:640, 1:8 to 1:1920 and 1:5 to 1:80, respectively.

Sanjiv and Kataria (2007) also reports titre of β-toxin (1:120) from *Staphylococcus aureus* of cattle mastitis origin. They recorded that out of 21 isolates, one isolate did not produce β-toxin and the titre produced by the rest 20 isolates ranged widely from 1:5 to 1:120 but any of the studied isolate did not produced δ-toxin.

Upadhyay and Kataria (2010) recorded titre for β- and δ-toxins α-toxins among 30 (20 from cattle and 10 from goats) isolates of *S. aureus* from cattle and goat the highest titres for β- and δ-toxins was similar (1:160) for cattle as well as goat isolates.

In a study by Nathawat (2013), the titres of α-toxin recorded were 1:320, 1:1280 and 1:2560 produced by 3, 10 and 14 *S. aureus* isolates from goat, respectively. The titre of β-toxin produced by *S. aureus* was much below than that of α-toxin, the highest titres being only 1:40.

Bhati (2013) investigated titres of alpha toxin produced by *S. aureus* isolates from H-F crossbred cattle as determined against rabbit erythrocytes ranged between 1:640, 1:1280, 1:2560 and 1:5120, and were produced by one, four, nine and two isolates, respectively. Hence, most of the isolates produced a titre of 1:2560. The titres of β-toxins by the isolates from both the breeds of cattle were almost similar and the highest titres for β-toxin in *S. aureus* isolates from both the breeds were same being 1:160.

Khichar and Kataria (2015) recorded in his investigation of 28 *S. aureus* isolates obtained from milk collected from matitic Holstein-Friesian crossbred (13) and Rathi (15) cattle that the titre of α-haemolysin from H-F isolates ranged between 1:40 and 1:1280, whereas for Rathi isolates it ranged between 1:160 and 1:2560. The titre of β-haemolysin was much less for isolates both from H-F (between 1:5 and 1:30) and Rathi (between 1:5 and 1:60) cattle while none of isolate was found to be delta (δ) toxin producing.

Yadav *et al.* (2015d) reported 32 *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis. All the 32 (100%) isolates from both cattle (16) and buffalo (16) produced (α-toxin, the maximum titre of which was 1: 5120 whereas beta-toxin was produced by 11 (68.75%) cattle isolates and by seven (43.75%) buffalo isolates with maximum titre of 1:1280 and 1:240, respectively. Delta toxin was detected to be produced by only five (15.62%) isolates, two from cattle and three from buffalo.

E. Antibiotic resistance pattern and MIC determination:

Amorena *et al.* (1999) reported MIC of slime producing *S. aureus* obtained from mastitis samples and found 0.031 mg/L, 2.0 mg/L, 0.5 mg/L, 1.0 mg/L, 0.25 mg/L and 0.12 mg/L MIC for penicillin, gentamicin, vancomycin, erythromycin, ciprofloxacin and cefazolin respectively.

In an investigation over 10 years (1995 to 2004) of period by Guler *et al.* (2005), a total of 29.8%, out of the two hundred sixty-five *S. aureus* strains from bovine clinical mastitic milk isolated from individual animals in different herds (235 herds), were susceptible to all antibiotics tested. The highest resistance was observed in 63.3% of the strains against β-lactam antibiotics, penicillin and ampicillin. Oxytetracycline resistance was also observed in 27.9% of the strains, either alone or in combination with beta-lactams. No resistance was detected for amoxicillin-clavulanate, oxacillin, enrofloxacin and kanamycin-cephalexin. Resistance against beta-lactams increased from 43.5% in 1995 to 58 in 1999 to 77% in 2004.

Barcia-Macay *et al.* (2006) studied pharmacodynamic evaluation of the intracellular activities of antibiotics against *S. aureus* (ATCC 25923) in a model of THP-1 macrophages model at pH 7.3 and found MIC 0.5 mg/liter, 0.5 mg/liter, 0.015 mg/liter, 0.125 mg/liter, 1.0 mg/liter and 0.125 mg/liter for azithromycin, gentamicin, penicillin v, oxacillin, vancomycin and ciprofloxacin respectively.

In an antibiogram study on *S. aureus* isolates from cattle clinical mastitis against 21 antibiotics, Sanjiv and Kataria (2006) recorded that 13 antibiotics in this study were effective against 90% or more isolates. All the isolates were sensitive to gentamicin, cloxacillin, cephalexin and methicillin, 98% isolates were susceptible to chloramphenicol, bacitracin, levofloxacin, 96% to ofloxacin, 94% to ciprofloxacin, streptomycin and neomycin, 92% to kanamycin and 90% to cotrimoxazole. Novobiocin was effective against 85% isolates. Of the total isolates 66% were resistant to oxytetracycline and 49% to penicillin.

Staphylococcus aureus isolates from mastitic cattle were resistant to cloxacillin (100%) followed by penicillin (87%) and ampicillin (62.5%) and amoxicillin (60%) as observed by Ebrahimi *et al.* (2007).

Mohanasoundaram and Lalitha (2008) studied 150 isolates of *S. aureus* from human clinical infections and high resistance was noted for erythromycin (85%), tetracycline (82%), gentamicin (88%), norfloxacin (100%), co-trimoxazole (97%) and ciprofloxacin (97%), moderate resistance to rifampicin (21%) and netilmicin (30.3%) and low rates of resistance to chloramphenicol (18%) and ofloxacin (15%). They also determined MIC of oxacillin by break point method as >32 mg/ml, 16-8 mg/ml and <4 mg/ml and isolates were classified as high (13.3%), moderate (6%) and low level resistant (2.7%), respectively.

Russi *et al.* (2008) isolated 95 *S. aureus* strains causing both clinical and subclinical bovine mastitis from 61 dairy farms in Argentina. Minimal inhibitory concentrations (MICs) of penicillin, oxacillin, gentamicin, erythromycin, enrofloxacin and florfenicol were estimated for all the isolates. The MIC50 and MIC90 were as follows: penicillin, 0.05 and 4 microg/ml; oxacillin, 0.25 and 0.25 microg/ml; gentamicin, 0.25 and 0.5 microg/ml; erythromycin 0.125 and 0.25 microg/ml; enrofloxacin 0.25 and 0.5 microg/ml, and florfenicol 4 and 8 microg/ml.

In an antibiogram study of 148 food origin isolates of *S. aureus* Pereira *et al.* (2009) recorded that strains were highly resistant to beta-lactams, ampicillin and penicillin. Seventy per cent of the isolates were resistant to beta-lactam antibiotics followed by ampicillin and penicillin (73%) and 38% of the isolates were resistant to oxacillin with >or= 6 microg/mL MIC.

The antibiogram observed for 30 *S. aureus* isolates (20 from cattle and 10 from goats suffering from clinical mastitis) revealed that azithromycin was the most effective antibiotic against all the isolates from both species of animals. Gentamicin, netilmicin, levofloxacin, trimethoprim, sparfloxacin, norfloxacin, bacitracin and chloramphenicol showed their efficacy in decreasing order. The highest resistance (100%) was shown towards cefixime followed by ampicillin (96%), cloxacillin (80%), azlocillin (50%) and vancomycin (40%). No difference was observed in antibiotic sensitivity or resistance patterns against isolates from cattle and goats (Upadhyay and Kataria 2009).

Among the 128 isolates of *S. aureus*, only 20 were susceptible to all the antibiotics used in the study by Kumar *et al*. (2010) and rest were resistant against at least one antibiotic. The isolates showed higher susceptibility percent proportion against vancomycin (100) followed by pristinomycin (91.4), amoxicillin-clavulanate (89.8), clindamycin (85.9), ciprofloxacin (85.9), ampicillin (84.4), lincomycin (83.6), cefixime (80.5) and erythromycin (77.3). The elevated resistance percent among the isolates was observed as tetracycline (36.7), gentamicin (30.5), streptomycin (26.6), kanamycin (25.8) and penicillin G (22.7). Thirteen isolates showed resistance against methicillin and oxacillin.

Japoni *et al.* (2010) studied 356 *S. aureus* isolates from the hospitalized patients and determined their MIC_{50} (µg/ml) and MIC_{90} (µg/ml) for oxacillin, vancomycin, ciprofloxacin, erythromycin and gentamicin. The values for MIC_{50} (µg/ml) were 256.0, 2.0, 32.0, 250.0 & 250.0 while MIC_{90} (µg/ml) were >256.0, 4.0, 32.0, >256.0 and 256.0 respectively for mentioned antibiotics.

Akindele *et al*. (2010) studied resistance pattern of 100 *S. aureus* strains obtained from human clinical infections and found that 90% of isolates were resistant to ampicillin, 96% to penicillin, 70% to cloxacillin, 25% to cephalexin, 16% to ceftriaxone, 39% to gentamicin, 30% to erythromycin, 48% to streptomycin, 71% to tetracycline and 34% isolates were resistant to chloramphenicol.

Antibiotic resistance in *S. aureus* isolated from mastitic Sahiwal cattle was studied by Kumar *et al.* (2011) who found that 13.1% of the isolates were methicillin resistant, 36.4% were resistant to streptomycin, 33.6% to oxytetracycline, 29.9% to gentamicin and 26.2% each to chloramphenicol, pristinomycin and ciprofloxacin.

A total of 115 milk samples from cattle (34.78%), buffalo (34.78%), goat (17.39%) and sheep (13.04%) were collected from which 25 *S. aureus* were isolated by Sharma *et al.* (2011) and they reported that almost 80-90% of the isolates showed multiple drug resistance to majority of the antimicrobial agents tested such as nalidixic

acid, amoxycillin+ sulbactam, cloxacillin, erythromycin, kanamycin and vancomycin. While several isolates were found susceptible to the ofloxacin, ampicillin, tetracycline oxacillin, streptomycin, sulphafurazole and ciprofloxacin.

Kreausukon *et al.* (2012) studied antibiogram for 36 *S. aureus* isolates from bulk milk of dairy herd and observed that all isolates were resistant to oxacillin and tetracycline. Resistance to clindamycin, erythromycin, kanamycin, and quinupristin/dalfopristin was detected at high rates in 21 (58.3%), 19 (52.8%), 10 (27.8%), and 13 (36.1%) isolates, respectively. Resistance to ciprofloxacin was found in 3 isolates (8.3%). Only one isolate was resistant to chloramphenicol. All isolates were susceptible to mupirocin, vancomycin, and linezolid.

Hussain *et al.* (2012) evaluated 23 isolates of *S. aureus* from mastitis through disk diffusion method for antibiotic susceptibility testing and recorded efficacy of cotrimoxazole (100%), oxytetracycline (95.65%), amoxicillin (86.95%), gentamicin (86.95%), ampicillin (82.60%), ciprofloxacin (82.60%) chloromphenicol (82.60%), enrofloxacin (69.56%) and novabiocin (60.86%).

Sixty three *S. aureus* isolates from bovine mastitic milk showed 100% resistance to penicillin and ampicillin whereas 41 isolates (65.1%) were resistant to amoxicillin/clavulanic. No resistance was detected for trimethoprim and gentamicin antibiotics (Saei, 2012).

Memon *et al.* (2012) obtained *S. aureus* isolates from bovine mastitis and found that 91% isolates were resistant to ampicillin, 59% to oxytetracycline and 29% to methicillin.

Of the 31 isolates, only nine isolates (29%) of *S. aureus* recovered from foods and food handlers in Spain were susceptible to all studied antibiotics, and none were resistant to chloramphenicol, ciprofloxacin, moxifloxacin, rifampisin, linezolid, vancomycin, tigecycline, trimethoprim, or trimethoprim-sulfamethoxazole. The resistance of isolates was 61.3% for ampicillin-penicillin, 6.5% for oxacillin-methicillin, 25.8% for erythromycin, 19.4% for clindamycin, 3.2% for tetracycline, 6.5% for amikacin-gentamicin-kanamycin-tobramycin, and 19.4% for mupirocin (Argudin *et al.*, 2012).

Alian *et al.* (2012) recorded an antibiogram for 46 *S. aureus* isolates obtained from 348 raw milk samples from cow, sheep and goat by disk diffusion method. All isolates were susceptible to methicillin, vancomycin, chloramphenicol and ciprofloxacin and most of the isolates (82.6%) were resistant to one or more antimicrobial agent while six isolates (13.0%) were resistant to single antibiotic and 16 isolates (34.8%) showed resistance to two antibiotics. Multi resistance was also found in 34.8% of *S. aureus* isolates. Maximum resistant was shown against ampicillin (54.3%), followed by oxacillin (28.3%), tetracycline (26.1%), penicillin G (23.9%), erythromycin (23.9%), trimethoprim-sulfamethoxazole (17.4%) and cephalotin (2.2%).

The study of Rathore and Kataria (2012) revealed linizolid as the most effective antibiotic against all the 15 *S. aureus* isolates from camel skin wounds, followed by azithromycin and gentamicin against which 93.33% of the isolates were sensitive. Methicillin, levofloxacin, rifampicin, ofloxacin and vancomycin were effective against 80% of the isolates, azlocillin (73.33%), bacitracin and norfloxacin (60.00%) and rest were less effective whereas ampicillin, cefexime, metronidazole and nalidixic acid were completely resistant.

Sharma *et al*. (2013) reported antibiogram for 15 *S. aureus* isolates from 46 nasal swabs from pneumonic camels and found that 100% isolates were resistant to cefepime, cefotaxime, nalidixic acid and penicillin, 93.33% to ampicillin, 53.34% to vancomycin, 40% to oxacillin, 33.33% to rifampicin and 20% isolates were resistant to cephalothin. The 100% isolates were sensitive to ciprofloxacin, carbenicillin, chloramphenicol, gentamicin, imipenem and ofloxacin followed by 93.33% to ampicillin/sulbactum co-trimoxazole and nitrofurantoin, 80% to colistin, 60% to clindamycin, neomycin and erythromycin and 53.33% isolates sensitive to minocycline and tetracycline.

Bhati *et al.* (2013) determined the efficacy of 32 different antibiotics against 38 *S. aureus* isolates obtained from milk samples from cattle with subclinical mastitis. Six antibiotics, i.e. azithromycin, gentamicin, chloramphenicol, tobramycin, netillin and neomycin were found 100% effective followed by methicillin and linezolid (94.74%), cotrimoxazole (92.11%), nitrofurazone (92.10%), enrofloxacin (89.47%), bacitracin (84.21%) and sparfloxacin, amoxyclav, cefaclor and amoxycillin (81.58%). The resistance was 71.05% for vancomycin and polymyxin-B, 50% for oxytetracycline, 47.37% for azlocillin and 36.84% for novobiocin whereas cefixime and metronidazole were completely (100%) ineffective against all the isolates.

Lindeman *et al.* (2013) in North America obtained approximately 8,000 different isolates of *Streptococci*, *S. aureus* and *E. coli*, between 2002 and 2010. The minimal inhibitory concentrations (MICs) of the beta-lactam drugs remained low against most of the Gram-positive strains tested, and no substantial changes in the MIC distributions were seen over time. However, *S. aureus* showed little change in erythromycin susceptibility over time.

Gundogan *et al.* (2013) studied the antibiotic resistance for 40 *S. aureus* isolates from meat and milk products in Ankara, Turkey. The isolates were resistant to penicillin, ampicillin, erythromycin, tetracycline, chloramphenicol and gentamicin in order of 77%, 67%, 30%, 25%, 20% and 17%, respectively but they were sensitive to vancomycin and teicoplanin.

Thaker *et al.* (2013) obtained *S. aureus* isolates from milk and milk products which showed highest sensitivity (100%) towards cephalothin, co-trimoxazole, cephalexin and methicillin followed by gentamicin (90%), ciprofloxacin (80%), oxacillin (70%), streptomycin (60.00 %) and ampicillin (60%). The study indicated that the high percent of *S. aureus* isolates were resistant to Penicillin-G (100%) followed by ampicillin (40%), oxytetracycline and oxacillin (20%) and gentamicin (10%).

Nathawat *et al.* (2013) subjected *S. aureus* isolates from mastitic milk of goat to antibiogram studies by using 32 antibiotics belonging to different categories and generations. The results revealed that the most effective antibiotics were gentamicin and tobramycin showing 100% susceptibility, followed by netillin, methicillin, cefalexin, chloramphenicol, enrofloxacin, azithromycin and Nitrofurazone in descending order of their efficacy. All isolates were resistant to cefixime and metronidazole.

Out of the 61 isolates of *S. aureus* isolated from Erzincan tulum cheeses, 49 were found resistant to oxacillin, 28 to penicillin, nine to methicillin, eight to cefoxitin, four to amoxicillin-clavulanic acid and none to vancomycin (Ozpinar and Gumussoy, 2013).

Memon *et al.* (2013) studied 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China. The recorded resistance to enrofloxacin was (3%), oxacillin (9%), ciprofloxacin (26%), methicillin (29%), chloramphenicol (32%), penicillin G (47%), trimethoprim (56%), oxytetracycline (59%), ampicillin (91%) and (100%) against erythromycin.

Ebrahimi *et al.* (2014) studied 40 (87%) isolates of *S. aureus* from sixty one human wound infections and found that 9 (22.5%) isolates were resistant to oxacillin, 5 (12.5%) to penicillin, 7 (17.5%) to methicillin, 5 (12.5%) to ofloxacin, 3 (7.5%) to ciprofloxacin, 2 (5%) to erythromycin, 6 (15%) to azithromycin 4 (10%) to clindamycin and 3 (8%) isolates were resistant to lincospectin.

Thirty two *S. aureus* isolates obtained from mastitis of cattle and buffalo were subjected to antibiogram studies using 33 antibiotics belonging to different categories and generations. Antibiotics such as doxycycline, gentamicin, methicillin, netiline, rifampicin and tobramycin were 100% effective against all isolates from both cattle and buffalo. The maximum resistance was exhibited against nalidixic, cephalexin, polymixin-B, cefexime, cephalexin and amoxicillin by isolates from both species (Yadav *et al.,* 2015e).

Sharma *et al.* (2015) reported resistance pattern of 27 *S. aureus* obtained from 80 samples of cattle and buffalo with clinical as well as sub-clinical mastitis. Drug sensitivity revealed 100% resistance against penicillins followed by vancomycin (88.89%), nalidixic acid (77.78%), cefixime, methicillin, novobiocin (66.67% each), amoxiclav, colistin, pipemidic acid (55.56% each), ofloxacin, streptomycin, sulphamethizole (44.44% each), ampicillin/sulbactam, cefalexin, cefazolin, cefoperazone, enrofloxacin, floxidin, meropenem (33.33% each) and cefuroxim, ciprofloxacin, clindamycin, gentamicin, levofloxacin, norfloxacin, tetracycline (22.22% each).

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During study the penicillin resistance was the most frequent resistance phenotype (82.1%), followed by resistance to streptomycin (46.4%), kanamycin and tobramycin (35.7%), cefoxitin and gentamicin (32.1%), erythromycin (14.3%), tetracycline (10.7%) and clindamycin (3.6%).

Roy *et al.* (2015) studied antibiotic resistance pattern of *S. aureus* obtained from hospital environment and volunteers working in the hospitals. The hospital isolates showed highest resistance was observed against erythromycin (88.89%) and ampicillin (83.33%), moderate resistance observed against ciprofloxacin (33.33%) and tetracycline (33.33%) and lowest resistance was shown against streptomycin (11.11%). On the other hand, volunteer's isolates were 100% sensitive against ciprofloxacin, erythromycin, tetracycline and streptomycin whereas 60% isolates were resistant to ampicillin.

F. Beta-lactamase (*blaZ* gene), ESBL, MRSA (*mecA* gene) and VRSA activity:

Murakami *et al.* (1991) determined MIC broth microdilution assay and developed identification method of Methicillin-Resistant Strains of Staphylococci by PCR. The breakpoints for susceptibility and resistance were ≤2 µg/ml and ≥4 µg/ml for oxacillin and ≤8 µg/ml and ≥16 µg/ml for methicillin, respectively. Of 99 *mecA*-negative *S. aureus* isolates, 100% of the strains were methicillin susceptible and 98% of the strains were oxacillin susceptible. Three strains of 111 *mecA*-positive *S. aureus* isolates exhibited almost the same susceptibility to β–lactams (resistant to both oxacillin and methicillin) as the *mecA*-negative ones and did not produce detectable amounts of PBP 2' despite the presence of the *mecA* gene.

MO and Qi-nan (1997) studied 184 strains of *S. aureus* obtained from human clinical infections for rapid detection of MRSA. A total of 57 of 58 oxacillin-resistant *S. aureus* strains were *mecA*-positive, whereas 3 of 126 oxacillin-susceptible strains were *mecA*-positive. Oxacillin MIC is ≥ 4 µg/ml and ≤ 2 µg/ml was observed for MRSA and MSSA respectively.

In a study Oliveira *et al.* (2000) determined minimum inhibitory concentration for 811 strains of *S. aureus* isolated from bovine mastitis in Europe and the Unites States. Of the strain tested, 35.6% were positive for β-lactamse on initial testing, with an additional 21.3% positive after induction of penicillin. The β-lactam combinations were very active against the *S. aureus* strains.

The *mec*A gene was detected in 27.9% (55/197) of the isolates of *S. aureus* obtained from patients with bloodstream, catheter tip, bone or joint, respiratory tract, ocular, soft tissue, wound, and skin human infections by Booth *et al.* (2001).

Lee (2003) studied various types of meat and clinical specimens from cattle, pigs, and chickens for the presence of methicillin (oxacillin)-resistant *S. aureus* (MRSA). Among 1,913 specimens collected from the animals, 421 contained *S. aureus*; of these, 28 contained *S. aureus* resistant to concentrations of oxacillin higher than 2 µg/ml considered phenotypically MRSA. Isolates from 15 of the 28 specimens were positive by PCR for the *mecA* gene. Of the 15 *mecA*-positive MRSA isolates, 12 were from dairy cows and 3 were from chickens. MIC is < 2 µg/ml and ≥ 16 µg/ml of vancomycin and oxacillin respectively observed in all 15 *mecA*-positive MRSA isolates.

Out of 68 *S. aureus* strains from bovine mastitc milk, 38 isolates (55.9%) were β-lactamase producers. The β-lactamase producing isolates were 21.1% resistant to methicillin, but were 100% susceptible to amoxicillin/clavulanic acid and 97.4% susceptible to ampicillin/sulbactam (Turutoglu *et al.*, 2006).

Arslan and Ozkardes (2007) reported 96 (74.4%) of 129 S. *aureus* strains from several clinical specimens were positive for β– lactamase enzyme. However, 78 (81.25%) of 96 β-lactamase positive *S. aureus* strains were β–lactamase positive oxacillin resistance *S. aureus* (ORSA) strains, but none of them had vancomycin resistance.

Mohanasoundaram and Lalitha (2008) studied 150 isolates of *S. aureus* from human clinical infections for MRSA detection by latex agglutination test (PBP 2a protein) and specific PCR (*mecA* gene). Out of 150, 34 isolates were found to be MRSA by both methods however, one isolate of MSSA revealed the presence of *mecA* gene having the potential to become MRSA in future.

Vindel *et al.* (2009) isolated 463 isolates of *S. aureus* from humans. The 135 MRSA isolates showed resistance to ciprofloxacin (93.3%), tobramycin (72.6%), gentamicin (20.0%), erythromycin (66.7%) and clindamycin (39.3%). Among the isolates resistant to erythromycin, 27.4% showed the M phenotype. All of the isolates were susceptible to glycopeptides. Twelve resistance patterns were found, of which four accounted for 65% of the isolates. Two genotypes were observed for the first time in *Spa*in. SCC*mec* type IV accounted for 6.7% of the isolates (70.1% were type IVa, 23.9% were type IVc, 0.9% were type IVd, and 5.1% were type IVh), and SCC*mec* type I and SCC*mec* type II accounted for 7.4% and 5.2% of the isolates, respectively. One isolate was non-typeable.

Russi *et al.* (2008) isolated 95 *S. aureus* strains causing both clinical and subclinical bovine mastitis from 61 dairy farms in Argentina. Beta-lactamase activity was detected in 89% of 46 penicillin-resistant strains.

Coelho *et al.* (2009) studied 21 genotypically confirmed *S. aureus* isolates and their antibiotic resistance pattern and found that 47% and 25% of strains exhibited resistance to penicillin and oxacillin respectively. All oxacillin-resistant isolates were *mecA* gene positive.

In an antibiogram study on 148 food origin isolates of *S. aureus* Pereira *et al.* (2009) recorded that 38% of the isolates were MRSA with resistant to oxacillin but only 0.68% showed the presence of *mecA* gene.

Turutoglu *et al.* (2009) studied 18 phenotypic methicillin resistant *S. aureus* isolates from bovine mastitis and compared *mecA* genes sequence with human isolates. Out of 18 *S. aureus* isolates (oxacillin MICs, ≥4 μg/ml), 3 were positive for *mecA* gene. In sequence analysis, all three *mecA* genes of these isolates were identical to that found in human MRSA strains, except a one-base substitution at nucleotide position 757. It may be concluded that MRSA isolated from bovine mastitis may be originated from human beings.

Capurro *et al.* (2010) found beta-lactamase production in 9% of the isolates (82 *S. aureus* isolates) from clinical mastitic milk of dairy cow.

Akindele *et al*. (2010) studied resistance pattern of 100 *S. aureus* strains obtained from human clinical infections and found that 80% were ß-lactamase producer. Among the ß-Lactamase producing organisms, susceptibility to antibiotics was: penicillin (100%), ampicillin (96%), erythromycin (82.5%), cephalexin (71%) ceftriaxone (70%), cloxacillin (66%).

Japoni *et al.* (2010) studied 356 *S. aureus* isolates from the hospitalized patients and 200, 137 and 19 were MSSA, MRSA, and intermediate MRSA strains, respectively were isolated.

Twenty seven MRSA positive samples were identified by Nordin *et al.* (2010) based on cultural, biochemical and antibiotic sensitivity assay. 14 (51.9%) of the MRSA isolates from blood samples, 9 (33.3%) from tracheal aspirate and three (11.1%) isolated were from nasal swabs and pus. Antibiogram analysis of the MRSA

collection resolved these strains into six groups designated (I-VI). Group II, which was resistant to cotrimoxazole, gentamicin, clindamycin and erythromycin, was the commonest accounting for 40.7% (11/27) of the isolates collected. Group I accounts for 33.3% (9/27) of the isolates and this group was resistant to cotrimoxazole, gentamicin and erythromycin. Group III accounts for 3.7% (1/27) of the isolates and this group was resistant to gentamicin, clindamycin and erythromycin. Group IV accounts for 3.7% (1) of the isolates and this group was resistant to fusidic acid, cotrimoxazole, rifampicin, gentamicin and erythromycin. Group V accounts for 11.1% (3/27) of the isolates and this group was resistant to fusidic acid, cotrimoxazole, rifampicin, gentamicin, clindamycin and erythromycin. Group VI accounts for 7.4% (2/27) of the isolates and this group was resistant to rifampicin, gentamicin, clindamycin and erythromycin.

Asfour and Darwish (2011) conducted phenotypic and genotypic detection of both *mecA* and *blaZ*- genes mediated β-lactam resistance in staphylococci isolated from bovine clinical and subclinical mastitis and found that only one and twelve isolates was positive for β- lactamase (*blaZ* gene) while none and five of isolates was positive for MRSA (*mecA* gene) from clinical and subclinical samples respectively.

Suleiman *et al.* (2012) developed antibiotic resistance profiles for 73 MRSA isolated from bovine mastitic milk against 12 antibiotics. They found 26 (35.6%) to be resistant to oxacillin (ORSA) and 10 other antibiotics. All the seventy three isolates were susceptible to vancomycin and resistant to penicillin, and also resistant to more than one antibiotic. PCR was also used to detect the gene, *mecA* gene was detected in two (7.6%) of the 26 ORSA, and the *blaZ* gene in all the 26 ORSA. The range of the oxacillin MICs for the *mecA* positive MRSA was between 2.5µg/ml and greater than10µg/ml. While the non- *mecA* isolates were between 1.25µg/ml and 2.5µg/ml.

Franca *et al.* (2012) identified antimicrobial resistance patterns and molecular resistance markers (*mec*A and *bla*Z gene) of *Staphylococcus* spp. (n=210) obtained from small ruminant mastitis in Brazil. The isolates were most resistant to amoxicillin (50.0%), streptomycin (42.8%), tetracycline (40.4%), lincomycin (39.0%) and erythromycin (33.8%). Although phenotypic resistance to oxacillin was observed in 12.8% of the isolates but none of the isolate harbored the *mec*A gene. However, 45.7% of the isolates harbored *bla*Z indicating that beta-lactamase production was the main mechanism associated with staphylococci resistance to beta-lactam antibiotics. In addition, the seventy three isolates (54 from goats and 19 from sheep) were negative for all resistance genes tested and 69 isolates presented two or more resistance genes.

Bagcigil *et al.* (2012) determined the location of the *blaZ* gene in the *S. aureus* isolates obtained from bovine clinical and subclinical mastitis in Finland and Sweden. Out of 147 isolates 78 beta-lactamase positive isolates were identified with positive *blaZ* gene. Based on the southern blotting, the *blaZ* gene was found to be located on the chromosome in 27 isolates and on plasmids in 51 isolates. Of the 27 isolates with chromosomally located *blaZ*, 12 (44.4%) were from clinical mastitis and 15 (55.6%) from subclinical mastitis. Of the 51 isolates with plasmid-located *blaZ*, 19 (37.3%) were from clinical and 32 (62.7%) from subclinical mastitis. The location of *blaZ* did not significantly differ between isolates from different type of mastitis (clinical vs. subclinical) ($p = 0.629$).

Memon *et al.* (2013) studied 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China and recorded 9% resistance to oxacillin and 29% against methicillin. However high resistance rate against methicillin was found but no isolate was positive for *mecA* gene, whereas *blaZ* gene was detected in 82% isolates.

Schaumburg *et al.* (2014) analyzed 151 chicken meat samples, 3% of the samples were found contaminated with ESBL producing *S. aureus* but none of the isolate carried MRSA as confirmed by the absence of *mecA* gene.

Gomez *et al.* (2014) studied 13 *S. aureus* in faecal samples from 101 wild small mammals and found that two isolates were methicillin-resistant *S. aureus* (MRSA) and 11 were methicillin-susceptible *S. aureus* (MSSA). Both MRSA isolates harboured the *mecC* gene and the novel *blaZ* gene.

Ebrahimi *et al.* (2014) studied 40 isolates (87%) of *S. aureus* from sixty one human wound infections and found 14 (28.6%) isolates to be β-lactamase producers.

Abd El-Hamid and Bendary (2015) studied the antibiotic susceptibility patterns of 133 identified *S. aureus* isolates of various clinical infections, which revealed that over 70% of the isolates were multi-drug resistant (MDR). Fifty MDR isolates were characterized using antibiotyping and *mecA* gene typing methods. Amplification of *mecA* gene confirmed 30 strains as methicillin-resistant *S. aureus* (MRSA) and 20 as methicillin-sensitive *S. aureus* (MSSA).

Breves *et al.* (2015) studied antimicrobial susceptibility profile of *S. aureus* on surfaces of medical articles and in professionals from two basic health units and found that among the 35 *S. aureus*, 31.42% were methicillin resistant (MRSA), and 2.8% were vancomycin resistant, characterized as VRSA. Further *mecA* gene was detected in 30.6% of studied strains.

Sharma *et al.* (2015) reported resistance pattern of 27 *S. aureus* obtained from 80 samples of cattle and buffalo with clinical as well as sub-clinical mastitis. Drug sensitivity revealed that 18 isolates were methicillin-resistant while nine were methicillin-susceptible. Similarly, 24 *S. aureus* isolates were intermediate to vancomycin while three were vancomycin susceptible and no isolate was resistant to vancomycin.

Out of 323 isolated *S. aureus* from human clinical samples from different hospitals, 97 strains were found to be Methicillin Resistant *S. aureus* (MRSA) by Debnath and Chikkaswamy (2015). They also reported that MRSA were 100% resistant to oxacillin, 90% resistant to ampicillin, 84.5% to penicillin-G, 76.2% to erythromycin, 71% to cephalexin, 59.79% to ciprofloxacin, 40.2% to co-trimoxazole, 28.87% to vancomycin and 7.22% resistant to linezolid.

Morcillo *et al.* (2015) obtained 256 MRSA isolates from swine samples and five MRSA isolates from pig worker samples. The antibiotic resistant pattern analysis revealed that 39 isolates were resistant to one antibiotic, 71 isolates were resistant to two antibiotics and 96 isolates were resistant to three or more antibiotics. Resistance levels to various antimicrobial for MRSA isolates measured was as follows: gentamicin (n=98, 38.3%), tobramycin (n=101, 39.4%), levofloxacin (n=34, 13.3%), erythromycin (n=86, 33.6%), clindamycin (n=129, 50.4%), nitrofurantoin (n=1, 0.5%) and trimethoprim-sulfomethoxazole (n=99, 38.7%). MRSA isolated from humans showed similar antibiotic resistance pattern to those isolated from pigs, excepting for erythromycin.

Roy *et al.* (2015) obtained 125 *S. aureus* isolates from the hospital and 45 isolates from the hand swab of volunteer's working in the hospitals to find vancomycin resistance pattern. In hospital none of the isolate was resistant to vancomycin, only 11.11% isolates were intermediates but 20% isolates from volunteers were resistant to vancomycin.

Wadekar *et al.* (2015) obtained 35 *S. aureus* isolates from 100 pus samples from patients with chronic osteomyelitis and detected 40% isolates as MRSA. Most of them were sensitive to vancomycin 14(100%), linezolid 14(100%), amikacin 11(78.5%) and cotrimoxozole 7(50.0%).

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During study the most prevalent antibiotic resistance gene was *blaZ* conferring the resistance to 23 (82.1%) isoaltes, followed by *mecA* to 10 (35.7%) isolates.

3. Molecular typing of *S. aureus* with Repetitive sequence-based PCR (rep-PCR):

The high degree of genetic relatedness between *S. aureus* strains has proven to be an obstacle to using routine bacteriological methods for epidemiologic analysis. Various methods have been used to monitor *S. aureus* epidemiology such as phage typing, serotyping, protein electrophoresis, esterase electrophoresis, capsule typing, plasmid profiling, plasmid restriction fragment patterns, restriction fragment length polymorphism (RFLP) of genomic DNA, riboprobing of southern blots of RFLP agarose gels, pulsed-field gel electrophoresis of macrorestriction fragments, random amplified polymorphic DNA analysis, PCR amplification of a variable region of the

staphylocoagulase gene along with restriction endonuclease analysis of the resulting amplicons and resistance governing genes analysis (Costas *et al.*, 1989; Goh *et al.*, 1992; Prevost, *et al.*, 1992; Saulnier, *et al.*, 1993).

A multiple copies of repetitive DNA sequences are continuously dispersed in naturally occurring bacterial genomes. Although the exact functions of these repetitive sequences remain unknown, their occurrence is very valuable for DNA fingerprinting of bacteria. In REP-PCR, primers complementary to those interspersed repetitive consensus DNA sequences are used to amplify DNA fragments between repetitive elements (Gilson *et al.*, 1984). Three families of repetitive sequences: the 35–40-bp repetitive extragenic palindromic (REP) sequence, the 124–127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154-bp BOX element sequences (Stern *et al.*, 1984; Lupski and Weinstock, 1992; Koeuth *et al.*, 1995), have frequently been used in REP-PCR assays. The corresponding protocols are referred to as REP-PCR, ERIC-PCR, and BOX-PCR genomic fingerprinting, respectively.

Recently, introduced PCR amplification of repeat DNA sequences found in prokaryotic chromosomes as a means of typing in conjunction with rep-PCR and FERP (Fluorophore-Enhanced Repetitive-Sequence PCR) fingerprinting methods, generated highly reproducible and discriminatory patterns for typing of *S. aureus* strains but unfortunately none of the single method is perfect and hence combining of two or three methods of molecular typing were suggested to be useful and discriminatory for differentiation and epidemiology of *S. aureus* strains (del Vecchio *et al.*, 1995).

Evaluation of 170 isolates of methicillin resistance in *S. aureus* (MRSA) and twenty-one isolates of methicillin-susceptible *S. aureus* (MSSA) obtained from nosocomial and community-acquired human infections was carried out by del Vecchio *et al.* (1995). Eight banding patterns or rep-PCR profiles (designated letter A to H) were observed among MRSA isolates collected from various sources. The various rep patterns allowed easy differentiation among the isolates. The MSSA isolates analyzed displayed fingerprints different from the eight patterns observed for MRSA. In conclusion, rep-PCR of MRSA could easily determine an outbreak source, and this could have important clinical and epidemiological ramifications. They suggested that it could be used in routine surveillance to enhance the potential for infection control.

To investigate whether certain rep-PCR fingerprints would have prognostic value for epidemic or nonepidemic MRSA strains, van der Zee *et al.* (1999) genotyped 46 *S. aureus* strains. Among 46 strains, 13 different rep-PCR fingerprints were obtained, designated as N to Z. The distribution of rep-PCR types N, O, P, Q, and X were found for more than one isolate and were more common among epidemic MRSA strains while Strains with rep-types R to Z were isolated less frequently.

A commercial kit, the REP-PCR DiversiLab microbial typing system (Spectral Genomics Inc., Houston, TX) was reported to be more convenient than manual methods (Shutt *et al.*, 2005).

The online DIVERSILAB software not only provides standardized comparisons among isolates almost instantaneously, but also generates user-friendly customized reports and provides a user-specific data storage and retrieval system (Healy *et al.*, 2005).

In addition, REP-PCR genomic fingerprinting protocols can be performed on whole cells of some species, obviating the need for DNA extraction (Shutt *et al.*, 2005). However, REP-PCR has disadvantages similar to other PCR-based assays, including the potential for contamination, artifacts, and the need for multiple controls.

The automated method using repetitive sequence-based PCR (rep-PCR) for microbial strain typing was used by Shutt *et al.* (2005) to characterize *S. aureus* isolates obtained from hospital outbreaks and found that correlation between rep-PCR and PFGE was quite good. For 15 of the 19 outbreaks in their study, rep-PCR and PFGE results were complete concordant, and for the remaining four, results of the two methods were partially concordant.

Gardella *et al.* (2005) studied twenty seven isolates (14 MRSA and 13 MSSA) of *S. aureus* recovered from hospitalized patients. The isolates were evaluated by rep PCR for their genetic characterization. Three type rep patterns (12 A1 and One of G and D1 each) were observed among MRSA strains and 11 type rep patterns (two A1, two B1 and one F, C, H, E1, D3, E2, A2, B2 and B3 of each) were observed among MSSA strains. The rep patterns of MRSA and MSSA were clearly differentiable.

Reinoso *et al.* (2007) designed a study to assess genetic relationship of 52 *S. aureus* strains isolated from mammary gland infections collected in four herds located in the central dairy region of Argentina. A total of 31 rep-profiles in the range size from 300 to 6000 bp were identified after rep-PCR analysis. At a first level of similarity (50%), isolates were grouped into 5 clusters namely I to V. Most of the strains (75%) were grouped in cluster I. they concluded that rep-PCR typing could successfully differentiate *S. aureus* strains of bovine origin.

Reinoso *et al.* (2008) analyzed 45 *S. aureus* strains genotypically by *rep*-PCR obtained from humans, bovine subclinical mastitis and food samples. The subsequent cluster analysis suggested the existence of 35 rep profiles which could be divided by dendrogram analyses into seven different groups at 60% of relative genetic similarity designated I–VII. The groups generally matched with the origin of the isolates. Group II consists of 13 (87%) human infection strains, groups III and IV of 14 (93%) bovine mastitis strains and groups V and VII of five (71%) strains isolated from anterior nares of healthy people. Six (75%) food sample strains were grouped together with the human strains in group II. The rep-PCR group of the remaining *S. aureus* strains did not match with the origin of the strains.

Nordin *et al.* (2010) conducted molecular characterizations of 27 MRSA by rep-PCR to differentiate them. MRSA positive samples were identified based on cultural, biochemical and antibiotic sensitivity assay. Results of rep-PCR showed 5 different pattern (designated as ‘a’ to ‘e’) of bands ranging in size from 210bp to 1150bp. The MSSA patterns were different from that of MRSA, where MSSA isolates lack the 650bp band that was seen in the MRSA isolates. Thus, rep-PCR was proved to be potential tool to determine genomic differences of nosocomial MRSA in resource limited settings.

Memon *et al.* (2013) studied 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China for REP-PCR generated banding patterns of *S. aureus* isolates which ranged from 150bp to 1800bp in size. The DNA polymorphism based genotype analysis suggested the existence of 34 REP-profiles, which were arranged by dendrogram analysis in seven distinct genotypes designated as A, B, C, D, E, F and G.

4. Genotypic characterization of *S. aureus* in relation to following virulence factors and their associated genes:

A. Adherence (*clfA, clfB, icaA, icaD, agr* and *trap* gene)

Staphylococcus aureus is an important pathogen of human and animals that is responsible for a wide range of infections ranging from superficial skin infections to more serious invasive diseases. The ability of *S. aureus* as a pathogen is due in part to its ability to adhere to a wide range of host tissues including host extracellular matrix proteins such as fibrinogen, fibronectin and collagen. Adhesion to host proteins is mediated by bacterial cell-wall-associated proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and secreted expanded-repertoire adhesive molecules (SERAMs). Expression of these binding proteins governed by various molecules and their associated genes *viz.* clumping factors A and B (*clfA* and *clfB* gene), intercellular adhesion molecule (*icaA* and *icaD* gene), accessory gene regulator molecule *(agr* gene group*)* and target of RNA III-activating molecule (*trap* gene) (Navarre and Schneewind, 1994; Foster and Hook, 1998).

Clumping factors A and B bind specifically to fibrinogen, ClfA binds to the extreme C terminus of the γ-chain protruding from D domain of fibrinogen while ClfB is only expressed on the cell surface during the exponential phase of growth and can only bind to A region of fibrinogen and cytokeratin 10 (Navarre and Schneewind, 1994; Ni Eidhin, *et al.*, 1998).

Fitzgerald *et al.,* (2000) studied *S. aureus* obtained from bovine intramammary infection. Of the 102 Irish isolates of *S. aureus* 77 (75.5%) were positive for *clf*A while all of the RAPD types isolates (7) were negative for *clf*A. And of the 42 American isolates 30 (71.4%) tested positive for *clf*A but the strains of RAPD types 10, 11 and 12 were negative for clumping factor. The sizes of the PCR products of the *clf*A region-R, varied from 780-1180bp.

The *clf*A gene was detected in 96.2% (51/53) of the isolates of *S. aureus* obtained from patients with bloodstream, catheter tip, bone or joint, respiratory tract, ocular, soft tissue, wound, and skin human infections by Booth *et al.* (2001).

Amplification of the clumping factor (*clf*A) gene resulted in a single amplicon with a size of approximately 980bp for all 34 *S. aureus* isolates recovered from cow mastitis indicating no size polymorphisms of this gene (Stephan *et al*., 2001).

Jarraud *et al.* (2002) investigated relationship between *agr* groups and human disease origin *S. aureus* by studying 198 *S. aureus* strains isolated from human with various clinical samples. The isolates were relatively evenly distributed among the four *agr* groups, with 61 strains belonging to *agr* group I, 49 belonging to group II, 43 belonging to group III, and 45 belonging to group IV. The principal coordinate analysis performed on the AFLP distance matrix divided the 198 strains into three main phylogenetic groups, AF1 group corresponding to strains of *agr* group IV, AF2 group corresponding to strains of *agr* groups I and II, and AF3 group corresponding to strains of *agr* group III. This indicated that the *agr* type was linked to the genetic background.

Gilot *et al.* (2002) carried out a study in which restriction maps constructed for *agr* variants allowed the linking of all types in an evolution scheme and their grouping in one of the four *agr* interference groups. The majority of strains isolated from cows with mastitis belonged to *agr* group1 (69.0%) and to *agr* group 2 (23.9%). Groups 3 and 4 contained only 2.8 and 1.4% of the analyzed strains, respectively. The analysis indicated that groups 2, 3, and 4 probably evolved from the more frequently encountered type, which belonged to group 1. The *agr* group 1 was also found to be the most prevalent. All studied isolates were also positive for *trap* gene.

Tristan *et al.* (2003) isolated 157 isolates from various human clinical samples for evaluation of *clfA* and *clfB* gene and found that *clfA* gene was present in 155 (99%) of the isolates whereas *clfB* gene was present in all (100%) the isolates.

Vasudevan *et al.* (2003) isolated 35 *S. aureus* strains from bovine mastitis. Thirty-two of the 35 isolates tested produced slime on Congo red agar, whereas only 24 of the isolates were found to produce biofilm in-vitro. However, all the 35 isolates possessed *icaA* and *icaD* genes. This study indicated a high prevalence of the *ica* genes among *S. aureus* mastitis isolates.

Lina *et al.* (2003) studied 65 *S. aureus* from 216 human nasal samples for *agr* typing and isolates were *agr* typed by a PCR method. Only one *S. aureus agr* (agr_{Sa}) allele was detected in each carrier. All 65 *S. aureus* isolates fell into one of the four previously described agr_{Sa} groups (alleles). Thirty four isolates belonged to *agr*-1_{Sa}, 19 belonged to *agr*-2_{Sa}, seven belonged to *agr*-3_{Sa}, and five belonged to *agr*-4_{Sa}. Multiple logistic regressions of the two most prevalent agr_{Sa} alleles (*agr*-1_{Sa} and *agr*-2_{Sa}) showed a specific influence of the *agr* system. To verify that each carrier harbored only one agr_{Sa} allele, PCR was also performed on total DNA extracted from brain heart infusion broth cultures of samples from 20 of the *S. aureus* carriers. In all cases, only one agr_{Sa} allele (identical to one of the four previously identified alleles) was detected in each culture by multiplex PCR.

In the study by Ando *et al.* (2004) on 109 MRSA isolates of *S. aureus* from human suffering from urinary tract infections one (0.9%), 99 (90.8%), two (1.8%) and zero (0%) strains showed presence of *agr*I, *agr*II, *agr*III and *agr*IV gene respectively. The *icaD* and *clfA* genes were also detected in 108 (99.1%) strains and 84 (77.1%) strains respectively.

Amplification of the *clf*A resulted in a single amplicon with a size of approximately 1000bp from all 35 *S. aureus* from bovine subclinical mastitis indicating no size polymorphisms in this gene (Salasia *et al*., 2004).

The amplification of *clfA* gene revealed two different sized amplicons of 900 and 1000bp. Among the *S. aureus* strains representing the clinical mastitic cow, 13 strains (54.2%) had an amplicon size of 900bp, while 11 strains (45.8%) had an amplicon size of 1000bp. From subclinical mastitis two (12.5%) strains had a *clfA* amplicon size of 900bp and 14 (87.5%) strains an amplicon size of 1000bp. The gene *agr* class I with a size of 360bp for six (25%) and 12 (75%) strains and the gene *agr* class II with a size of 470bp for 16 (66.6%) and four (25%) strains of the clinical and subclinical *S. aureus* group, respectively. Two (8.3%) *S. aureus* strains could not be characterized by the use of the oligonucleotide primers specific for *agr* class I and II (El-Sayed *et al.*, 2006).

Yazdani *et al*. (2006) evaluated the biofilm forming capacity of *S. aureus* isolated from wound infections and also the presence of *icaAD* gene among these isolates by polymerase chain reaction (PCR) method. Out of the fifty strains identified, 54% produced black colonies on CRA plate, 52% were positive for biofilm formation, and all strains carried the *icaAD* gene.

A total of 37 strains of *S. aureus* were isolated during processing of 552 milk samples from 140 cows with subclinical mastitis. All of the isolates were found to produce an amplicon with a size of approximately 1,042bp of the *clf*A gene without any size polymorphisms (Kalorey *et al*., 2007).

Reinoso *et al.* (2008) analyzed 45 *S. aureus* strains genotypically obtained from humans, bovine subclinical mastitis and food samples. The PCR amplification of the clumping factor gene *clfA* yielded an amplicon size of 1000bp for 40 of the investigated *S. aureus*. Four strains from bovine origin and one strain from food sample had an amplicon size of 900bp. With the oligonucleotide primers for the genes *agr*-classes I–III, eight (36%) human strains were *agr* class III, the remaining 14 strains belonged to *agr* classes I and II (14% and 27%, respectively). Seven (47%) bovine mastitis strains were assigned to *agr* class II and the remaining eight strains belonged to *agr* classes I and II (27% and 13%, respectively). Ten (22%) *S. aureus* strains were negative for the *agr*-classes I to III.

Vindel *et al.* (2009) conducted *agr* typing for 135 MRSA isolated from human clinical infections. The *agr*-2 type was most prevalent with 111(82.2%) isolates followed by *agr*-1 type with 20 (14.8%) isolates and *agr*-3 type with 4(3.0%) isolates. The *agr*-2 type contain 14 sporadic MRSA strains, *agr*-1 type having eight sporadic MRSA strains and *agr*-3 type was found in one sporadic MRSA strain. None of the MRSA strains presented *agr*-4 type.

Peerayeh *et al.* (2009) studied 212 *S. aureus* strains isolated from human clinical infections for *agr* group typing**.** The majority of 55.1% (117) isolates belonged to *agr*-I group, followed by *agr*-II group 16.9% (36 isolates), *agr*-III group 16.5% (35 isolates), and *agr*-IV group 9.4% (20 isolates). The rates of failure to detect the *agr* locus were 1.8% (4 isolates). Although *agr*-I group was much prevalent in all specimens, *agr*-II group was dominant in nasal swabs (30%), *agr*-III group was prevalent in respiratory tract specimens (26%), and *agr*-IV group was higher in cutaneous specimens (12.1%).

In the study of Bhanderi *et al*. (2009) on 43 *S. aureus* isolates from bovine clinical and subclinical mastitis, the *clf*A gene amplification in single amplicon with a size of approximately 980bp product without any size polymorphism.

Duran *et al.* (2010) studied the presence of adhesion and slime production genes among *S. aureus* strains obtained from surgical wounds by multiplex PCR. The occurrence rate of adhesion genes (*clfA*) was 45 strains of 88 (51.1%) and slime genes (*icaA* and *icaD*) was 56 strains of 88 (63.6%) among the *S. aureus* isolates.

Cotar *et al.* (2010) in a study on *S. aureus* strains isolated from patients with infections (blood cultures) with associated cardiovascular devices, showed that all *S. aureus* strains possess both *clfA* and *clfB* genes without any size polymorphism.

Zmantar *et al.* (2010) studied quantitative and qualitative properties of biofilm producing *S. aureus* isolated from auricular infection (n = 46) and it was found that 78.26% of strains possessed *icaA* and *icaD* gene.

Proietti *et al.* (2010) carried out amplification of the *clf*A and obtained a single amplicon (approximately 1,042bp) in all (170) the isolates of *S. aureus* from bovine mastitic milk samples.

In the investigation of intramammary infections in bovines by Salem-Bekhit *et al*. (2010) amplification of the clumping factor (*clf*A) gene resulted in a single amplicon with a size of approximately 985bp for all 68 *S. aureus* strains indicating no size polymorphisms in this gene.

Dhanawade *et al.* (2010) reported 102 *S. aureus* isolates from bovine subclinical mastitis. Among studied isolates, 36 (35.29%) strains revealed the presence of *icaA* and *icaD* genes.

Momtaz *et al.* (2010) characterized 86 *S. aureus* isolates from 360 mastitic milk samples of which 63 contained *clfA* gene without any polymorphism, 10 isolates contained *agr*-I gene, 42 contained *agr*-II gene, 19 contained *agr*-III gene and 15 isolates contained *agr*-IV gene.

Stutz *et al.* (2011) studied 79 PCR-confirmed bovine mastitis-derived *S. aureus* isolates and found that all isolates were positive for *clfA* and *clfB* genes. The size polymorphism is not detected but variation in their expression noted by quantitative PCR. The a*gr* typing also conducted in the present investigation and it was found that all of the Staphaurex-negative isolates were of *agr* type II. In contrast, the majority (84%) of Staphaurex-positive isolates were of *agr* type I, apart from the isolates harboring *spa* type t045, t7007, or t7013, which were of *agr* type II. The agr III and agr IV was not detected among any of the isolate.

Among all microbial surface components recognizing adhesive matrix molecules encoded genes, the genes *clf*A and *clf*B are found in most of the *S. aureus* strains. The isolates tested positive for the *clf*A and *clf*B were 96.9% each (Ote *et al.*, 2011).

Salasia *et al.* (2011) detected *clf*A gene in all (41) isolates of bovines, humans, and food source samples with amplicon of 1000bp without any size polymorphism.

Karahan *et al.* (2011) reported that 91.3% (84/92) of *coa* positive *S. aureus* isolates of bovine subclinical mastitis origin carried *clf*A gene. Sixty-eight (81.0%) of the *clf*A gene positive isolate produced amplicons of approximately 1000bp and remaining 16 isolates (19.0%) produced amplicons of approximately 900bp.

Kumar *et al.* (2011) studied 107 strains of *S. aureus* from mastitic milk samples from 195 infected udders of Sahiwal cattle. The proportions of *agr* genes I, II, III and IV were found to be 39.2%, 27.1%, 21.5% and 12.1%, respectively. Polymorphic band patterns were also observed in *clfA*, *viz.* 950 (3.7%), 1000 (70.0%) and 1100bp (20.5%).

Xie *et al.* (2011) studied a total of 108 *S. aureus* isolates obtained from human clinical infections and detected four *agr* groups (I – IV) by multiplex PCR based on the amplicon size. The *agr* group I was the most predominant as detected in 60.2% (65/108) of the strains and groups of *agr* III, II, and IV were less common and found in 19.4%, 16.7%, and 2.8% of the isolates, respectively. One isolate repeatedly yielded negative result for any of the *agr* types tested.

Chen *et al.* (2012) analyzed 100 *S. aureus* isolates obtained from nasal carriage and 34 isolates from community-onset infection, which revealed that *agr* group I was the most common *agr* type for both nasal carriage (65%) and community infection (74%) isolates, followed by *agr* group II for infection isolates (21%) and *agr* group IV for nasal carriage isolates (18%). The *agr* group III was not detected in their study.

Gharsa *et al.* (2012) studied 50 *S. aureus* strains isolated from 100 nasal swabs from donkeys. Amplification of the *agr* locus showed that *agr* group I was predominant (74%), the remaining isolates being ascribed to *agr* group III (14%), *agr* group II (8%), and *agr* group IV (4%).

Franca *et al.* (2012) identified biofilm production of *Staphylococcus* spp. (n=210) obtained from small ruminant mastitis in Brazil. The isolates were subjected to Congo red agar and *ica*D gene specific PCR and found that only 16 (7.6%) isolates (15 isolates from goat and one from sheep) were phenotypically biofilm producing while biofilm-encoding gene *ica*D was detected in total 69 (33.0%) isolates.

Atshan *et al.* (2012) studied 30 methicillin-sensitive and 30 methicillin-resistant *S. aureus* isolates. The *clfA, clfB icaA* and *icaD* genes were present in all the 60 isolates tested indicating a high prevalence.

The gene *clf*A was found in all the 55 *S. aureus* isolates by Contreras *et al.* (2012) showing 100% prevalence whereas Yang *et al.* (2012) reported 24 (62%) *clfA* positive isolates from 39 *S. aureus* strains isolated from bovine clinical mastitis in Guangxi by polymerase chain reaction amplification.

Li-li *et al.* (2012) studied the biofilm forming ability and distribution of biofilm associated genes in the clinically isolated bovine mastitis *Staphylococcus* genes. Out of 137 studied isolates, *icaAD* gene was present in 43 isolates, *clfA* in 76 and *clfB* was present in 50 isolates.

Bnyan (2013) reported 100% presence of adhesion genes (*clfA*) and slime production genes (*icaA* and *icaD*) among six *S. aureus* isolates obtained from cases of septicemia in hemodialysis patients.

Fifteen isolates of *S. aureus* from animal and human clinical infection were typed with *agr* group specific genes which revealed the predominance of *agr* group I, being present in 14 out of 15 *S. aureus* isolated from all types of clinical samples (93.33%). On the other hand, only one strain from pus sample was found to harbor *agr* group III (6.67%) and none was positive for both *agr* types II and IV (Abd El-Hamid and Bendary, 2013).

The *agr* locus specificity groups were studied in 43 *S. aureus* isolates obtained from ewes with mastitis in three regions in the Northwest of Iran. Identification of *agr* groups, using *agr* group-specific multiplex PCR, classified the majority of isolates into *agr* group I (51.16%) followed by *agr* group IV (44.19%) and only one isolate (2.23%) for each of the *agr* groups II and III was found to be positive (Saei *et al.*, 2013).

Memon *et al.* (2013) investigated 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China for *clfA* gene which present in 59% isolates with 900bp-1000bp size.

Almeida *et al.* (2013) studied 18 isolates, 6 from clinical mastitis samples and 12 from subclinical mastitis samples of sheep flocks. All (18) were positive for *clfA* while negative for both *icaA* and *icaD.* The *agr* group typing revealed that 50% isolates were positive for *agr* groups I and II each while *agr* groups III and IV were not detected in any of the isolate.

Tang *et al.* (2013) studied *S. aureus* obtained from nosocomial infection and cross-contamination of foods and it was found that 100% isolates carried *clfB*, 87.50% *icaA* and *icaD* gene and 25% *clfA* gene.

Memon *et al.* (2013) reported that the amplicon size of *clfA* gene varied from 900bp to 1000bp in *S. aureus* isolates associated with bovine mastitis. The clumping factor encoding *clfA* gene was detected in 59% of isolates.

Havaei *et al.* (2014) studied 50 *S. aureus* isolated from various human clinical specimens for *agr* typing and reported that 45 (90%) isolates were *agr* type I, 2 (4%) were *agr* type III, and 3 (6%) were nontypeable.

Fabres-Klein *et al.* (2015) studied 54 *S. aureus* strains obtained from cows with subclinical mastitis. The bacteria belonged to *agr* groups I (12/54), II (34/54), III (6/54), and IV (2/54), and bacteria in *agr* group III were found to be stronger biofilm producers than those in groups I and II. Again, milk had a significant influence only on slime-positive *agr* I and II isolates, revealing an association between milk and slime.

Barbieri *et al.* (2015) studied breast peri-implant infections causing 13 *S. aureus* isolates. All the isolates carried *icaA, icaD* and *clfA* gene.

Khoramian *et al.* (2015) studied 215 *S. aureus* strains collected from human and dairy cow's infections. The most of the isolates were positive for *icaA* (87.9%) and *icaD* (88.4%) genes.

Castelani *et al.* (2015) studied 110 *S. aureus* isolates from mammary gland secretions of heifers and cows with mastitis and reported presence of *icaA* and *icaD* genes in 98% and 100% of isolates respectively, while only 55.5% of all isolates produced a biofilm on Congo red agar phenotypically.

A total of 209 *S. aureus* isolates were obtained from human patients. Out of these, sixty-four (30.6%) isolates were methicillin-resistant, among which thirty-six (56.2%) were MDR. The majority of MDR-MRSA was belonged to *agr*-I (67%, n=24), followed by *agr* –II (17%, n=6), *agr*-IV (11%, n=4) and *agr*-III (5.5%, n=2). Among the biofilm related genes, the frequency of *icaA* and *icaD* genes in MDR-MRSA were 75% (n=27) and 61% (n=22) respectively. Furthermore, the prevalence of *clfA* and *clfB* was 100% for each. The relation between the *agr* specific groups and the antibiotic resistance was not confirmed. There was not a confirmed relationship between the *agr* groups and antibiotic susceptibility pattern or biofilm production by the isolates (Ghasemian *et al.*, 2015).

Nathawat *et al.* (2015) characterized 27 *S. aureus* isolates of milk origin from goat clinical mastitis for *clfA* gene. Twenty five isolates produced single amplicon of 1050bp indicating no polymorphism in *clfA* gene while two isolates did not produce any PCR amplicon.

Yadav *et al.* (2015c) studied 32 *S. aureus* isolates obtained from milk of cattle and buffalo with clinical mastitis. The *clfA* gene was amplified in 27 isolates producing two different amplicons of 900 and 1000bp sizes showing polymorphism. The most (71.80%) of the isolates produced amplicons of 900bp while amplicon size of 1000bp was produced by four (12.5%) of the isolates.

Mohsenzadeh *et al.* (2015) evaluated 31 isolates of *S. aureus* obtained from mastitis in dairy cattle for *agr* gene polymorphism by specific primers. Most of the isolates belonged to *agr* group I (54.8%), followed by *agr* group III (25.8%) and *agr* group II (19.4%). There was not any isolates belonging to *agr* group IV.

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. They detected presence of *clfA, clfB, icaD, agr*-1 and *agr*-2 genes in 25 (89.3%), 24 (85.7%), 20 (71.4), 18 (64.3%) and 5 (17.9%) isolates respectively, while the *icaA, agr*-3, *agr*-4 genes were not detected in any of the isolates.

B. Antiphagocytosis (*cap5K* and *cap8K* gene)

Capsule production by *Staphylococcus aureus was* first described by Gilbert in 1931. The encapsulation has been demonstrated to be very important trait for survival of this organism in the host systems as the capsulated organism are able to protect themselves from being phagocytosed. There have been 11 different capsular types demonstrated on the basis of serological or agglutination reaction with monospecific antisera. Among 11 capsular serotypes capsular type 5 and 8 have been reported to be most common in *S. aureus* isolates from human sources. However, there seems to be great variation in capsular type from animal sources. The information concerning the geographical distribution of capsular serotypes is important for the rational design and use of vaccine against *S. aurues* causing mastitis based on capsular typing (Gilbert, 1931; Karakawa *et al.*, 1988).

Poutrel *et al*. (1988) carried out serotyping using monoclonal antibodies to identify *S. aureus* capsular polysaccharide types 5 and 8 in 71.5% and 78.8% of the isolates, respectively.

Similarly Naidu *et al*. (1991) also reported that 70% of *S. aureus* isolates from bovines with mastitis were either CP5 or CP8 producers.

Sau *et al*. (1997) determined the nucleotide sequences of both *cap5* and *cap8* gene cluster. The *cap5* and *cap8* loci are chromosomal and allelic and comprise a ≈17.5 kb region of the chromosome. The each gene locus contains 16 closely linked genes, *cap5A* (*cap8A*) through *cap5P* (*cap8P*), transcribed in one orientation. The predicted amino acid sequence of 12 of the 16 ORFs of *cap5* and *cap8* gene clusters are almost similar and 4 ORFs located in the central region are type-specific.

In experiments in a mouse infection model of bacteremia, Luong and Lee (2002) recorded that CP8 overproducing strains of *S. aureus* persisted longer in the bloodstream. Their results indicated that CP8 was an antiphagocytic virulence factor of *S. aureus.*

The capsular typing of *S. aureus* from cow, the genes *cap5* with a size of 880bp were observed for 20 (83.3%) and 13 (81.3%) strains and *cap8* with a size of 1150bp for 4 (16.7%) and 3 (18.8%) strains of the clinical and subclinical *S. aureus* group, respectively (El-Sayed *et al.*, 2006).

Verdier *et al.* (2007) studied the capsular phenotypes and genotypes of 195 *S. aureus* isolates representative of all clinical syndromes that encompassed both hospital and community-acquired infections. In this population, most of clinical isolates (87%) expressed either capsular polysaccharide type 5 (42%) or 8 (45%), whereas 13% were nontypeable by the serotyping method with antibodies specific to capsular polysaccharide type 5 or 8. While PCR method allowed genotyping of 100% of strains, and all strains carried either the *cap5* (46%) or *cap8* locus (54%).

The capsular typing for *cap*5 and *cap*8 genes in *S. aureus* strains isolated from bovine, ovine and caprine ruminants was carried out by Alves *et al.* (2008). In order to find out the host specificity for the isolates, they concluded that the nature of *S. aureus* strains differs between large and small ruminants and suggested existence of a host rather than tissue specificity. In their study they recorded CP type 8 with an overall prevalence of 83.1% in ovine and caprine isolates.

Reinoso *et al.* (2008) analyzed 45 *S. aureus* strains genotypically obtained from humans, bovine subclinical mastitis and food samples. The gene *cap5* was observed in 21 (47%) *S. aureus* strains (11 human, nine bovine and one food sample strain), whereas the gene *cap8* in seven (15%) *S. aureus* strains (four human and three food sample strains). Seventeen cultures (38%) were non-*cap5* or *cap8*. All bovine strains were negative with capsular polysaccharide type 8 oligonucleotide primers.

Proietti *et al.* (2010) characterized 170 *S. aureus* from bovine milk collected from 7 dairy farms in Italy for *cap*5*K* and *cap*8*K* genes. Of the 170 isolates, they recorded 140 isolates to possess *cap*5 gene producing PCR amplicon of 880bp. They did not record any of the isolates with *cap*8 gene.

The capsular typing of *S. aureus* of bovine (20 isolates) and caprine (10 isolates) origin was carried out by polymerase chain reaction using primers specific for *cap5K* and *cap8K* genes. From cattle mastitis 60% of *S. aureus* were found to possess *cap5K* gene and 20% of the isolates possessed *cap8K* gene. The rest 20% isolates did not produce amplicon with these primers and were considered non-CP5 and non-CP8 isolates. From goats, 30% of the isolates showed presence of *cap5K* gene and 20% of the isolates exhibited presence of *cap8K* gene whereas 50% of the isolates were non-typable for *cap5K* or *cap8K* genes (Upadhyay *et al.*, 2010a).

Capsular typing of 107, 128 and 111 *S. aureus* isolated from intra-mammary infections in Sahiwal, Karan-Fries cattle and Murrah buffaloes, respectively was carried out by Singh *et al.* (2011). The study revealed that 86.8% (60.7 % *cap*5K and 26.1% *cap*8K), 79.0% (56.3% cap5K and 22.7% *cap*8K) and 81.1% (22.2% *cap*5K and 18.9% *cap*8K) *S. aureus* isolates from Sahiwal cattle, Karan-Fries cattle and Murrah buffaloes, respectively were capsulated.

Khichar and Kataria (2014) characterize 28 *S. aureus* isolates obtained from 59 mastitis samples of Holstein-Friesian crossbred and Rathi cattle for capsular genotyping (*cap5K* and *cap8K*). The detection of capsular gene fragments was investigated by PCR where in all the isolates were found to possess either *cap5K* or *cap8K* genes responsible for CP5 or CP8 types, respectively. In this study 26 isolates (92.86%) produced amplicons of 361bp indicating presence of *cap5K* gene and two isolates (7.14%) produced 173bp amplicon indicating presence of *cap8K* gene.

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During the study the *cap5* and *cap8* was found in 13 (46.4%) and 11 (39.3%) isolates, respectively.

Yadav *et al.* (2015a) studied 32 *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis for capsular gene typing. The genotyping for capsular genes (*cap5K* and *cap8K*) revealed that 22 isolates (68.75%) were positive for *cap5K* and seven isolates (21.87%) were recorded positive for *cap8K* genes whereas three isolates (9.37%) were found non-typable for both *cap5K* and *cap8K*.

Nathawat *et al.* (2015) characterized 27 *S. aureus* isolates of milk origin from goat clinical mastitis. Of the 27 isolates, 17 (68.38%) showed single amplicon of approximately 350bp representing presence of *cap5K* gene responsible for capsular type 5 whereas nine (34.61%) of the isolates produced amplicons of approximately 200bp representing presence of *cap8K* genes responsible for production of capsular type 8, whereas one isolate was non-typable for any of the two genes.

C. Exoenzymes (*coa* and *aur* gene)

Goh *et al*. (1992) characterized *S. aureus* isolates on the basis of PCR amplification of variable region of coagulase gene followed by *Alu*I enzyme digestion to analyze the restriction fragment length polymorphic DNA. The sizes of PCR products obtained were between 440 to 950bp. They observed ten distinct RFLP patterns among 30 different isolates and were successful in tracing the source of *S. aureus* outbreak. They also observed an excellent correlation between RFLP patterns and multiple locus enzyme electrophoresis.

A typing procedure for *S. aureus* was developed based on improved PCR amplification of the coagulase gene and restriction fragment length polymorphism (RFLP) analysis by Hookey *et al.* (1998). All coagulase positive staphylococci produced a single PCR amplification product of 875, 660, 603, or 547bp and ten distinct RFLP patterns were found by enzyme *Alu*I among 85 isolates of methicillin-resistant *S. aureus* (MRSA) and 10 methicillin-sensitive *S. aureus* (MSSA) examined.

Genotyping of 453 *S. aureus* isolates obtained from bovine mastitic milk by RFLP of *coa* gene was done by Su *et al*. (1999) wherein they grouped these isolates into 40 genotypes. They further recorded that coagulase genotypes differed among geographic locations, and only a few genotypes prevailed in each location. They also found that the predominant genotypes were more resistant to neutrophil bactericidal activities than rare genotypes.

Lange *et al.* (1999) studied 66 isolates of *Staphylococcus aureus* obtained from milk samples of dairy cows suffering from subclinical mastitis analyzed by analysis of coagulase (*coa*) gene polymorphisms by PCR amplification of the 3' terminal region of the *coa* gene to discriminate between the isolates. The *coa* gene amplification was found to be suitable for isolate differentiation with good discriminatory index value (D =0.82).

Sabat *et al.* (2000) studied variations, distribution and copy number of the *aur* gene among 53 *S. aureus* strains isolated from healthy as well as diseased persons. All strains were found positive for *aur* gene with amplicons of 1.5 kb. The RFLP with four different restriction enzymes, *Apo*I, *Hinc*II, *Hinf*I and *Nde*I of *aur* gene revealed only two distinct restriction patterns, designated I and II, respectively. The PCR-RFLP pattern I contained 16 isolates, (including seven from the nasal passages

of healthy individuals, four from patients with furuncles and five from patients with atopic dermatitis) while pattern II contained 37 isolates (including seven from healthy individuals, 13 from patients with furuncles and 17 from patients with atopic dermatitis). They suggested that type II was more often isolated from skin diseases than type I, although the differences observed are not statistically significant (Fisher exact test, $P = 0.09$). In order to compare the aureolysin encoded by the two PCR-RFLP types of the *aur* gene, strain of type I and type II were selected for sequencing of the *aur* gene. The deduced amino acid sequences of the open reading frames were almost identical within each of the two *aur* PCR-RFLP types, whereas homology at the gene level between the two types was only 89%.

Nimmo *et al*. (2000) generated coagulase gene RFLP patterns of 31 gentamicin and methicillin resistant *S. aureus*. Coagulase gene RFLP patterns of the 31 GS-MRSA isolates were divided into four types (A to D), with types A and B being further divided into four (I to IV) and two (I and II) closely related subtypes, respectively. All but one of the community-acquired isolates fell into subtypes AI and AII, and conversely only three of the health care facility-acquired isolates belonged to subtype AI. Seven different sized PCR products ranging between 580 and 1060bp were obtained and the product of approximately 730bp was observed in 20 isolates.

Thirty four strains of *S. aureus* obtained from milk samples of 34 dairy cows suffering from mastitis from different farms were identified and characterized for *coa* gene and found single band for each of the isolates. Two different sized PCR products of 580 and 660bp were distinguished of which former was present in 33 strains (Stephan *et al*., 2001).

Ninety-two *S. aureus* strains from human soft tissue infections were cultivated on casein agar plates in order to study extracellular protease production. Twenty-one strains (23%) produced a zone of precipitation around the bacterial streak showing protease activity. Twelve protease-negative and five protease-positive strains were also tested for the presence for *aur* (aureolysin) gene which revealed that all tested strains were positive (Karlsson and Arvidson, 2002) for *aur* gene.

The gene (*aur*) encoding the metalloprotease (aureolysin) of *S. aureus* from domestic animals was analyzed by PCR and PCR-RFLP by *Hinc*II enzyme and sequencing. The *aur* gene was detected in all 74 isolates from cows, pigs and chickens by PCR amplification and was classified into types I and II by PCR- RFLP patterns. The type II *aur* gene was contained in 36 (94.7%) of 38 protease-positive isolates as judged by skim milk agar plate culture, while type I was contained in 28 (77.8%) of 36 protease-negative isolates. The deduced amino acid sequences of aureolysins from type I and II isolates were almost identical with those of the published data (Takeuchi *et al.*, 2002).

Schlegelova *et al.* (2003) studied the 86 *S. aureus* isolates recovered from clinical infections of cattle and humans sharing an infected environment and reported polymorphism in *coa* gene having variable product size of 650bp, 730bp, 810bp and 1050bp. Further genetic diversity among isolates was observed by restriction fragment length polymorphism of the coagulase gene. Molecular analyses identified ten polymorphism types with prevalent presentation of type II in isolates from cow's milk and type IV in isolates from people coming into contact with dairy cows on the farm (the cattlemen) and the other farm personnel. Seven further genotypes were identified among the isolates from the cattlemen.

Scherrer *et al.* (2004) studied 293 isolates of *S. aureus* obtained from 127 bulk-tank milk samples of goats and sheep. The PCR amplification of the 3' end of the coagulase gene showed a single amplicon of 500bp in eight isolates (2.7%), of 580bp in 45 (15.4%), of 660bp in 95 (32.4%), of 740bp in 68 (23.2%) and of 820bp in 69 (23.5%) of the isolates. Coagulase gene restriction profile analysis of the 145 isolates revealed six different patterns using *Alu*I and five different patterns using *Hae*III.

Amplification of *coa* gene yielded two different PCR products of 600bp and 850bp for four and 12 of the *S. aureus* isolates from bovine subclinical mastitis in Central Java, Indonesia, while five different PCR products with sizes of 510, 600, 680, 740 and 850bp were found for one, ten, two, one and five of the *S. aureus* isolated in Hesse, Germany, respectively (Salasia *et al*., 2004).

Katsuda *et al*. (2005) carried out molecular typing of 270 *S. aureus* isolated from bovine mastitis on the basis of *coa* gene and observed that isolates produced one of the five types of amplicons 420, 580, 660, 740 and 820bp. In their study the amplicon of 580bp was present in 60% of the isolates. They observed 15 distinct RFLP patterns after *Alu*I digestion.

One hundred twenty-five strains of *S. aureus* from bovine mastitc milk were examined for *coa* gene polymorphism by Guler *et al*. (2005). They grouped these isolates into four subtypes and 60.8% of the isolates predominantly observed 1000bp PCR product showing that a few types of *S. aureus* were responsible for the majority of bovine clinical mastitis cases in one area of Central Anatolia region, Turkey.

A typing procedure based on polymorphism of the *coa* gene was carried out by da Silva and da Silva (2005). They obtained 27 different PCR products from 64 *S. aureus* isolates of cow mastitis of which 60 produced only one amplicon while remaining four produced two amplicons. On the basis of PCR-RFLP of *coa* gene they further grouped these isolates into 49 types.

Vimercati *et al.* (2006) carried out amplification of *coa* gene and RFLP of 116 *S. aureus* isolates from cows, goats and sheep and found seven different *coa* types. Further on the basis of PCR-RFLP, 29 different *coa* subtypes were identified.

Karahan and Cetinkaya (2007) used PCR-RFLP method to study the genetic relatedness of *coa* positive *S. aureus* isolated from cows with subclinical mastitis in Turkey. Out of 200 isolates of *S. aureus* identified by species specific PCR 161 isolates carried *coa* gene as confirmed by PCR of 3' end of the *coa* gene. Most of the isolates produced a single band of *coa* amplicon with molecular sizes ranging from 500 to 1400bp and a small number of isolates produced two bands. The detection of two bands suggested that milking personnel could play a role in the transmission of *S. aureus*. Analysis of *coa*-RFLP using *Alu*I generated 23 band patterns.

Aslantas *et al*. (2007) recorded that most of the isolates (98.7%) in their study produced a single band of *coa* amplicon with molecular sizes ranging from 730 to 1050bp whereas only one isolate yielded two amplification products (1.3%). The products of 730 and 970bp size were the most frequent and accounted for 38.8% and 41.3% of the isolates, respectively.

Thirty seven *S. aureus* isolates of milk origin strains were characterized genotypically by PCR using oligonucleotide primers that amplified genes encoding coagulase (*coa*) by Kalorey *et al.* (2007) which yielded three different products of 627, 710, and 910bp for 20, 10, and seven isolates, respectively.

Moon *et al*. (2007) subjected 696 *S. aureus* isolates from bovine mastitis to PCR amplification of the *coa* gene and recorded a single band ranging between 620 and 809 bp for each isolate. Restriction enzyme digestion with *Alu*I of amplicons generated 1 to 4 fragments of 126 to 670bp and the digestion gave 10 different RFLP patterns.

Tiwari *et al.* (2008) designed the forward primer *coa* F (5'-GGGATAACAAAGCAGATGCGATAG-3') and the reverse primer *coa* R (5'-ACGTTGATTCAGTACCTTGTGG-3') for the amplification of hyper variable region of *coa* gene and found that out of the 288 *S. aureus* strains from different clinical specimens, 237 were *coa* gene PCR positive with the PCR products of 1456, 1150 and 710bp size while rest 51 strains were *coa* gene negative.

An epidemiologically and genetically diverse collection of *S. aureus* strains was used by Sabat *et al.* (2008) to determine the range of aureolysin (*aur*) gene polymorphism. Sequence analysis of *aur* gene confirmed that all investigated MRSA and MSSA strains possessed *aur* gene of the same length of 1530bp. This study found 24 nucleotide alleles of *aur* gene which translated to 15 amino acid sequences. Sequencing analysis supported their conclusion that *aur* gene occurs in two distinct groups (type I and type II) of related sequences. There were 195 (12.7%) polymorphic nucleotide sites, which resulted in 43 (8.4%) variable inferred amino acid positions. Type I consisted of 9 nucleotide alleles which translated to 6 amino acid sequences, and type II consisted of 15 nucleotide alleles which translated to 9 amino acid sequences. Pairwise differences in nucleotide sequences between representative pairs of alleles ranged from 1 to 40 (0.1–2.6%) nucleotide sites within type I, and from 1 to 13 (0.1–0.8%) nucleotide sites within type II, whereas between the types the differences ranged from 146 to 170 nucleotide sites (9.5–11.1%). The deduced amino acid diversity of preproaureolysin varied from 1 to 6 (0.2–1.2%) amino acid positions within type I, and from 1 to 4 (0.2–0.8%) amino acid positions within type II, whereas between types the difference was from 31 to 38 (6.1–7.5%) amino acid positions.

Twenty one *S. aureus* isolates of bovine mastitic milk were analyzed by Sanjiv *et al.* (2008) for their *coa* gene products and RFLP patterns. The isolates produced three different types of *coa* gene products (600, 680 or 850bp) and three distinct RFLP patterns. Amplicons of 600bp produced only one fragment of 300bp (Pattern I), amplicons of 680bp produced two fragments of 210 and 260bp (pattern II) and amplicons of 850bp produced three bands of 170, 290 and 390bp (pattern III).

Saei *et al.* (2009) carried out study on *coa* gene polymorphism in 58 *S. aureus* isolates recovered from 350 milk samples of cows with clinical and subclinical mastitis in east and west Azerbaijan provinces, Iran. Amplification of the variable region of *coa* gene produced five different PCR products ranging in size from approximately 490bp to 850bp. The RFLP patterns of the PCR products digested with *Hae*III revealed nine RFLP patterns, numbered I–IX with 23 isolates (39.66%) assigned to RFLP pattern I and 14 isolates (24.14%) assigned to RFLP pattern III. Five out of nine patterns were found in both regions and four of nine patterns were only found in one region.

The amplification of *coa* gene displayed four different size polymorphisms with approximately 400bp for one (4%) strain, 600bp for 12 (57%) strains, 700bp for two (9%) strains and 900bp for six (28%) strains of *S. aureus* from bovine mastitis (Coelho *et al.*, 2009).

Bhanderi *et al.* (2009) also reported polymorphism as they obtained two types of *coa* PCR products from 23 coagulase positive isolates obtained from 43 *S. aureus* isolates of bovine clinical and subclinical mastitis. Of the total isolates 16produced 670bp while seven isolates produced 850bp product.

Himabindu *et al.* (2009) carried out molecular analysis for coagulase gene polymorphism in clinical isolates of methicillin resistant *S. aureus* by RFLP based genotyping. The sizes of PCR products obtained after amplification ranged between 650-1000bp and were classified into three band classes. Further the products were digested with *Alu*I and electrophoresed. They observed bands in multiples of 81bp and were divisible into nine band classes.

El-Jakee *et al*. (2010) studied 19 *S. aureus* isolates (9 from bovine and 10 from human sources) and recorded polymorphism of *coa* genes by PCR gene analysis wherein the coagulase gene product ranged from 423bp to 658bp.

In a study Upadhyay *et al*. (2010b) reported that all 30 isolates from mastitic milk of cattles and goats produced one of the three types of *coa* gene product. Out of these isolates, 10 produced amplicon of 600bp, 15 of 680bp and five of 850bp sizes. They did not observe any difference in the RFLP patterns of *Alu*I digests of *coa* amplicons of cattle and goat isolates.

Isolation of *S. aureus* from normal quarter's milk sample and from contact dairy workers was carried out by Abeer *et al.* (2010) for detection of *coa* gene. They recorded presence of gene 40.45% and 69.56% of the milk and dairy workers, respectively. The polymorphic amplicons ranged from 400 to 800bp.

Sindhu *et al.* (2010) detected *S. aureus* directly from mastitic milk of Murrah buffaloes using coagulase gene based specific polymerase chain reaction assay. Out of 628 samples, a total of 140 samples were found positive for the gene with four amplified products of size 960bp, 870bp, 740bp and 610bp in 8.57, 19.28, 29.29 and 42.85% of the milk samples, respectively.

The study of Salem-Bekhit *et al.* (2010) carried out on *coa* gene in 68 *S. aureus* from bovine mastitis from different farms indicated polymorphism of the gene. Three different products were observed in a pattern, as 22 isolates produced amplicon size of 910bp, 11 isolates produced amplicon size of 710bp and eight isolates produced amplicon size of 627bp.

Laarman *et al.* (2011) studied the metalloprotease aureolysin as a potent complement inhibitor. They showed that aureolysin inhibited the deposition of C3b on bacterial surfaces and the release of the chemoattractant C5a.

Restriction fragment length polymorphism was used to determine the polymorphism in the coagulase gene in 42 strains of *S. aureus* (Momtaz *et al.*, 2011). The enzymatic digestion with *Alu*I of the 920bp products in 31 strains produced three bands of 490, 320, and 160bp and 11 strains with 730bp amplicon produced two bands of 490 and 240bp.

All of the confirmed 50 *S. aureus* samples from bovine mastitis were PCR positive for the *coa* gene and displayed three different size polymorphisms (Coelho *et al.*, 2011).

Molecular typing of *S. aureus* strains isolated from mastitic milk samples was carried out by Karakulska *et al*. (2011) based on PCR-RFLP of *coa* gene. They obtained only single type of amplicon in all the 43 *S. aureus* strains of about 1130bp length and only one restriction pattern consisting of four bands of about 470, 300, 170 and 90bp.

Genetic analysis by Linage *et al.* (2012) of 151 isolates was done for amplification of the *coa* gene in which 149 (97.39%) of the isolates showed the presence of *coa* gene.

Saei (2012) reported five different *coa* types of *S. aureus* isolates obtained from clinical and subclinical bovine mastitis milk specimens in which most *coa* types were type I (38.1% of the 63 isolates) and type III (36.51%). They indicated that a few coagulase gene types of *S. aureus* were responsible for the majority of bovine mastitis cases.

Khichar *et al*. (2012) carried out *coa* gene typing of *S. aureus* of cattle mastitis origin and found polymorphism in *coa* gene. They obtained one amplicon in each isolate either of 510, 600, 710 or 850bp in size. They further characterized these isolates for their RFLP patterns using *Alu*I restriction endonuclease. The number of fragments generated upon *Alu*I digestion of *coa* amplicons varied from one to three and their sizes varied between 150 and 390bp.

Zdzalik *et al.* (2012) studied 167 *S. aureus* strains originated from human subjects suffering from the following *S. aureus* caused or complicated conditions: cystic fibrosis (27 strains), pneumonia (26 strains), wound infection (24 strains), nosocomial infection (23 strains), skin infection (15 strains), sepsis (10 strains), urinary tract infection (eight strains), bone infection (seven strains), eye infection (seven strains), upper respiratory tract infection (six strains), central nervous system infection (three strains), and otitis (two strains). Nine commensal strains isolated from asymptomatic carriers were also included in the study. The analysis revealed high abundances (99%) of the aureolysin encoding *aur* gene.

The coagulase positive *S. aureus* isolates of human and animal sources were analyzed by Gharib *et al.* (2013) for detection of *coa* gene. The resulted amplicons were of 648, 723, 812 and 913bp sizes. Of which 812bp product was the most frequent and accounted for 5/15 (33.3%) of the isolates followed by 648bp (26.6%), 723bp (13.3%) and 913bp (6.6%).

Monecke *et al.* (2013) studied postmortem samples of the two hedgehogs. Coagulase-positive *S. aureus* was detected in pure culture from the brain and kidneys samples from hedgehog and found to be positive for *aur* gene.

Bhati *et al.* (2014) subjected 38 isolates of *S. aureus* recovered from Holstein-Friesian (H-F) crossbred and Rathi cattle with subclinical mastitis were subjected to *coa* gene amplification and RFLP. Out of 38 isolates, 35 strains produced single amplicon of either 400, 490, 510, 550, 600, 710, 760, 810 or 850bp while three isolates did not produce any *coa* amplicon. Results showed higher *coa* gene polymorphism in *S. aureus* isolated from native breed (eight *coa* types) as compared to that in H-F crossbred cattle (three *coa* types). Subsequently, the *coa* products were digested with restriction enzyme *Alu*I for RFLP typing which generated four and seven RFLP patterns with isolates from H-F crossbred and Rathi cattle, respectively. The RFLP patterns obtained from similar amplicons in isolates from these two breeds did not show any variation.

Eed *et al.* (2015) reported *coa*-RFLP patterns for 58 MRSA (40 hospital-acquired MRSA/HA-MRSA and 18 community-acquired MRSA/CA-MRSA). The HA-MRSA strains showed seven distinct RFLP patterns; the most frequent being pattern 2 (15 isolates), followed by patterns 1 and 4 (5 isolates each) and CA-MRSA strains showed five RFLP patterns; the most frequent being pattern 3 (7 isolates) followed by pattern 8 (6 isolates).

Puacz *et al.* (2015) studied 21 *S. aureus* strains isolated from mastitic milk of cows living in 12 different farms with infected mammary glands. All studied isolates were found positive for *aur* gene.

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. All of the isolates carried *coa* gene with size polymorphism.

Abd El-Hamid and Bendary (2015) studied genotypic discrimination of methicillin resistant and susceptible 133 *S. aureus* strains obtained from various clinical infections. Coagulase (*coa*) typing discriminated the isolates into eight different groups, while *coa*-RFLP yielded ten distinct RFLP banding patterns.

A typing procedure based on polymorphism of the *coa* gene was used to discriminate 32 *S. aureus* isolates obtained from cattle (16) and buffalo (16) mastitic milk. Amplification of *coa* gene produced four different *coa* PCR products (400bp, 510bp, 600bp and 650bp) from cattle isolates and five different products (400bp, 510bp, 600bp, 650bp and 680bp) from buffalo isolates. From cattle and buffalo isolates five and six RFLP patterns were obtained, respectively. The *coa* gene amplicon of 600bp was produced by the maximum number of isolates (Yadav *et al.,* 2015b).

D. Immune evasion (*spa-X, spa-IgG, chp* and *scn* gene)

Frenay *et al.* (1994) used amplification of region-X of *spa* gene encoding for C-terminal cell wall-binding protein A by PCR to discriminate between epidemic and non-epidemic MRSA strains and suggested that a longer X-region resulted in a better exposition of the F_c-binding region of protein A thereby facilitating colonization of host surfaces and contributing to the epidemic phenotype. Most epidemic MRSA strains (24 of 33) harbored more than seven repeats, while most nonepidemic MRSA strains (10 of 14) contained seven or fewer repeats. They suggested that a longer X-region resulted in a better exposition of the Fc-binding region of protein A, thereby facilitating colonization of host surfaces and contributing to the epidemic phenotype.

Seki *et al.* (1998) subjected 119 strains of *S. aureus* from healthy individual to an investigation on the producibility of surface protein A. Among these strains, 69, 43, 3 and I strains were found to have the protein-A gene containing 5, 4, 3 and 2 IgG-binding domains respectively. On the other hand, only one strain was devoid of the protein-A gene.

In a study on genotyping of *S. aureus* isolated from bovine mastitis involving *spa-X* and *spa-IgG* region of protein A gene Annemuller *et al*. (1999) obtained amplified fragments of 120, 150, 170, 250 and 300bp with calculated number of repeats of 3, 4, 5, 8 and 10, respectively for *spa-X* gene. Amplification of the *spa-Ig* gene encoding the IgG binding region revealed sizes of 620bp for 20 of the isolates and 280bp for four isolates indicating, for the latter, a deletion of segments in this region.

Indrajulianto (2000) studied polymorphism of *spa* gene in 16 *S. aureus* isolates from bovine and human sources. A gene yielded amplicons with a size of 100, 150, 200, 250, 280, 300 and 330bp by that correspond with the number of repeats of 3, 4, 6, 8, 9, 10 and 11, respectively. It was of interest that the sizes of the amplified fragments from human isolates were longer than from bovine isolates.

Stephan *et al*. (2001) recorded the *spa-X* and *spa-IgG* region of the protein A of *S. aureus* strains isolated from bovine mastitis *spa-X* region amplicons of 100, 200, 280 and 300bp with repeats of 2, 6, 10 and 11, respectively. The PCR amplification of the gene encoding the IgG-binding region of protein A revealed a size of 920bp for most of the isolates investigated. However, the protein A gene of three cultures revealed an amplicon size of 750bp.

Molecular typing of methicillin resistant *S. aureus* by protein A gene sequencing was carried out by Mitani *et al*. (2002) who suggested that *spa* typing would prove useful in epidemiological study in MRSA isolates.

Salasia *et al*. (2004) studied *S. aureus* strains obtained from bovine mastitis for variations in *spa-X* and *spa-IgG* regions. Amplification of the X-region of *spa* gene of the *S. aureus* isolated from bovine from Central Java, Indonesia showed two different sized amplicons of 270 and 320bp for six and ten isolates, respectively. On the other hand, nine different sized amplicons of 100, 150, 200, 230, 240, 250, 270, 290 and 340bp were observed for 8, 1, 1, 1, 2, 1, 1, 2 and 2, respectively for *S. aureus* isolated in Hesse, Germany. PCR amplification of the gene segment encoding the IgG-binding region of protein A revealed a size of 900bp from 32 of the isolates investigated from Central Java, Indonesia and Hesse, Germany. However, the protein A gene of three cultures from Hesse, Germany revealed an amplicon size of 780bp.

On the basis of the size of corresponding PCR products 100-320bp of amplified *spa* gene, 2-11 repeats were supposed to be present in the genes investigated. The isolates showed 8, 9, 10 and 11 repeats most frequently (20.39%, 15.53%, 14.56% and 16.50%, respectively) and seven or less repeats were found only in 33% of the isolates by Kuzma *et al.* (2005).

Rooijakkers *et al.* (2005) showed that *scn* is human specific and does not affect cow, goat or sheep complement. The chemotaxis inhibitory protein (CHIP) encodes a modulator of chemokine responses that prevents neutrophil chemotaxis and activation.

van Wamel *et al.* (2006) studied 85 randomly selected clinical strains and five classical lab strains to assess the frequency distribution of β-hemolysin (*hlb*)-converting bacteriophages (βC-φs). The βC-φs were found in 80 (88.9%) of the *S. aureus* strains, containing seven different IEC types named A through G. Type B (*sak-chp-scn*) showed the highest prevalence and was found in 24 (26.7%) strains., The CHIPS was present in 51 (56.6%) of these strains, and staphylococcal complement inhibitor (SCIN) was present in all βC-φ-containing strains. The *scn* and *chp* genes are found in 6/7 and 3/7 sequenced *S. aureus* strains, respectively. Analyses of these sequenced genomes showed that both *scn* and *chp* are carried by βC-φs, formerly known as double- and triple-converting phages.

Molecular characterization of 84 *S. aureus* isolates from mastitic milk of buffalo, cattle, ovine and caprine origin by pulsed-field gel electrophoresis (PAGE), *spa* typing, and multi-locus sequence typing revealed five distinct clonal *spa* types (Aires-de-Sousa *et al.,* 2007).

Kalorey *et al*., (2007) studied a total of 37 strains of *S. aureus* were isolated during processing of 552 milk samples from 140 cows suffering with subclinical mastitis. The amplification of the gene segment encoding the IgG binding region of protein A (*spa*) revealed a size of 590, 810, and 970 bp in 12, 15 and 7 isolates from 5, 5 and 4 farms, respectively, and gene polymorphism was noted in isolates from four farms. The X-region binding of the *spa* gene produced an amplicon of 220, 253, and 315bp in 10, 9, and 7 isolates, respectively.

Kumagai *et al.* (2007) studied quadruple or quintuple conversion of *scn* and *chp* genes by bacteriophages in non-beta-hemolysin-producing bovine isolates of *S. aureus*. In 13 of 43 non-beta-hemolysin-producing bovine isolates, the *scn* and *chp* genes were detected in 13 and 4 of the 13 isolates, respectively.

Sung *et al.* (2008) compared 56 *S. aureus* isolates that caused infection in cows, horses, goats, sheep and a camel with 161 human *S. aureus* isolates from healthy carriers and community acquired infections in the UK for genes carried on mobile genetic elements (MGEs) such as *scn* and *chp*. The *scn* and *chp* gene was found in 154 (96 %) and 134 (83%) of human isolates and 12 (21 %) and 8 (14 %) of animal origin isolates, respectively. This finding suggested that handful of such genes or gene combinations might be responsible for host specificity.

Faria *et al.* (2008) carried out major *spa* typing methods for a diverse collection of 198 *S. aureus* strains and found 98 distinct *spa* types.

Reinoso *et al.* (2008) analyzed 45 *S. aureus* strains genotypically obtained from humans, bovine subclinical mastitis and food samples. The PCR amplification of the gene segment encoding IgG binding region of protein A revealed a size of approximately 900bp for 42 isolates and a size of approximately 700bp (four repeats) for three of the investigated isolates. The amplification of the gene segments encoding the X region of protein A revealed typical size polymorphisms ranging from 100 to 315bp (2–10 repeats). Strains isolated from humans had different size amplicons and consequently different numbers of repeats compared to the strains isolated from bovines and food samples. Twelve (80%) bovine mastitis strains showed 300 and 280 bp indicating that strains of this origin possessed 10 and 9 repeats. Six (75%) strains isolated from food samples had 300 and 315bp amplified fragment indicating that food samples strains possessed 10 and 11 repeats.

Coelho *et al.* (2009) studied *S. aureus* strains obtained from bovine mastitis. The amplification of X-region yielded a single amplicon for each isolate with the prevalent amplicon size being of 250bp for 10 strains (38%), 280bp for nine strains (14%) and 180bp for eight strains (47%) and the PCR amplification of the gene encoding the IgG-binding region of protein A revealed 700bp bands for most of isolates and only two isolates with 900bp.

Bystron *et al*. (2009) characterized 68 *S. aureus* from cow's milk and reported *spa* types containing three and six to 14 repeats. Isolates harbored eight, ten, and twelve repeats were observed most frequently (33.82%, 29.41%, and 13.23% respectively).

Mehndiratta *et al.* (2009) carried out molecular typing of methicillin resistant *S. aureus* strains by PCR-RFLP of *spa* gene, each RFLP pattern confirmed the length of these restriction fragments due to variation in 24 and 174 tandem repeats.

Bhanderi *et al.* (2009) studied total 43 *S. aureus* were isolated from 121 clinical and subclinical mastitis cases of cows and buffaloes. The PCR amplification for the *spa-IgG* gene encoding the IgG binding region of protein A yielded a product size of 920bp for 38 isolates while 4 isolates revealed a product size of 730bp indicating polymorphism of this gene.

Momtaz *et al.* (2010) studied 86 *S. aureus* from bovine mastitis milk samples, of which 69 isolates contained IgG binding region gene (*spa-IgG*) with 920bp size and 22 isolates contained X region coding gene (*spa-X*) protein A with 320bp size without any polymorphism.

Protein A encoded by *spa* gene, is one of the virulence factors involved in the staphylococcal pathogenesis. It has been suggested that the number of 24-bp tandem repeat units along the X region of the *spa* gene correlates with the virulence level of the strains. They reported *spa* typing of MSSA isolates with amplicon size of 170-350bp while *spa* typing of MRSA isolates ranges from 210 to 370bp (Kurlenda *et al*., 2010).

Salem-Bekhit *et al.* (2010) investigated 68 *S. aureus* strains isolated from 456 milk samples obtained from 120 dairy cows from eight different farms for *spa* gene. A single DNA band of 920bp was developed with the PCR amplification of the *spa-IgG* gene segment in most investigated isolates. However, three cultures revealed an amplicon size of 750bp. The PCR amplification of the X-region of the *spa-X* gene yielded a single amplicon in each isolate. Four different sized amplicons of 290, 280, 200 and 100bp were developed in 33, 10, 9 and 7 out of 68 isolates, respectively.

Ikawaty *et al.* (2010) investigated 76 isolates of the *S. aureus* isolated from bovine mastitis where one isolate was found to be positive for *scn and chp* genes.

Proietti *et al.* (2010) studied characteristics of 170 *S. aureus* isolates from 1257 quarter milk samples collected from 320 cows from seven dairy farms suffering with subclinical mastitis. The PCR amplification of the *spa-IgG* gene segment revealed 80 (47%) and 70 (41%) strains to produce 970bp and 810bp amplicon respectively. However, PCR amplification of *spa-X* gene yielded a single amplicon of 253bp in all isolates.

Kumar *et al.* (2011) isolated 107 strains of *S. aureus* from mastitic milk samples from 195 infected udders of sahiwal cattle. Amplification of the *spa* gene (X-region) showed seven amplicons of 3, 4, 7, 8, 9, 10 and 11 repeats. The most frequent number of repeats was eight (in 57.9% of the isolates) followed by seven (17.7%)

and nine (15.8%). Polymorphic band patterns were also observed in *spa-IgG* gene. The sizes of the amplicons for Ig-binding gene were 500 (in 14.9% of the isolates), 1000 (55.1%) and 1100bp (29.9%).

Karahan *et al*. (2011) studied 92 *S. aureus* isolated from 400 subclinical mastitis affected bovines. In PCR amplification of *spa-IgG*, most of the isolates (n=67, 72.8%) produced amplicons at the molecular length of approximately 920bp, while the remaining 25 isolates (27.2%) formed bands at the size of approximately 750bp and PCR amplification of *spa* X-region gene recorded amplicons of approximately 110, 140, 170, 190, 220, 240, 270, 290 and 320bp with 3, 4, 5, 6, 7, 8, 9, 10 and 11 repeat units, respectively.

El-Jakee *et al.* (2011) studied 19 *S. aureus* isolates (9 from bovine and 10 from human) for *spa* gene and obtained different PCR products in size ranging from approximately 423 to 658bp and 396 to 462bp in bovine and human isolates respectively.

Coelho *et al.* (2011) studied 50 *S. aureus* isolates obtained from milk samples from subclinical mastitis cases identified in 15 dairy cattle farms. The amplification of the *spa*-X gene yielded a single amplicon of 315bp for each isolate.

Verkaik *et al.* (2011) studied the presence of the IEC-carrying bacteriophages in human and animal *S. aureus* isolates, using PCR for the gene encoding SCIN (*scn*). The *S. aureus* immune evasion cluster (IEC), located on β-haemolysin-converting bacteriophages (βC-Φs), encodes the immune-modulating proteins chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin A and staphylokinase. Human isolates were obtained by collecting serial nasal swabs from 21 persistent carriers. *S. aureus* strains from 19 (90%) persistent carriers contained an IEC while 77 infectious animal strains included in the study, only 26 strains (34%) were IEC-positive.

Singh *et al*. (2011) carried out *spa* typing of *S. aureus* isolates from intra-mammary infections of cattle and buffalo. They recorded variations in variable number of repeats in *spa* gene in all the isolates. Amplification of *spa* gene showed different amplicon (3-12 repeats), the most common number of repeat was eight (57.9%) followed by seven (17.7%) and nine (15.8%) in Sahiwal cattle whereas in Karan fries the frequent number of repeats was seven (45.3%) followed by eight (33.6%) and nine (7.0%). In Murrah buffalo the frequent number of repeats was seven (53.2%) followed by eight (27.9%) and six (10.8%).

From bovine mastitis 58 isolates of *S. aureus* were typed by Saei and Ahmadi (2012). They on the basis of polymorphism characterization of the gene encoding for the X-region of protein A (*spa*) observed five differently sized amplicons of approximately 1,200bp to 1,410bp. The *spa* gene *Hin6I* digestion produced a total of eight distinct patterns, designated as S1–S8. A distinct genotype (S9) had no amplification of the gene. The majority of isolates were classified into *spa* types S2 (24.14%) and S6 (24.14%).

Suleiman *et al.* (2012) studied 20 *S. aureus* isolated from milk samples from the cows. All the 20 strains yielded uniform amplicon sizes of approximately 300bp for *spa-X* gene whereas 18 strains yielded amplicon size of 900bp and two strains 750bp for *spa-lgG* gene.

The analysis of the size polymorphism of fragment-X of *spa* gene revealed high genetic differentiation of the analyzed group of isolates. The number of repeats of the 24 nucleotide sequence varied between 2 and 14 in the analyzed group of isolates and most prevalent strains, constituting 19.7%, 20.5% and 13.6% of all strains tested, had 10, 11 or 12 repeats, respectively among 132 *S. aureus* strains isolated from cows with mastitis (Szweda *et al.*, 2012).

Khichar *et al*. (2012) in a study on characterization of *S. aureus* of cattle mastitis origin carried out PCR amplification of X-region for *spa* gene wherein they obtained seven different sizes of amplicons (206, 243, 262, 277, 292, 306 and 339bp) with calculated number of 7, 8, 9, 10, 10, 11 and 12 repeats. They reported all these isolates to be highly pathogenic on the basis of number of repeats.

Cuny *et al.* (2013) reported that methicillin-susceptible *S. aureus* (MSSA) attributed to clonal complex (CC) 398 and exhibiting *spa*-type t571 received attention in Europe and in the USA for being associated with severe infections in humans. They suggested that as this spa-type was exhibited by livestock-associated (LA) methicillin-resistant *S. aureus* (MRSA) as well, it was important to discriminate LA- and human-derived strains by PCR-based methods. The MSSA t571 contained phage int3 carrying *scn* and *chp* genes, whereas LA-MRSA t571 lacked these markers.

The *spa* gene was found in all of the isolates of *S. aureus* from bovine mastitis, with varying amplicons sizes, 300bp being the prevalent size by Marques *et al.* (2013).

Memon *et al.* (2013) studied 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China. The *spaX* gene was found in all isolates with 150bp - 315bp product size and *spa-IgG* gene found in 79% isolates with 900bp -1000bp size.

In a study by Nathawat (2013), the variable amplicon sizes of 200, 240, 250, 290, 300, 320, 330, 350, 380, 400 and 650bp with calculated number of repeats of 7, 9, 9, 11, 11, 12, 12, 13, 14, 15, and 26, respectively were recovered for *spa-X* gene in *S. aureus* isolates from caprine mastitic milk.

Bhati (2013) investigated *spa-X* gene in *S. aureus* from subclinical mastitis of crossbred cattle and Rathi cattle. Of the 16 isolates from crossbred cattle, 15 produced *spa* amplicon with 200bp, 160bp and 150bp size and calculated number of 6, 5, 4 repeats, respectively. Whereas, 22 isolates from Rathi cattle were of greater variability than that in isolates from crossbred cattle as the sizes of *spa* amplicons were 280, 250, 240, 200, 190, 180, 170, 150 and 140bp with 10, 8, 8, 6, 6, 6, 5, 4 and 4 repeats, respectively.

Gomez *et al.* (2014) studied 13 *S. aureus* of faecal origin from 101 wild small mammals. Out of 13 isolates two were MRSA and 11 were MSSA. Three isolates were positive for *scn* (two MRSA and one MSSA) and one MSSA isolate was found to be positive for *chp* gene.

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During the study the *spa-X* and *spa-IgG* was found in 27 (96.4%) and 28 (100%) isolates with polymorphism of variable product size respectively.

Yadav *et al.* (2015c) studied 32 isolates from cattle (16) and buffalo (16) which were divisible into seven *spa-X* types with amplicon sizes ranging between 120 and 380bp. The cattle isolates produced seven different spa amplicons of 120, 150, 200, 250, 280, 300, and 330bp with 3, 4, 6, 8, 10, 11 and 12 number of tandem repeats, respectively. Whereas buffalo isolates were divisible into five spa types with amplicons of 150, 200, 250, 330 and 380bp having calculated number of repeats of 5, 7, 9, 12, and 14, respectively. Of the total isolates, 24 were considered pathogenic on the basis of more than seven tandem repeats.

Puacz *et al.* (2015) studied 21 *Staphylococcus aureus* strains isolated from mastitic milk of cows living in 12 different farms with infected mammary glands. Out of total isolates, nine (42.85%) and six (28.57%) isolates possessed *chp* and *scn* gene, respectively.

Sarrou *et al.* (2015) studied a large collection of *S. aureus* including 745 clinically significant isolates from animals and humans. Based on the molecular markers, the 17 CC398 strains were equally placed in the livestock-associated (9 isolates) and in the human clades (8 isolates), which were further characterized for *scn* and *chp* gene. Out of total 17 isolates, all livestock associated isolates were negative for both *scn* and *chp* genes while eight and five isolates of human origin were positive for *chp* and *scn* genes respectively.

E. Plasminogen activator/ Staphylokinase (*sak* gene)

Staphylococcus aureus has many specific proteins (SAK, SCIN, CHIPS, SpA and Sbi) that can have profound impact on the innate and adaptive immune system. The staphylokinase (SAK)/ plasminogen activator factor is a one of them important protein. Staphylokinase not only function by binding to α-defensins to prevent their bactericidal activities but also forms an equimolar complex with human plasmin (huPli) catalyzing the further activation of plasminogen (Behnke, *et al.,* 1987). Expression of staphylokinase govern by the *sak* gene, the *sak* gene has a highly conserved sequence and is carried on a bacteriophage containing other genes with an important function in immune evasion, such as complement inhibitory factors and enterotoxins. SAK was shown to enhance the breaching of tissue barriers in vitro (Coleman *et al.,* 1989; Kim *et al.,* 1997).

Although the direct role of SAK as a potential virulence factor in *S. aureus* disease remains unresolved but the role of SAK is present in the large majority of *S. aureus* strains causing human infection. In contrast, *S. aureus* strains from veterinary sources commonly lack SAK production (Jin *et al.,* 2003).

van Wamel *et al.* (2006) studied 85 randomly selected clinical strains and 5 classical lab strains to assess the frequency distribution of βC-φs. The βC-φs were found in 80 (88.9%) of the *S. aureus* strains, containing seven different IEC types named A through G. Type B (*sak-chp-scn*) showed the highest prevalence and was found in 24 (26.7%) strains. Concerning the virulence factors, SAK was present in 69 (76.6%) strains.

Kumagai *et al.* (2007) studied quadruple or quintuple conversion of *sak* genes by bacteriophages in non-beta-hemolysin-producing bovine isolates of *Staphylococcus aureus*. In 13 of 43 non-beta-hemolysin-producing bovine isolates, the staphylokinase (*sak*) gene was detected in all 13 isolates.

Monecke *et al.* (2007) studied that *Staphylococcus aureus* is a common pathogen which can colonise and infect not only man, but also domestic animals. Especially, infection of cattle is of high economic relevance as *S. aureus* is an important causal agent of bovine mastitis. One hundred and twenty-eight isolates from bovine mastitis were studied. Out of total isolates 89.1% were found negative for *sak* gene.

Sung *et al.* (2008) compared 56 UK *S. aureus* isolates that caused infection in cows, horses, goats, sheep and a camel with 161 human *S. aureus* isolates from healthy carriers and community acquired infections in the UK for *sak* gene carried on mobile genetic elements (MGEs). The *sak* gene was found in 134 (83%) of human isolates and 11 (20 %) of animal origin isolates. Interestingly, *sak* gene carried on mobile genetic elements (MGEs) was less common in animal *S. aureus* isolates. These finding may also suggested that handful of such genes or gene combinations may be responsible for host specificity.

Ikawaty *et al.* (2010) investigated 76 isolates of the *S. aureus* isolated from bovine mastitis. Only one isolate was found to be positive for *sak* genes.

Stastkova *et al.* (2011) reported that *sak* gene was found in all (45) studied *S. aureus* isolates producing food-borne intoxication from retal meat products from year 2005 to 2008.

Verkaik *et al.* (2011) studied the presence *sak* gene by locating *Staphylococcus aureus* immune evasion cluster (IEC) carrying *sak* gene, among human and animal origin isolates. Nasal swabs from 21 persistent carriers. *S. aureus* strains from 19 (90%) persistent carriers contained a *sak* gene while 77 infectious animal strains included in the study, only 26 strains (34%) were *sak* gene positive.

Cuny *et al.* (2013) studied methicillin susceptible *Staphylococcus aureus* (MSSA) attributed to clonal complex (CC) 398 and exhibiting *spa*-type t571 associated with livestock and human and reported that none of the human and animal origin isolate was positive for *sak* gene.

Peetermans *et al.* (2014) studied the role of plasminogen activation by SAK in a skin infection model in mice and found that SAK-mediated plasmin activity increased the local invasiveness of S. aureus, leading to larger lesions with skin disruption as well as decreased bacterial clearance by the host.

Gomez *et al.* (2014) studied *Staphylococcus aureus* faecal carriage in 101 wild small mammals. *S. aureus* was detected in 13/101 studied faecal samples. Out of thirteen isolates two were MRSA and 11 were MSSA. Two MRSA isolates were positive for *sak* while all MSSA isolate were found negative for *sak* gene.

Sarrou *et al.* (2015) studied a large collection of *Staphylococcus aureus* including 745 clinically significant isolates from animals and human origin. Based on the molecular markers, the 17 CC398 strains were equally placed in the livestock-associated (9 isolates) and in the human clades (8 isolates), which further characterize for *sak* gene. Out of total 17 isolates, all livestock associated isolates were negative while three isolates from human origin were found to be positive for *sak* genes.

Puacz *et al.* (2015) studied 21 *Staphylococcus aureus* strains isolated from mastitic milk of cows living in 12 different farms with infected mammary glands. Out of total isolates, six (28.57%) isolates were positive for *sak* gene.

F. Toxins (*hla, hlb, hld* and *tst* gene)

Aarestrup *et al.* (1999) found that the *hla* gene encoding α-haemolysin was detected in all isolates from human and bovine sources with a significant difference in the prevalence of the *hlb* gene encoding β-haemolysin between the bovine (96%), human carrier (56%) and isolates from septicemia (57%).

The *hla, hlb and tst* genes were detected in 100% (128/128), 38% (77/200) and 28.6% (45/157) of the studied *S. aureus* isolates obtained from patients with bloodstream, catheter tip, bone or joint, respiratory tract, ocular, soft tissue, wound, and skin human infections by Booth *et al.* (2001).

Jarraud *et al.* (2002) studied 198 S. aureus isolates obtained from human disease samples and it was found that 100 % isolates were carry *hla* and *hld* gene while only 27% and 10% isolates were positive for *tst* and *hlb* gene respectively.

All the *S. aureus* isolates of bovine mastitic milk samples from Central java, Indonesia (n=16) and Hesse (n=19), Germany possessed *hla* gene while *hlb* gene was found for six cultures from Central Java, and 15 cultures from Hesse. For the *tst* gene, 11 isolates of Hesse were positive while none of the isolate from Central Java was found to be positive (Salasia *et al.*, 2004).

Ando *et al.* (2004) studied 109 MRSA isolates from human suffering from urinary tract infections. Frequency of *hla, hlb* and *tst* gene was recorded as 89 (81.7%), 73 (67%) and 79 (72.5%) respectively.

Marconi *et al.* (2005) demonstrated the presence of the *hld* gene in all three (100%) *S. aureus* strains obtained from human clinical samples.

In an investigation El-Sayed *et al*. (2006) detected that none of clinical and subclinical mastitis isolate having *tst* gene while all (100%) were positive for *hla*. The *hlb* gene was found in all 24 *S. aureus* isolates (100%) obtained from clinical mastitis and in 13 isolates (81.3%) of subclinical mastitis.

The genes for haemolysins alpha, beta delta, and gamma were found in 97.4%, 76.7%, 87.9, and 88.8% of the 116 strains isolated at the onset of mastitis and 64.6% of the *S. aureus* isolates were positive for all genes, and 81.9% for *hlb*, *hld*, and *hlg* (Haveri *et al.*, 2007).

Staphylococcus aureus isolates collected from intramammary infections from two separately managed dairy herds were analyzed by Haveri *et al.* (2008) for molecular typing and genetic profiling. The most prevalent genes were *hla* and *hlg* in herd I and *hla, hld* and *hlg* genes in herd II. The other genes were also linked within the herds. No connection between specific virulence genes and the origins of isolates was found in the study.

Ohkura *et al.* (2009) studied 208 MRSA strains of human origin from 100 hospitals throughout Japan. The occurrence of *hld, hla, hlb and tst* genes were recorded as 203 (97.6%), 202 (97.1%), 176 (84.6%) and 167 (80.3%) of the studied isolates respectively.

Ikawaty *et al.* (2010) investigated 76 isolates of the *S. aureus* isolated from bovine mastitis. The *hlb* gene was reported in 100% isolates while *tst* gene was found in only 15 (19.7%) isolates.

Kateete *et al.* (2011) studied 41 *S. aureus* isolates from human clinical infections and it was found that 100% (41) isolates were carry *hla* and *hld* gene while only 10% (4) isolates had *tst* gene.

Argudin *et al.* (2011) studied 62 *Staphylococcus aureus* from bloodstream (n=31) and wound (n=31) infections in geriatric patients attending a long-term care Spanish hospital from 1996 to 2006 and reported 62 (100%), 40 (64.5%), 62 (100%) and 18 (29.0%) occurrence of *hla, hlb, hld* and *tst* toxin gene respectively.

Singh *et al.* (2011) identified 107 *S. aureus* isolates from sahiwal, 128 isolates from Karan fries and 111 isolates from Murrah buffalo and further checked the prevalence of toxic genes. The *hla* gene responsible for alpha haemolysis was found in all the isolates with 550 bp amplicon size, whereas *hlb* gene was most predominantly found in Sahiwal (94.6%), followed by Karan-fries (82%) and Murrah (72.1%) with similar amplicon size of 840bp.

Delgado *et al.* (2011) studied 30 human and bovine mastitic milk origin *S. aureus* isolates for toxin and other virulent genes. It was found that, all (100%) isolates were positive for *hla* and *hld* genes while only 15 (50%) isolates having *hlb* gene and four (13.3%) isolates were positive for *tst* gene.

Characterization of pathogenic factors of 107 strains of *S. aureus* isolated from milk samples was carried out by Kumar *et al.* (2011) who recorded that more than 90% of the isolates possessed *hla* and *hlb* gene with amplicon size of 550 and 840bp, respectively while none of the isolate was found positive for *tsst-1* gene.

Coelho *et al.* (2011) found 24 and 16% *S. aureus* isolates from bovine subclinical mastitic milk positive for *hla* and *hlb* genes, respectively.

In the study by Salasia *et al.* (2011), of the 41 *S. aureus* isolates from bovines, humans, and food origin, 84% of the isolates from bovine origin and 73% of the isolates from human origin harbored *hla* gene whereas all the Strains (100%) from food sources were positive for *hla*.

Almost all isolates of *S. aureus* associated with bovine mastitis harbored *hla* (98.7%), *hlb* (99.1%) and *hld* (100%) genes, coding for the alpha, beta, and delta-haemolysins, respectively (Ote *et al.*, 2011).

Amplification of the gene encoding haemolysin of *Staphylococcus aureus* was carried out by Ariyanti *et al.* (2011) with specific primers and detected nine isolates (81.81%) to possess *hla* gene and two isolates (18.18%) with both *hla* and *hlb* genes. The *hla* and *hlb* had a single amplicon with a size of approximately 534bp and 833 bp, respectively.

The study of 129 *S. aureus* isolates for detection of the hemolysin gene type by Wang *et al.* (2011) revealed that 47 (34.88%) of the isolates possessed *hla* gene while 55 (42.6%) isolates possessed *hlb* gene, 27 isolates had both the genes (20.93%) while 52 (40.20%) isolates had neither *hla* and *hlb* genes.

Yang *et al*. (2012) studied 39 *S. aureus* strains isolated from bovine clinical mastitis. The *hla* gene was the most widespread gene found in 33 (85%), followed by *hlb* gene 32 (82%) and 1(3%) isolates carry *tst* gene.

In the study by Gharsa *et al.* (2012) 50 *S. aureus* strains were isolated from nasal swabs of donkey. The *hla* and *hld* genes were found in 100% isolates while *hlb* gene was present in 70% isolates and *tst* gene was detected in the 12% isolates

A number of 34 *S. aureus* were isolated from bovine subclinical mastitis from Eastern China by Memon *et al. (*2013) for detection of various genes associated with virulence factors. They found that *hla* and *hlb* genes were detected in 85% and 71% of the isolates, respectively.

Alfatemi *et al.* (2014) isolated 345 isolates of *S. aureus* from clinical specimens of patients referred to teaching hospitals of Shiraz. The frequency of the *hla, hld* and *tst* genes were 93.15%, 84.24% and 11.64% respectively. The greatest coexistence of genes was observed for the hla + *hld* gene combination (48.83%).

Kim and Lee (2015) studied 41 *Staphylococcus aureus* isolated from periodontitis patients. The frequency of toxin genes were reported as 41 (100%), 40 (98%) and 4 (10%) for *hld, hla* and *hlb* gene respectively.

Xu *et al.* (2015) studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During the study the *hla* and *hlb* was found in 27 (96.4%) and 26 (92.9) isolates respectively. But none of the isolates carry super antigen *tsst-1* gene responsible for Toxic shock syndrome toxin-1.

Tkalec *et al.* (2015) isolated 47 *Staphylococcus aureus* strains from mastitic milk of cows and found that all (100%) strains were positive for *hla* and *hld* genes followed by 35 strains (74.5 %) had the *hlb* gene and ten strains (21.3 %) were positive for *tst* gene.

Yang *et al.* (2015) studied 37 strains of *S. aureus* resistant to penicillin obtained from bovine mastitis. Their investigation found frequency of *tst, hla, hlb,* and *hld* gene as 5.4%, 70.3%, 73.0%, and 70.3% respectively.

Oliveira *et al.* (2015) studied a 123 *S. aureus* isolates obtained from hospitalized patients. Total 112 (91.1%) isolates were positive for *hlb* and *hld* gene while 16 (13%) and 42 (34.1%) isolates were positive for *hla* and *tst* genes respectively.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

1. Agar-agar type 1 (SRL)
2. Agarose, for molecular biology (SRL)
3. *Alu*I restriction endonuclease (Promega)
4. Barium chloride (Glaxo Lab. Chemical division)
5. Basic fuchsin
6. Beef extract (Titan biotech)
7. Boric acid, Mol. Biology grade (SRL)
8. Bromocresol purple solution(0.05%w/v)(Sarabhai Chemicals)
9. Bromophenol blue (s.d. Fine Chemical Pvt. Ltd.)
10. Bromothymol blue - pH indicator, powder (BDH)
11. Buffer tablets, pH 7.0 (Glaxo Lab., Fine Chemicals)
12. Cefoxitin supplement (FD259)
13. Cetyl trimethyl ammonium bromide (SRL)
14. Chloroform (Qualigens Fine Chemicals, Glaxo Lab.)
15. Citric acid (Merck)
16. Crystal violet (Glaxo Lab., Chemical Division)
17. Dipotassium hydrogen phosphate (Central Drug House Pvt. Ltd.)
18. Dipotassium phosphate (Glaxo)
19. EDTA 99% (Sigma)
20. Ethidium bromide (SRL)
21. Ethyl alcohol (Jai Chemical & Pharma Works)
22. Ethylene diamine tetra acetic acid disodium salt (EDTA)
23. Formaldehyde (Qualigens Fine Chemicals, Glaxo Lab.)
24. Formamide (Hi-Media)
25. Go Taq® Green Master Mix (M7123) (Promega)
26. Glacial acetic acid (Qualigens Fine Chemicals, Glaxo Lab.)

27. Glycerol (Hi- Media)
28. D-Glucose (Sarabhai M. Chemicals)
29. *Hinf*I restriction endonuclease (Promega)
30. Hydrochloric acid (Qualigens Fine Chemicals, Glaxo Lab.)
31. Hydrogen peroxide 3 per cent (BDH)
32. Isoamyl alcohol (Merck)
33. Lysozyme (SRL)
34. Magnesium chloride (Bangalore Genei)
35. Mannitol salt agar (Sarabhai M. Chemicals)
36. D-Mannitol, G.R. (Hi-Media)
37. β-Mercaptoethanol (Sigma)
38. MeReSa Selective Supplement (FD229)
39. Methanol (Qualigens Fine Chemicals – Glaxo division)
40. Molecular marker, 20bp DNA ladder (Bangalore Genei)
41. Molecular marker 50 bp DNA ladder (Promega)
42. Molecular marker 100 bp DNA ladder (Promega)
43. Molecular marker 500 bp DNA ladder (Promega)
44. Molecular marker 1kb DNA ladder (Promega)
45. Mueller-Hinton agar medium (Hi-media)
46. α-naphthol
47. Normal saline solution
48. Peptone - Bacteriological (s.d. Fine. Chemical Pvt. Ltd.)
49. Phenol (Qualigens Fine Chemicals, Glaxo Lab.)
50. Phenol red – pH indicator powder (s.d. Fine. Chemical Pvt. Ltd.)
51. Potassium Hydroxide (s.d. Fine Chemical Pvt. Ltd.)
52. Primers (Table No.-3) (Sigma)
53. Propan-2-ol (Qualigens Fine Chemicals, Glaxo Lab.)
54. Proteinase K (SRL)
55. Safranine (s.d. Fine Chemical Pvt. Ltd.)
56. Sodium carbonate (Hi-Media)
57. Sodium hydroxide (1N) solution

58. Sodium chloride (Glaxo Lab. Chemical Division)
59. Sodium citrate (British drug houses, Lab. Chemical Division)
60. Sodium hydroxide pellets (CDH)
61. Sodium perchlorate (1M)
62. Sulphuric acid (Qualigens Fine chemical, Glaxo Lab.)
63. N'N'N'N'-tetramethyl-p-Phenylenediaminedihydrochloride (Himedia)
64. Tris (Hydroxymethylaminomethane) (SRL)
65. Xylene cyanol (SRL)

3.1.2 Other material

1. Plasmas from different animal species such as sheep, goat, poultry, cattle, camel and human for coagulase test.
2. Sheep blood for blood agar
3. Antibiotic discs (Table No.-1, Hi-Media)
4. MIC strip of mentioned antibiotics (Table No.-2, Hi-Media)
5. Cattle and Horse erythrocyte

3.1.3 Stains

1. Gram's stain

3.1.4 Bacteriological Media

(a) Nutrient agar medium

Following ingredients were dissolved in one litre of double glass distilled water to prepare the medium:

Peptone	10.0 g
Beef extract	5.0 g
Sodium chloride	5.0 g

The pH of the medium was adjusted to 7.2 and agar-agar type 1 was added at the rate of 2% and it was autoclaved at 121^{o}C at 15 lb pressure for 15 min. The prepared medium was then dispersed in glass petridishes and stored in refrigerator at 4^{o}C. The plates were incubated for 24 h to check the sterility before storing in the refrigerator.

(b) Sheep Blood agar medium

Nutrient agar medium was prepared and autoclaved as above to prepare blood agar. Sheep blood collected aseptically was mixed well in the nutrient agar at the rate of 5% (vol/vol) after the temperature of the medium was brought down to 50^{o}C. The medium was then distributed in sterilized petri plates.

3.1.5 Media used for metabolic and biochemical reactions

(a) Mannitol salt agar medium

The following ingredients were mixed and dissolved in 700 ml of glass water:

Peptone	10.0 g
Beef extract	1.0 g
Sodium chloride	75.0 g
Phenol red	0.025 g

The pH of the medium was adjusted to 7.4. Agar-agar type 1 was added at the rate of 2% for final volume and volume of the medium was made to 800 ml with distilled water, then autoclaved at 121^{o}C for 15 min. A 200 ml of 5% D-mannitol solution prepared in glass distilled water and sterilized separately at 10 lbs pressure for 15 min, was added to the basal medium before pouring in sterilized petri dishes.

(b) Medium for haemolysis

To study the pattern of haemolysis sheep blood agar medium was prepared.

(c) Medium for toxin production (Semi-solid agar medium)

The medium was prepared by dissolving the following in 800 ml of double glass distilled water:

Peptone	10.0 g
Sodium chloride	5.0 g
Beef extract	10.0 g

The pH of the medium was adjusted to 7.2 and agar-agar type 1 was added at the rate of 0.75% for final volume of medium. The volume was made to one litre and then autoclaved at 121^{o}C for 20 min. After sterilization, it was allowed to cool down to 45^{o}C in a water bath before pouring into the sterile petri-dishes.

(d) Mueller-Hinton agar medium

Readymade Mueller-Hinton agar medium used for antibiotic sensitivity tests and MIC pattern, was prepared as per the instructions of manufacturer and autoclaved at 121^{o}C for 15 min, distributed in sterile petridishes and stored in refrigerator at 4^{o}C until used.

(e) Medium for slime production (Congo red agar)

The medium was prepared by dissolving the following in 800 ml of double glass distilled water:

Blood Agar Base 2	40.0 g

Glucose/Dextrose	10.0 g
Congored dye	0.4 g

The pH of the medium was adjusted to 7.2 and agar-agar type 1 was added at the rate of 2% for final volume of medium. The volume was made to one litre and then autoclaved at 121^{o}C for 20 min. After sterilization, it was allowed to cool down to 45^{o}C in a water bath before pouring into the sterile petri-dishes.

(f) MeReSa Agar Base

Readymade medium supplied by Hi-Media with following ingredients was used

Casein enzymic hydrolysate	10.0 g/l
Meat extract	5.0 g/l
Glycine	10.0 g/l
Sodium pyruvate	10.0 g/l
Lithium chloride	5.0 g/l
Mannitol	10.0 g/l
Sodium chloride	10.0 g/l
Indicator mixture	0.13 g/l
Agar	20.0 g/l
Final pH (at 25°C)	7.1±0.2

In 500ml distilled water 40.06 grams of medium was suspended and then heated medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 45-50°C and aseptically added sterile rehydrated contents of 1 vial of MeReSa selective supplement (FD229) and Cefoxitin supplement (FD259) both in combination for more selectivity. Mixed well and poured into sterile petri plates.

3.1.6 Antibiotic discs (Hi-Media)

In the present investigation various categories of antibiotics were used in order to study the antibiogram for *S. aureus* isolates as described below:

i. **Aminoglycosides:** Gentamicin and Tobramycin

ii. **Beta-lactam antibiotics:**

a. Beta-lactamase resistant penicillins - cloxacillin, methicillin and oxacillin

b. Broad spectrum penicillins- ampicillin and piperacillin

c. Narrow spectrum penicillins- penicillin G

d. Carbapenems- imipenem, faropenem and meropenem

e. Monobactams- aztreonam

f. Carboxypenicillin- ticarcillin

iii. **Cephalosporins:**
    a. First generation- cefalothin and cefzolin
    b. Second generation- cefaclor and cefoxitin
    c. Third generation- cefotaxime, ceftriaxone, cefoparazone, ceftazidime and cefixime
    d. Fourth generation- cefepime

iv. **Chloramphenicol**

v. **Fluoroquinolones:**
    a. Second generation- ciprofloxacin and norfloxacin
    b. New generation fluoroquinolones- levofloxacin

vi. **Glycopeptides:** vancomycin

vii. **Macrolides:** azithromycin and clindamycin

viii. **Nitrofurans:** nitrofurantoin

ix. **Oxazolidinone:** linezolid

x. **Polypeptide:** polymxin-B

xi. **Tetracyclines:** tetracycline and doxycycline hydrochloride

xii. **Antibiotics in combination:**
    a. ampicillin + sulbactum
    b. cefixime + clavulanic acid
    c. cefoparazone + sulbactam
    d. cefotaxime + clavulanic acid
    e. ceftazidime + clavulanic acid
    f. piperacillin + tazobactam
    g. ticarcillin + clavulanic acid

The antibiotic discs mentioned in (Table 1) were used for the determination of antibiogram against *S. aureus* isolates. The concentration of antibiotic per disc is given along with. For all the antibiotics concentration is given in mcg except for bacitracin and polymyxin B for which concentration is expressed in units.

Table 1: List of antibiotics used for antibiogram study against *S. aureus* isolates obtained in the present study.

3.1.7 Multiple Antibiotic Resistance Index (MAR) value

All Multidrug resistant isolates were evaluated for their Multiple Antibiotic Resistance (MAR) index. In an effort for risk assessment of MDR isolates this index was calculated as per method given by Krumperman, (1983).

MAR Index of single isolate = a/b, where a -represents the number of antibiotics to which the isolate was resistant and b -represents the number of antibiotics to which the isolate was exposed.

Group MAR index value = a/b*c), where a is the aggregate antibiotic resistance score of all isolates in the group, b is the number of antibiotics to which the isolate was exposed and c is the total number of isolates in the group

3.1.8 Antibiotic Ezy MIC™ Strip (Hi-Media)

The antibiotic Ezy MIC™ Strip determination of minimum inhibitory concentrations. The concentration of antibiotic per strip is given along with. For all the antibiotics concentration is given in mcg/ml.

Table 2: List of antibiotics Ezy MIC™ strips used to determine minimum inhibitory concentration (MIC) against *S. aureus* isolates.

S. No	Antibiotic/Symbol (interpretation zone in mm- R/I/S)	Disc content (mcg)	S. No	Antibiotic/Symbol (interpretation zone in mm- R/I/S)	Disc content (mcg)
1.	Ampicillin/AMP^{10}(28/-/29)	10	22.	Cloxacillin/COX^{10} (23/24-33/34)	10
2.	Ampicillin + Sulbactum, / A/S$^{10/10}$ (11/12-14/15)	10/10	23.	Doxycycline Hydrochloride/ DO^{30} (12/13-15/16)	30
3.	Azithromycin, AZM^{15} (13/14-17/18)	15	24.	Faropenem/ FAR^{5} (27/-/34)	5
4.	Aztreonam, AT^{30} (17/18-20/21)	30	25.	Gentamicin/ GEN^{10} (12/13-14/15)	10
5.	Cefaclor/ CF^{30} (14/15-17/18)	30	26.	Imipenem/ IPM^{10} (13/14-15/16)	10
5.	Cefalothin/CEP^{30} (14/15-17/18)	30	27.	Levofloxacin/ LE^{5} (15/16-18/19)	5
7.	Cefepime/CPM^{30} (14/15-17/18)	30	28.	Linezolid/ LZ^{15} (20/-/21)	15
8.	Cefixime/CFM^{5} (15/16-18/19)	5	29.	Meropenem/ MRP^{10} (13/14-15/16)	10
9.	Cefixime + Clavulanic Acid/ CMC$^{5/10}$ (15/16-18/19)	5/10	30.	Methicillin/ MET^{5} (9/10-13/14)	5
10.	Cefoparazone/CPZ^{75} (15/16-20/21)	75	31.	Nitrofurantoin (NIT^{300}) 14/15-16/17	300
11.	Cefoparazone + Sulbactam /CFS$^{75/10}$ (23/-/30)	75/10	32.	Norfloxacin/ NX^{10} (12/13-16/17)	10
12.	Cefotaxime/CTX^{30} (14/15-22/23)	30	33.	Oxacillin/ OX (10/11-12/13)	1
13.	Cefotaxime + Clavulanic Acid CEC$^{30/10}$ 18/-/22	30/10	34.	Penicillin –G/ P^{10}U) (28/-/29)	10 unit
14.	Cefoxitin/CX^{30} (21/-/22)	30	35.	Piperacillin/ PI^{100} (17/18-20/21)	100
15.	Ceftazidime (CAZ^{30}) 14/15-17/18	30	36.	Piperacillin + Tazobactam/ PIT$^{100/10}$ (17/-/18)	100/10
16.	Ceftazidime + Clavulanic Acid/ CAC$^{30/10}$ (18/-/22)	30/10	37.	Polymxin –B (PB^{300}U) (11/-/12)	300 unit
17.	Ceftriaxone/CTR^{30} (13/14-20/21)	30	38.	Tetracycline/ TE^{30} (14/15-18/19)	30
18.	Cefzolin/CZ^{30} (14/15-17/18)	30	39.	Ticarcillin (TI^{75}) (14/15-19/20)	75
19.	Chloramphenicol/C^{30} (12/13-17/18)	30	40.	Ticarcillin + Clavulanic Acid/ TCC$^{75/10}$ (22/-/23)	75/10
20.	Ciprofloxacin/CIP^{5} (15/16-20/21)	5	41.	Tobramycin/ TOB^{10} (12/13-14/15)	10
21.	Clindamycin/CD^{2} (14/15-20/21)	2	42.	Vancomycin/ VA^{30} (14/15-16/17)	30

S. No.	Antibiotics Ezy MIC™ strips (Symbol)	Strip content (mcg/ml)	Interpretative criteria in mcg conc. (S/I/R)
1.	Azithromycin (AZI)	0.016-256	<2/ 4/ >8
2.	Ceftriaxone (CTR)	0.016-256	<8/ 16-32/ >64
3.	Chloramphenicol (CHL)	0.016-256	<2/ 4-8/ >16
4.	Ciprofloxacin (CIP)	0.002-32	<1/ 2/ >4
5.	Gentamicin (HLG)	0.064-1024	<4/ 8/ >16
6.	Oxacillin (OXA)	0.016-256	<2/ -/ >4
7.	Penicillin (PEN)	0.002-32	<0.12/ -/ >0.25
8.	Vancomycin (VAN)	0.016-256	<2/ 4-8/ >16

3.1.9 Animals and humans for blood collection

For separation of erythrocytes, the blood was collected from cattle and horse, for blood agar preparation from sheep and for coagulase tube test, blood was collected from following species for plasma separation:

Cattle: Blood was collected from two healthy cows of non-descript breed.
Sheep: Blood was collected from healthy sheep brought to slaughter house.
Goat: Blood was collected from two healthy goat brought to slaughter house.
Camel: Blood was collected from two healthy camels.
Chicken: Blood was collected from two poultry birds before slaughtering.
Human: Blood was collected from three human volunteers.

3.1.10 Primer sets (Merck):

Following primers and their conditions were used in the present investigation

Table 3: Detail of primers used in the present study.

S. No.	Gene	Forward primer sequence (5´ to 3´)	Size	Annealing Temp. (°C)	Reference
1.	*23S*	F-5'-ACG GAG TTA CAA AGG ACG AC-3'	1250bp	55°C	(Straub *et al.*, 1999)

	rRNA	R-5'-AGC TCA GCC TTA ACG AGT AC-3'			
2.	*rep PCR*	5'-TCG CTC AAA ACA ACG ACA CC- 3'	Variable	37°C	(van Belkum *et al.*, 1995)
3.	*clfA*	F-5'-GGC TTC AGT GCT TGT AGG-3' R-5'-TTT TCA GGG TCA ATA TAA GC-3'	1000bp	52°C	(Stephan *et al.*, 2001)
4.	*clfB*	F-5'-ACA TCA GTA ATA GTA GGG GGC AAC-3' R-5'-TTC GCA CTG TTT GTG TTT GCA C-3'	205bp	55°C	(Tristan *et al.*, 2003)
5.	*icaD*	F-5'-AAA CGT AAG AGA GGT GG-3' R-5'-GGC AAT ATG ATC AAG ATA C-3'	381bp	49°C	(Vasudevan *et al.*, 2003)
6.	*icaA*	F-5'-CCT AAC TAA CGA AAG GTA G-3' R-5'-AAG ATA TAG CGA TAA GTG C-3'	1315bp	49°C	(Vasudevan *et al.*, 2003)
7.	*trap*	F-5'-ACA TAA GGG GGA CCT TTC AT-3' R-5'-ACC AAT GGA AGT TTT CTT CG-3'	504	52°C	(Gilot *et al.*, 2002)
8.	*agr-F*	F-5'-ATG CAC ATG GTG CAC ATG C-3'		55°C	(Gilot *et al.*, 2002)
	*agr*I	R-5'-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3'	441bp		
	*agr*II	R-5'-GTC ACA AGT ACT ATA AGC TGC GAT-3'	575bp		
	*agr*III	R-5'-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3'	323bp		
	*agr*IV	R-5'-CGA TAA TGC CGT AAT ACC CG-3'	659bp		
9.	*cap5K*	F-5'-GTC AAA GAT TAT GTG ATG CTA CTG AG-3' F-5'-ACT TCG AAT ATA AAC TTG AAT CAA TGT TAT ACA G-3'	361bp	55°C	(Verdier *et al.*, 2007)
10.	*cap8K*	F-5'-GCC TTA TGT TAG GTG ATA AAC C-3' R-5'-GGA AAA ACA CTA TCA TAG CAG G-3'	173bp		(Verdier *et al.*, 2007)
11.	*coa*	F-5'-ATA GAG ATG CTG GTA CAG G-3' R-5'GCT TCC GAT TGT TCG ATG C-3'	Variable	57°C	(Hookey *et al.*, 1998)
12.	*aur*	F-5'-GTG AGG AAA TTT TCA AGA TAT GC-3' R-5'-CCA CGC CTA CTT CAT TCC ATG C-3'	1526bp	51°C	(Zdzalik *et al.*, 2012)
13.	*sak*	F-5'-TGA GGT AAG TGC ATC AAG TTC A-3' R-5'-CCT TTG TAA TTA AGT TGA ATC CAG G-3'	403bp	55°C	(Sung *et al.*, 2008)
14.	*spa- X*	F-5'-CAA GCA CCA AAA GAG GAA-3' R-5'-CAC CAG GTT TAA CGA CAT-3'	Variable	55°C	(Frenay *et al.*, 1996)
15.	*spa-IgG*	F-5'-CAC CTG CTG CAA ATG CTG CG-3' R-5'-GGC TTG TTG TTG TCT TCC TC-3'	Variable	58°C	(Seki *et al.*, 1998)
16.	*scn*	F-5'-ATA CTT GCG GGA ACT TTA GCA A-3' R-5'-TTT TAG TGC TTC GTC AAT TTC G-3'	320bp	55°C	(Sung *et al.*, 2008)
17.	*chp*	F-5'-TTT TTA ACG GCA GGA ATC AGT A-3' R-5'-TGC ATA TTC ATT AGT TTT TCC AGG-3'	404bp	55°C	(Sung *et al.*, 2008)
18.	*hla*	F-5' GGT TTA GCC TGG CCT TC 3' R-5' CAT CAC GAA CTC GTT CG 3'	534 bp	53 °C	(Booth *et al.*, 2001)
19.	*hlb*	F-5' GCC AAA GCC GAA TCT AAG 3' R-5' CGC ATA TAC ATC CCA TGG C 3'	833 bp	53 °C	(Booth *et al.*, 2001)
20	*hld*	F-5'- AAG AAT TTT TAT CTT AAT TAA GGA AGG AGT G-3' R-5'-TTA GTG AAT TTG TTC ACT GTG TCG A-3'	111bp	55°C	(Jarraud *et al.*, 2002)
21.	*tst*	F-5'-ATG GCA GCA TCA GCT TGA TA-3' R-5'-TTT CCA ATA ACC ACC CGT TT-3'	350bp	55°C	(Johnson *et al.*, 1991)
22.	*mecA*	F-5'-AAA ATC GAT GGT AAA GGT TGG C-3'	533bp	52°C	(Murakami *et al.*, 1991)

		R-5'-AGT TCT GCA GTA CCG GAT TTT GC-3'			
23.	*blaZ*	F-5'-AAG AGA TTT GCC TAT GCT TC-3' R-5'-GCT TGA CCA CTT TTA TCA GC-3'	517bp	50°C	(Sawant *et al.*, 2009)

3.2 METHODS

3.2.1. Collection of samples

A total of 517 clinical and non-clinical samples from human, meat and various animal sources were collected as describe in Table 4. These sample sources belonged to two different localities *viz*. Bikaner (Rajasthan) and Rewari (Haryana). The samples were collected in the morning and were immediately taken thereafter to the laboratory on ice for further processing.

3.2.2. Isolation and identification of bacteria

Each sample was swabed on nutrient agar media and then allowed to incubate overnight at 37ºC. Next day bacterial colonies were closely observed for their morphology, color and consistency. Gram's staining was used as primary identification test and suspected colonies were streaked on mannitol salt agar in primary, secondary and tertiary fashion in order to obtain isolated colonies of bacteria. These Petri plates were incubated for 24 h at 37^{o}C for aerobic cultivation. Next day the growth was examined for the colonial morphology and pigmentation and different types of colonies were sub-cultured on separate plates in order to obtain pure cultures.

The smears prepared from each of the sub-cultured colony were fixed by gentle heat, stained by Gram's-method and examined under oil immersion in order to check the purity of the cultures. After ascertaining the pure growth, the bacterial isolates were allocated code numbers accordingly and the colonies were transferred to paired nutrient slants. One of the slants was kept under refrigeration at 4^{o}C after proper sealing with paraffin wax and the other was used for various tests. The organisms were isolated and identified as described by Buchanan and Gibbons (1974), Cowan and Steel (1975) and Quinn *et al*. (2000).

3.2.3. Tests for Generic identification of Bacteria

(a) Growth in air

Growth in air was studied to confirm the ability of bacterial isolates to grow under aerobic conditions.

(b) Gram's reaction

Smears prepared from young culture of bacterial isolates were stained by modified Gram's Method of staining.

(d) Morphology

Morphology of bacterial isolates was recorded after staining by Gram's-method.

(e) Motility

Six hour old broth culture of bacterial isolates incubated at 37^{o}C was examined in the hanging drop preparation under 40 x magnifications to examine bacterial motility.

(f) Catalase activity (Quinn *et al*., 2000)

A loopful of young culture of bacterial isolate was mixed with a drop of 3% hydrogen peroxide over a clean glass slide. The production of gas bubbles or any effervescence within a few seconds was considered as catalase positive and absence of gas bubbles as catalase negative.

(g) Oxidase test (Faller and Schleifer, 1981)

Immediately after placing one drop of oxidase reagent on a filter paper, a loopful of test bacterial culture was rubbed against the wet surface. Appearance of dark purple colour within few seconds was noted as oxidase positive. Absence of colour or delay in appearance of any colour was considered as negative.

3.2.4. Metabolic and biochemical reactions (Secondary tests)

(a) Mannitol fermentation

In order to see the fermentation of mannitol, the mannitol salt agar (MSA) plates were streaked with the test culture, incubated for 24-48 h and observed for the change in colour of the medium. The mannitol fermenting organisms changed the colour of medium to yellow, whereas non-fermenting organisms did not.

3.2.5. Phenotypic characterization of *Staphylococcus aureus*

(a) Coagulase production

The test was carried out in sterile tubes for production of free enzyme using plasmas from different animal species *viz.* catttle, sheep, goat, chicken, camel and human. Required blood was collected aseptically in anticoagulant coated test tube. It was centrifuged at 2500 rpm for 15-20 min and top layer of clear plasma was obtained which was transferred into another sterilized test tube for use in test.

The plasma was diluted to 1:10 in physiological saline solution and 0.5 ml of reconstituted plasma was taken in three serological tubes. A 0.1 ml of an overnight broth culture of the *S. aureus* was added to one tube and 0.1 ml of broth culture of *S. epidermidis* was added to second tube (negative control) and remaining third un-inoculated tube was kept as control. The tubes were rotated gently to mix the contents and incubated in water bath at 37^{o}C. By slowly tilting the tube at 90^{o} angle, the tubes were examined at 5h. and compared with the control tube. Clotting of plasma within 5 h was recorded as a positive for "free" coagulase enzyme.

The results for coagulation of plasma from different animal species and human were also recorded for comparison for relative suitability of plasma in this test.

(b) Haemolysis production

Sheep blood agar medium was used to test haemolysis properties by bacterial isolates and the reactions were categorized as complete-haemolysis, partial-haemolysis, both or non haemolysis.

(c) Toxins of *S. aureus*

The method described by Sanjiv and Kataria (2007) was used in order to type and titrate the toxins.

Toxin Production

The test culture suspension (about 1-2 ml, 24 h old) was poured, spread well onto surface of semisolid nutrient agar plate and then plates were incubated at 37^{o}C in an atmosphere of 20% carbon dioxide tension for 48 h in carbon dioxide incubator. Following incubation, the agar medium was sliced into small pieces and the plates were then transferred to deep freezer at -20^{o}C for 30 min. Alternate freezing and thawing was carried out to obtain the fluid from culture. It was then centrifuged at 4000 rpm in refrigerated centrifuge machine for 45 min. The supernatant having the toxin was collected in screw capped test tubes and was stored at -20^{o}C in deep freezer till use for titration of beta and delta haemolysins.

Erythrocyte Preparation

Blood from cattle and horse was collected aseptically in double the amount of Alsever's solution and kept in refrigerator for 3 days for stabilization of erythrocytes. After 3 days the suspension was centrifuged at 500 rpm for 5 min and supernatant was removed. The erythrocytes were washed with normal saline solution three times and finally were suspended in normal saline solution to make 1% suspension. The cattle and horse erythrocytes were used for titration of beta and delta haemolysins, respectively.

Beta haemolysin titration

To titrate beta haemolysin double fold serial dilutions of extracted haemolysin starting from 1:5 to 1:2560 were prepared in 0.1 ml amounts. The washed 1% cattle erythrocyte suspension in normal saline solution was added in 0.1 ml amount to various dilutions of the toxin. The serological tubes were initially incubated at 37^{o}C for 2 h in a water bath and then examined for haemolytic activity. The record of the end point at 50% level was made by visual reading.

After the primary reading, the serological tubes were kept in refrigerator at 4^{o}C for 24 h. The tubes were again examined and the highest dilution showing 50% lysis was recorded as the end titre of the haemolysin. The difference between the second and initial reading was taken as final haemolysin titre.

Delta haemolysin titration

To titrate delta haemolysin double fold serial dilutions of the toxin filtrate starting from 1:5 to 1:2560 was made in normal saline in 0.1 ml quantity in serological tubes and then 1% washed horse erythrocyte suspension in 0.5 ml amount was added to each dilution. All the serological tubes were incubated at 37^{o}C in a water bath for 2 h. After incubation, tubes were examined and the highest dilution showing 50% lysis was recorded as the end titre of the haemolysis on visual reading. The horse erythrocyte saline control was also run parallel to the test.

(d) Antibiotic sensitivity test

To determine the antibiogram of the isolates against different antibiotics the method of Bauer *et al*. (1966) was followed. In brief, the isolates were inoculated in sterile 5 ml nutrient broth tube, incubated for 18 h at 37^{o}C and then the opacity was adjusted to 0.5 McFarland opacity standards (Quinn *et al*., 2000). The inoculum was well spread over the agar surface with the help of sterilized swab. Plates were allowed to dry for 10 min at 37^{o}C and then antibiotic discs were carefully placed on the surface with enough space around each disc for diffusion of the antibiotic. Plates were incubated for 24 h at 37^{o}C and the zone of inhibition of growth of the organism around each disc was measured in millimeters and compared with standard chart provided by disc manufacturer.

(e) Minimum inhibitory concentration (Ezy MIC™ Strip method)

The Mueller Hinton Agar medium in petri plates was streaked with the test inoculum. The Ezy MIC™ strip taken out from freeze was kept at room temperature for 30 minutes before placing it on the medium. The strip was not repositioned after placed. Inoculated plates were incubated for 24 h at 37^{o}C.

The result was read where the ellipse intersected the MIC scale on the strip after incubation. Since Ezy MIC™ strip has continuous gradient, MIC values "in-between" two fold dilutions can be obtained. If the ellipse intersects the strip in between 2 dilutions, read the MIC as the value which is nearest to the zone.

Note:- In the present study sometime isolate showed complete resistant to Ezy MIC™ strip, in that condition highest concentration of Ezy MIC™ strip took in statistical calculations.

(f) Beta-lactamase activity (Acidimetric method)

Hydrolysis of the β-lactam ring generates a carboxyl group, acidifying unbuffered systems. The resulting acidity can be tested in tubes. The method described by Livermore and Brown (2001) was used in which 2 ml of 0.5% (w/v) aqueous phenol red solution was diluted with 16.6ml distilled water, and 1.2g of benzylpenicillin was added to it. The pH was adjusted to 8.5 with 1M NaOH. The resulting solution (violet in color) was stored at -20^{o}C. Before use, 100µl portions was distributed into microtitre wells and inoculated with bacteria from culture paltes (not broth) to produce dense suspensions. A yellow color within 5 min indicated β-lactamase activity. Positive and negative controls were run in parallel.

(g) Methicillin resistant *S. aureus* (MRSA) Activity

Methicillin resistant *S. aureus* (MRSA) activity among *S. aureus* isolates was detected by two methods (i) MeReSa Agar Base method and (ii) Methicillin disc method

(i) MeReSa Agar Base method: This method included observation of colony growth on MeReSe agar base with MeReSa selective supplement (FD229) and Cefoxitin supplement (FD259). After inoculation of testing isolate, the MRSA positive strain grew as luxuriant greenish pink colour colony after an incubation at 35-37°C for 18-48 hours.

(ii) Methicillin disc method: During antibiotic sensitivity test, isolates with less than 9 mm diameter of inhibition zone for 5 mcg methicillin disc were considered as MRSA strains.

(h) Extended spectrum beta-lactamase (ESBL) Activity

Extended spectrum beta-lactamase activity among *S. aureus* isolates was detected by combined disc method as described by Livermore and Brown (2001). This method included comparing the zone given by discs containing an extended- spectrum cephalosporin with and without clavulanic acid. If an ESBL is produced, the zones

are increases ≥5mm for the discs containing clavulanic acid. This method recommends comparison of the zone given by cefotaxime 30µg versus cefotaxime 30 +clavulanic acid 10µg and ceftazidime 30µg versus œftazidime 30 +clavulanic acid 10µg. The readymade (HiMedia) discs were used for this test as described.

(i) Vancomycin resistant *S. aureus* (VRSA) activity

Vancomycin resistant *S. aureus* determine by antibiotic disc method. In disc method isolates, with less than 14 mm diameter of inhibition zone for 30 mcg vancomycin disc was considered phenotypically as VRSA strains.

3.2.6 Genotypic characterization of *Staphylococcus aureus*

(a) 23S rRNA gene based genotypic identification

The ribotyping/ genotypic confirmation through 23S rRNA was carried out as per the method described by Straub *et al*. (1999). The following sequences for the two primers were used:

Primer F – 5' ACG GAG TTA CAA AGG ACG AC 3'

Primer R – 5' AGC TCA GCC TTA ACG AGT AC 3'

Preparation of bacterial cell culture

a. Bacterial culture was grown overnight in 25 ml nutrient broth in shaker incubator at 37^{o}C.
b. The bacterial culture was then centrifuged at 5000 rpm to obtain pellet and supernatant was discarded.

Isolation of DNA

DNA isolation was carried out as per the method of Nachimuttu *et al.* (2001) with some modifications.

a. Bacterial cultures were pelleted and washed two times with PBS.
b. Final cell pellet was re-suspended in 1 ml of Tris-EDTA (TE) solution.
c. One hundred µl of lysozyme solution (conc. 3 mg/ml) was added and mixture was then incubated at 37^{o}C in water bath for 15 min.
d. One hundred fifty µl of 10% SDS solution and 2 µl of Proteinase K (10 mg/ml) solution was added and incubated at 60^{o}C in water bath for 1 h with gentle mixing of the contents at every 10 min interval.
e. After 1 h of incubation 0.75 ml of DNA extraction buffer was added and further incubated for 30 min at 60^{o}C in water bath.
f. Chloroform: Isoamyl alcohol mixture (24:1) was prepared and 0.5 ml of it was added to the DNA preparation and mixed gently for about 10-15 min.
g. Above mixture was centrifuged at 15000 rpm for 15 min at 20^{o}C and upper aqueous phase containing DNA was transferred to another tube.
h. To this mixture 0.5 ml of cold isopropanol was added and the tube was replaced in ice for 15 min and then centrifuged at 15000 rpm for 10 min at 20^{o}C.
i. The supernatant was discarded and the pellet was dissolved in 0.5 ml of cold 70% ethanol and centrifuged at 10000 rpm for 10 min at 20^{o}C.
j. Supernatant was discarded and the tubes were inverted on a filter paper for 5 min. The tubes were then covered with parafilm with a small hole in it and left at room temperature for overnight to allow evaporation of alcohol
k. Next morning the pellet was redissolved in 50 µl of TE buffer and left for 24 h for dissolution of the pellet.

Agarose gel electrophoresis

To check the integrity of DNA, agarose gel electrophoresis was carried out in a horizontal, submerged electrophoresis unit. Each DNA sample was mixed with one-fifth volume of the gel loading buffer. For electrophoresis, 0.8 per cent agarose gel in TBE buffer containing ethidium bromide (0.5-1.0 µg) was used. Uncut λ DNA was used as a molecular marker.

Each sample was loaded in the well of gel after adding trekking dye (bromophenol blue), and electrophoresis was carried out at 100 V at room temperature for about 1-2 h depending upon the length of the gel or till the dye migrated more than half of the length of the gel. At the end of the electrophoresis, the gel was visualized under UV transilluminator and photographs were obtained.

Quantification of DNA

DNA quantification was carried out in spectrophotometer in following steps:

a. By taking one ml TE buffer in a cuvette, the spectrophotometer was calibrated at 260 nm as well as at 280 nm wavelength.
b. In another cuvette 10 µl of DNA was added to 990 µl of TE buffer, genomic DNA was diluted to a final concentration of 25 ng/µl in TE buffer, mixed properly and optical density (OD) was recorded both at 260 and 280 nm and A_{260}/A_{280} ratio of around 1.9 (1.85-1.95) indicated best quality of DNA.
c. DNA concentration was estimated (Sambrook *et al.*, 1989) by using following formula :

$$\text{Amount of DNA (µg/µl)} = \frac{\text{(OD) } 260 \times 50 \times \text{dilution factor}}{1000}$$

d. Quantified DNA was diluted to a final concentration of 25 mg/µl in TE buffer.

Amplification of DNA

The reaction mixture (total volume 25.0 µl.) was prepared by mixing:

Go Taq® Green Master Mix, 2X	12.5 µl
Primer-F (25 pM/µl)	0.5 µl
Primer-R (25 pM/µl)	0.5 µl
DNA template	3.0 µl
Nuclease free water to make	25.0 µl

The PCR was performed in Veriti Thermal Cycler (Applied biosystem) using following cycling parameters:

Cycles	Step	Temperature (^oC)	Time
Cycle 1	Denaturation	94	5 min
	Primer annealing	55	30 s

	Primer extension	72	1.15 min
Cycle 2-37	Denaturation	94	40 s
	Primer annealing	55	1.00 min
	Primer extension	72	1.15 min
Cycle 38	Denaturation	94	1 min
	Primer annealing	55	1 min
	Primer extension	72	3 min

The PCR products were resolved in 1.2% agarose gels prepared in 1x TBE buffer containing 0.5 µg/ml of ethidium bromide and 1kb ladder was used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 V. The gel was then visualized under gel documentation system (ENDURO GDS).

(b) Antibiotic resistance genes (*MecA* and *blaZ*):

Antibiotic resistance gene (*mecA*) was detected by method of Murakami *et al.* (1991) and *blaZ* gene was detected in duplex PCR with *icaD* gene according to Sawant *et al.* (2009).

The reaction mixture for both resistance genes (total volume 25.0 µl.) was prepared by mixing:

Go Taq® Green Master Mix, 2X	12.5 µl
Primer-F (25 pM/µl)	0.5 µl
Primer-R (25 pM/µl)	0.5 µl
DNA template	3.0 µl
Nuclease free water to make	25.0 µl

DNA amplification of both gene consisted of a cycle of pre denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C denaturation, 1 min at annealing (Table 3) and 1 min at 72°C. A final extension step of 72ºC for 10 min was included. Amplified products were separated by electrophoresis in a 1.5% agarose gel in 1X TBE buffer at a constant voltage of 4 V/cm and stained using ethidium bromide (0.5 µg/ml).

(c) Molecular typing by rep PCR:

According to method of van Belkum *et al.* (1995) DNA amplification consisted of a cycle of pre denaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 93°C, 1.30 min at 37°C and 1 min at 72°C. A final extension step of 72ºC for 10 min was included. A negative and a positive control were also included. Each isolate was tested under the same conditions at least twice with the selected oligonucleotide (Table: 3). Amplified products were separated by electrophoresis in a 1.5% Metaphore- agarose gel in 1X TBE buffer at a constant voltage of 4 V/cm and stained using ethidium bromide (0.5 µg/ml). The PCR pattern analysis was performed as described by Reinoso *et al.* (2004).

(d) Virulence factors and their associated genes:

i. Adherence [*clfA, clfB, icaA, icaD, agr* (*agr*I, *agr*II, *agr*III and *agr*IV) and *trap* gene]
ii. Antiphagocytosis (*cap5K* and *cap8K* gene)
iii. Exoenzymes (*coa* and *aur* gene)
iv. Immune evasion (*spa-X, spa-IgG, chp* and *scn* gene)
v. Plasminogen activator/ Staphylokinase (*sak* gene)
vi. Toxins (*hla, hlb, hld* and *tst* gene)

According to primers, annealing temperature and reference mentioned in the Table 3 *clfA, icaA, trap, coa, aur, spa-X, spa-Ig, chp, hla, hlb, hld* and *tst* gene genes were amplified with simple PCR while *icaD* and *blaZ* in one duplex PCR and *cap5K* and *cap8K* amplified in another duplex PCR. The *scn, sak* and *clfB* were amplified in one multiplex PCR and *agr* genes were amplified in *agr* group specific multiplex PCR.

The reaction mixture for all above genes (total volume 25.0 µl.) was prepared by mixing:

Go Taq® Green Master Mix, 2X	12.5 µl
Primer-F (25 pM/µl)	0.5 µl
Primer-R (25 pM/µl)	0.5 µl
DNA template	3.0 µl
Nuclease free water to make	25.0 µl

DNA amplification of all these genes was consisted of a cycle of pre denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C denaturation, 1 min at corresponding annealing (Table 3) and 1 min at 72°C. A final extension step of 72ºC for 10 min was included. Amplified products were separated by electrophoresis in a 1.5% agarose gel in 1X TBE buffer at a constant voltage of 4 V/ cm and stained using ethidium bromide (0.5 µg/ml).

(e) Restriction Fragment Length Polymorphism (RFLP) of *coa* and *aur* gene product

Restriction fragment length polymorphism of PCR *coa* (Hookey *et al*., 1998) and *aur* (Sabat *et al.,* 2000) gene product digested by *Alu*I and *Hinf*I, respectively was carried out. The PCR product (10 µl) was added with nuclease free water (5 µl), 10x Buffer (2 µl) and *Alu*I and *Hinf*I (2 units, conc. of stock enzyme was 5 U/µl), mixed gently and incubated in water bath at 37°C for 3 h. After addition of 2 µl of trekking dye, the digest was resolved in 2% Metaphore agarose gel prepared in 1x TBE buffer containing 0.5 µg/ml of ethidium bromide and 100bp, 50bp and 20bp DNA ladder was used as molecular marker. The amplification products were electrophoresed for 1 h at 100 V. The gel was then visualized under gel documentation system (UVP GDS) and photographs were taken.

(f) Gene Sequencing of some selected isolates

Some of the representative isolates were selected for sequencing of genes *viz. spa-X, aur, tst, sak* and *mecA* to compare isolates of present study and that with the database at Genbank. For this, the PCR products were got sequenced commercially (DNA Sequencing Facility, Delhi University). The sequences obtained were analyzed

in the Gene tool software to build a complete sequence from forward and reverse sequences of each sample. The complete sequences were subjected to nucleotide BLAST (Basic Local Alignment Search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at the server of the National Centre for Biotechnology information (NCBI) to determine the similarity with the already prevalent gene sequences. The sequences were also aligned using Bioedit, Gene tool and Mega-6 version software to display the variations in the nucleotide sequences.

(g) Data Analysis:

All statistical analysis were carried out by IBM SPSS Advanced Statistics 20.0 version and The discriminatory power of the different typing system i.e. their ability to distinguish between unrelated strains was determined by the number of types defined by the test method and the relative frequency of their types. The numerical index of discrimination was calculated using the formula given by Hunter and Gaston (1988).

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

Where,

D = Discriminatory index, S = Total number of type used, nj = Number of strains belonging to jth type, N = Total number of strains.

4. RESULT AND DISCUSSION

Staphylococcus aureus is an important pathogen of various clinical and sub clinical infections in human causing pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, boils, furuncles, styes, impetigo, toxic shock syndrome and meningitis, and are often associated with hospitalized patients and in animals causing various conditions of which mastitis is the commonest condition, other condition being suppurative infections, septicemia, toxinoses, udder impetigo, endometritis, pyoderma, cystits, dermatitis, botryomycosis of mammary gland, arthritis, scirrhous cord, bumble foot and tick pyaemia. It is responsible for heavy socio economic losses to human society and dairy industry throughout the world. The organism is endowed with many virulence properties and is prone to swift changes in its antibiogram patterns and other properties. Therefore, an early and precise characterization of *S. aureus* based on accurate species identification is essential. Since some host specific factors and environmental factors may influence changes in biochemical and phenotypic properties of the organisms hence, modern diagnostic tools are preferentially required. Various DNA based technologies are being used for identification and characterization as they are less time consuming, precise, specific and sensitive. In the present study *S. aureus* isolates from various sources were investigated for their phenotypic properties, virulence associated genes and antibiogram patterns along with their resistance genes.

5. *Staphylococcus aureus* isolation and genotypic confirmation

In the present investigation, 517 samples were collected from clinical and non-clinical sources *viz.* clinical infections of human (80), meat pieces (80), horse wounds (17), pig nasal cavity (17), camel wounds (26), dog skin infections (23), clinical infections of sheep (35), mastitic milk of buffalos (85), mastitic milk of goats (90) and mastitic milk sample of cattle (70).The detail of sampling including sample number from each source and place, and recovery of *S. aureus* from each source along with percent recovery has been depicted in table 4. The overall prevalence of *S. aureus* from various human and animal origin was 30.3% (157/517) with highest prevalence in human samples. The genotypic confirmation of *S. aureus* was done by polymerase chain reaction using species-specific 23S rRNA based primers with 1250bp size amplicon (Fig.1) according to method of Straub *et al.* (1999). Similar genotypic method of *S. aureus* identification have been used by Sanjiv *et al.* (2008); Upadhyay *et al.* (2010b); and Rathore and Kataria (2012), Bhati *et al.* (2013) for the isolates from this study area successfully and Stephan *et al.* (2001); Salasia *et al.* (2004), Bhanderi *et al.* (2009), Momtaz *et al.* (2011), Ariyanti *et al.* (2011), Suleiman *et al.* (2012) and Vazquez *et al.* (2013) for *S. aureus* isolates from elsewhere.

Similar prevalence of *S. aureus* isolation was also reported by Yadav *et al.* (2015a) who found 32 (16 of each cattle and buffalo) *S. aureus* isolates from 89 mastitic milk samples (41 were from cattle and 48 were from buffalo) with similar method and same study area. Close to present study, Nathawat *et al.* (2013) determined 38.03% of *S. aureus* from71 mastitic milk samples of goat from same study area. Similarly, 38.8% prevalence was also reported by Khichar *et al.*, (2012) from cattle mastitic milk samples.

Momtaz *et al.* (2010) detected 23.88% *S. aureus* from bovine mastitis while Abd El-Hamid and Bendary (2013) were confirmed as *S. aureus* with 10.6 % recovery from animal and human clinical samples. Both these recovery rates are lower than present study.

These isolates were studied for their phenotypic properties including growth on different media, colony characteristics, haemolysis on blood agar, production of coagulase in tube test, slime production, production, typing and titration of haemolysins, antibiotic resistance pattern with determination of minimum inhibitory concentration (MIC) of some antibiotics including screening of MRSA, VRSA, beta-lactamase and ESBL activity. The *S. aureus* were also characterize with detection of *mecA* and *blaZ* gene including sequence analysis of *mecA* gene.

Molecular typing of *S. aureus* isolates done with *rep* PCR. Genetic differentiation of isolates conducted on the basis of virulence factors and their associated genes such as adherence factors (*clfA, clfB, icaA, icaD, agr* typing *(agr*I, *agr*II, *agr*IIIand *agr*IVgene*)* and *trap* gene), capsule as antiphagocytic factor (*cap5K* and *cap8K* gene), exoenzymes (*coa* and *aur* gene), immune evasion factor (*spa-X, spa-IgG, chp* and *scn* gene), staphylokinase as plasminogen activator factor (*sak* gene) and toxin secretory system (*hla, hlb, hld* and *tst* gene). Variations among the strains were detected by restriction fragment length polymorphism (RFLP) patterns obtained from *Alu*I digests of *coa* amplicons and *Hinf*I digest of *aur* amplicons and sequence analysis was also conducted for *spa-X*, *sak*, *aur* and *tst* genes.

Table 4: Detail of samples, sources of samples and recovery of *S. aureus* isolates from various sources.

S. No.	Total No. of samples	Place of sampling	Source of Sample	Isolate I.D.	No. of Isolate	Total No. of isolate (%)
1.	80	Hospitals and Laboratories , Bikaner	Human various wound infections	H5, H6, H7, H8 H11, H12, H13, H14, H16, H21, H25, H28, H29, H30, H31, H33, H34, H41, H45, H48	20	35 (43.75)
			Urinary tract infection	H1, H2, H22, H24, H37, H39, H44, H46	8	
			Post-operative infection	H3, H4,H9, H10, H15, H27, H40	7	
2.	80	Meat shops at Kote Gate area, Bikaner	Meat pieces (Mixed of goat, sheep and poultry)	Mt 2, Mt 3,Mt 4, Mt 9, Mt 10, Mt 11, Mt 12, Mt 13, Mt 14, Mt 15, Mt 19, Mt 20, Mt 22, Mt 24, Mt 25, Mt 26, Mt 27, Mt 28, Mt 31	20	20 (25.0)
3.	17	Clinical complex, CVAS	Horse wounds	Hrs1, Hrs3, Hrs4	3	3 (17.6)
4.	11	Piggery farm, Bikaner	Pig nasal swabs	Pg2, Pg4	2	2 (17.1)
5.	26	Clinical complex, CVAS	Camel wounds	J2,J3, J4, J9, J10, J14,J15, J18	8	8 (30.6)
6.	23	Clinical complex, CVAS	Dog skin swabs	D4, D6, D7, D9, D10, D13	6	6 (26.0)
7.	35	Sheep farm Beechwal and Clinical complex, CVAS	Sheep vaginal swabs	SV2, SV3, SV4,	3	6 (17.1)
			Nasal swabs from pneumonic sheep	SN3, SN4, SN14	3	
8.	85	Rewari, Hariyana	Mastitic milk sample of buffalo	B1, B5, B10, B19, B21, B23, B24, B26, B27, B28, B29, B30, B31, B34, B36, B39, B42, B43, B46, B55, B57	21	21 (24.7)
9.	90	Clinical complex, CVAS Court Gate area and Hasanpura Bikaner	Mastitic milk sample of goat	AG5, AG6, AG8, AG10, AG13, AG15, AG17, G1, G2, G7, G9, G10, G11, G16, G21, G24, G29, G35, G37, G39, G40, G41, G43, G45, G46, G47, G49, G55	28	28 (31.1)
10.	70	Clinical complex of College, M.P. Colony, Beechwal area cattle dairies and Rewari, Hariyana	Mastitic milk sample of cattle	C2, C2R, C3R, C5R, C7, C8, C9, C11, C12, C13, C15, C17, C20, C22, C23, C26, C29, C34, C36, C37, C39, C40, C41, C43, C46, C47, C50, C52	28	28 (40.0)
Total	517	157 (30.3)				

6. Phenotypic characterization of *S. aureus*:

A. Cultural and biochemical properties:

All the 157 *S. aureus* isolates were subjected to aerobic cultivation on nutrient agar to study the cultural properties on incubation at 37˚C for 24 h. The colonies obtained were round, smooth and glistening and the color of the colonies was variable as pale yellow 73 (46.5%), whitish 50 (31.8%), golden yellow 25 (15.9%), and mustard yellow 9 (5.7%) as described in table 5 and Fig. 2. The color of the colonies of *S. aureus* is due to a pigment namely staphyloxanthin which has been considered as important factor in alleviating stress to the organisms. Our observations are in conformity to those of other workers who also recorded variation in the colony pigmentation. Salasia *et al.* (2004) recorded three types of pigments (orange, yellow and pale yellow) produced by *S. aureus* from bovine sub-clinical mastitis from different countries. A variation in the colony pigmentation of *S. aureus* from skin wounds in camel was also reported by Qureshi and Kataria (2012) as golden yellow, yellow and white.

Similarly Islam *et al.* (2007) characterized 28 coagulase positive *S. aureus* of bovine origin and recorded that 46.4% of isolates produced golden yellow pigment, 39.3% produced yellow pigment and 14.3% produced white pigment on nutrient agar. Sanjiv *et al.* (2008) recorded *S. aureus* of bovine mastitis milk origin obtained from different locations producing golden yellow pigmentation on nutrient agar by 95.23% of the isolates and they also recorded white colored colonies of the isolates.

El-Jakee *et al.* (2010) isolated *S. aureus* from mastitic cows and buffalo and obtained three types of pigments *viz.* golden yellow, creamy and white. Likewise, Khichar (2011) also recorded variation in the pigment production by *S. aureus* isolates of bovine mastitic origin. The pigment recorded was golden yellow and mustard yellow. Sun *et al.* (2012) studied growth properties of *S. aureus* in biofilm formed on polystyrene plate and obtained nearly colorless colonies. Qureshi and Kataria (2012) characterized 40 *S. aureus* from camel wound samples and recorded 24 (60.0%) to produce yellow, 15 (37.5%) isolates to produce golden yellow and one (2.5%) to produce white colony. Yadav, (2014) also reported three type colony variants as golden yellow, pale yellow and yellowish white among *S. aureus* isolates from msatitic cattle and buffalo.

Mannitol fermentation is also another important biochemical character of *S. aureus* and various strains were recorded to show two different fermentation pattern. The color of mannitol salt agar (Fig.3) changed to yellow after incubation indicating fermentation of the mannitol. In the present investigation, 153 (97.5%) isolates were mannitol fermenter while only four isolates (H1, SV4 B34 and G2) were mannitol non fermenter as mentioned in table 5. Similar to present study, other authors namely Shittu *et al.* (2007), Kateete *et al.* (2010) and Santos *et al.* (2015) had also reported mannitol-negative *S. aureus* isolates with different prevalence from various sources.

Kateete *et al* (2010) reported two (6%) mannitol-negative *S. aureus* isolates from patients' blood, cerebral spinal fluid, anterior nares, skin and wound swabs while high prevalence (15%) of mannitol-negative *S. aureus* isolates was detected by Santos *et al.* (2015) among nasal swab specimens.

Our observations were close to report of Singh (2006) who evaluated mannitol fermentation by *S. aureus* isolates from cattle subclinical mastitis and found that all isolates were mannitol fermenter. Similarly, Sanjiv (2006), Upadhyay (2009), Singh *et al.* (2011) and Sharma *et al.* (2013) also reported that 100% isolates from cattle, goat, buffalo and camel were mannitol fermenter.

Table 5: Properties of *S. aureus* isolates on Nutrient Agar and Mannitol Salt Agar

S. No.	Source of Isolate	Total No.	Growth on NA (%)				Growth on MSA (%)	
			PY	W	GY	MY	F	NF
1.	Human	35	15 (42.9)	20 (57.1)	0 (0)	0 (0)	34 (97.2)	1 (2.8)
2.	Meat piece	20	10 (50)	6 (30)	4 (20)	0 (0)	20 (100)	0 (0)
3.	Horse	3	2 (66.7)	0 (0)	1 (33.3)	0 (0)	3 (100)	0 (0)
4.	Pig	2	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)
5.	Camel	8	3 (37.5)	2 (25)	2 (25)	1 (12.5)	8 (100)	0 (0)
6.	Dog	6	2 (33.3)	1 (16.7)	2 (33.3)	1 (16.7)	6 (100)	0 (0)
7.	Sheep	6	1 (16.7)	3 (50)	2 (33.3)	0 (0)	5 (83.4)	1 (16.6)
8.	Buffalo	21	11 (52.3)	6 (28.6)	3 (14.3)	1 (4.8)	20 (95.2)	1 (4.8)
9.	Goat	28	12 (42.9)	5 (17.8)	7 (25)	4 (14.3)	27 (96.4)	1 (3.6)
10.	Cattle	28	15 (53.6)	7 (25)	4 (14.3)	2 (7.1)	28 (100)	0 (0)
Total		**157**	**73 (46.5)**	**50 (31.8)**	**25 (15.9)**	**9 (5.7)[a]**	**153 (97.5)**	**4 (2.5)[b]**

Abbreviations:- NA- Nutrient Agar, MSA- Mannitol Salt Agar, PY- Pale Yellow, W-White, GY- Golden Yellow, MY-Mustard Yellow, F- Fermenter, NF- Non Fermenter
Superscripts:- a-Mustard Yellow colony showed isolates (J10, D7, AG5, AG6, AG8, G37,C2R and C36),
b- Mannitol non fermenter (H1, SV4 B34 and G2)

B. Coagulase production:

Coagulase is an extracellular protein encoded by *coa* gene and is considered as one of the important criteria for identification of this organism. In present investigation, production of coagulase was accessed in tube using plasma from six species of animals and human (Fig. 4). The purpose of incorporating different plasma was to access the suitability and interaction between source of isolates and plasma from a different species for use in tube coagulase test.

In the present investigation out of 157 isolates, 148 (94.2%) isolates were found to be coagulase positive while nine (5.7%) isolates were coagulase negative (H8, H48, Mt31, J4, G29, C12, C13, C15 and C50) at 5h reading but of nine isolates four isolates (H8, H48, Mt31 and C50) showed positive reaction at 24h. reading and remaining five isolates were negative at 24h. In these coagulase non producer isolates, coagulase reaction was not recorded in any of the studied plasma (Table 6).

Though coagulase production has been considered to be important criterion in the identification of *S. aureus* but similar to present study, coagulase negative *S. aureus* isolates have also been reported earlier. Jasper *et al*. (1985) isolated *S. aureus* from cows and found that 1-2% of the organisms did not produce tube coagulase. Singh *et al.* (2011) had reported coagulase production by only 78.5, 88.3 and 90.7% *S. aureus* isolates obtained from intramammary infections in Sahiwal cattle, Karan Fries cattle and Murrah buffalo, respectively.

In agreement to present investigation, some of the isolates showed weak reaction even after 5 h of incubation but showed good reaction at 24 h reading. Rayman *et al.* (1975) and Turkyilmaz and Kaya (2006) also reported the coagulation of plasma after 24 h. Similarly, Pennell *et al.* (1984) recorded reaction at 4 and 24 h incubation. Boerlin *et al*. (2003) had also reported that 24 h incubation is necessary for the full sensitivity of coagulase test. They also recorded conversion of weak reactions as positive after 24h.

In support to present study, coagulase negative *S. aureus* had also been reported by Jasper *et al.* (1985), Bedidi-Madani *et al.* (1998), Citak *et al.* (2003), Wani and Bhatt (2003), Turkyilmaz and Kaya (2006), Sanjiv *et al.* (2008), Kateete *et al.* (2010), Oliveira *et al.* (2010) and Kenar *et al.* (2011) with variable percentage and from various sources.

In the present study human plasma showed the best coagulation reaction followed by plasma from camel, poultry, sheep, goat and cattle in decreasing order for all studied isolates. Our results suggested use of human plasma for the coagulase test for *S. aureus.* The analysis of reactions suggested that coagulation reaction depends on both, source of isolate and source of plasma with a possible correlation between source of isolate and plasma. In the present study, more positive reactions were observed between isolate and plasma obtained from same species in comparison to when both belonged to different sources as shown in table 6.

Close to our results Abd El-Hamid and Bendary (2013) isolated 46 staphylococci from 141 human origin samples. Among these 46 isolates, 26 were coagulase positive *S. aureus* and 20 were identified as coagulase negative while Sharma *et al*. (2013) reported 15 (100%) coagulase producing *S. aureus* isolates obtained from 46 nasal swabs from pneumonic camels and found human plasma superior to cattle plasma as in the present study.

Our results are in agreement to the observation of Orth *et al.* (1971) who achieved best coagulation with plasma from human followed by pig, dog, poultry, sheep, goat, camel and buffalo. They assessed suitability of plasma from several animals in coagulase testing and observed that coagulase-reacting factor (CRF) activities of the different plasma had following relative concentrations of CRF: human > pig > rabbit > horse > bovine, chicken, and lamb.

Results of present investigation were also in agreement to those of Bhati (2013) and Kateete *et al.* (2010), where in human plasma was recorded to give the best coagulation. Our finding are supported by observation of Duthie and Lorenz (1952) who recorded that plasma of human, pig and fowl contained relatively more amount of coagulase activator.

Our results except for dog plasma were also in accordance with those of Adesiyun and Shehu (1985) who evaluated effect of sources of *S. aureus* and plasmas and recorded that the value of plasmas in order of superiority was human and rabbit > pig > donkey > chicken > cattle > duck > goat > dog. Difference in coagulation of plasma from various species was observed by Qureshi *et al.* (2002) who recorded that *S. aureus* isolates coagulated the plasma from rabbit, human, buffalo, horse, cattle, goat, camel and sheep in decreasing order of superiority. Similar to present investigation coagulase reaction with human plasma was more (91%) as compared to that with sheep plasma (81%) in identifying *S. aureus* (Kateete *et al.,* 2010). Though most of the workers have defined the coagulase reaction in terms of firmness of the clot, superiority of plasma for a particular species but Turner and Schwartz (1958) suggested that any degree of clotting in the coagulase plasma should be considered as a positive reaction.

In agreement to present investigation, Yadav *et al.* (2015a) also found 32 (100%) coagulase positive *S. aureus* from 89 mastitic milk sample of both cattle and buffalo. Strongest coagulation reaction recorded for human plasma followed by pig, rabbit, horse, bovine, chicken, and lamb in decreasing order. Similarly, Khichar and Kataria (2015) characterized 28 *S. aureus* isolates obtained from 59 mastitis samples of Holstein-Friesian crossbred and Rathi cattle for coagulase production and the overall strongest coagulation reaction in regards to early onset and firmness of clot was recorded with plasma from rabbit followed by buffalo, cattle, camel, human, goat, sheep, dog, horse, chicken and pig in decreasing order.

Table 6: Comparison of coagulation of plasmas from different species of animals and human by *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Total coagulase (%)		Plasma for coagulase production (%) at 5h.											
			P	N	Human		Cattle		Poultry		Sheep		Goat		Camel	
					P	N	P	N	P	N	P	N	P	N	P	N
1.	Human	35	33 (94.3)	2 (5.7)	33 (94.3)	2 (5.7)	0 (0)	35 (100)	1 (2.9)	34 (97.1)	0 (0)	35 (100)	0 (0)	35 (100)	22 (62.9)	13 (37.1)
2.	Meat piece	20	19 (95.0)	1 (5.0)	19 (95)	1 (5)	0 (0)	20 (100)	2 (10)	18 (90)	0 (0)	20 (100)	0 (0)	20 (100)	16 (80)	4 (20)
3.	Horse	3	3 (100)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	3 (100)	0 (0)	3 (100)	0 (0)	3 (100)	3 (100)	0 (0)
4.	Pig	2	2 (100)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)	2 (100)	0 (0)
5.	Camel	8	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	0 (0)	8 (100)	0 (0)	8 (100)	1 (12.5)	7 (87.5)	1 (12.5)	7 (87.5)	7 (87.5)	1 (12.5)
6.	Dog	6	6 (100)	0 (0)	6 (100)	0 (0)	0 (0)	6 (100)	3 (50)	3 (50)	0 (0)	6 (100)	0 (0)	6 (100)	6 (100)	0 (0)
7.	Sheep	6	6 (100)	0 (0)	6 (100)	0 (0)	1 (16.7)	5 (83.3)	6 (100)	0 (0)	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)	6 (100)	0 (0)
8.	Buffalo	21	21 (100)	0 (0)	21 (100)	0 (0)	4 (19)	17 (81)	2 (9.5)	19 (90.5)	1 (4.7)	20 (95.2)	1 (4.8)	20 (95.2)	21 (100)	0 (0)
9.	Goat	28	27 (96.4)	1 (3.6)	27 (96.4)	1 (3.6)	11 (39.2)	17 (60.8)	12 (42.9)	16 (57.1)	13 (46.4)	15 (53.6)	13 (46.4)	15 (53.6)	27 (96.4)	1 (3.5)
10.	Cattle	28	24 (85.7)	4 (14.3)	24 (85.7)	4 (14.3)	12 (42.9)	16 (57.1)	19 (67.9)	9 (32.1)	12 (42.9)	16 (57.1)	11 (39.3)	17 (60.7)	23 (82.1)	5 (17.8)
Total		**157 (100)**	**148 (94.2)**	**9 (5.7)[a]**	**148 (94.2)**	**9 (5.7)**	**28 (17.8)**	**129 (82.1)**	**45 (28.6)**	**112 (71.3)**	**29 (18.4)**	**128 (81.5)**	**28 (17.8)**	**129 (82.1)**	**133 (84.7)**	**24 (15.2)**

Abbreviations:- P- Positive, N- Negative
Superscript:- a- Coagulase Negative (H8, H48, Mt31, J4, C12, C13, C15 and C50) at 5h but at 24h reading H8, H48, Mt31 and C50 showed positive reaction.

C. Slime production:

Slime production is an important trait of *S. aureus* responsible for virulence of the organism. However, this trait is not possessed by all the *S. aureus* strains and a variable percentage of slime producing *S. aureus* recovered from various sources has been reported by many workers.

In the present investigation out of 157 isolates, 145 (92.4%) produced slime while 12 (7.6%) isolates were found to be negative for slime production (H8, H31, J4, G37, G39, G43, C2, C9, C23, C36, C39 and C40) as described in table 6. The positive colonies were detected as black in color on Congo red agar (Fig. 5). Our results showed presence of very high percentage (92.4%) of slime producing bacteria (Table 7). Similarly, Vasudevan *et al.* (2003) also obtained *S. aureus* from bovine mastitis and found 91.4% isolates to be slime producer on Congo red agar. Yazdani *et al.* (2006) detected high prevalence of biofilm forming capacity of *S. aureus* isolated from wound infections phenotypically by Congo red agar (CRA). Gundogan *et al.* (2006) detected slime production in 52.7% *S. aureus* isolates from clinical samples wherein Arslan and Ozkardes (2007) reported, 31% isolates were slime positive from 129 isolates of several clinical specimens with Congo red agar method.

Close to present study, Ebrahimi *et al.* (2014) reported 83.7% isolates from sixty one human wound infections were biofilm producers. Similarly Barbieri *et al.* (2015) also reported high prevalence (100%) of slime producer *S. aureus* from breast peri-implant infections.

Yadav *et al.* (2015a) reported 96.87% *S. aureus* isolates from mastitic milk of cattle and buffalo were slime producer while Fabres-Klein *et al.* (2015) reported 35.18% *S. aureus* slime producer strains from cows with subclinical mastitis. Khoramian *et al.* (2015) reported 70% of the isolates produced biofilm. Among these, 59.3% were producers of weakly adherent biofilms while 34.8% and 5.8% produced moderate and strong biofilms, respectively from 215 *S. aureus* strains of human and

dairy cow's infections. Similarly 60.65% slime producers were reported by Ozpinar and Gumussoy (2013) and 55.5% positivity was detected by Castelani *et al.* (2015) among mammary gland infections.

Likewise, Singh *et al.* (2011) reported slime production in 65.4%, 83.6% and 81.4% *S. aureus* isolates from Sahiwal cattle, Karan fries cattle and Murrah buffalo, respectively with intramammary infections. Melo *et al.* (2013) also reported 85% of the *S. aureus* from bovine subclinical mastitis to produce slime on Congo red agar.

Similarly, Marques *et al.* (2013) also recorded that 176 out of 250 (76.8%) isolates from bovine mastitic milk were slime producers. In a study by Gundogan *et al.* (2013), a high proportion of *S. aureus* (80%) isolated from raw calf meat (minced), chicken drumsticks, raw milk, ice cream and white cheese samples were detected as slime producer.

Many researchers also found slime producer *S. aureus* in their studies but prevalence was lower than that obtained in our study. In study by Liberto *et al.* (2009), 16 strains (55.2%) were positive to the CRA test by giving characteristic black color colony. In a study by Turkyilmaz and Kaya (2006), out of 180 strains of *S. aureus*, 77.8% coagulase positive *S. aureus* showed positive slime production activity. In continuation, 42.4% and 5.1% of *S. aureus* of goat mastitic milks and raw milk reported as slime producers by Bedidi-Madani *et al.* (1998) and Citak *et al.* (2003) respectively. This high to low slime production variability among *S. aureus* isolates may indicate diversity among *S. aureus* for slime production which may be due to variations in source of isolation or geographical variations among *S. aureus* isolates.

Table 7: Detection of slime production among *S. aureus* isolates.

S. No.	Source of Isolate	Total No.	Slime Production (%)	
			P	N
1.	Human	35	33 (94.3)	2 (5.7)
2.	Meat piece	20	20 (100)	0 (0)
3.	Horse	3	3 (100)	0 (0)
4.	Pig	2	2 (100)	0 (0)
5.	Camel	8	7 (87.5)	1 (12.5)
6.	Dog	6	6 (100)	0 (0)
7.	Sheep	6	6 (100)	0 (0)
8.	Buffalo	21	21 (100)	0 (0)
9.	Goat	28	25 (89.3)	3 (10.7)
10.	Cattle	28	22 (78.6)	6 (21.4)
Total		**157**	**145 (92.4)**	**12 (7.6)[a]**

Abbreviations:- P- Positive, N- Negative.
Superscripts:- a- Negative for slime production (H8, H31, J4, G37, G39, G43, C2, C9, C23, C36, C39 and C40)

D. Haemolytic properties and Toxin Assay:

Haemolysis is an important property of *S. aureus* and plays role in virulence. In present investigation all the 157 isolates from various clinical and non-clinical human and animals sources were checked for complete, partial or no haemolysis on 5% sheep blood agar (Fig.6). Of the 157 isolates 108 (68.8%) isolates exhibited

complete haemolysis, 39 (24.8%) isolates exhibited incomplete / partial haemolysis, two (1.3%) isolates were both (complete and partial hemolytic) and 8 (5.1%) isolates (H2, H6, H8, G29, G37, G39, C2R and C13) were ahaemolytic (Table 8).

From 39 (100%) isolates with partial haemolysied, 34 (87.2%) showed phenomenon of hot-cold lysis while four (10.2%) isolates (H30, Mt19, J9, D7 and C12) remained did not shoed hot-cold lysis (Table 9).

Similar to present study high prevalence of complete hemolysis and low prevalence of non-hemolytic was also reported by Graber *et al.,* (2013), who reported that all clinical isolates *S. aureus* showing complete, incomplete or double hemolysis while non-hemolytic strains of *S. aureus* showed very low prevalence (0–2%) and Chu *et al.* (2013) also reported 100% isolates to produce complete haemolysis on sheep and horse blood agar.

Similarly Upadhyay and Kataria (2010) also recorded that of the 21 of the isolates (14 from cattle, seven from goats) all isolates produced complete haemolysis, three produced partial haemolysis (two from cattle and one from goat), and six isolates (four from cattle and two from goats) produced both haemolysis and in close to our results, Qureshi and Kataria (2012) also obtained 40 *S. aureus* isolates from clinical infections of camel and reported that 16 isolates showed complete haemolysis, 11 showed incomplete and 13 exhibited both types haemolysis on sheep blood agar.

Yadav *et al.* (2015d) reported 32 *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis and revealed that five (15.62%) isolates to show complete haemolysis, 20 (62.50%) isolates to show partial haemolysis, four (12.50%) isolates to show both complete and partial and similar to present investigation, three (9.37%) did not show any haemolysis while Helal *et al.* (2015) studied 72 *S. aureus* from 100 samples of human and animal (poultry, cow and sheep) source and All isolates were found to be hemolytic in contrary to our results none of the isolate was non hemolytic.

Similar to our results, Solanki, (2004) reported that more isolates were complete hemolytic in comparison to partial or non hemolytic who studied 46 *S. aureus* of camel origin and demonstrated that 21 isolates showed complete haemolysis, 13 isolates exhibited incomplete haemolysis and 12 isolates did not show any haemolysis on blood agar. Similarly Matsunaga *et al.* (1993) reported more complete haemolysis (74.1%) and partial haemolysis (65.5%) *S. aureus* isolates from bovine mastitic milk. In contrary to these results Younis *et al.* (2000) recorded that majority (62.7%) of the isolates were non-haemolytic including 23.2% complete haemolysis and 12.5% incomplete haemolysis isolates from dairy animals.

Although haemolysis factor is an important virulence governing property of *S. aureus,* many variations were recorded by various researchers such as Jasper *et al.* (1985) who recorded 99% of the isolates to produce haemolysis. Aarestrup *et al.* (1999) recorded 72% *S. aureus* of bovine mastitic origin to produce incomplete haemolysis. In contrast to these observations Boerlin *et al.* (2003) did not detect incomplete haemolysis on blood agar plate by *S. aureus* isolates. Islam *et al.* (2007) also recorded more 86.3% *S. aureus* from cattle showing incomplete haemolysis and Annemuller *et al.* (1999) who recorded production of complete haemolysis by eight isolates and partial haemolysis by 13 out of 25 bovine mastitis *S. aureus* isolates.

Sharma *et al.* (2013) also reported that 12 out of 15 isolates showed partial haemolysis of which four isolates later showed hot-cold lysis whereas three isolated showed complete haemolysis and Khichar and Kataria (2015) recorded that of the 28 *S. aureus* isolates from cattle mastitis, 15 produced incomplete haemolysis which turned into complete haemolysis when the plates were further incubated at 4^0C overnight (Hot-cold lysis) and 13 showed complete zone surrounded by incomplete zone of haemolysis both of these not recorded none hemolytic isolate while El-Jakee, *et al.* (2008) studied 78 isolates of *S. aureus* obtained from 409 samples of human and animal origin and found 70 isolates were haemolytic on sheep blood agar and 8 were non haemolytic.

Similar to our observations of obtaining ahaemolytic isolates, Salasia *et al.* (2004) also reported 10 non-haemolytic *S. aureus* isolates out of 35 *S. aureus* isolates from bovine subclinical mastitis, and in the study of Ariyanti *et al.* (2011), the types of haemolysins of *S. aureus* on the sheep blood agar plate, revealed complete haemolysis for two isolates (18.18%), partial haemolysis for three isolates (27.27%) and no haemolysis for six isolates (54.55%).

These recorded variations in hemolysis pattern of *S. aureus* in present or previous studies may indicate diversity existed among isolates in regards to hemolysis property. It may warrant the urgency to study the genes related with hemolysis factor.

Table 8: Haemolysis pattern of *Staphylococcus aureus* isolates on sheep blood agar in the present study.

S. No.	Source of Isolate	Total no of isolate	Haemolysis on incubation at 37ºC, 24h. (%)				Haemolysis on incubation at 4ºC (%) (Hot - Cold Lysis)			
			C	P	C+P	N	C	P	C+P	N
1.	Human	35	24 (68.6)	6 (17.1)	2 (5.7)	3 (8.6)	30 (85.7)	2 (5.7)	0 (0.0)	3 (8.6)
2.	Meat piece	20	19 (95.0)	1 (5.0)	0 (0.0)	0 (0.0)	19 (95.0)	1 (5.0)	0 (0.0)	0 (0.0)
3.	Horse	3	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
4.	Pig	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
5.	Camel	8	3 (37.5)	5 (62.5)	0 (0.0)	0 (0.0)	7 (87.5)	1 (12.5)	0 (0.0)	0 (0.0)
6.	Dog	6	4 (66.7)	2 (33.3)	0 (0.0)	0 (0.0)	5 (83.3)	1 (16.7)	0 (0.0)	0 (0.0)
7.	Sheep	6	6 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
8.	Buffalo	21	16 (76.2)	5 (23.8)	0 (0.0)	0 (0.0)	20 (95.2)	0 (0.0)	1 (4.8)	0 (0.0)
9.	Goat	28	19 (67.9)	6 (17.9)	0 (0.0)	3 (10.7)	22 (78.6)	0 (0.0)	3 (10.7)	3 (10.7)
10.	Cattle	28	12 (42.9)	14 (50.0)	0 (0.0)	2 (7.1)	16 (57.1)	1 (3.6)	9 (32.1)	2 (7.1)
Total		**157**	**108 (68.8)**	**39 (24.8)**	**2 (1.3)**	**8 (5.1)**	**130 (82.8)**	**6 (3.8)**	**13 (8.3)**	**8 (5.1)[a]**

Abbreviations:- C- Complete haemolysis, P- Partial haemolysis, C+P- both complete and partial hemolysis, N- Non haemolytic
Superscript:- Non haemolytic isolates (H2, H6, H8, G29, G37, G39, C2R and C13)

Table 9: Changes in haemolysis pattern of *Staphylococcus aureus* isolates during Hot – Cold lysis phenomena.

S. No.	Source of Isolate	No. of isolate showed partial hemolysis	Isolate I.D.	Hot-Cold lysis		No. of Isolate showed variations (%)	Total no. isolates showed variations (%)
				Initial	Final		
1.	Human	6	H7, H16 and H28	P	C	3 (8.5)	5 (14.3)
			H25 and H34	C+P	C	2 (5.7)	
			H30[a]	P	P	-	

2.	Meat piece	1	Mt19[a]	P	P	-	-
3.	Horse	0	-	-	-	-	-
4.	Pig	0	-	-	-	-	-
5.	Camel	5	J2, J3, J4 and J10	P	C	4 (50.0)	4 (50.0)
			J9[a]	P	P	-	
6.	Dog	2	D6	P	C	1 (16.7)	1 (16.7)
			D7[a]	P	P	-	
7.	Sheep	0	-	-	-	-	-
8.	Buffalo	5	B1, B19, B24 and B42	P	C	4 (19.0)	5 (23.8)
			B28	P	C+P	1 (4.8)	
9.	Goat	6	G10, G16 and G21	P	C+P	3 (10.7)	6 (21.4)
			AG8, G47 and G49	P	C	3 (10.7)	
10.	Cattle	14	C7, C22, C26, C34, C36, C37, C39, C40 C41	P	C+P	9 (32.1)	13 (46.4)
			C8, C11, C15 and C23	P	C	4 (14.2)	
			C12[a]	P	P	-	
Total		**39 (100.0)**					**34 (87.2)**
Abbreviations:- C- Complete haemolysis, P- Partial haemolysis, C+P- both complete and partial hemolysis, N- Non haemolytic Superscript:- a- Isolates were not changed during hot- cold lysis (H30, Mt19, J9, D7 and C12)							

Toxin assay

Toxin production is considered to be related to pathogenicity of *S. aureus*. To study the qualitative and quantitative production of toxins, all the isolates were subjected to haemolytic assays using erythrocytes from cattle and horse for beta- and delta-toxins, respectively.

Qualitative Assay

In the present investigation of the 157 isolates, 75 (47.8%) isolates haemolysed cattle erythrocytes indicating presence of beta-toxin (Fig. 7) and 121 (77.1%) isolates haemolysed horse erythrocytes indicating delta-toxin activity (Fig. 8). Most of the isolates from human, camel, goat and cattle showed beta toxin activity (Table 10). Lesser number of cattle isolates produced delta- toxin as compared to isolates from other sources (Table 11). Similar to present study, more delta toxin activity was also reported by Garcia *et al.* (1980) and Bedidi-Madani *et al.* (1998).

Close to our result, Garcia *et al.* (1980) reported 82.4% delta-hemolysin (δ) and 78.8% beta-hemolysin (β) activity while 7.0% isolates were negative for any activity. Similarly, Bedidi-Madani *et al.* (1998) reported 76.3% and 56.9% isolates exhibited δ- and β-haemolysin activity respectively from goat milk. Capurro *et al.* (2010) and Ariyanti *et al*. (2011) revealed 34% and 27.27% beta-haemolysin activity, respectively among *S. aureus* isolates, which is slightly lower than the present study. Similar to these findings, Solanki (2004) reported 26.66% isolates were β-toxin producer while only 6.66% produced δ- toxin activity for camel origin isolates.

Quantitative Assay

The results of the quantitative assays of beta and delta toxin have been depicted in tables 10 and 11. The production of beta-toxin was shown by lesser number of isolates and the titres of beta-toxin were also much less with titre ranging from 1:5 to 1:320. In this study 48 (30.6%) isolates showed β toxin activity with 1:5 titre, 14 (8.9%) isolates showed 1:20 titre and rest of positive isolates showed variable titres as described in table 10.

In our investigation delta toxin was detected to be produced by more isolates as compared to beta toxin production with titre ranging from 1:5 to 1:40. The 60 (38.2%) isolates showed 1:5 titre, 45 (28.7%) were positive with 1:10 titre and remaining other positive isolates possessed more than 1:10 titre as described in table 11.

Close to our results Khichar and Kataria (2015) recorded the titre of β-toxin from cattle and goat isolates ranged between 1:20 and 1:160 and titre of δ-toxin is 1:20 titre of cattle mastitis isolates similarly, goat isolates also produced the δ-toxin but the titres remained towards lower side. Upadhyay and Kataria (2010) recorded titre for β- and δ-toxins was similar (1:160) for cattle as well as goat isolates from mastitis. Qureshi *et al.* (2002) found that all *S. aureus* isolates from camel produced β- and δ-toxin either in pure or in combinations and the titre of β- and δ-toxin ranged between 1:8 to 1:1920 and 1:5 to 1:80, respectively. In comparison to present study Singh (2006) reported 1:640 titre of β-toxin but did not record production of δ-toxin from the isolates from sub-clinical mastitis in cattle.

Lesser quantity β-toxin and absence of δ-toxin was also recorded by Sanjiv and Kataria (2007) who reported titre of 1:120 from cattle mastitis origin for β-toxin but they did not record any production of δ-toxin among any of the isolates. In the study of *S. aureus* from goat mastitis by Nathawat (2013) the titres of β-toxin was much below than that ofα-toxin, the highest titres being only 1:40 recorded. Similarly, in the study by Bhati (2013), titres of β-toxin produced by *S. aureus* isolates from H-F cattle and crossbred cattle recorded and found that both the breeds of cattle were almost similar and the highest titres for β-toxin in *S. aureus* isolates from both the breeds were same being 1:160.

Table 10: Titration of beta (β) toxin produced by *S. aureus* isolates against cattle erythrocytes after 2 h incubation at 37^0 C followed by overnight refrigeration at 4^0C

S. No.	Source of isolate	Total No. of isolate	Negative for toxin activity (%)	Toxin titre (%)						
				1:5	1:10	1:20	1:40	1:80	1:160	1:320
1.	Human	35	17 (48.6)	18 (51.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2.	Meat piece	20	16 (80.0)	4 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
3.	Horse	3	3 (100.0)*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
4.	Pig	2	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
5.	Camel	8	4 (50.0)	4 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
6.	Dog	6	1 (16.7)	5 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
7.	Sheep	6	1 (16.7)	5 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
8.	Buffalo	21	21 (100.0)*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
9.	Goat	28	9 (32.1)	8 (28.6)	3 (10.7)	3 (10.7)	0 (0.0)	3 (10.7)	1 (3.6)	1 (3.6)
10.	Cattle	28	9 (32.1)	3 (10.7)	1 (3.6)	11 (39.4)	2 (7.1)	2 (7.1)	0 (0.0)	0 (0.0)
Total		**157**	**82 (52.2)**	**48 (30.6)**	**4 (2.6)[a]**	**14 (8.9)[b]**	**2 (1.4)[c]**	**5 (3.2)[d]**	**1 (0.64)[e]**	**1 (0.64)[f]**

*= None of the horse and buffalo isolate showed β toxin activity

Superscripts:- a - Isolate with β toxin titre 1:10 (AG13, AG15, G45 and C11)
b - Isolate with β toxin titre 1:20 (G11, G24, G40, C2, C3R, C20, C22, C34, C36, C39, C40, C41, C47and C50)
c - Isolate with β toxin titre 1:40 (C9 and C52), d - isolate with β toxin titre 1:80 (G7, G10, G16, C8 and C26)
e - Isolate with β toxin titre 1:160 (G21), f - isolate with β toxin titre 1:320 (G35)

Table 11: Titration of delta (δ) toxin produced by *S. aureus* isolates against horse erythrocytes after 3 h incubation at 37^0 C.

S. No.	Source of isolate	Total no. of isolate	Negative for toxin activity (%)	Toxin titre (%)			
				1:5	1:10	1:20	1:40
1.	Human	35	7 (20.0)	9 (25.7)	13 (37.1)	6 (17.2)	0 (0.0)
2.	Meat piece	20	1 (5.0)[b]	11 (55.0)	8 (40.0)	0 (0.0)	0 (0.0)
3.	Horse	3	0 (0.0)[a]	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)
4.	Pig	2	0 (0.0)[a]	1 (50.0)	0 (0.0)	0 (0.0)	1 (50.0)[c]
5.	Camel	8	1 (12.5)[b]	7 (87.5)	0 (0.0)	0 (0.0)	0 (0.0)
6.	Dog	6	0 (0.0)[a]	6 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
7.	Sheep	6	1 (16.6)[b]	4 (66.8)	1 (16.6)	0 (0.0)	0 (0.0)
8.	Buffalo	21	5 (23.8)	5 (23.8)	8 (38.1)	3 (14.3)	0 (0.0)
9.	Goat	28	7 (25.0)	10 (35.7)	8 (28.6)	3 (10.7)	0 (0.0)
10.	Cattle	28	14 (50.0)	6 (21.4)	6 (21.4)	2 (7.2)	0 (0.0)
Total		**157**	**36 (22.9)**	**60 (38.2)**	**45 (28.7)**	**15 (9.6)**	**1 (0.60)**

Superscripts:- a – None of the isolate was negative for δ toxin titre (Horse, Pig and Dog)
b – One isolate of each was negative for δ toxin titre (Meat piece-Mt13, Camel-J14, Sheep-SN3)
c – Only one isolate with δ toxin titre 1:40 (Pg2)

E. Antibiotic resistance pattern and MIC determination:

Over the last few decades, there was a sudden increase in the use of antibiotics in veterinary as well as medical science not only to control disease but also as prophylactic measure to prevent bacterial infections secondary to viral infections. *Staphylococcus aureus* is able to produce a host of structural changes and keeps on developing resistance against the most commonly used antibiotics. These resistant microorganisms become part of the environment and are transmitted from animals to humans and vice-versa. Presently there is growing concern among scientists in regards to increasing resistance in pathogens. The concerns are multifaceted *viz.* inaccurate diagnosis, defective dosage, indiscriminate use, development of new drugs etc.

Indiscriminate antibiotic use in dairy animals leads to treatment failure, escalated treatment costs and development of resistance to antimicrobials. The multiplicity of the cause and emergence of resistance due to indiscriminate and prolonged use of antibiotics in absence of antibiogram is a major hurdle in physical, chemical and microbiological control of mastitis.

In the present investigation *S. aureus* isolates were subjected to antibiogram studies using 42 antibiotics belonging to different categories and generations (Fig.9) where in huge variations were recorded in sensitivity patterns (Table 12). All (100%) isolates were sensitive to meropenem, chloramphenicol (except one human isolate, H28), and nitrofurantoin (except one cattle isolate, C39), in continuation more than 85% isolates were sensitive to nine antibiotic namely ampicillin+ sulbactum (96.2%) (only three isolates from human (H2, H3 and H4) were resistant), imipenem (95.5%), cefalothin (91.7%), piperacillin+ tazobactam (91.7%), tobramycin (89.8%), doxycycline hydrochlorid (89.2%), ceftazidime+ clavulanic Acid (88.5%), oxacillin (87.3%) and polymxin –B (86.0%).

More than 95.0% isolates were resistant to ampicillin (one isolate from Goat- AG17 and three isolates from cattle-C3R, C9 and C17 were sensitive) and penicillin –G (one isolate from sheep- SV4, one from buffalo-B36 and one isolate from cattle C3R were sensitive).

Since many variations were found in the antibiogram of *S. aureus* from different sources thus the statistical analysis was carried out and comparison of diameter of inhibition zone (mm) of each antibiotics between each source of isolate was made. The isolates from different sources showed highly significant ($p \leq 0.01$) variation in their resistance patterns for 39 antibiotics, significant variation ($p \leq 0.05$) for levofloxacin and nitrofurantoin, and no significant variation ($p > 0.05$) for clindamycin as described in table 13.

Table 12: Sensitivity patterns for *S. aureus* isolates against various tested antibiotics

S. No.	Name of Antibiotic	Antibiogram pattern of *S. aureus* isolates from various sources (%)																	
		Human			Meat piece			Horse			Pig			Camel			Dog		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
1.	Ampicillin	35 (100.0)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)
2.	Ampicillin+ Sulbactum	3 (8.6)	3 (8.6)	29 (82.8)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
3.	Azithromycin	33 (94.3)	0 (0.0)	2 (5.7)	20 (100.0)	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)	2 (100)	7 (87.5)	0 (0.0)	1 (12.5)	2 (33.3)	0 (0.0)	4 (66.7)
4.	Aztreonam	17 (48.6)	9 (25.7)	9 (25.7)	0 (0.0)	4 (20.0)	16 (80.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	2 (25.0)	2 (25.0)	4 (50.0)	0 (0.0)	0 (0.0)	6 (100.0)
5.	Cefaclor	23 (65.7)	5 (14.3)	7 (20.0)	9 (45.0)	4 (20.0)	7 (35.0)	2 (66.7)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	2 (100.0)	4 (50.0)	2 (25.0)	2 (25.0)	0 (0.0)	0 (0.0)	6 (100.0)
6.	Cefalothin	9 (25.7)	1 (2.9)	25 (71.4)	0 (0.0)	2 (10.0)	18 (90.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
7.	Cefepime	11 (31.4)	9 (25.7)	15 (42.9)	0 (0.0)	6 (30.0)	14 (70.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	1 (12.5)	1 (12.5)	6 (75.0)	0 (0.0)	0 (0.0)	6 (100.0)
8.	Cefixime	30 (85.7)	2 (5.7)	3 (8.6)	11 (55.0)	1 (5.0)	8 (40.0)	3 (100.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	2 (33.3)	3 (50.0)	1 (16.7)
9.	Cefixime+ Clavulanic Acid	27 (77.1)	2 (5.7)	6 (17.2)	7 (35.0)	6 (30.0)	7 (35.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	3 (37.5)	3 (37.5)	2 (25.0)	0 (0.0)	2 (33.3)	4 (66.7)
10.	Cefoparazone	14 (40.0)	18 (51.4)	3 (8.6)	10 (50.0)	7 (35.0)	3 (15.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	2 (100.0)	0 (0.0)	2 (25.0)	4 (50.0)	2 (25.0)	0 (0.0)	3 (50.0)	3 (50.0)
11.	Cefoparazone+ Sulbactam	6 (17.1)	19 (54.3)	10 (28.6)	0 (0.0)	6 (30.0)	14 (70.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	3 (37.5)	5 (62.4)	0 (0.0)	0 (0.0)	6 (100.0)
12.	Cefotaxime	11 (31.4)	15 (42.9)	9 (25.7)	1 (5.0)	6 (30.0)	13 (65.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	1 (50.0)	1 (50.0)	1 (12.5)	4 (50.0)	3 (37.5)	0 (0.0)	0 (0.0)	6 (100.0)
13.	Cefotaxime+ Clavulanic Acid	8 (22.9)	15 (42.9)	12 (34.3)	1 (5.0)	2 (10.0)	17 (85.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	3 (37.5)	5 (62.5)	0 (0.0)	0 (0.0)	6 (100.0)
14.	Cefoxitin	26 (31.4)	0 (0.0)	9 (25.7)	7 (35.0)	0 (0.0)	13 (65.0)	1 (33.3)	0 (0.0)	2 (66.7)	1 (50.0)	0 (0.0)	1 (50.0)	4 (50.0)	0 (0.0)	4 (50.0)	0 (0.0)	0 (0.0)	6 (100.0)
15.	Ceftazidime	21 (60.0)	6 (17.1)	8 (22.9)	4 (20.0)	9 (45.0)	7 (35.0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)	1 (50.0)	1 (50.0)	3 (37.5)	4 (50.0)	1 (12.5)	0 (0.0)	3 (50.0)	3 (50.0)
16.	Ceftazidime+ Clavulanic Acid	8 (22.9)	3 (8.6)	24 (68.6)	0 (0.0)	2 (10.0)	18 (90.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (12.5)	7 (87.5)	0 (0.0)	0 (0.0)	6 (100.0)
17.	Ceftriaxone	8 (22.9)	18 (51.4)	9 (25.7)	0 (0.0)	5 (25.0)	15 (75.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	1 (12.5)	3 (37.5)	4 (50.0)	0 (0.0)	0 (0.0)	6 (100.0)
18.	Cefzolin	12 (34.3)	4 (11.4)	19 (54.3)	2 (10.0)	4 (20.0)	14 (70.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	4 (50.0)	4 (50.0)	0 (0.0)	0 (0.0)	6 (100.0)
19.	Chloramphenicol	1 (2.9)	0 (0.0)	34 (97.1)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
20.	Ciprofloxacin	19 (54.3)	15 (42.9)	1 (2.9)	6 (30.0)	13 (65.0)	1 (5.0)	0 (0.0)	3 (100.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	1 (12.5)	6 (75.0)	1 (12.5)	0 (0.0)	6 (100.0)	0 (0.0)
21.	Clindamycin	2 (5.7)	13 (37.1)	20 (57.2)	0 (0.0)	4 (20.0)	16 (80.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	5 (62.5)	3 (37.5)	0 (0.0)	0 (0.0)	6 (100.0)
22.	Cloxacillin	14	20	1 (2.9)	1 (5.0)	16	3 (15.0)	0 (0.0)	2	1 (33.3)	2	0 (0.0)	0 (0.0)	1 (12.5)	6	1 (12.5)	0 (0.0)	0 (0.0)	6

S. No.	Name of Antibiotic	Antibiogram pattern of *S. aureus* isolates from various sources (%)																	
		Human			Meat piece			Horse			Pig			Camel			Dog		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
		(40.0)	(57.2)			(80.0)			(66.7)		(100.0)				(75.0)				(100.0)
23.	Doxycycline Hydrochloride	3 (8.6)	5 (14.3)	27 (77.1)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (12.5)	7 (87.5)	0 (0.0)	0 (0.0)	6 (100.0)
24.	Faropenem	17 (48.6)	14 (40.0)	4 (11.4)	5 (25.0)	12 (60.0)	3 (15.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	1 (50.0)	1 (50.0)	1 (12.5)	5 (62.5)	2 (25.0)	0 (0.0)	0 (0.0)	6 (100.0)
25.	Gentamicin	8 (22.9)	2 (5.7)	25 (71.4)	3 (15.0)	0 (0.0)	17 (85.0)	1 (33.3)	0 (0.0)	2 (66.7)	0 (0.0)	1 (50.0)	1 (50.0)	5 (62.5)	2 (25.0)	1 (12.5)	0 (0.0)	0 (0.0)	6 (100.0)
26.	Imipenem	5 (14.3)	2 (5.7)	28 (80.0)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
27.	Levofloxacin	0 (0.0)	5 (14.3)	30 (85.7)	1 (5.0)	5 (25.0)	14 (70.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	3 (37.5)	5 (62.5)	0 (0.0)	0 (0.0)	6 (100.0)
28.	Linezolid	3 (8.6)	0 (0.0)	32 (91.4)	1 (5.0)	0 (0.0)	19 (95.0)	0 (0.0)	0 (0.0)	3 (100.0)	2 (100.0)	0 (0.0)	0 (0.0)	5 (62.5)	0 (0.0)	3 (37.5)	0 (0.0)	0 (0.0)	6 (100.0)
29.	Meropenem	0 (0.0)	0 (0.0)	35 (100.0)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
30.	Methicillin	10 (28.6)	15 (42.9)	10 (28.6)	2 (10.0)	11 (55.0)	7 (35.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	1 (12.5)	2 (25.0)	5 (62.5)	0 (0.0)	1 (16.7)	5 (83.3)
31.	Nitrofurantoin	0 (0.0)	1 (2.9)	34 (97.1)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
32.	Norfloxacin	29 (89.9)	5 (14.3)	1 (2.9)	16 (80.0)	3 (15.0)	1 (5.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	1(12.5)	7 (87.5)	0 (0.0)	0 (0.0)	4 (66.7)	2 (33.3)
33.	Oxacillin	13 (37.1)	1 (2.9)	21 (60.0)	2 (10.0)	0 (0.0)	18 (90.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
34.	Penicillin –G	35 (100)	0 (0.0)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)
35.	Piperacillin	34 (97.1)	1 (2.9)	0 (0.0)	20 (100.0)	0 (0.0)	0 (0.0)	3	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)
36.	Piperacillin+ Tazobactam	11 (31.4)	0 (0.0)	24 (68.6)	2 (10.0)	0 (0.0)	18 (90.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
37.	Polymxin –B	5 (14.3)	0 (0.0)	30 (85.7)	4 (20.0)	0 (0.0)	16 (80.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	3 (37.5)	0 (0.0)	5 (62.5)	1 (16.7)	0 (0.0)	5 (83.3)
38.	Tetracycline	6 (17.1)	15 (42.9)	14 (40.0)	0 (0.0)	1 (5.0)	19 (95.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	3 (37.5)	1 (12.5)	4 (50.0)	0 (0.0)	0 (0.0)	6 (100.0)
39.	Ticarcillin	7 (20.0)	16 (45.7)	12 (34.3)	0 (0.0)	3 (15.0)	17 (85.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (12.5)	7 (87.5)	0 (0.0)	0 (0.0)	6 (100.0)
40.	Ticarcillin+ Clavulanic Acid	28 (80.0)	0 (0.0)	7 (20.0)	12 (60.0)	0 (0.0)	8 (40.0)	1 (33.3)	0 (0.0)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	3 (37.5)	0 (0.0)	5 (62.5)	0 (0.0)	0 (0.0)	6 (100.0)
41.	Tobramycin	15 (42.9)	0 (0.0)	20 (57.2)	0 (0.0)	0 (0.0)	20 (100.0)	1 (33.3)	0 (0.0)	2 (66.7)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
42.	Vancomycin	18 (51.4)	16 (45.7)	1 (2.9)	5 (25.0)	13 (65.0)	2 (10.0)	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	7 (87.5)	1 (12.5)	0 (0.0)	4 (66.7)	2 (33.3)

contd....

S. No.	Name of Antibiotic	Antibiogram pattern of *S. aureus* isolates from various sources (%)															
		Sheep			Buffalo			Goat			Cattle			Total			
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	
1.	Ampicillin	6 (100.0)	0 (0.0)	0 (0.0)	21 (100)	0 (0.0)	0 (0.0)	27 (96.4)	0 (0.0)	1 (3.5)	25 (89.3)	0 (0.0)	3 (10.7)	153 (97.5)[a]	0 (0.0)	4 (2.5)	
2.	Ampicillin+ Sulbactum	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	3 (1.9)	3 (1.9)	151 (96.2)[c]	
3.	Azithromycin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	4 (21.0)	17 (80.0)	3 (10.7)	4 (14.3)	21 (75.0)	10 (35.7)	5 (17.9)	13 (46.4)	76 (48.4)	14 (8.9)	67 (42.7)	
4.	Aztreonam	0 (0.0)	1 (16.7)	5 (83.3)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	3 (10.7)	25 (89.3)	4 (14.3)	6 (21.4)	18 (64.3)	24 (15.3)	28 (17.8)	105 (66.9)	
5.	Cefaclor	0 (0.0)	0 (0.0)	6 (100.0)	1 (4.8)	3 (14.3)	17 (81.0)	1 (3.6)	4 (14.3)	23 (82.1)	5 (17.9)	6 (21.4)	17 (60.7)	45 (28.6)	24 (15.3)	88 (56.1)	
6.	Cefalothin	0 (0.0)	0 (0.0)	6 (100.0)	1 (4.8)	0 (0.0)	20 (95.2)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	10 (6.4)	3 (1.9)	144 (91.7)[c]	
7.	Cefepime	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	1 (3.6)	6 (21.4)	21 (75.0)	13 (8.3)	23 (14.6)	121 (77.1)	
8.	Cefixime	0 (0.0)	1 (16.7)	5 (83.3)	6 (28.6)	12 (57.1)	3 (14.3)	1 (3.6)	13 (46.4)	14 (50.0)	14 (50.0)	6 (21.4)	8 (28.6)	76 (48.4)	39 (24.8)	42 (26.8)	
9.	Cefixime+ Clavulanic Acid	0 (0.0)	0 (0.0)	6 (100.0)	4 (19.0)	7 (33.3)	10 (47.6)	0 (0.0)	2 (7.1)	26 (92.9)	7 (25.0)	6 (21.4)	15 (53.6)	48 (30.6)	28 (17.8)	81 (51.6)	
10.	Cefoparazone	0 (0.0)	1 (16.7)	5 (83.3)	1 (4.8)	12 (57.1)	8 (38.1)	1 (3.6)	11 (39.3)	16 (57.1)	3 (10.7)	9 (32.1)	16 (57.1)	31 (19.7)	69 (43.9)	57 (36.3)	
11.	Cefoparazone+ Sulbactam	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	1 (4.8)	20 (95.2)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	9 (32.1)	19 (67.1)	6 (3.8)[d]	39 (24.8)	112 (71.3)	
12.	Cefotaxime	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	4 (19.0)	17 (81.0)	0 (0.0)	2 (7.1)	26 (92.9)	0 (0.0)	10 (35.1)	18 (64.3)	13 (8.3)	43 (27.4)	101 (64.1)	
13.	Cefotaxime+ Clavulanic Acid	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	6 (21.4)	22 (78.6)	9 (5.7)[d]	27 (17.2)	121 (77.1)	
14.	Cefoxitin	0 (0.0)	0 (0.0)	6 (100.0)	2 (9.5)	0 (0.0)	19 (90.5)	2 (7.1)	0 (0.0)	26 (92.9)	12 (42.9)	0 (0.0)	16 (57.1)	55 (5.0)	0 (0.0)	102 (65.0)	
15.	Ceftazidime	0 (0.0)	0 (0.0)	6 (100.0)	1 (4.8)	4 (19.0)	16 (76.2)	1 (3.6)	5 (17.9)	22 (78.6)	6 (21.4)	8 (28.6)	14 (50.0)	37 (23.6)	41 (26.1)	79 (50.3)	
16.	Ceftazidime+ Clavulanic Acid	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	3 (7.1)	25 (89.3)	8 (5.1)	10 (6.4)	139 (88.5)[c]	
17.	Ceftrioxane	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	2 (9.5)	19 (90.5)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	7 (25.0)	21 (75.0)	9 (5.7)[d]	36 (22.9)	112 (71.3)	
18.	Cefzolin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21(100.0)	0 (0.0)	1 (3.6)	27	0 (0.0)	1 (3.6)	27 (94.4)	14 (8.9)	15 (9.6)	128 (81.5)	
19.	Chloramphenicol	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	1 (0.6)	0 (0.0)	156 (99.5)[b]	
20.	Ciprofloxacin	0 (0.0)	5 (83.3)	1 (16.7)	1 (4.8)	16 (76.2)	4 (19.0)	3 (10.7)	18 (64.3)	7 (25.0)	3 (10.7)	11 (39.3)	14 (50.0)	34 (21.7)	94 (59.9)	29 (18.5)	
21.	Clindamycin	0 (0.0)	2 (33.3)	4 (66.7)	0 (0.0)	8 (38.1)	13 (61.9)	0 (0.0)	10 (35.7)	18 (64.3)	1 (3.6)	10 (35.1)	17 (60.7)	3 (1.9)[d]	54 (34.4)	100 (63.7)	
22.	Cloxacillin	0 (0.0)	5 (83.3)	1 (16.7)	2 (9.5)	8 (38.1)	11 (52.4)	0 (0.0)	14 (50.0)	14 (50.0)	4 (14.3)	18 (64.3)	6 (21.4)	24 (15.3)	89 (56.7)	44 (28.0)	

S. No.	Name of Antibiotic	Antibiogram pattern of *S. aureus* isolates from various sources (%)														
		Sheep			Buffalo			Goat			Cattle			Total		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
23.	Doxycyline Hydrochlorid	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	1 (3.6)	27 (94.4)	5 (17.9)	2 (7.1)	21 (75.0)	8 (5.1)	9 (5.7)	140 (89.2)[c]
24.	Faropenem	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	4 (19.0)	17 (81.0)	0 (0.0)	3 (10.7)	25 (89.3)	2 (7.1)	9 (32.1)	17 (60.7)	25 (15.9)	50 (31.8)	82 (52.2)
25.	Gentamicin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	2 (9.5)	19 (90.5)	0 (0.0)	0 (0.0)	28 (100.0)	4 (14.3)	5 (17.9)	19 (67.9)	21 (13.4)	12 (7.6)	124 (79.0)
26.	Imipenem	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	5 (3.2)	2 (1.3)	150 (95.5)[c]
27.	Levofloxacin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	4 (19.0)	17 (81.0)	0 (0.0)	2 (7.1)	26 (92.9)	2 (7.1)	3 (10.7)	23 (82.1)	3 (1.9)[d]	23 (14.6)	131 (83.4)
28.	Linezolid	0 (0.0)	0 (0.0)	6 (100.0)	1 (4.8)	0 (0.0)	20 (95.2)	3 (10.7)	0 (0.0)	25 (89.3)	9 (32.1)	0 (0.0)	19 (67.9)	24 (15.3)	0 (0.0)	133 (84.7)
29.	Meropenem	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	157 (100.0)[b]
30.	Methicillin	0 (0.0)	0 (0.0)	6 (100.0)	1 (4.8)	7 (33.3)	13 (61.9)	0 (0.0)	2 (7.1)	26 (92.9)	5 (17.9)	5 (17.9)	18 (64.3)	19 (12.1)	44 (28.0)	94 (59.9)
31.	Nitrofurantoin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21(100.0)	0 (0.0)	0 (0.0)	28 (100.0)	1 (3.6)	0 (0.0)	27 (96.4)	1 (0.6)	1 (0.6)	155 (98.7)[b]
32.	Norfloxacin	0 (0.0)	3 (50.0)	3 (50.0)	2 (9.5)	9 (42.9)	10 ()	5 (17.9)	9 (32.1)	14 (50.0)	5 (17.9)	6 (21.4)	17 (60.7)	63 (40.1)	46 (29.3)	48 (30.6)
33.	Oxacillin	0 (0.0)	0 (0.0)	6 (100.0)	2 (9.5)	0 (0.0)	19 (90.5)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	2 (7.1)	26 (92.9)	17 (10.8)	3 (1.9)	137 (87.3)[c]
34.	Penicillin –G	5 (83.3.0)	0 (0.0)	1 (1.6)	20 (95.2)	0 (0.0)	1 (4.8)	28 (100.0)	0 (0.0)	0 (0.0)	27	0 (0.0)	1 (3.6)	155 (98.1)[a]	0 (0.0)	3 (1.9)
35.	Piperacillin	1 (16.7)	0 (0.0)	5 (83.3)	16 (76.1)	1 (4.8)	4 (19.0)	11 (39.3)	9 (32.1)	8 (28.6)	14 (50.0)	3 (10.7)	11 (39.3)	115 (73.2)	14 (8.9)	28 (17.8)
36.	Piperacillin+ Tazobactam	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	13 (8.3)	0 (0.0)	144 (91.7)[c]
37.	Polymxin –B	1 (16.7)	0 (0.0)	5 (83.3)	2 (9.5)	0 (0.0)	19 (90.5)	6 (21.4)	0 (0.0)	22 (78.6)	0 (0.0)	0 (0.0)	28 (100.0)	22 (14.0)	0 (0.0)	135 (86.0)[c]
38.	Tetracycline	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	1 (4.8)	20 (95.2)	0 (0.0)	2 (7.1)	26 (78.6)	8 (28.6)	4 (14.3)	16 (57.1)	17 (10.8)	24 (15.3)	116 (73.9)
39.	Ticarcillin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	1 (3.6)	3 (10.7)	24 (85.7)	8 (5.1)[d]	25 (15.9)	124 (79.0)
40.	Ticarcillin+ Clavulanic Acid	0 (0.0)	0 (0.0)	6 (100.0)	3 (14.3)	0 (0.0)	18 (85.7)	2 (7.1)	0 (0.0)	26 (92.9)	6 (21.4)	0 (0.0)	22 (78.6)	55 (35.0)	0 (0.0)	102 (65.0)
41.	Tobramycin	0 (0.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	21 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	0 (0.0)	0 (0.0)	28 (100.0)	16 (10.1)	0 (0.0)	141 (89.8)[c]
42.	Vancomycin	6 (100.0)	0 (0.0)	0 (0.0)	12 (57.1)	9 (42.9)	0 (0.0)	6 (21.4)	22 (78.6)	0 (0.0)	3 (10.7)	23 (82.1)	2 (7.1)	50 (31.8)	97 (61.8)	10 (6.4)

Abbreviations:- R- Resistant, I- Intermediate, S- Sensitive

Superscript:- a- More than 95.0% isolates were resistant for Ampicillin (One isolate from Goat- AG17 and three isolates from cattle-C3R, C9 and C17 were sensitive) and Penicillin –G (one isolate from sheep- SV4, one from buffalo-B36 and one isolate from cattle C3R were sensitive), b- 100 % isolates were sensitive for Chloramphenicol (except one Human isolate (H28) was resistant), Meropenem and Nitrofurantoin (except one cattle isolate (C39) was resistant), c- More than 85% isolates were sensitive for Ampicillin+ Sulbactum (only three isolates from human (H2, H3 and H4) were resistant), Cefalothin, Ceftazidime+ Clavulanic Acid, Doxycyline Hydrochlorid, Imipenem, Oxacillin, Piperacillin+ Tazobactam, Polymxin –B and Tobramycin, d- Isolate which having more no. of intermediates and sensitives but less no. of resistant for Cefoparazone+ Sulbactam, Cefotaxime+ Clavulanic Acid, Ceftrioxane, Clindamycin, Levofloxacin and Ticarcillin

Table. 13: Analysis of variance of diameter of inhibition zone (mm) of antibiotics for *S. aureus* isolates.

S. No.	Source of variation	MSS
1.	Ampicillin	288.043**
2.	Ampicillin+ Sulbactum	271.987**
3.	Azithromycin	789.142**
4.	Aztreonam	157.118**
5.	Cefaclor	429.189**
6.	Cefalothin	353.234**
7.	Cefepime	180.461**
8.	Cefixime	411.040**
9.	Cefixime+ Clavulanic Acid	298.578**
10.	Cefoparazone	152.219**
11.	Cefoparazone+ Sulbactam	165.105**
12.	Cefotaxime	268.889**
13.	Cefotaxime+ Clavulanic Acid	207.341**
14.	Cefoxitin	304.429**
15.	Ceftazidime	134.165**
16.	Ceftazidime+ Clavulanic Acid	163.499**
17.	Ceftrioxane	302.930**
18.	Cefzolin	349.944**
19.	Chloramphenicol	25.004**
20.	Ciprofloxacin	64.327**
21.	Clindamycin	9.628^{NS}
22.	Cloxacillin	476.205**
23.	Doxycycline Hydrochloride	141.901**
24.	Faropenem	536.097**
25.	Gentamicin	50.272**
26.	Imipenem	937.524**
27.	Levofloxacin	17.739*
28.	Linezolid	41.257**
29.	Meropenem	121.492**
30.	Methicillin	160.922**

31.	Nitrofurantoin	11.777*
32.	Norfloxacin	194.456**
33.	Oxacillin	351.371**
34.	Penicillin –G	256.907**
35.	Piperacillin	399.747**
36.	Piperacillin+ Tazobactam	149.886**
37.	Polymxin –B	24.501**
38.	Tetracycline	197.135**
39.	Ticarcillin	322.626**
40.	Ticarcillin+ Clavulanic Acid	350.513**
41.	Tobramycin	77.792**
42.	Vancomycin	10.179**

i. Degree of freedom (*df*)= 9
ii. * = Significant ($p \leq 0.05$)
iii. ** Highly significant ($p \leq 0.01$)
iv. NS = Non-significant ($p > 0.05$)
v. MSS = Mean Sum of Square

In the present study highly significant ($p \leq 0.01$) variation were recorded among *S. aureus* isolates for most of the antibiotics thus except clindamycin ($p > 0.05$), all sources of isolates were further subjected to Duncan's Homogeneous Subsets analysis (DMRT) of Mean± SEM values of diameter of inhibition zone (mm) of antibiotics at level of 0.05, different superscripts indicating significant variations at ($p \leq 0.05$) level (Table 14). In the DMRT analysis maximum five subsets were found for five antibiotic namely cefaclor, cefixime, cefixime+ clavulanic acid, imipenem and ticarcillin and four subset were found for 13 antibiotic namely ampicillin, ampicillin+ sulbactum, cefoparazone, ceftazidime+ clavulanic acid, doxycycline hydrochloride, linezolid, nitrofurantoin, norfloxacin, penicillin–G, piperacillin, piperacillin+ tazobactam, tetracycline and ticarcillin+ clavulanic acid among *S. aureus* isolates. For rest of other antibiotic two or three subsets were existed among *S. aureus* isolates as described in the table 14.

Further the Bonferroni correction was carried out to find out exact value of probability level of variance among sources of *S. aureus* isolates. It was found that *S. aureus* isolates from human sources were significantly variable with $p \leq 0.0001$ probability level of variance with other meat piece and animal origin sources for most of antibiotic (Table 15). Isolates from horse, camel and pig sources were non-significantly variable with each other for most of antibiotic and the isolates of sheep, goat, buffalo and cattle are non-significantly variable with each other for most of antibiotics (Table 15).

Hierarchical ascendant cluster analysis of antibiotic sensitivity pattern through mean values of diameter of inhibition zone (mm) of antibiotics against *S. aureus* isolates was carried out by Squared Euclidean Distance (SED) and the between-groups linkage method. The isolates were clustered into three groups at 10.0 rescaled cluster distance, one cluster comprising of buffalo, cattle, sheep, dog and goat, second cluster included horse, camel, meat piece and pig while third cluster included human isolates (Fig. 10). All three clusters have significant variation ($p \leq 0.05$) among each other. First cluster of buffalo, cattle, sheep, dog and goat exhibited lowest resistance and third cluster of human isolates showed highest resistance for most of antibiotics (Table 12). Overall lower resistance was recorded among animal origin isolates in comparison to human origin isolates.

Similar to present study Guler *et al.* (2005) also recorded highest resistance against β-lactam antibiotics, penicillin and ampicillin for *S. aureus* isolates from bovine clinical mastitic milk isolated from individual animals. Similarly, Turutoglu *et al.* (2006), Ebrahimi *et al.* (2007) and Hussain *et al.* (2012) also recorded maximum resistance for beta-lactam antibiotics against *S. aureus* isolates from various sources.

Similar results were reported Pereira *et al.* (2009) in an antibiogram study of 148 food origin isolates of *S. aureus* who recorded that strains were highly resistant to beta-lactams, ampicillin and penicillin, respectively. Seventy per cent of the isolates were resistant to beta-lactam antibiotics followed by ampicillin and penicillin (73%)

Close to our results, Khichar (2011) used 27 different antibiotics against 28 *S. aureus* isolates of cattle mastitis origin wherein they recorded that most effective antibiotic was azithromycin (92.86%) followed by cloxacillin and methicillin (89.29%). He also recorded resistance towards ampicillin, cefixime, metronidazole and nalidixic acid for all the isolates. Tobramycin was one of the most effective antibiotics against all the isolates in our study was contrary with the study of El-Jakee *et al.* (2011), where 20 % resistance was also reported.

Sanjiv and Kataria (2006) recorded antibiogram of *S. aureus* isolates for 13 antibiotics and complete agreement was reported for gentamicin, methicillin, chloramphenicol, ciprofloxacin and kanamycin with more than 90% sensitivity. And similar to present study 66% isolates were resistant to tetracycline group and comparative lower resistance (49%) found toward penicillin.

Mohanasoundaram and Lalitha (2008) studied 150 isolates of *S. aureus* from human clinical infections and similar results were reported for norfloxacin (100% resistance) and chloramphenicol (18% resistance) but higher resistance towards tetracycline (82%), gentamicin (88%) and ciprofloxacin (97%) was reported in compare to present study.

Similar to present study non observable difference was observed in antibiotic sensitivity or resistance patterns against isolates from cattle and goats (Upadhyay and Kataria 2009).

Table. 14: Duncan's Homogeneous Subsets analysis of Mean± SEM values of diameter of inhibition zone of antibiotics among *S. aureus* isolates.

S. No.	Name of Antibiotics	Mean± SEM values of diameter of inhibition zone (mm) of *S. aureus* isolates (No. of isolate)									
		Human (35)	Meat piece (20)	Horse (3)	Pig (2)	Camel (8)	Dog (6)	Sheep (6)	Buffalo (21)	Goat (28)	Cattle (28)
1.	Ampicillin	8.51± 0.611^{ab}	9.80± 1.002^{abc}	7.33± 3.712^{a}	10.50± 0.500^{abc}	6.63± 1.981^{a}	14.67± 0.760^{bc}	22.50± 2.078^{d}	15.38± 1.027^{c}	16.21± 1.077^{c}	16.18± 1.535^{c}
2.	Ampicillin+ Sulbactum	22.23± 1.127^{a}	29.10± 0.695^{bc}	27.67± 2.186^{abc}	25.50± 1.500^{ab}	27.13± 2.074^{abc}	36.00± 0.931^{d}	31.67± 1.687^{cd}	31.57± 0.909^{bcd}	31.32± 0.694^{bcd}	31.39± 0.991^{bcd}
3.	Azithromycin	2.83± 1.054^{a}	3.85± 1.009^{a}	12.00± 6.028^{b}	18.50± 0.500^{b}	3.75± 2.569^{a}	16.17± 3.701^{b}	18.67± 0.333^{b}	19.29± 0.464^{b}	17.61± 1.270^{b}	12.04± 1.778^{b}
4.	Aztreonam	15.69± 1.448^{a}	22.50± 0.478^{b}	20.33± 1.453^{ab}	22.50± 2.500^{b}	21.25± 1.509^{b}	24.67± 0.803^{b}	23.67± 0.955^{b}	23.67± 0.326^{b}	22.89± 0.323^{b}	21.21± 0.525^{b}
5.	Cefaclor	10.14± 1.307^{a}	14.90± 1.005^{abc}	16.67± 3.712^{abcd}	16.00± 1.000^{abcd}	14.38± 1.401^{ab}	21.83± 0.980^{cde}	24.83± 1.447^{e}	21.86± 1.435^{cde}	22.25± 0.827^{de}	20.75± 1.172^{bcde}
6.	Cefalothin	18.77± 1.426^{a}	25.35± 0.963^{ab}	24.33± 0.882^{ab}	23.50± 0.500^{ab}	25.75± 1.461^{ab}	28.33± 0.882^{b}	29.33± 0.989^{b}	26.43± 1.629^{b}	30.36± 0.720^{b}	30.96± 1.032^{b}
7.	Cefepime	13.94± 1.438^{a}	20.40± 0.812^{b}	22.00± 2.517^{b}	23.00± 1.000^{b}	19.75± 1.398^{b}	24.17± 0.167^{b}	21.17± 0.543^{b}	22.00± 0.602^{b}	22.18± 0.321^{b}	19.96± 0.668^{b}
8.	Cefixime	5.51± 1.195^{a}	11.65± 1.995^{abcd}	7.67± 3.844^{ab}	15.00± 1.000^{bcde}	10.13± 1.641^{abc}	17.50± 1.565^{de}	21.17± 1.447^{e}	16.33± 0.527^{cde}	18.32± 0.389^{de}	12.96± 1.373^{bcd}
9.	Cefixime+ Clavulanic Acid	11.94± 0.906^{a}	16.35± 1.282^{abc}	14.00± 0.577^{ab}	13.50± 0.500^{ab}	16.63± 1.085^{abc}	20.33± 0.803^{cde}	26.00± 1.342^{e}	19.19± 0.875^{bcd}	22.89± 0.833^{de}	20.14± 1.235^{cde}
10.	Cefoparazone	15.80± 0.719^{a}	15.80± 0.756^{a}	17.67± 1.453^{abc}	17.00± 1.000^{ab}	18.00± 1.018^{abc}	22.17± 0.980^{bcd}	24.83± 1.447^{d}	20.33± 0.929^{abcd}	22.32± 0.951^{cd}	21.64± 0.933^{bcd}
11.	Cefoparazone+ Sulbactam	17.63± 1.040^{a}	22.00± 0.761^{ab}	22.00± 2.517^{ab}	22.50± 0.500^{b}	23.13± 1.109^{b}	24.67± 0.760^{b}	25.83± 1.352^{b}	25.57± 0.678^{b}	25.79± 0.441^{b}	24.11± 0.765^{b}
12.	Cefotaxime	15.97± 1.587^{a}	23.15± 1.011^{bc}	22.67± 2.963^{bc}	23.50± 1.500^{bc}	19.88± 1.726^{ab}	27.33± 0.494^{c}	26.83± 0.401^{c}	25.38± 0.627^{bc}	26.39± 0.500^{bc}	23.14± 0.795^{bc}
13.	Cefotaxime+ Clavulanic Acid	20.31± 1.063^{a}	26.35± 1.047^{bc}	24.00± 2.309^{ab}	27.00± 1.000^{bc}	24.13± 1.076^{ab}	30.00± 1.000^{c}	31.00± 1.033^{c}	28.43± 0.702^{bc}	29.21± 0.538^{bc}	27.07± 0.959^{bc}
14.	Cefoxitin	15.54± 1.605^{a}	22.65± 0.792^{b}	22.67± 1.202^{b}	22.50± 1.500^{b}	21.13± 1.381^{ab}	27.50± 1.118^{b}	26.33± 0.558^{b}	27.05± 0.671^{b}	26.07± 0.454^{b}	22.32± 1.142^{b}
15.	Ceftazidime	11.60± 1.192^{a}	16.65± 0.504^{bc}	16.00± 2.309^{abc}	16.50± 1.500^{bc}	14.38± 1.413^{ab}	18.00± 0.632^{bc}	20.33± 0.422^{c}	18.52± 0.481^{bc}	18.71± 0.363^{bc}	17.14± 0.590^{bc}
16.	Ceftazidime+ Clavulanic Acid	18.46± 0.915^{a}	22.60± 0.690^{abcd}	21.67± 2.848^{abc}	25.50± 0.500^{bcd}	21.38± 1.034^{ab}	25.33± 1.116^{bcd}	26.83± 1.815^{cd}	25.24± 0.883^{bcd}	27.07± 0.527^{d}	24.07± 0.901^{bcd}
17.	Ceftrioxane	15.09± 1.560^{a}	22.50± 0.822^{b}	22.00± 1.732^{b}	24.50± 0.500^{b}	19.50± 1.690^{ab}	26.00± 0.931^{b}	24.50± 0.342^{b}	23.76± 0.487^{b}	26.43± 0.492^{b}	24.96± 1.137^{b}
18.	Cefzolin	16.26± 1.746^{a}	20.15± 0.963^{ab}	19.67± 2.186^{ab}	21.00± 1.000^{ab}	19.50± 1.282^{ab}	26.17± 1.447^{b}	27.33± 1.358^{b}	26.48± 1.050^{b}	27.29± 0.917^{b}	26.36± 1.236^{b}
19.	Chloramphenicol	22.49± 0.576^{a}	24.05± 0.462^{ab}	26.00± 0.000^{bc}	25.00± 1.000^{ab}	24.63± 0.800^{ab}	28.00± 0.516^{c}	22.00± 0.730^{a}	24.10± 0.601^{ab}	24.11± 0.365^{ab}	23.14± 0.363^{ab}
20.	Ciprofloxacin	15.80± 0.393^{ab}	16.30± 0.603^{ab}	17.67± 0.882^{abc}	15.50± 0.500^{a}	17.38± 1.164^{abc}	18.17± 0.307^{abc}	19.17± 0.703^{abc}	18.43± 0.510^{abc}	19.64± 0.681^{bc}	21.00± 0.903^{c}
21.	Clindamycin	20.66± 0.462	21.05± 0.320	22.00± 0.000	19.50± 0.500	20.00± 0.378	24.17± 0.601	20.67± 0.211	21.43± 0.519	21.39± 0.458	21.29± 0.646
22.	Cloxacillin	20.20± 2.165^{ab}	29.80± 0.826^{cd}	30.33± 1.856^{cd}	19.50± 0.500^{a}	27.75± 2.403^{bc}	37.33± 0.422^{d}	32.33± 0.558^{cd}	32.24± 0.956^{cd}	33.89± 0.422^{cd}	28.25± 1.118^{bc}
23.	Doxycyline Hydrochlorid	19.17± 0.958^{ab}	22.75± 0.507^{abcd}	22.67± 2.404^{abcd}	21.00± 1.000^{abc}	19.13± 0.934^{ab}	26.83± 1.014^{d}	23.50± 0.500^{bcd}	25.00± 0.632^{cd}	24.46± 0.607^{cd}	18.14± 0.960^{a}
24.	Faropenem	22.31± 2.148^{a}	29.60± 0.634^{b}	32.67± 1.202^{bc}	34.00± 1.000^{bc}	31.38± 1.426^{bc}	39.00± 0.856^{c}	36.50± 0.563^{bc}	36.00± 0.644^{bc}	36.46± 0.508^{bc}	34.07± 0.783^{bc}

S. No.	Name of Antibiotics	Mean± SEM values of diameter of inhibition zone (mm) of *S. aureus* isolates (No. of isolate)									
		Human (35)	Meat piece (20)	Horse (3)	Pig (2)	Camel (8)	Dog (6)	Sheep (6)	Buffalo (21)	Goat (28)	Cattle (28)
25.	Gentamicin	14.89± 0.878^{abc}	16.7± 0.700^{abc}	16.67± 2.404^{abc}	14.5± 0.500^{ab}	13.13± 1.060^{a}	18.33± 0.989^{bc}	17.83± 0.307^{bc}	17.1± 0.487^{abc}	19.36± 0.499^{c}	16.89± 0.796^{abc}
26.	Imipenem	27.60± 1.496^{a}	32.95± 0.478^{ab}	40.33± 0.333^{cde}	38.00± 1.000^{bcd}	35.13± 1.563^{bc}	44.67± 1.520 ^{de}	43.83± 1.138^{de}	43.95± 1.279^{de}	46.32± 0.584^{e}	43.96± 1.032^{de}
27.	Levofloxacin	20.34± 0.281^{ab}	19.40± 0.505^{ab}	21.00± 0.577^{ab}	18.50± 0.500^{a}	19.25± 0.773^{ab}	20.00± 0.000^{ab}	21.33± 0.494^{ab}	22.29± 0.834^{b}	21.75± 0.586^{ab}	21.29± 0.640^{ab}
28.	Linezolid	24.29± 0.409^{cd}	24.15± 0.499^{cd}	23.00± 1.155^{bc}	17.50± 0.500^{a}	19.75± 0.796^{ab}	27.83± 0.946^{d}	24.83± 0.401^{cd}	24.24± 1.327^{cd}	23.54± 0.444^{bc}	22.57± 0.789^{bc}
29.	Meropenem	23.77± 0.519^{a}	26.00± 0.503^{ab}	25.00± 2.517^{ab}	27.00± 1.000^{abc}	23.88± 1.817^{a}	30.67± 2.813^{c}	28.00± 0.931^{abc}	29.00± 0.680^{bc}	30.93± 0.430^{c}	28.00± 0.934^{abc}
30.	Methicillin	10.20± 0.970^{a}	12.90± 0.584^{ab}	14.67± 2.404^{abc}	15.50± 0.500^{abc}	12.88± 2.117^{ab}	15.83± 0.946^{abc}	19.17± 1.222^{c}	15.62± 1.170^{abc}	18.82± 0.607^{bc}	15.68± 1.101^{abc}
31.	Nitrofurantoin	18.94± 0.224^{c}	19.25± 0.260^{c}	21.00± 0.000^{c}	19.00± 0.000^{a}	20.63± 0.263^{ab}	20.83± 0.401^{d}	19.33± 0.333^{cd}	19.48± 0.356^{cd}	19.89± 0.327^{c}	21.18± 0.834^{abc}
32.	Norfloxacin	10.11± 0.757^{a}	10.75± 0.894^{a}	11.33± 0.333^{ab}	10.50± 0.500^{a}	12.25± 1.770^{abc}	17.00± 2.708^{cd}	16.33± 1.145^{bcd}	16.10± 0.700^{bcd}	17.07± 0.907^{cd}	18.29± 0.955^{d}
33.	Oxacillin	12.66± 1.505^{a}	19.15± 1.084^{ab}	18.67± 2.333^{ab}	22.50± 0.500^{b}	18.25± 1.264^{ab}	24.67± 1.202^{b}	25.50± 0.671^{b}	23.10± 1.481^{b}	24.36± 0.458^{b}	22.54± 1.252^{b}
34.	Penicillin –G	8.49± 0.633^{ab}	8.10± 0.957^{a}	10.33± 0.333^{abcd}	9.50± 0.500^{abc}	7.63± 1.802^{a}	16.33± 0.989^{d}	23.50± 2.320^{e}	14.52± 1.247^{bcd}	14.68± 0.977^{cd}	14.50± 1.245^{bcd}
35.	Piperacillin	8.69± 0.659^{ab}	8.25± 0.885^{ab}	10.00± 1.155^{ab}	10.00± 0.000^{ab}	6.63± 1.954^{a}	14.00± 0.775^{bc}	24.00± 2.049^{d}	15.33± 1.379^{bc}	17.46± 1.293^{cd}	18.21± 1.504^{cd}
36.	Piperacillin+ Tazobactam	19.89± 0.694^{a}	23.25± 0.771^{abc}	22.67± 1.333^{ab}	25.00± 1.000^{bcd}	24.63± 1.362^{bcd}	25.83± 0.946^{bcd}	26.33± 1.116^{bcd}	25.52± 0.639^{bcd}	27.86± 0.601^{d}	27.14± 0.723^{cd}
37.	Polymxin –B	13.40± 0.250^{ab}	14.05±0.50^{ab}	15.67± 0.882^{bc}	15.50± 0.500^{abc}	13.25± 1.013^{ab}	13.50± 0.847^{ab}	13.00± 0.730^{a}	13.71± 0.443^{ab}	13.71± 0.506^{ab}	16.57± 0.350^{c}
38.	Tetracycline	18.54± 0.856^{ab}	25.05± 0.663^{cd}	24.33± 3.180^{cd}	22.50± 0.500^{bc}	17.38± 2.035^{a}	28.00± 0.894^{d}	24.17± 0.543^{cd}	25.62± 0.667^{cd}	23.5± 0.618^{cd}	18.21± 0.948^{ab}
39.	Ticarcillin	17.09± 1.176^{a}	22.60± 0.690^{abcd}	19.67± 1.202^{ab}	21.00± 1.000^{abc}	22.38± 1.085^{abc}	25.50± 1.478^{bccde}	28.67± 1.382^{de}	25.71± 0.753^{bcde}	29.18± 0.841^{e}	26.25± 1.166^{cde}
40.	Ticarcillin+ Clavulanic Acid	17.74± 1.191^{a}	22.25± 0.820^{ab}	21.33± 1.856^{ab}	21.00± 1.000^{ab}	22.50± 1.254^{abc}	27.00± 1.693^{bcd}	28.67± 1.308^{cd}	27.48± 0.748^{bcd}	29.86± 0.912^{d}	27.43± 1.133^{bcd}
41.	Tobramycin	13.91± 0.882^{a}	18.1± 0.410 ^{b}	17.00± 1.155^{ab}	18.5± 0.500^{b}	15.88± 0.611^{ab}	19.83± 0.872^{b}	18.00± 0.365^{b}	19.1± 0.507^{b}	19.0± 0.466^{b}	19.07± 0.615^{b}
42.	Vancomycin	14.46± 0.257^{ab}	15.55± 0.473^{ab}	14.00± 0.577^{a}	15.00± 0.000^{ab}	15.38± 0.263^{ab}	16.33± 0.211^{b}	13.50± 0.224^{a}	13.62± 0.722^{a}	14.82± 0.200^{ab}	15.61± 0.188^{ab}

i. Duncan's Homogeneous Subsets (DMRT) analysis of Mean± SEM values of diameter of inhibition zone performed at level of 0.05, different superscripts indicating significant variations at ($p \leq 0.05$) level
ii. Value in parenthesis indicates no. of isolates
iii. Mean comparisons have been made for a row
iv. Mean superscribed by similar alphabet show non-significant difference ($p > 0.05$)
v. Mean superscribed by different alphabet show Significant ($p \leq 0.05$)
vi. Mean not superscribed by alphabet indicates, that mean value has not processed for DMRT

Table. 15: Analysis of significance level of probability of variance of diameter of inhibition zone (mm) of antibiotics by Bonferroni correction test for *S. aureus* isolates.

S. No.	Name of Antibiotics	Comparison of probability level of variance among sources of *S. aureus* isolates									
		Human	Meat piece	Horse	Pig	Camel	Dog	Sheep	Buffalo	Goat	Cattle
1.	Ampicillin	$S^{0.000}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$S^{0.000}$, $G^{0.004}$ $C^{0.005}$	$S^{0.006}$	NS	$S^{0.000}$, $B^{0.008}$, $G^{0.001}$, $C^{0.001}$	NS	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.00}$, $J^{0.000}$	$H^{0.000}$, $J^{0.008}$	$H^{0.000}$, $Mt^{0.004}$, $J^{0.001}$	$H^{0.000}$, $Mt^{0.005}$, $J^{0.001}$
2.	Ampicillin+ Sulbactum	$Mt^{0.000}$,$D^{0.000}$,$S^{0.001}$,$B^{0.000}$ $G^{0.000}$, $C^{0.000}$	$H^{0.000}$	NS	NS	$D^{0.046}$	$H^{0.000}$, $J^{0.046}$	$H^{0.001}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
3.	Azithromycin	$D^{0.000}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$D^{0.004}$, $S^{0.000}$ $B^{0.000}$,$G^{0.000}$ $C^{0.002}$	NS	NS	$D^{0.030}$, $S^{0.002}$ $B^{0.000}$ $G^{0.000}$	$H^{0.000}$, $Mt^{0.004}$ $J^{0.030}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.002}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.000}$, $C^{0.009}$	$H^{0.000}$, $Mt^{0.000}$ $J^{0.000}$	$H^{0.000}$, $Mt^{0.002}$ $B^{0.009}$
4.	Aztreonam	$Mt^{0.000}$, $D^{0.001}$, $S^{0.006}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.000}$	NS	NS	NS	$H^{0.001}$	$H^{0.006}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
5.	Cefaclor	$D^{0.001}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$S^{0.019}$, $B^{0.011}$, $G^{0.002}$, $C^{0.041}$	NS	NS	$G^{0.050}$	$H^{0.001}$	$H^{0.001}$, $Mt^{0.019}$	$H^{0.000}$, $Mt^{0.011}$	$H^{0.000}$, $Mt^{0.002}$ $J^{0.050}$	$H^{0.000}$, $Mt^{0.041}$
6.	Cefalothin	$Mt^{0.006}$, $D^{0.018}$ $S^{0.005}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.006}$	NS	NS	NS	$H^{0.018}$	$H^{0.005}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
7.	Cefepime	$Mt^{0.000}$, $D^{0.000}$ $S^{0.042}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.000}$	NS	NS	NS	$H^{0.000}$	$H^{0.042}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
8.	Cefixime	$Mt^{0.016}$, $D^{0.001}$ $S^{0.000}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.016}$, $S^{0.036}$ $G^{0.009}$	NS	NS	$G^{0.036}$, $S^{0.036}$	$H^{0.001}$	$H^{0.000}$, $Mt^{0.036}$, $J^{0.036}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.009}$, $J^{0.036}$	$H^{0.000}$, $G^{0.045}$
9.	Cefixime+ Clavulanic Acid	$D^{0.011}$, $S^{0.000}$ $B^{0.00}$, $G^{0.000}$, $C^{0.000}$	$S^{0.003}$, $G^{0.001}$	$S^{0.044}$	NS	$S^{0.034}$	$H^{0.011}$	$H^{0.000}$, $Mt^{0.003}$, $Hrs^{0.04}$, $J^{0.034}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.001}$	$H^{0.000}$
10.	Cefoparazone	$D^{0.045}$, $S^{0.000}$ $B^{0.008}$, $G^{0.000}$ $C^{0.000}$	$S^{0.001}$, $B^{0.041}$, $G^{0.000}$, $C^{0.000}$	NS	NS	NS	$H^{0.045}$	$H^{0.000}$, $Mt^{0.001}$	$H^{0.008}$, $Mt^{0.041}$	$H^{0.000}$, $Mt^{0.000}$	$H^{0.000}$, $Mt^{0.000}$
11.	Cefoparazone+ Sulbactam	$Mt^{0.009}$, $J^{0.036}$, $D^{0.007}$, $S^{0.001}$, $B^{0.000}$, $G^{0.000}$ $C^{0.00}$	$H^{0.009}$	NS	NS	$H^{0.036}$	$H^{0.007}$	$H^{0.001}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
12.	Cefotaxime	$Mt^{0.000}$, $D^{0.000}$, $S^{0.001}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.000}$	NS	NS	NS	$H^{0.000}$	$H^{0.001}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
13.	Cefotaxime+ Clavulanic Acid	$Mt^{0.000}$, $D^{0.000}$, $S^{0.000}$, $B^{0.00}$, $G^{0.000}$, $C^{0.00}$	$H^{0.000}$	NS	NS	NS	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$

S. No.	Name of Antibiotics	Comparison of probability level of variance among sources of *S. aureus* isolates									
		Human	Meat piece	Horse	Pig	Camel	Dog	Sheep	Buffalo	Goat	Cattle
14.	Cefoxitin	$Mt^{0.001}$, $D^{0.000}$, $S^{0.002}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.001}$	NS	NS	NS	$H^{0.000}$	$H^{0.002}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
15.	Ceftazidime	$Mt^{0.001}$, $D^{0.021}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.001}$	NS	NS	NS	$H^{0.021}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
16.	Ceftazidime+ Clavulanic Acid	$Mt^{0.025}$, $D^{0.013}$, $S^{0.001}$, $B^{0.000}$, $G^{0.000}$,$C^{0.000}$	$H^{0.025}$ $G^{0.016}$	NS	NS	$G^{0.040}$	$H^{0.013}$	$H^{0.001}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.016}$, $J^{0.040}$	$H^{0.000}$
17.	Ceftrioxane	$Mt^{0.000}$, $D^{0.001}$, $S^{0.010}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$Mt^{0.000}$	NS	NS	NS	$H^{0.001}$	$H^{0.010}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
18.	Cefzolin	$D^{0.042}$, $S^{0.010}$, $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$G^{0.015}$	NS	NS	NS	$H^{0.042}$	$H^{0.010}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.015}$	$H^{0.000}$
19.	Chloramphenicol	$D^{0.000}$	$D^{0.033}$	NS	NS	NS	$H^{0.000}$, $Mt^{0.033}$, $S^{0.002}$, $B^{0.035}$ $G^{0.026}$, $C^{0.001}$	$D^{0.002}$	$D^{0.035}$	$D^{0.026}$	$D^{0.001}$
20.	Ciprofloxacin	$G^{0.000}$, $C^{0.000}$	$G^{0.021}C^{0.000}$	NS	NS	NS	NS	NS	NS	$H^{0.000}$, $Mt^{0.021}$	$H^{0.000}$, $Mt^{0.000}$
21.	Clindamycin	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
22.	Cloxacillin	$Mt^{0.000}$, $D^{0.000}$, $S^{0.009}$, $B^{0.000}$, $G^{0.000}$, $C^{0.001}$	$H^{0.000}$	NS	NS	NS	$H^{0.000}$	$H^{0.009}$	$H^{0.000}$	$H^{0.000}$	$H^{0.001}$
23.	Doxycyline Hydrochlorid	$D^{0.002}$, $B^{0.000}$ $G^{0.000}$	$C^{0.008}$	NS	NS	$D^{0.029}$, $B^{0.032}$	$H^{0.002}$, $J^{0.029}$, $C^{0.000}$	NS	$H^{0.000}$, $J^{0.032}$ $C^{0.000}$	$H^{0.000}$, $C^{0.000}$	$Mt^{0.008}$, $D^{0.000}$, $B^{0.000}$, $G^{0.000}$
24.	Faropenem	$Mt^{0.0007}$, $J^{0.034}$, $D^{0.000}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$H^{0.007}$ $G^{0.029}$	NS	NS	$H^{0.034}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.029}$	$H^{0.000}$
25.	Gentamicin	$G^{0.000}$	NS	NS	NS	$G^{0.002}$	NS	NS	NS	NS	$H^{0.000}$, $J^{0.002}$
26.	Imipenem	$Mt^{0.045}$, $Hrs^{0.013}$, $J^{0.042}$, $D^{0.000}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$H^{0.045}$, $D^{0.001}$, $S^{0.003}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.013}$	NS	$H^{0.042}$, $B^{0.012}$, $G^{0.000}$, $C^{0.007}$	$H^{0.000}$, $Mt^{0.001}$	$H^{0.000}$, $Mt^{0.003}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.012}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.007}$
27.	Levofloxacin	NS	$B^{0.041}$	NS	NS	NS	NS	NS	$Mt^{0.041}$	NS	NS
28.	Linezolid	$J^{0.043}$	NS	NS	$D^{0.014}$	$H^{0.043}$ $D^{0.001}$	$P^{0.014}$, $J^{0.001}$ $C^{0.038}$	NS	NS	NS	$D^{0.038}$
29.	Meropenem	$D^{0.001}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$G^{0.00}$	NS	NS	$D^{0.028}$, $B^{0.035}$ $G^{0.000}$	$H^{0.001}$, $J^{0.028}$	NS	$H^{0.000}$ $J^{0.035}$	$H^{0.000}$, $Mt^{0.000}$ $J^{0.000}$	$H^{0.000}$
30.	Methicillin	$S^{0.002}$, $B^{0.003}$, $G^{0.000}$,	$G^{0.002}$	NS	NS	NS	NS	$H^{0.002}$	$H^{0.003}$	$H^{0.000}$	$H^{0.001}$

S. No.	Name of Antibiotics	Comparison of probability level of variance among sources of *S. aureus* isolates									
		Human	Meat piece	Horse	Pig	Camel	Dog	Sheep	Buffalo	Goat	Cattle
		$C^{0.001}$								$Mt^{0.002}$	
31.	Nitrofurantoin	$C^{0.007}$	NS	NS	NS	NS	NS	NS	NS	NS	$H^{0.007}$
32.	Norfloxacin	$D^{0.029}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$B^{0.009}$, $G^{0.000}$ $C^{0.000}$	NS	NS	$C^{0.044}$	$H^{0.029}$	NS	$H^{0.000}$, $Mt^{0.009}$	$H^{0.000}$, $Mt^{0.000}$	$H^{0.000}$, $Mt^{0.000}$ $J^{0.044}$
33.	Oxacillin	$Mt^{0.011}$, $D^{0.001}$ $S^{0.000}$, $B^{0.000}$, $G^{0.000}$,$C^{0.000}$	$H^{0.011}$	NS	NS	NS	$H^{0.001}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
34.	Penicillin –G	$D^{0.026}$, $S^{0.000}$, $B^{0.001}$, $G^{0.000}$ $C^{0.000}$	$D^{0.027}$, $S^{0.000}$, $B^{0.003}$, $G^{0.001}$ $C^{0.001}$	$S^{0.014}$	$S^{0.039}$	$S^{0.000}$, $G^{0.029}$ $C^{0.039}$	$H^{0.026}$, $Mt^{0.027}$	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.01}$, $P^{0.039}$, $J^{0.000}$, $B^{0.008,}$ $G^{0.007}$, $C^{0.005}$	$H^{0.001}$ $Mt^{0.003}$ $S^{0.008}$	$H^{0.000}$, $Mt^{0.001}$, $J^{0.029}$, $S^{0.007}$	$H^{0.000}$, $Mt^{0.0001}$, $J^{0.039}$, $S^{0.005}$
35.	Piperacillin	$S^{0.000}$, $B^{0.003}$, $G^{0.000}$, $C^{0.000}$	$S^{0.000}$, $B^{0.006}$, $G^{0.000}$, $C^{0.000}$	$S^{0.038}$	NS	$S^{0.000}$, $B^{0.019}$, $G^{0.000}$, $C^{0.000}$	NS	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.038}$, $J^{0.000}$	$H^{0.003}$, $Mt^{0.006}$, $J^{0.019}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.000}$
36.	Piperacillin+ Tazobactam	$Mt^{0.038}$, $J^{0.034}$, $D^{0.009}$, $S^{0.003}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.038}$, $G^{0.001}$, $C^{0.010}$	NS	NS	$H^{0.034}$	$H^{0.009}$	$H^{0.003}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.001}$	$H^{0.000}$, $Mt^{0.010}$
37.	Polymxin –B	$C^{0.000}$	$C^{0.003}$	NS	NS	$C^{0.005}$	NS	$C^{0.009}$	$C^{0.000}$	$C^{0.000}$	$H^{0.000}$, $Mt^{0.003}$, $J^{0.005}$, $S^{0.009}$, $B^{0.000}$,$G^{0.000}$
38.	Tetracycline	$Mt^{0.000}$, $D^{0.000}$, $B^{0.000}$, $G^{0.000}$	$H^{0.000}$, $J^{0.001}$ $C^{0.000}$	NS	NS	$Mt^{0.001}$, $D^{0.000}$, $B^{0.000}$ $G^{0.013}$	$H^{0.000}$, $J^{0.000}$, $C^{0.000}$	NS	$H^{0.000}$, $J^{0.000}$,$C^{0.000}$	$H^{0.000}$, $J^{0.013}$, $C^{0.000}$	$Mt^{0.000}$, $D^{0.000}$, $B^{0.000}$, $G^{0.000}$
39.	Ticarcillin	$Mt^{0.008}$, $D^{0.012}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.008}$ $G^{0.001}$	NS	NS	NS	$H^{0.012}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.001}$	$H^{0.000}$
40.	Ticarcillin+ Clavulanic Acid	$D^{0.004}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$G^{0.000}$, $C^{0.042}$	NS	NS	$G^{0.027}$	$H^{0.004}$	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$, $Mt^{0.000}$, $J^{0.027}$	$H^{0.000}$, $Mt^{0.042}$
41.	Tobramycin	$Mt,^{0.001}$ $D,^{0.004}$ $B^{0.000}$, $G^{0.000}$ $C^{0.000}$	$H^{0.001}$	NS	NS	NS	$H^{0.004}$	NS	$H^{0.000}$	$H^{0.000}$	$H^{0.000}$
42.	Vancomycin	NS	$B^{0.024}$	NS	NS	NS	$B^{0.045}$	NS	$Mt^{0.024}$, $D^{0.045}$, $C^{0.006}$	NS	$B^{0.006}$

H = Human, Mt = Meat piece, Hrs = Horse, P = Pig, J = Camel, D = Dog, S = Sheep, B = Buffalo, G = Goat, C = Cattle

Superscript showing exact value of probability level of variance

Among the 128 isolates of *S. aureus*, Kumar *et al*. (2010) reported similar antibiogram for tetracycline, gentamicin, kanamycin, methicillin and oxacillin with less resistance but contrary to present study, they reported vancomycin (100% sensitivity), ampicillin (84.4% sensitivity) and penicillin G (22.7% resistance).

Akindele *et al*. (2010) reported resistance pattern of 100 *S. aureus* strains obtained from human clinical infections and found that 90% of isolates were resistant to ampicillin, 96% to penicillin, 16% to ceftriaxone, 39% resistant to gentamicin similar to present study but 71% resistance to tetracycline and 34% resistance to chloramphenicol are higher resistance in comparison to present study.

Similar antibiogram was reported from a total of 115 milk samples from cattle (34.78%), buffalo (34.78%), goat (17.39%) and sheep (13.04%) from which 25 *S. aureus* were isolated by Sharma *et al.* (2011). Almost 80-90% of the isolates showed multiple drug resistance to majority of the antimicrobial agents tested such as ampicillin, cloxacillin, kanamycin and vancomycin. While several isolates were found susceptible to the tetracycline oxacillin and ciprofloxacin.

Sharma *et al*. (2013) reported antibiogram study of 15 *S. aureus* isolates from 46 nasal swabs from pneumonic camels and found that higher resistance toward penicillin-G and ampicillin and higher sensitivity for ciprofloxacin, chloramphenicol, gentamicin, imipenem, ampicillin/Sulbactum, nitrofurantoin and tetracycline like present investigation.

Roy *et al.* (2015) studied antibiotic resistance pattern of *S. aureus* obtained from hospital environment and volunteers working in the hospitals and close to present study results they reported In case of hospital isolates, higher resistance was observed against ampicillin (83.33%). Moderate resistance observed against ciprofloxacin (33.33%) and tetracycline (33.33%).

Yadav *et al.* (2015e) reported similar antibiogram from same area of study, who studied thirty two *S. aureus* isolates obtained from mastitis of cattle and buffalo using 33 antibiotics belonging to different categories and generations. Antibiotics such as doxycycline, gentamicin, methicillin and tobramycin were more effective against all isolates. In case of resistance pattern, maximum resistance was exhibited against polymxin-B and cefixime like present study.

In the present investigation significant difference in the antibiogram pattern among different sources of *S. aureus* may indicates pattern and frequency of use of various antibiotic among human and animals. The antibiotics of initial generations showed lower efficacies than the antibiotics of latest generations. The analysis of the antibiogram revealed that the susceptibility and resistance shown by the isolates was dependent on use of the antibiotics and source of sample i.e. lesser the use more the susceptibility of the isolates was detected. Many workers have worked with *S. aureus* of various origins in regards to their antibiogram patterns and found that *S. aureus* are endowed with capability of developing resistance towards an antibiotic against which isolates are exposed even for shorter periods. Further if exposure to same antibiotics is removed, the isolates become susceptible to that antibiotic against which they were previously resistant.

Multiple Antibiotic Resistance Index (MAR) value

In the present investigation, all the *S. aureus* isolates were multidrug resistant (MDR) isolates (Except one cattle/C3R). In an effort for risk assessment of MDR isolates this index was given by Krumperman (1983). This index is an epidemiological tool which is used for risk analysis of environment through bacterial contamination and now a days it is used to assess whether the group of isolates/ individual isolate has originated from an environment where several antibiotics have been used or not. Index of isolated group of bacteria/individual bacteria if greater than 0.2, implies that strains of such bacteria originate from an environment where several antibiotics were used and more than 0.2 MAR group is an high risk potential source of spread MDR.

In the present study all multidrug resistant isolates were evaluated for their both group and individual Multiple Antibiotic Resistance (MAR) index. In the group MAR, *S. aureus* isolates from human sources (0.40) had highest MAR index and sources from camel (0.25), meat pieces (0.24), pig (0.23) and horse (0.21) has more than 0.20 MAR in decreasing order. Other group of *S. aureus* isolates of animal sources such as cattle, dog, buffalo, goat and sheep had less than 0.20 MAR as described in table 16. In the individual isolate MAR index, total of the 157 isolates 66 (42%) isolates had 0.2 or more than 0.2 MAR index value with high risk potential source of

spread MDR. These isolates comprised most of human and meat piece isolates while 91 (58%) isolates had less than 0.2 MAR index value with less risk source of MDR containing most of animal origin isolates (Table 17).

Similar to present study Adeyemi *et al* (2015) reported more than 0.2 MAR index among *S. aureus* isolates obtained from diseased human individuals. Similarly, Vijayalakshmi *et al*, (2013) screened 12 *S. aureus* isolates from human wound samples for nine different group of antibiotics and found that 100% isolates were multidrug resistant with more than 0.22 MAR index. Close to our results, Udobi *et al.* (2013) reported MAR index *S. aureus* isolates obtained from various clinical sample of human sources and detected that 79.6%, 60.6%, and 76.5% of wound, skin, and bed isolates had MAR index greater than 0.25%.

Shamila-Syuhada *et al.* (2016) studied the antibiotics resistance among *S. aureus* isolates isolated from raw milk samples obtained from small scale dairy farms and reported similar results with present study that MAR index ranging from 0.08 to 0.67. Ali *et al.* (2015) studied *S. aureus* isolates from mastitic milk samples of buffalo in Egypt and found that most of isolates having more than 0.28 MAR index in comparison to present study while only few isolates having less than 0.2 MAR.

Table 16: Detection of group Multiple Antibiotic Resistance Index (MAR) value among sources of *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Aggregate antibiotic resistance score	Group MAR index value	Significance
1.	Human	35	585	0.40	Greater than 0.2 MAR Index of group indicates that several antibiotics were used in that group and more than 0.2 MAR group is an high risk potential source of spread MDR
2.	Meat piece	20	192	0.24	
3.	Horse	3	27	0.21	
4.	Pig	2	19	0.23	
5.	Camel	8	84	0.25	
6.	Dog	6	23	0.10	
7.	Sheep	6	19	0.08	
8.	Buffalo	21	89	0.10	
9.	Goat	28	100	0.09	
10.	Cattle	28	187	0.16	
Decreasing Order of MAR index Value: - Human > Camel > Meat pieces > Pig > Horse > Cattle > Dog = Buffalo >Goat > Sheep.					

Table 17: Detection and Distribution of Multiple Antibiotic Resistance Index (MAR) value among individual *S. aureus* isolates.

S. No.	MAR Index Value Type (MAR)	Isolate I.D.	No. of Isolate	No. of antibiotic, which the isolate was resistant	MAR Index Value	Significance
1.	MAR1	H2	1	34	0.81	66 (42%) isolates had 0.2 or more than 0.2 MAR index value with high risk potential source of spread MDR
2.	MAR2	H1 and H8	2	32	0.76	
3.	MAR3	H3 and H4	2	31	0.74	
4.	MAR4	H5	1	29	0.69	
5.	MAR5	H29	1	28	0.67	
6.	MAR6	H6	1	25	0.6	
7.	MAR7	H27	1	23	0.55	
8.	MAR8	J4	1	21	0.50	
9.	MAR9	H25, Mt26	2	20	0.48	
10	MAR10	H7 and H48	2	19	0.45	
11.	MAR11	H24, H39, H46 and C29	4	18	0.43	
12.	MAR12	H40	1	17	0.40	
13	MAR13	H30 and Mt2	2	16	0.38	

14.	MAR14	H28, J14 and C39	3	15	0.36	
15.	MAR15	H33, H37, H45, Mt3, C37 and C43	6	14	0.33	
16.	MAR16	Mt4, Mt9 and J18	3	13	0.31	
17.	MAR17	Hrs3 and C12	2	12	0.29	
18.	MAR18	H12, H21, H34, H44, Mt27, Pg2 and C52	7	11	0.26	
19.	MAR19	H14, H22, Mt19, J3, B24, C13, C41 and C46	8	10	0.24	
20.	MAR20	H11, H15, Mt13, C34 and Hrs4	5	9	0.21	
21.	MAR21	H41, Mt1, Mt10, Mt15, Mt22, Mt24, Mt25, Mt28, Pg4, B27 and C5R	11	8	0.20	
22.	MAR22	H13, Mt14, Mt20, Hrs1, J9, J10, B1, B46, B55, C36 and C40	11	7	0.17	91 (58%) isolates had less than 0.2 MAR index value with less risk source of MDR
23.	MAR23	H10, Mt11, Mt12, J15, AG8 and G9	6	6	0.14	
24.	MAR24	H9, H16, H31, J2, D7, D9, SN4, B23, B26, B30, B39, AG15, G24, G29, G39, G46, G49, C23 and C26	19	5	0.12	
25.	MAR25	Mt31, D4, B21, B28, B31, B34, B42, B43, B57, AG13, G1, G7, G16 and G21	14	4	0.10	
26.	MAR26	D6, D10, D13, SV2, SV3, SN3, SN14, B29, B36, AG5, AG6, AG17, G2, G11, G40, G41, G55, C2R, C7, C8, C11, C17, C20 and C50	24	3	0.07	
27.	MAR27	SV4, B5, B10, B19, AG10, G10, G35, G37, G43, G45, G47, C2, C9, C15, C22 and C47	16	2	0.05	
28.	MAR28	C3R	1	1	0.02	

MIC determination of *S. aureus* isolates

Antimicrobial resistance is increasing day-by-day and is a major problem for diseases management so it is very necessary to know the exact dose of antibiotic which can be used for avoiding indiscriminate or misuse of antibiotic for disease management. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antibiotics that will inhibit the visible growth of a microorganism after overnight incubation Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate (Andrews, 2006).

In the present investigation, all 157 isolates were subjected to MIC determination for eight antibiotics by Ezy MIC™ Strip method (Fig. 11). Average MIC for the eight antibiotics in the present study are shown in the table 18.

Among all sources of isolates, highest MIC value of azithromycin (185.43 mcg/ml), vancomycin (1.52 mcg/ml), gentamicin (36.22 mcg/ml), chloramphenicol (12.20 mcg/ml) and penicillin (32.00 mcg/ml, equal with horse) was recorded for human isolates, value of oxacillin (32.60 mcg/ml) for camel isolates, value of ciprofloxacin (32.0 mcg/ml) for equally horse and pig and highest MIC value of ceftriaxone (42.30 mcg/ml) was found for buffalo isolates. While lowest MIC value of azithromycin (3.0 mcg/ml) for pig, value of vancomycin (0.90 mcg/ml), oxacillin (0.06 mcg/ml), ciprofloxacin (5.75 mcg/ml), ceftriaxone (2.17 mcg/ml) and penicillin (0.74 mcg/ml) for sheep isolates, value of gentamicin (1.31 mcg/ml) for goat isolates and lowest MIC value of chloramphenicol (1.83 mcg/ml) was found for horse isolates.

Since many variations and wide ranges of highest and lowest were found in the MICs value of studied antibiotics for *S. aureus* isolates from different sources thus the statistical analysis was carried out and comparison of MIC value of each antibiotics between each source of isolate was made. The isolates from different sources

showed highly significant ($p \leq 0.01$) variation in their MIC values for three antibiotics namely azithromycin, ciprofloxacin and penicillin, significant variation ($p \leq 0.05$) for vancomycin, and no significant variation ($p > 0.05$) for ceftriaxone, chloramphenicol, gentamicin and oxacillin as described in table 19.

In the present study significant ($p \leq 0.05$) variations for MIC values of four antibiotics thus except ceftriaxone, chloramphenicol, gentamicin and oxacillin ($p > 0.05$), all sources of isolates were further subjected to Duncan's Homogeneous Subsets analysis (DMRT) of Mean± SEM values of MIC of antibiotics at level of 0.05, different superscripts indicating significant variations at ($p \leq 0.05$) level (Table 14). In the DMRT analysis maximum three subsets were detected for ciprofloxacin and two subset were reported for azithromycin, penicillin and vancomycin (Table 20). Further the Bonferroni correction was carried out to find out exact value of probability level of variance of MIC values among different sources of *S. aureus* isolates. It was found that *S. aureus* isolates from human and meat piece sources were significantly variable with $p \leq 0.0001$ probability level of variance with other animal origin sources for azithromycin, ciprofloxacin and penicillin (Table 21).

Hierarchical ascendant cluster analysis of MICs values through mean values of MICs of antibiotics against *S. aureus* isolates was carried out by Squared Euclidean Distance (SED) and the between-groups linkage method. The isolates were clustered into three groups at 5.0 rescaled cluster distance, one cluster comprising of buffalo, cattle, sheep, dog, pig and goat isolates, second cluster included horse, camel and meat piece isolates while third cluster included human isolates (Fig. 12). All three clusters had significant variation ($p \leq 0.05$) among each other. Overall lower MICs of studied antibiotics were recorded among animal origin isolates in comparison to human origin isolates.

Amorena *et al.* (1999) reported MIC of slime producing *S. aureus* obtained from mastitis samples and found MIC value of penicillin (0.031 mg/L), gentamicin (2.0) mg/L and vancomycin (1.0 mg/L) and MIC value of ciprofloxacin (0.25 mg/L). They detected lower values of MIC of studied antibiotic from same mastitis source in comparison to present study. It indicates that with the difference of time MICs of antibiotic showed increasing pattern. Barcia-Macay *et al.* (2006) studied standard strain of *S. aureus* (ATCC 25923) and found lowered MIC in comparison to present study such as MIC of azithromycin (0.5 mg/liter), gentamicin (0.5 mg/liter), penicillin (0.015 mg/liter), oxacillin (0.125 mg/liter), vancomycin (1.0 mg/liter) and MIC of ciprofloxacin (0.125 mg/liter). Higher values of MICs of same antibiotic in the present study may indicate the exposure of field strains with antibiotics in routine.

Table 18: Determination and Distribution of Minimum Inhibitory Concentration (MIC) of following antibiotics for studied *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Antibiotic with average value of MIC in mcg/ml							
			Azithromycin (0.016-256 mcg/ml)	Vancomycin (0.016-256 mcg/ml)	Oxacillin (0.016-256 mcg/ml)	Gentamicin (0.064-1024 mcg/ml)	Ciprofloxacin (0.002-32 mcg/ml)	Chloramphenicol (0.016-256 mcg/ml)	Ceftriaxone (0.016-256 mcg/ml)	Penicillin (0.002-32 mcg/ml)
1.	Human	35	185.43[a]	1.52[a]	3.89	36.22[a]	28.19	12.20[a]	20.17	32.00[a]
2.	Meat piece	20	97.20	1.47	3.95	5.93	31.20	3.60	11.15	30.10
3.	Horse	3	86.34	1.17	0.50	8.00	32.00[a]	1.83[b]	6.00	26.67
4.	Pig	2	3.00[b]	1.50	0.32	11.00	32.00[a]	5.00	4.00	32.00[a]
5.	Camel	8	76.48	1.25	32.60[a]	17.00	26.50	3.38	16.04	26.00
6.	Dog	6	34.58	1.0	0.30	5.21	20.67	2.17	4.19	4.42
7.	Sheep	6	12.50	0.90[b]	0.06[b]	1.42	5.75[b]	2.33	2.17[b]	0.74[b]
8.	Buffalo	21	5.19	0.92	12.32	3.43	12.77	9.12	42.30[a]	2.60
9.	Goat	28	14.10	1.12	0.20	1.31[b]	10.31	2.65	23.78	5.83
10.	Cattle	28	51.21	1.20	1.04	8.10	6.70	3.00	8.50	8.70
Total no. of isolates		157	Average value of MIC of each antibiotic for total isolates							
			73.40	1.22	4.92	12.35	18.38	5.85	18.55	16.35

Superscript: a – Highest value of MIC of Azithromycin, Vancomycin, Gentamicin, Chloramphenicol and Penicillin (equal with horse) for Human, MIC of Oxacillin for Camel, MIC of Ciprofloxacin for equally horse and pig and Highest value of MIC of Ceftrioxane for buffalo.

b- Lowest value of MIC of Azithromycin for pig, MIC of Vancomycin, Oxacillin, Ciprofloxacin, Ceftrioxane and Penicillin for Sheep, MIC of Gentamicin for Goat and lowest value of MIC of Chloramphenicol for horse.

Table 19: Analysis of variance of Mean± SEM values of Minimum Inhibitory Concentration (MIC) of antibiotics for *S. aureus* isolates.

S. No.	Source of variation	MSS
1.	Azithromycin	78144.592**
2.	Ceftriaxone	2271.111^{NS}
3.	Chloramphenicol	278.629^{NS}
4.	Ciprofloxacin	1710.811**
5.	Gentamicin	3071.996^{NS}
6.	Oxacillin	971.922^{NS}
7.	Penicillin	2770.524**
8.	Vancomycin	0.787*

i. Degree of freedom (*df*)= 9
ii. * = Significant ($p \leq 0.05$)
iii. ** Highly significant ($p \leq 0.01$)
iv. NS = Non-significant ($p > 0.05$)
v. MSS = Mean Sum of Square

Table 20 : Duncan's Homogeneous Subsets analysis of Mean± SEM values of Minimum Inhibitory Concentration (MIC) of following antibiotics for *S. aureus* isolates.

S. No.	Name of Antibiotics	MIC Mean± SEM values of isolate (No. of isolate)									
		Human (35)	Meat piece (20)	Horse (3)	Pig (2)	Camel (8)	Dog (6)	Sheep (6)	Buffalo (21)	Goat (28)	Cattle (28)
1.	Azithromycin	185.43± 17.430[b]	97.20± 21.514[a]	86.33± 84.667[a]	3.00± 0.000[a]	76.37± 17.779[a]	34.58± 31.482[a]	12.50± 10.301[a]	4.98± 2.948[a]	13.98± 9.139[a]	51.20± 16.686[a]
2.	Ceftrioxane	20.17± 7.176	11.15± 2.892	6.00± 3.000	4.00± 0.000	16.04± 11.440	4.18± 0.989	2.17± 0.307	42.29± 19.570	23.78± 12.472	8.45± 1.467

3.	Chloramphenicol	12.20± 7.179	3.60± 0.245	1.83± 0.000	5.00± 1.000	3.38± 0.183	2.17± 0.307	2.33± 0.333	9.11± 4.461	2.64± 0.203	2.99± 0.286
4.	Ciprofloxacin	28.18± 1.616^{c}	31.20± 0.800^{c}	32.00± 0.000^{c}	32.00± 0.000^{c}	26.50± 2.922^{c}	20.67± 5.103^{bc}	5.75± 5.167^{a}	12.76± 2.718^{ab}	10.30± 2.294^{ab}	6.70± 1.888^{a}
5.	Gentamicin	36.22± 29.088	5.92± 1.331	8.00± 4.000	11.00± 5.000	17.00± 3.273	5.20± 2.789	1.41± 0.167	3.42± 0.938	1.31± 0.254	7.96± 2.055
6.	Oxacillin	3.88± 1.064	3.95± 1.890	0.47± 0.000	0.31± 0.000	32.58± 31.857	0.29± 0.000	0.05± 0.000	12.32± 12.143	0.19± 0.000	1.03± 0.296
7.	Penicillin	32.00± 0.000^{b}	30.10± 1.532^{b}	26.67± 5.33^{b}	32.00± 0.000^{b}	26.00± 2.928^{b}	4.41± 1.667^{a}	0.74± 0.333^{a}	2.59± 0.713^{a}	5.82± 1.537^{a}	8.70± 2.176^{a}
8.	Vancomycin	1.51± 0.060^{b}	1.37± 0.105^{b}	1.16± 0.333^{b}	1.50± 0.000^{b}	1.25± 0.189^{b}	0.95± 0.167^{b}	0.87± 0.000^{b}	0.91± 0.000^{a}	1.11± 0.090^{b}	1.18± 0.262^{b}

i. Duncan's Homogeneous Subsets (DMRT) analysis of Mean± SEM values of diameter of inhibition zone performed at level of 0.05, different superscripts indicating significant variations at ($p \leq 0.05$) level
ii. Value in parenthesis indicates no. of isolates
iii. Mean comparisons have been made for a row
iv. Mean superscribed by similar alphabet show non-significant difference ($p > 0.05$)
v. Mean superscribed by different alphabet show Significant ($p \leq 0.05$)
vi. Mean not superscribed by alphabet indicates, that mean value has not processed for DMRT

Table 21: Analysis of significance level of variance of Minimum Inhibitory Concentration (MIC) of antibiotics by Bonferroni test for *S. aureus* isolates.

S. No	Name of Antibiotics	Comparison of significance level among Sources of *S. aureus* isolates									
		Human	Meat piece	Horse	Pig	Camel	Dog	Sheep	Buffalo	Goat	Cattle
1.	Azithromycin	$Mt^{0.004}$, $J^{0.24}$, $D^{0.001}$ $S^{0.000,}$ $B^{0.000}$, $G^{0.00}$, $C^{0.000}$	$H^{0.004,}$ $B^{0.011,}$ $G^{0.019}$	NS	NS	$H^{0.024}$	$H^{0.001}$	$H^{0.000}$	$H^{0.000,}$ $Mt^{0.011}$	$H^{0.000}$, $Mt^{0.019}$	$H^{0.000}$
2.	Ceftriaxone	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3.	Chloramphenicol	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4.	Ciprofloxacin	$S^{0.000,}$ $B^{0.000}$, $G^{0.00}$, $C^{0.000}$	$S^{0.000}$ $B^{0.000}$, $G^{0.00}$, $C^{0.000}$	$S^{0.020}$, $G^{0.033}$ $C^{0.004}$	$C^{0.048}$	$S^{0.013}$, $G^{0.006}$, $C^{0.000}$	NS	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.020}$, $J^{0.013}$	$H^{0.000}$, $Mt^{0.000}$	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.033}$, $J^{0.006}$	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.004}$ $P^{0.048}$, $J^{0.000}$
5.	Gentamicin	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
6.	Oxacillin	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7.	Penicillin	$D^{0.000}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$D^{0.000}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$D^{0.001}$, $S^{0.000}$, $B^{0.000}$, $G^{0.000}$, $C^{0.002}$	$D^{0.000}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$, $C^{0.001}$	$D^{0.000}$, $S^{0.000}$ $B^{0.000}$, $G^{0.000}$, $C^{0.000}$	$H^{0.000}$, $Mt^{0.000}$, $Hrs^{0.001}$, $P^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$ $Hrs^{0.000}$, $P^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$ $Hrs^{0.000}$, $P^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$ $Hrs^{0.000}$, $P^{0.000}$, $J^{0.000}$	$H^{0.000}$, $Mt^{0.000}$ $Hrs^{0.002}$, $P^{0.001}$, $J^{0.000}$
8.	Vancomycin	$B^{0.038}$	NS	NS	NS	NS	NS	NS	$H^{0.038}$,	NS	NS

H = Human, Mt = Meat piece, Hrs = Horse, P = Pig, J = Camel, D = Dog, S = Sheep, B = Buffalo, G = Goat, C = Cattle
Superscript denotes exact value of level of significance

Russi *et al*. (2008) reported similar MICs for *S. aureus* isolates from mastitis as in the present study. They isolated 95 *S. aureus* strains causing both clinical and subclinical bovine mastitis from 61 dairy farms in Argentina and detected MIC50 and MIC90 as follows: penicillin, 0.05 and 4 microg/ml; oxacillin, 0.25 and 0.25 microg/ml; gentamicin, 0.25 and 0.5 microg/ml. Similar to present study in an MIC study of 148 food origin isolates of *S. aureus* Pereira *et al*. (2009) found that 38% of the isolates were resistant to oxacillin with >or= 6 microg/mL MIC. Similar MICs were also reported by Japoni *et al*. (2010) during study of 356 *S. aureus* isolates from the hospitalized patients and determined their MIC_{50} (µg/ml) and MIC_{90} (µg/ml) for oxacillin, vancomycin, ciprofloxacin, and gentamicin. MIC_{50} (µg/ml) were 256.0, 2.0, 32.0 and 250.0 while MIC_{90} (µg/ml) were >256.0, 4.0, 32.0 and 256.0 respectively for mentioned antibiotics.

F. MRSA (*mecA* gene), Beta-lactamase (*blaZ* gene) ESBL and VRSA activity:

Since 1970s, *S. aureus* strains have acquired resistance to the penicillinase-stable penicillins and become a major problem worldwide. The resistance is the result of a penicillin binding protein (PBP-2a) encoded by the chromosomal *mec*A gene. These strains historically are termed methicillin resistant *S. aureus* (MRSA) and are resistant to beta-lactam antibiotics. These strains produced penicillinases (beta lactamase) encoded by the chromosomal *blaZ* gene and other Extended-spectrum beta-lactamase (ESBL) enzymes and become not only resistant to penicillin but also to cephalosporins, monobactams and carbapenems antibiotics. Researchers found the way to combat MRSA through glycopeptide antibiotic vancomycin and increasing prevalence of MRSA infections has led to the extensive use of vancomycin. In-fact, vancomycin was the treatment of choice for MRSA infections. However, the overuse of this antibiotic has led to the emergence of vancomycin resistant *S. aureus* strains

(VRSA). In the present study the phenotypic and genotypic characteristic of *S. aureus* isolates in regards to MRSA, beta lactamase, ESBL and VRSA activities were investigated.

Of the 157 isolates, phenotypically, 85 (54.1%) were detected as MRSA by MeReSa agar base method (MeReSa Selective Supplement having Methicillin 2mg/ml + Cefoxitin 3mg/ml in 100 ml media) (Fig.13A) and 19 (12.1%) isolates were positive by methicillin disk (5mcg) methods (Fig.13B). Of the all studied sources, human, meat piece and cattle sources were found more positive for MRSA by both phenotypic methods (Table 22). The differences in the prevalence of MRSA by two methods may be because of different concentration of antibiotic in the mentioned methods.

Vancomycin resistant *S. aureus* activity was detected in 52 (33.1%) isolates by vancomycin disc (30 mcg). Maximum VRSA activity (100%) was found in sheep isolates followed by horse 66.6 % (2/3), buffalo 57.1% (12/21) and human 51.4 (18/35) in decreasing order and other sources were positive with variable percentage as described in table 22 but none of the isolate from pig, camel and dog was found to be positive for VRSA.

Beta-lactamase activity was present in 142 (90.4%) isolates (Fig.14). The activity was shown by 100% isolates from human, meat piece, horse, pig and dog. Extended-spectrum beta-lactamase (ESBL) activity was exhibited by 108 (68.8%) isolates, with more than 85% isolates from human, pig and camel. Least activity of ESBL was detected in buffalo isolates and other sources were positive for Beta-lactamase and ESBL activity with variable percentage as described in table 22.

In the present study, *mecA* gene was found in 37 (23.6%) isolates with single amplicon of 533bp (Fig. 15). Highest prevalence was recorded in human (51.4%) followed by horse (66.6%) meat piece (40.0%) and camel (37.5%) in decreasing order and none of the isolates from pig, dog and goat was positive. The *blaZ* gene was found in 137 (83.3%) isolates with single amplicon of 517bp (Fig. 16). Isolates from human, horse, pig were 100% positive while other isolates was found to be positive with variable percentage (table 23).

In the present study three isolates were MRSA positive by both phenotypic methods but *mecA* gene was absent (H46, Mt24 and C41) and five isolates were MRSA negative by both phenotypic methods but *mecA* gene was present (H31, Mt10, Mt11, Mt14 and C47).

Similar to present study, Murakami *et al.* (1991) reported that three strains (3%) of 111 *mecA*-positive *S. aureus* isolates exhibited resistance to both oxacillin and methicillin as the *mecA*-negative ones and did not produce detectable amounts of PBP 2' despite the presence of the *mecA* gene. Similarly, MO and Qi-nan (1997) studied 184 strains of *S. aureus* obtained from human clinical infections for rapid detection of MRSA. A total of 57 of 58 oxacillin-resistant *S. aureus* strains were *mecA*-positive, whereas 3 of 126 oxacillin-susceptible strains were *mecA*-positive.

Memon *et al.* (2013) studied 34 *S. aureus* isolated from bovine subclinical mastitis from Eastern China. The recorded (29%) resistance against methicillin. However high resistance rate against methicillin was found but no isolate was positive for *mecA* gene, whereas *blaZ* gene was detected in 82% isolates.

Franca *et al.* (2012) identified molecular resistance markers (*mec*A and *bla*Z gene) of *Staphylococcus* spp. (n=210) from small ruminant mastitis in Brazil. Although phenotypic resistance to oxacillin was observed in 12.8% of the isolates but none of the isolate harbored the *mec*A gene. However, 45.7% of the isolates harbored *bla*Z indicating that beta-lactamase production was the main mechanism associated with staphylococci resistance to beta-lactam antibiotics.

Mohanasoundaram and Lalitha (2008) reported 34 (22.6%) MRSA isolates by both phenotypic and specific PCR (*mecA* gene) from 150 isolates of human clinical infections. Similar to present study they also reported, one isolate of MSSA revealed the presence of *mecA* gene having the potential to become MRSA in future. It may be possible due to non-expression of *mecA* gene and phenotypically MRSA detection instead of absence of *mecA* gene is indicating possibilities of presence of *mecA* gene in a plasmid or presence of other uncommon methicillin resistance governing gene such as *fem, mecI, mecC* and *mecR1*(Lowy, 2003).

Oliveira *et al.* (2000) reported less prevalence of β-lactamase, they studied 811 strains of *S. aureus* isolated from bovine mastitis in Europe and the Unites States. Of the strain tested, 35.6% were positive for β-lactamase on initial testing, with an additional 21.3% positive after induction of penicillin.

Similar to present study, Booth *et al.* (2001) reported 27.9% (55/197) prevalence of the *mec*Agene among *S. aureus* isolates obtained from patients with bloodstream, catheter tip, bone or joint, respiratory tract, ocular, soft tissue, wound and human skin infections. Lee (2003) studied various types of meat and clinical specimens from cattle, pigs, and chickens for the presence of methicillin (oxacillin)-resistant *S. aureus* (MRSA) and found very low prevalence of MRSA and *mecA* gene

in comparison to present study. They reported 28 MRSA from 421 *S. aureus* isolates phenotypically. Isolates from 15 of the 28 specimens were positive by PCR for the *mecA* gene. Of the 15 *mecA*-positive MRSA isolates, 12 were from dairy cows and 3 were from chickens.

Arslan and Ozkardes (2007) reported, similar results with ninety six (74.4%) positive strains of 129 S. *aureus* isolates for β-lactamase enzyme from human clinical samples and close to present study, Turutoglu *et al.* (2006) also reported 38 isolates (55.9%) were β-lactamase producers from 68 *S. aureus* strains from bovine mastitc milk.

Similar to present study, Akindele *et al*. (2010) reported 80% isolates were β-lactamase producer from 100 *S. aureus* strains obtained from human clinical infections while Capurro *et al.* (2010) found only 9% of the isolates (from 82 *S. aureus* isolates) of clinical mastitic milk of dairy cow. Asfour and Darwish (2011) conducted phenotypic and genotypic detection of both *mecA* and *blaZ* genes mediated β-lactam resistance in staphylococci isolated from bovine clinical and subclinical mastitis and found that only one and twelve isolates was positive for β-lactamase (*blaZ* gene) while none and five of isolates were positive for MRSA (*mecA* gene) from clinical and subclinical samples, respectively.

Suleiman *et al.* (2012) detected prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in bovine mastitic milk. From 103 *S. aureus* isolates 26 isolates were found to be MRSA. Among those MRSA, the gene *mecA* was found in two (7.6%) and the *blaZ* gene in all the MRSA isolates. Schaumburg *et al.* (2014) analyzed 151 chicken meat samples where in 3% were found to be contaminated with ESBL producing *S. aureus* but none of the isolate carried MRSA as confirmed by the absence of *mecA* gene.

Similar to present study, Breves *et al.* (2015) studied antimicrobial susceptibility profile of *S. aureus* on surfaces of medical articles and in professionals from two basic health units and found that among the 35 *S. aureus*, 31.42% were methicillin resistant (MRSA), and 2.8% were vancomycin resistant (VRSA). Further *mecA* gene was detected in 30.6% of studied strains. Sharma *et al.* (2015) reported resistance pattern of 27 *S. aureus* obtained from 80 samples of cattle and buffalo with clinical as well as sub-clinical mastitis. Drug sensitivity revealed that 18 isolates were methicillin-resistant, while the remaining (09) were methicillin-susceptible. Similarly, 24 *S. aureus* isolates were intermediate to vancomycin but no isolate was resistant to vancomycin in comparison to present study.

Xu *et al.* (2015) reported results similar to our study, who studied 28 *S. aureus* isolates obtained from 209 mastitis milk samples of cow. During study the most prevalent antibiotic resistance gene was *blaZ* conferring the resistance to 23 (82.1%) isolates, followed by *mecA* to 10 (35.7%) isolates. Roy *et al.* (2015) isolated 125 *S. aureus* isolates from the hospital and 45 isolates from the hand swab of volunteer's working in the hospitals to find vancomycin resistance pattern. In hospital none of the isolate was resistant to vancomycin and only 11.11% isolates were intermediates while similar to our results, 20% isolates from volunteers were resistant to vancomycin. Wadekar *et al.* (2015) studied 35 *S. aureus* isolates from 100 pus samples of chronic osteomyelitis suffered patients and similar to present study, MRSA was detected in 14(40%) isolates but contrary to present study most of them were sensitive to vancomycin.

Table 22: Detection of MRSA, VRSA, β-lactamase production and ESBL activity among *S. aureus* isolates.

S. No.	Source of Isolate	Total no of isolate	MRSA activity (%)				VRSA activity (%)		β-lactamase production(%)		ESBL Production (%)	
			MeReSa Agar Base method		Methicillin disc method							
			P	N	P	N	P	N	P	N	P	N
1.	Human	35	28 (80.0)	7 (20.0)	10 (28.5)	25 (71.4)	18 (51.4)	17 (48.5)	35 (100.0)	0 (0.0)	31 (88.5)	4 (11.4)
2.	Meat piece	20	14 (70.0)	6 (30.0)	2 (10.0)	18 (90.0)	5 (25.0)	15 (75.0)	20 (100.0)	0 (0.0)	14 (70.0)	6 (30.0)
3.	Horse	3	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	2 (66.6)	1 (33.3)	3 (100.0)	0 (0.0)	1 (33.3)	2 (66.6)
4.	Pig	2	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	2 (100.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)

5.	Camel	8	4 (50.0)	4 (50.0)	1 (12.5)	7 (87.5)	0 (0.0)	8 (100.0)	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)
6.	Dog	6	2 (33.3)	4 (66.6)	0 (0.0)	6 (100.0)	0 (0.0)	6 (100.0)	6 (100.0)	0 (0.0)	3 (50.0)	3 (50.0)
7.	Sheep	6	4 (66.6)	2 (33.3)	0 (0.0)	6 (100.0)	6 (100.0)	0 (0.0)	5 (83.3)	1 (16.6)	3 (50.0)	3 (50.0)
8.	Buffalo	21	7 (33.3)	14 (66.6)	1 (4.7)	20 (95.2)	12 (57.14)	9 (42.8)	16 (76.1)	5 (23.8)	9 (42.8)	12 (57.14)
9.	Goat	28	10 (35.7)	18 (64.2)	0 (0.0)	28 (100.0)	6 (21.4)	22 (78.5)	21 (75.0)	7 (25.0)	20 (71.4)	8 (28.5)
10.	Cattle	28	11 (39.2)	17 (60.7)	5 (17.8)	23 (82.14)	3 (10.7)	25 (89.2)	27 (96.4)	1 (3.5)	18 (64.2)	10 (35.7)
Total		**157**	**85 (54.1)**	**72 (45.9)**	**19 (12.1)**	**138 (87.9)**	**52 (33.1)**	**105 (66.9)**	**142 (90.4)**	**15 (9.5)**	**108 (68.8)**	**49 (31.2)**
Abbreviations:- P- Positive, N- Negative												

Table 23 Detection of *mecA* (MRSA) and *blaZ* (β-lactamase) gene among *S. aureus* isolates.

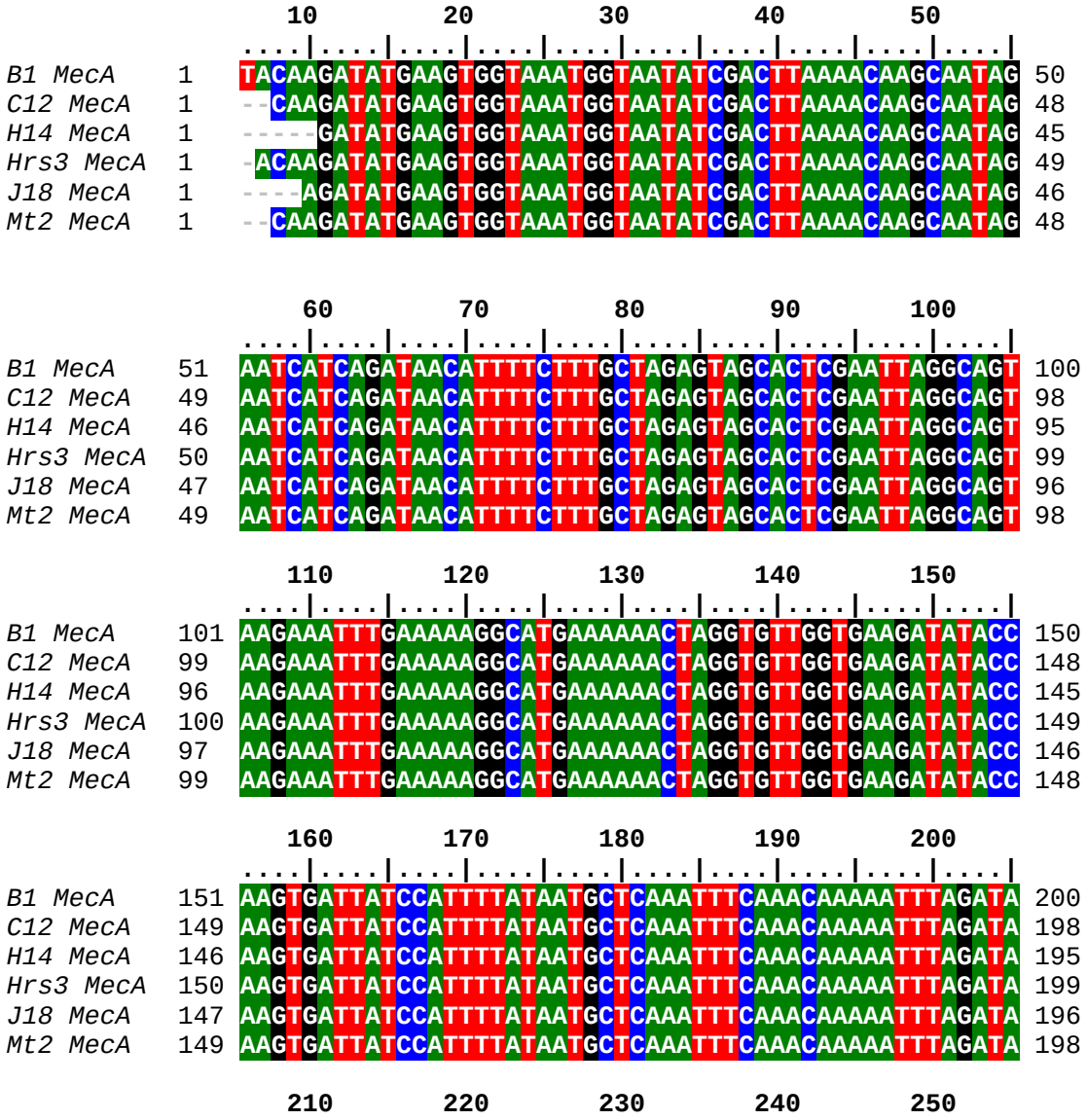
S. No.	Source of Isolate	Total no of isolate	*mecA* gene (%) (533bp)		*blaZ* gene (%) (517bp)	
			P	N	P	N
1.	Human	35	18 (51.4)	17 (48.5)	35 (100.0)	0 (0.0)
2.	Meat piece	20	8 (40.0)	12 (60.0)	19 (95.0)	1 (5.0)
3.	Horse	3	2 (66.6)	1 (33.3)	3 (100.0)	0 (0.0)
4.	Pig	2	0 (0.0)	2 (100.0)	2 (100.0)	0 (0.0)
5.	Camel	8	3 (37.5)	5 (62.5)	7 (87.5)	1 (12.5)
6.	Dog	6	0 (0.0)	6 (100.0)	5 (83.3)	1 (16.6)
7.	Sheep	6	0 (0.0)	6 (100.0)	5 (83.3)	1 (16.6)
8.	Buffalo	21	1 (4.7)	20 (95.2)	15 (71.4)	6 (28.5)
9.	Goat	28	0 (0.0)	28 (100.0)	19 (67.85)	9 (32.14)
10.	Cattle	28	5 (17.8)	23 (82.14)	27 (96.4)	1 (3.5)
Total		**157**	**37 (23.6)[b]**	**120 (76.4)**	**137 (87.3)**	**20 (12.7)**

Abbreviations:- P- Positive, N- Negative
Superscript:- a- Isolates were MRSA positive by both phenotypic methods but *mecA* gene was absent (H46, Mt24 and C41), b- Isolates were MRSA negative by both phenotypic methods but *mecA* gene was present (H31, Mt10, Mt11, Mt14 and C47)

mecA gene sequence analysis

The six *mecA* gene positive isolates from this study, namely H14 from human, B1 from buffalo, C12 from cattle, Hrs3 from horse, J18 from camel and Mt2 from meat piece were got sequenced. First of all the sequences were BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed as *mecA* gene sequences of *S. aureus* strains. During BLAST of *mecA* gene sequences it was found that sequences of present study not only align with 100% similarities and zero gap with *S. aureus* strains but also with *S. epidermidis* (KP265313), *S. schleiferi* (AP014944), *S. haemolyticus* (KM369884) and some other *Staphylococcus* spp. etc. (http://blast.ncbi.nlm.nih.gov/Blast.cgi#928189965). It may indicates the interspecies transmission of *mecA* gene and methicillin resistance among environment of bacterial habitat.

The sequence analysis of *mecA* gene revealed some nucleotide variations, one at 316 (cytosine replaced by adenine) position, another at 378 (adenine replaced by thymine) position and additional 16 nucleotide insertion (GGTACTGGCAGAAACA) from 467 to 481 position in cattle (C12) sequence as mentioned in figure 17. These variations were found to be non-significant ($p > 0.05$) during analysis of codon based test of neutrality. Two distinct clusters with 0.0022 scaled distance were found in phylogenetic tree analysis of *mecA* gene sequences of the present study, first cluster was including sequence of cattle (C12) isolate and second cluster comprising sequences of remaining other isolates as depicting in figure 18.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H14 MecA 1 -----GATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 45
Hrs3 MecA 1 -ACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 49
J18 MecA 1 ----AGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 46
Mt2 MecA 1 --CAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 48
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C12 MecA 49 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 98
H14 MecA 46 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 95
Hrs3 MecA 50 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 99
J18 MecA 47 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 96
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Hrs3 MecA 150 AAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATA 199
J18 MecA 147 AAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATA 196
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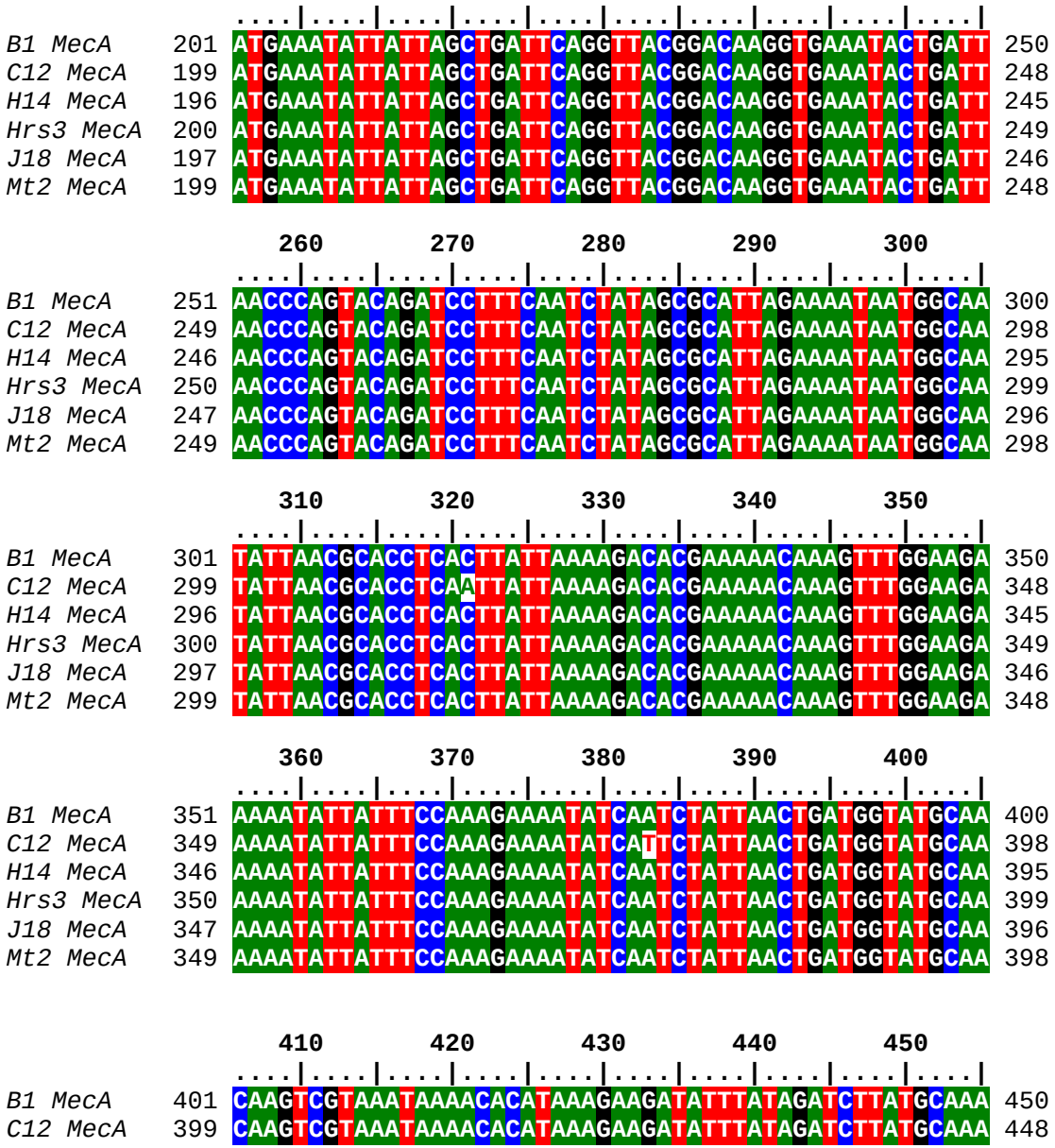
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Hrs3 MecA 200 ATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGATT 249
J18 MecA 197 ATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGATT 246
Mt2 MecA 199 ATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGATT 248
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C12 MecA 249 AACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGCAA 298
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Hrs3 MecA 250 AACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGCAA 299
J18 MecA 247 AACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGCAA 296
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C12 MecA 299 TATTAACGCACCTCAATTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 348
H14 MecA 296 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 345
Hrs3 MecA 300 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 349
J18 MecA 297 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 346
Mt2 MecA 299 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 348
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C12 MecA 349 AAAATATTATTTCCAAAGAAAATATCATTCTATTAACTGATGGTATGCAA 398
H14 MecA 346 AAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAA 395
Hrs3 MecA 350 AAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAA 399
J18 MecA 347 AAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAA 396
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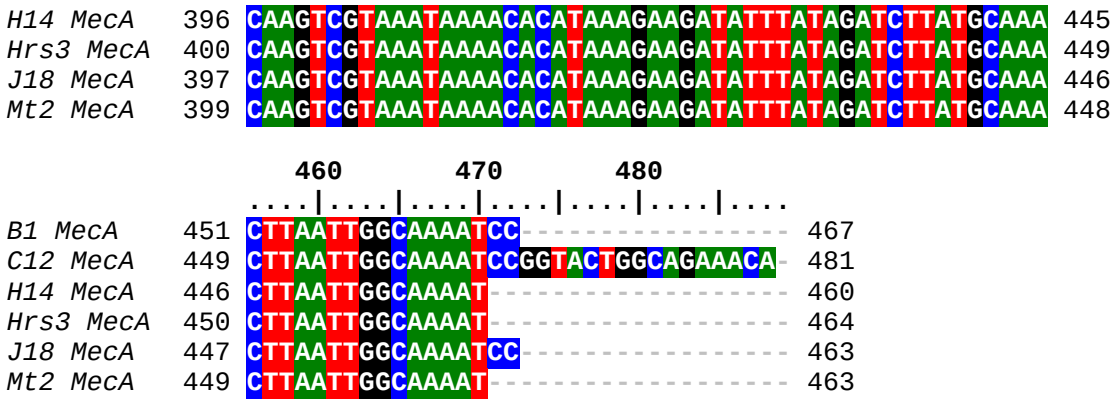
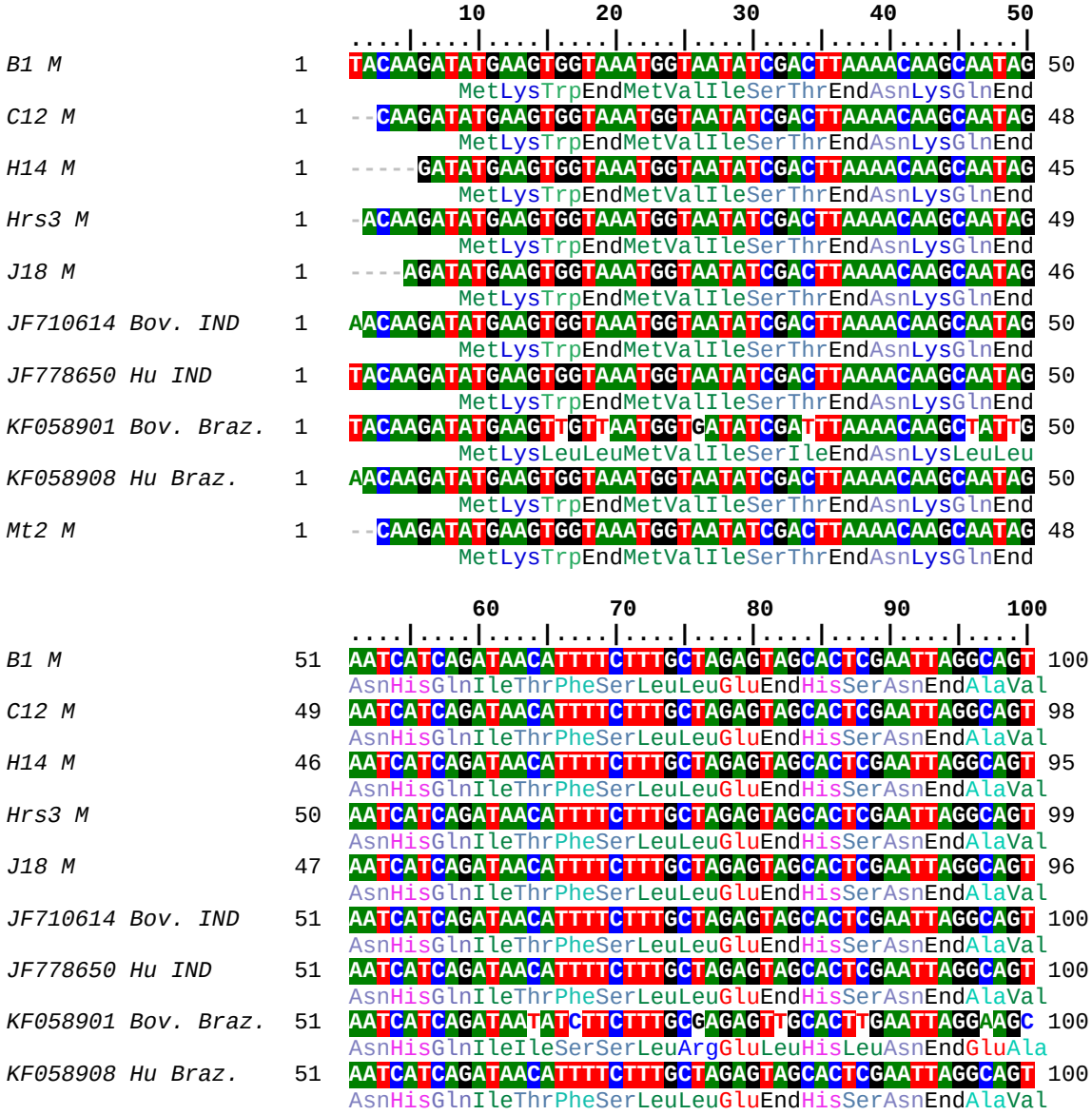


Fig. 17:The sequence analysis *mecA* gene obtained from present study.

Multiple sequence alignment of present study sequences was conducted with reference sequences of NCBI database to find out similarities and variations between our sequences and reference NCBI sequences. For this purpose, four sequences of NCBI database such as Sequence ID- JF710614 and JF778650 from bovine and human strain of India, respectively and Sequence ID- KF058901 and KF058908 from bovine and human strain of Brazil, respectively (de Melo *et al.*, 2014) were chosen according to source of isolation and geographic variations. The multiple sequence alignment of all sequences revealed 100% similarities (except cattle strain of present study as describe earlier and one position at 474 in human strain India). But bovine strain of India having six nucleotide variation (one gap at 202 position, and single nucleotide variation at 210 position, 215, 464, 465 and at 474 position) and bovine strain from Brazil having more than 50 nucleotide variations at various positions in comparison to other studied strains as described in the figure 19. After multiple sequence alignment, it is concluded that nucleotide variations not only depend on source of isolation but also on geographic variations and close similarities between animal and human origin sequences in the present study may indicate close interaction and exchange of bacterial genetic material in between isolates from human, animals and cross infections (meat piece).

The statistic for codon-based test of neutrality between sequences conducted The probability of rejecting the null hypothesis of strict-neutrality $d_N = d_S$ (d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively) and non-significant ($p > 0.05$) variations were found between all aligned sequences except bovine strain of brazil was significantly ($p \leq 0.05$) variable with all other sequences as described in table 24. Phylogenetic tree analysis of multiple align *mecA* gene sequences revealed that Brazilian bovine strain is most far with 0.0941 scaled distance and having maximum differences from all other align sequences. Cattle (C12) and bovine strain from India are closer and separated from other sequences with 0.0039 and 0.0033 scaled distances respectively. Sequence of human (H14) strain, meat piece (Mt2) and human strain from India are more closer in compare to other sequences while sequence of camel (J18) strain, horse (Hrs3), buffalo (B1) and Brazilian human strain are more closer as depicting in figure 20.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MetLysTrpEndMetValIleSerThrEndAsnLysGlnEnd
C12 M 1 --CAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 48
MetLysTrpEndMetValIleSerThrEndAsnLysGlnEnd
H14 M 1 -----GATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 45
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KF058908 Hu Braz. 1 AACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAG 50
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MetLysTrpEndMetValIleSerThrEndAsnLysGlnEnd
60 70 80 90 100
B1 M 51 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 100
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C12 M 49 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 98
AsnHisGlnIleThrPheSerLeuLeuGluEndHisSerAsnEndAlaVal
H14 M 46 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 95
AsnHisGlnIleThrPheSerLeuLeuGluEndHisSerAsnEndAlaVal
Hrs3 M 50 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 99
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J18 M 47 AATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGT 96
AsnHisGlnIleThrPheSerLeuLeuGluEndHisSerAsnEndAlaVal
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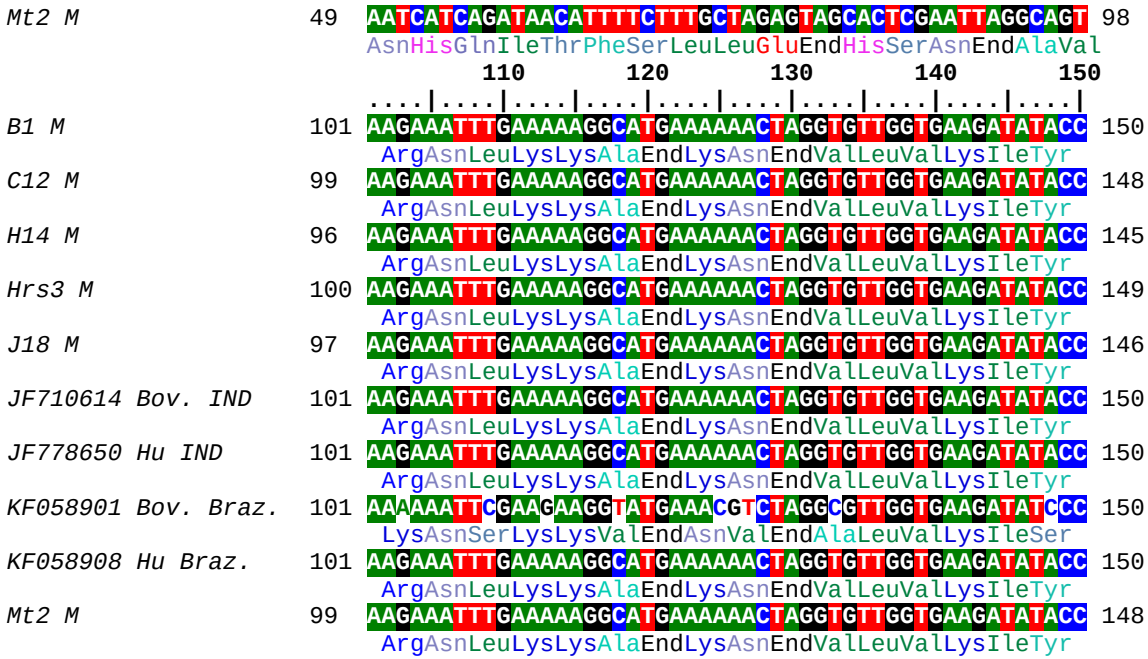
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B1 M 101 AAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACC 150
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C12 M 99 AAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACC 148
ArgAsnLeuLysLysAlaEndLysAsnEndValLeuValLysIleTyr
H14 M 96 AAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACC 145
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Hrs3 M 100 AAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACC 149
ArgAsnLeuLysLysAlaEndLysAsnEndValLeuValLysIleTyr
J18 M 97 AAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACC 146
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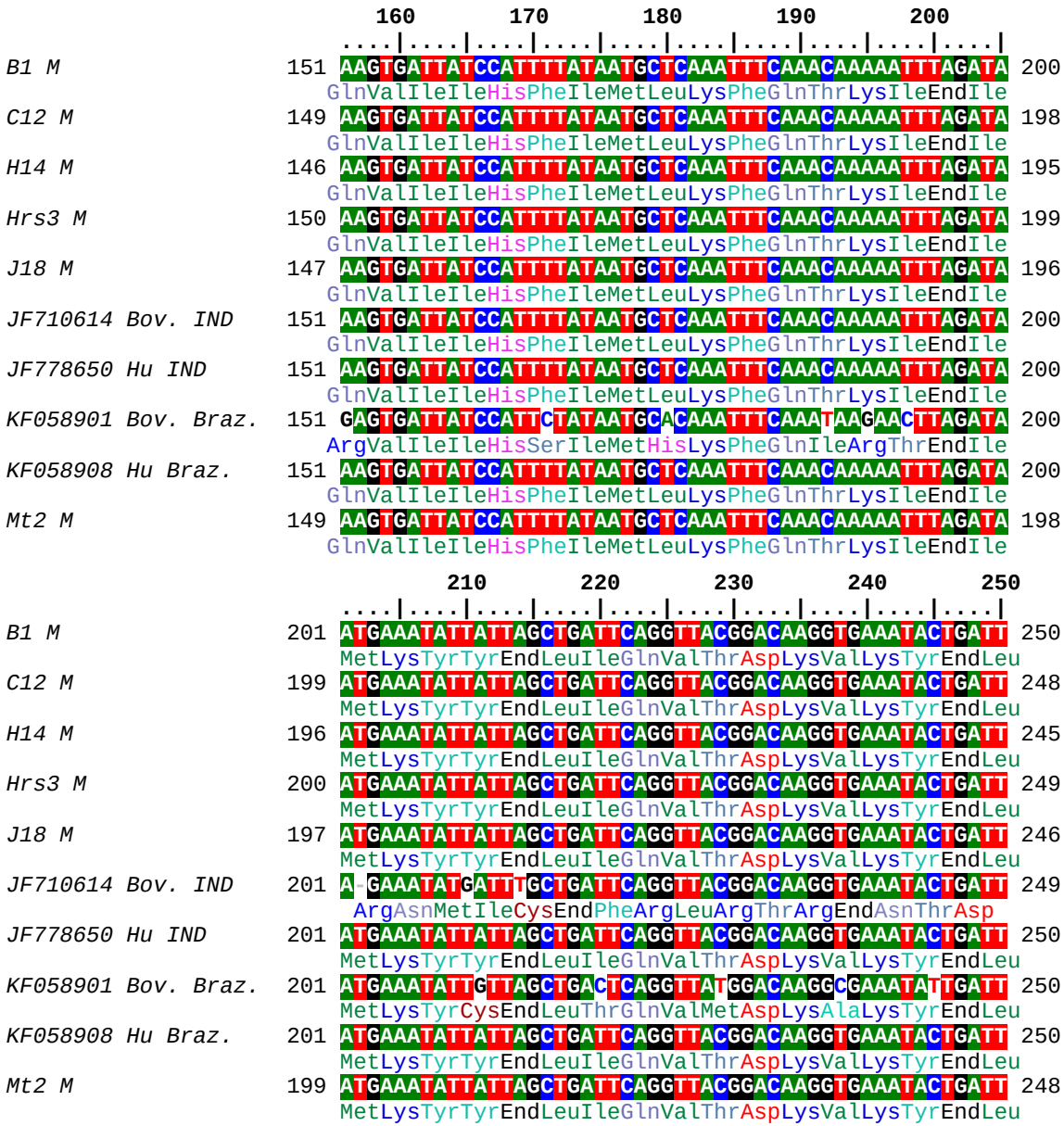
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GlnValIleIleHisPheIleMetLeuLysPheGlnThrLysIleEndIle
H14 M 146 AAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATA 195
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J18 M 147 AAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATA 196
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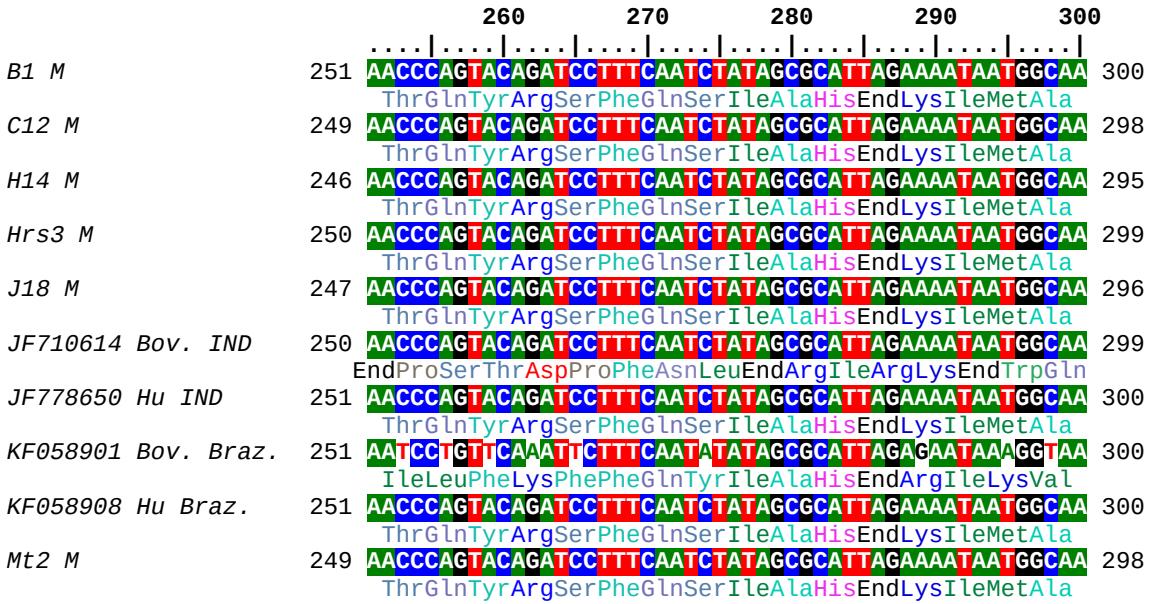
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H14 M 246 AACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGCAA 295
ThrGlnTyrArgSerPheGlnSerIleAlaHisEndLysIleMetAla
Hrs3 M 250 AACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGCAA 299
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ThrGlnTyrArgSerPheGlnSerIleAlaHisEndLysIleMetAla
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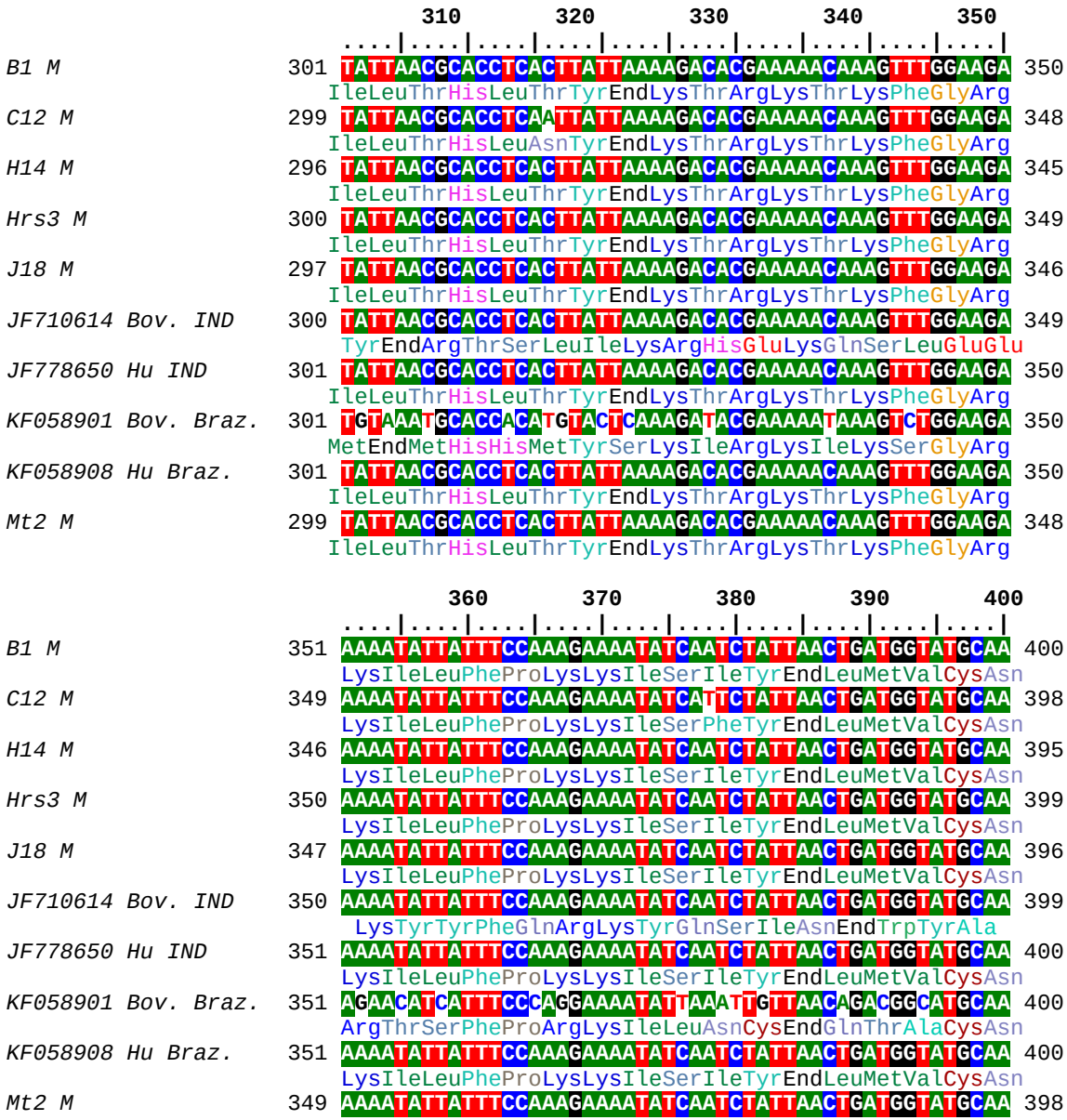
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IleLeuThrHisLeuAsnTyrEndLysThrArgLysThrLysPheGlyArg
H14 M 296 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 345
IleLeuThrHisLeuThrTyrEndLysThrArgLysThrLysPheGlyArg
Hrs3 M 300 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 349
IleLeuThrHisLeuThrTyrEndLysThrArgLysThrLysPheGlyArg
J18 M 297 TATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGAAGA 346
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C12 M 349 AAAATATTATTTCCAAAGAAAATATCATTCTATTAACTGATGGTATGCAA 398
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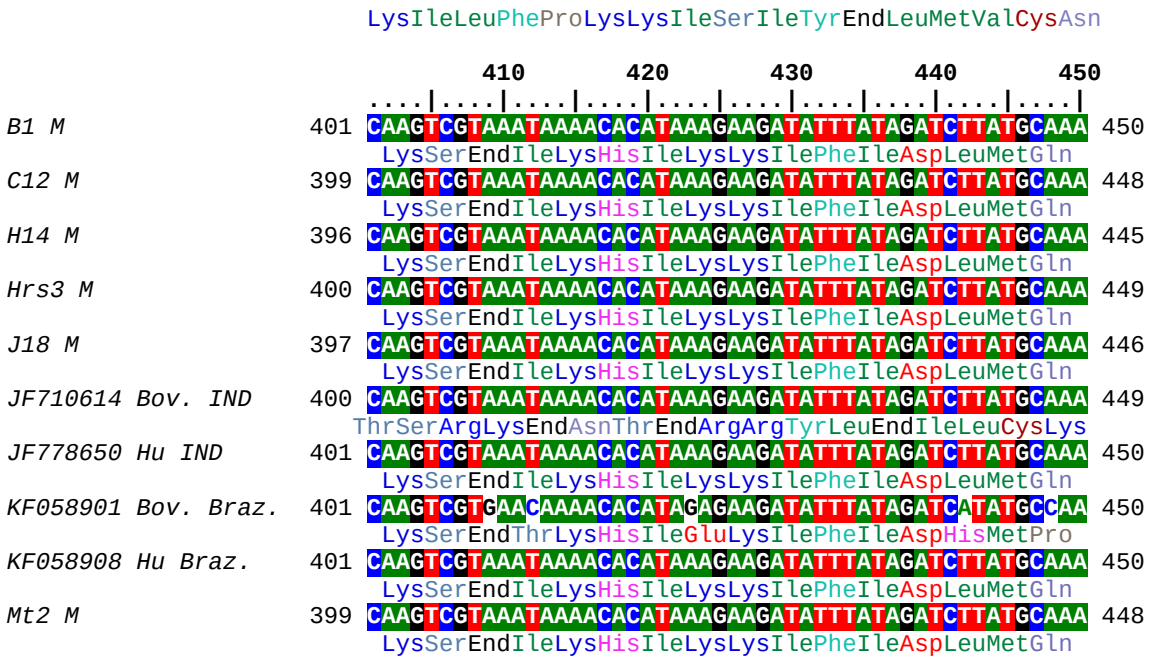
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C12 M 399 CAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAA 448
LysSerEndIleLysHisIleLysLysIlePheIleAspLeuMetGln
H14 M 396 CAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAA 445
LysSerEndIleLysHisIleLysLysIlePheIleAspLeuMetGln
Hrs3 M 400 CAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAA 449
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J18 M 397 CAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTTATGCAAA 446
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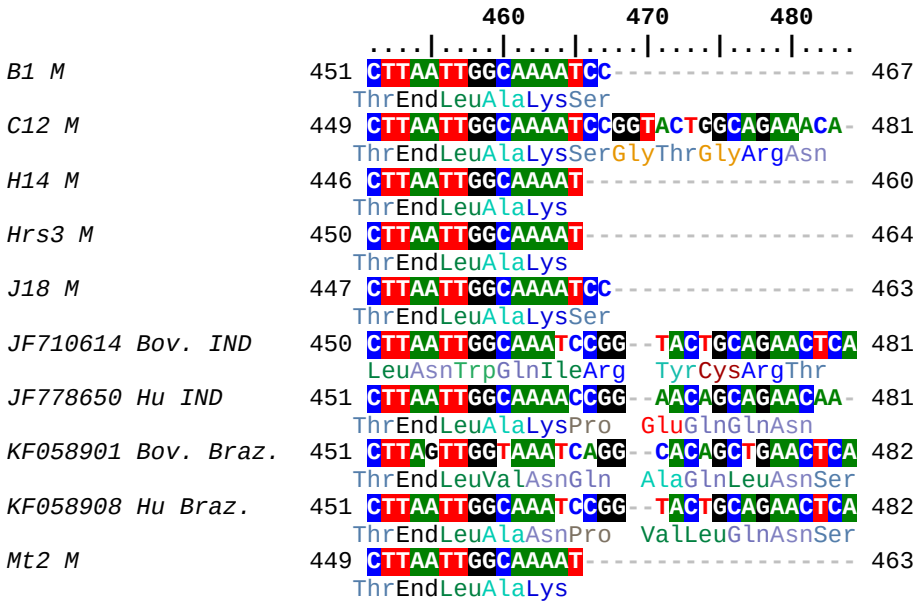


Fig. 19: Multiple sequence alignment of *mecA* gene sequences of present study with reference sequences of NCBI database.

Table 24: Codon-based Test of Neutrality for multiple alignment analysis between sequences of present study with reference sequences of NCBI database.

M6: Z-Test of Neutral Evolution (C:\Users\SANDEE~1\AppData\Local\Temp\PhyloAnalysis-1.meg)

File Display Caption Help

	1	2	3	4	5	6	7	8	9	10
1. B1 M		1.418	0.000	0.000	0.000	1.001	0.000	-11.390	0.000	0.000
2. C12 M	0.159		1.418	1.418	1.418	1.739	1.418	-11.136	1.418	1.418
3. H14 M	1.000	0.159		0.000	0.000	1.001	0.000	-11.390	0.000	0.000
4. Hrs3 M	1.000	0.159	1.000		0.000	1.001	0.000	-11.390	0.000	0.000
5. J18 M	1.000	0.159	1.000	1.000		1.001	0.000	-11.390	0.000	0.000
6. JF710614 bovine strain from india	0.319	0.085	0.319	0.319	0.319		1.001	-11.336	1.001	1.001
7. JF778650 human strain from india	1.000	0.159	1.000	1.000	1.000	0.319		-11.390	0.000	0.000
8. KF058901 Bovine strain from Brazil	0.000	0.000	0.000	0.000	0.000	0.000	0.000		-11.390	-11.390
9. KF058908 human strain from brazil	1.000	0.159	1.000	1.000	1.000	0.319	1.000	0.000		0.000
10. Mt2 M	1.000	0.159	1.000	1.000	1.000	0.319	1.000	0.000	1.000	

The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) (below diagonal) is shown. Values of *P* less than 0.05 are considered significant at the 5% level and are yellow highlighted. The test statistic ($d_N - d_S$) is shown above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. Analyses were conducted using the Nei-Gojobori method. The analysis involved 10 nucleotide

7. Molecular typing of *S. aureus* with *rep* PCR:

The high degree of genetic relatedness between *S. aureus* strains has been established obstacle to using routine bacteriological methods for epidemiologic analysis. Thus repetitive sequence-based PCR (*rep*-PCR) is found suitable and potential tool which permits differentiation of isolates to species, subspecies and strain level. Because of the low cost of the materials and the rapidity, ease of use, and low labor inputs, *rep*-PCR may be a valuable method for bacterial strain typing.

In the present investigation, 36 different *rep* patterns comprising in the range size from 300 to 1400bp with different arrangements of band sizes (Fig. 22) were recorded among all 157 isolates by *rep*-PCR (table 25) with 0.8892 discriminatory index (D.I.). This discriminatory index indicates the good differentiations of *S. aureus* isolates by *rep* PCR. One pattern (rep30) was most common with 47 cattle and goat origin isolates followed by rep17 with 13 isolates which belonged to sheep, horse and camel then rep7 with 10 isolates of human origin and other remaining rep-patterns comprising with less than seven isolates of different origins. The obtaining of large numbers and separate rep-patterns from different origin of isolates in the present study indicated usefulness of the method in differentiating *S. aureus* from various sources.

Table 25: *Staphylococcus aureus* typing on the basis of overall distribution of *rep* patterns (repetitive element sequence-based PCR) among isolates.

S. No.	*rep* pattern I.D.	*rep* pattern	Number of Isolates	*S. aureus* Isolate I.D.
1.	rep1	500bp, 600bp	1	H1
2.	rep2	300bp, 500bp, 600bp, 700bp, 1000bp, 1050bp	1	H2
3.	rep3	300bp, 700bp, 1000bp, 1050bp	6	H3, H4, H15, H21, H24, Mt15
4.	rep4	300bp, 500bp, 600bp,700bp	1	H5
5.	rep5	300bp, 600bp, 900 bp,1000bp, 1400bp	5	H6, H9, H10, H11, H28
6.	rep6	300bp, 700bp, 1000bp	4	H14, Mt31, J2, B26
7.	rep7	300bp, 700bp, 750bp, 1000bp, 1050bp	10	H29, H33,H37,H39,H40,H41,H46,H48,Mt2,Mt9
8.	rep8	300bp, 700bp, 800bp, 1000bp, 1050bp	2	Mt1,Mt4
9.	rep9	300bp, 600bp, 700bp, 750bp, 1000bp, 1050bp	1	H45
10.	rep10	300bp, 600bp, 700bp, 1000bp, 1050bp	5	Mt3, Mt10,Mt11, Mt13, Mt14
11.	rep11	300bp, 400bp, 600bp, 700bp, 1000bp	2	D13, B46
12.	rep12	300bp, 400bp, 600bp, 700bp, 1050bp	3	B5, B19, B21
13.	rep13	300bp, 500bp, 700bp, 750bp, 1000bp, 1050bp	1	H12
14.	rep14	600bp, 900bp, 1000bp, 1400bp	1	H8
15.	rep15	400bp, 700bp, 1000bp, 1050bp	1	H31
16.	rep16	300bp, 600bp,700bp, 750bp	2	H30 ,H34
17.	rep17	300bp, 600bp, 700bp, 1000bp	13	Mt19, Hrs3, Hrs4, Pg2, J14,J18, D10, SV2, SV3, SV4, SN3,SN4, SN14
18.	rep18	300bp, 600bp, 1000bp, 1050bp	2	Mt20, Mt24
19.	rep19	300bp, 400bp, 700bp, 1000bp	2	D7, B24
20.	rep20	300bp, 400bp, 700bp, 750bp, 1000bp	1	D9
21.	rep21	300bp, 400bp, 600bp, 1000bp	7	B27, B28, B29, B30, B31, B34, B36,
22.	rep22	300bp, 400bp, 600bp, 700bp	6	B10, B39, B42, B43, B55, B57
23.	rep23	300bp, 350bp, 600bp, 700bp	6	AG8, G29, C3R, C8, C17, C22
24.	rep24	300bp, 350bp, 600bp, 1000bp	2	G46, C15
25.	rep25	300bp, 700bp, 1400bp	1	H27
26.	rep26	300bp, 600bp, 700bp	3	H44, Mt27, B23
27.	rep27	300bp, 800bp, 1000bp	1	Hrs1
28.	rep28	600bp, 700bp, 1000bp	1	J4
29.	rep29	300bp, 400bp, 700bp	1	J15
30.	rep30	300bp, 350bp, 600bp	47	AG5, AG6, AG10, AG13, AG15, AG17, G1,G2,G7,G9, G10, G11, G16, G21, G24, G35, G37,G39, G40, G41, G43, G45, G47, G49, G55, C2, C5R, C7, C9, C11, C12, C13, C20, C23, C26, C29, C34, C36, C37, C39, C40, C41, C43, C46, C47, C50, C52
31.	rep31	300bp, 700bp	5	H7, H16, H22, H25, J10
32.	rep32	400bp, 1000bp	1	H13
33.	rep33	300bp, 600bp	7	Mt22, Mt25, Mt28, J3, D4, D6, C2R
34.	rep34	300bp, 1000bp	1	Mt26
35.	rep35	600bp	2	Mt12, Pg4
36.	rep36	300 bp	1	B1

In the source-wise analysis of rep- patterns it was found that human isolates were differentiated into maximum rep-profiles (16 rep-profiles) indicating more variations while sheep isolates were grouped only into one rep-pattern (rep17) indicating no variations. Other sources including variable number of rep profiles *i.e.* meat piece (11 rep-patterns), buffalo (8 rep-patterns), camel (6 rep-patterns) dog (5 rep- patterns) and remaining sources having less than four rep-patterns with less variabilities as described in table 26. Exceptionally one isolate (J9) from camel was non-typeable with rep PCR. During phylogenetic cluster analysis of *S. aureus* rep-patterns in the present study, all 36 rep-patterns were divided into five clusters on the basis of 80% genetic similarity. First cluster was made up with seven isolates (most of buffalo and dog) and five rep-patterns (rep11, rep15, rep19, rep20 and rep32), second cluster comprising six human isolates and two rep patterns (rep5 and rep14), third cluster including 29 isolates (most of human, meat piece and one of horse, pig and camel each) and ten rep- patterns (rep2, rep3, rep6, rep7, rep8, rep9, rep13, rep18, rep27 and rep34), fourth cluster comprising 41 isolates (human, meat piece and few of sheep and buffalo) and 11 rep-patterns (rep4, rep10, rep12, rep16, rep17, rep22, rep25, rep26, rep29, rep31 and rep36) and fifth cluster including 73 isolates (most of cattle, goat, buffalo and few of dog, camel and meat piece) and eight rep-patterns (rep1, rep21, rep23, rep24, rep28, rep30, rep33 and rep35) as depicting in figure 21. The cluster analysis indicated that most of animal origin isolates were separately clustered (I^{st} and V^{th} cluster) in comparison to human origin isolates and meat piece origin isolates which fell into both human and animal clusters with indication of cross contamination from humans and animal environments. It may also be concluded that human isolates could be separately differentiated from animal origin isolates even up to 80% similarity level.

Similar results were reported by Reinoso *et al.* (2008) who analyzed 45 *S. aureus* strains by *rep*-PCR obtained from humans, bovine subclinical mastitis and food samples. The subsequent cluster analysis suggested the existence of 35 rep profiles which could be divided by dendrogram analyses into seven different groups at 60% of relative genetic similarity designated I–VII. The groups generally matched with the origin of the isolates. Group II consists of 13 (87%) human infection strains, groups III and IV of 14 (93%) bovine mastitis strains and groups V and VII of five (71%) strains isolated from anterior nares of healthy people. Six (75%) food sample strains were grouped together with the human strains in group II.

Reinoso *et al.* (2007) assessed genetic relationship of 52 *S. aureus* strains isolated from mammary gland infections collected in four herds located in the central dairy region of Argentina. A total of 31 rep-profiles in the range size from 300 to 6000 bp were identified after rep-PCR analysis. At a first level of similarity (50%), it could be defined 5 clusters namely I to V. Most of the strains (75%) were grouped in cluster I and suggested that rep-PCR typing successfully differentiated *S. aureus* strains of bovine origin.

Many researchers like del Vecchio *et al.* (1995), van der Zee *et al.* (1999), Gardella *et al.* (2005) and Nordin *et al.* (2010) etc. analyzed MRSA and MSSA strains from various clinical infections to differentiate them from each other and to investigate whether certain rep-PCR fingerprints would have prognostic value for routine clinical and epidemiological surveillance of *S. aureus* strains. Similar to the present study, they also found that rep-PCR was potential tool to determine genomic differences among *S. aureus* strains from different sources.

Table 26: GroupWise distribution of *rep* patterns (repetitive element sequence-based PCR) among isolates.

S. No.	Source of Isolate	S. No.	*rep* pattern I.D.	*rep* pattern	Number of Isolates	Isolate I.D.	Total No. of Isolate
1.	Human	1.	rep1	500bp, 600bp	1	H1	35
		2.	rep2	300bp, 500bp, 600bp, 700bp, 1000bp, 1050bp	1	H2	
		3.	rep3	300bp, 700bp, 1000bp, 1050bp	5	H3, H4, H15, H21 and H24	
		4.	rep4	300bp, 500bp, 600bp, 700bp	1	H5	

		5.	rep5	300bp, 600bp, 900bp, 1000bp, 1400bp	5	H6, H9, H10, H11 and H28	
		6.	rep6	300bp, 700bp, 1000bp	1	H14	
		7.	rep7	300bp, 700bp, 750bp, 1000bp, 1050bp	8	H29, H33, H37, H39, H40, H41, H46 and H48	
		8.	rep9	300bp, 600bp, 700bp,750bp, 1000bp, 1050bp	1	H45	
		9.	rep13	300bp, 500bp, 700bp, 750bp, 1000bp, 1050bp	1	H12	
		10.	rep14	600bp, 900bp, 1000bp, 1400bp	1	H8	
		11.	rep15	400bp, 700bp, 1000bp, 1050bp	1	H31	
		12.	rep16	300bp, 600bp, 700bp, 750bp	2	H30 ,H34	
		13.	rep25	300bp, 700bp, 1400bp	1	H27	
		14.	rep26	300bp, 600bp, 700bp	1	H44	
		15.	rep31	300bp, 700bp	4	H7, H16, H22 and H25	
		16.	rep32	400bp, 1000bp	1	H13	
2.	Meat Piece	1.	rep3	300bp, 700bp, 1000bp, 1050bp	1	Mt15	20
		2.	rep6	300bp, 700bp, 1000bp	1	Mt31	
		3.	rep7	300bp, 700bp, 750bp, 1000bp, 1050bp	2	Mt2 and Mt9	
		4.	rep8	300bp, 700bp, 800bp, 1000bp, 1050bp	2	Mt1 and Mt4	
		5.	rep10	300bp, 600bp, 700bp, 1000bp, 1050bp	5	Mt3, Mt10, Mt11, Mt13 and Mt14	
		6.	rep17	300bp, 600bp, 700bp, 1000bp	1	Mt19	
		7.	rep18	300bp, 600bp, 1000bp, 1050bp	2	Mt20 and Mt24	
		8.	rep26	300bp, 600bp,700bp	1	Mt27	
		9.	rep33	300bp, 600bp	3	Mt22, Mt25 and Mt28	
		10.	rep34	300bp, 1000bp	1	Mt26	
		11.	rep35	600bp	1	Mt12	
3.	Horse	1.	rep17	300bp, 600bp, 700bp, 1000bp	2	Hrs3 and Hrs4	3
		2.	rep27	300bp, 800bp, 1000bp	1	Hrs1	
4.	Pig	1.	rep17	300bp, 600bp, 700bp, 1000bp	1	Pg2	2
		2.	rep35	600bp	1	Pg4	
5.	Camel	1.	rep6	300bp, 700bp, 1000bp	1	J2	8
		2.	rep17	300bp, 600bp, 700bp, 1000bp	2	J14 and J18	
		3.	rep28	600bp, 700bp, 1000bp	1	J4	
		4.	rep29	300bp, 400bp, 700bp	1	J15	
		5.	rep31	300bp, 700bp	1	J10	
		6.	rep33	300bp, 600bp	1	J3	
		7.	-	-	1	J9	
6.	Dog	1.	rep11	300bp, 400bp, 600bp, 700bp, 1000bp	1	D13	6
		2.	rep17	300bp, 600bp, 700bp, 1000bp	1	D10	
		3.	rep19	300bp, 400bp, 700bp, 1000bp	1	D7	
		4.	rep20	300bp, 400bp, 700bp, 750bp, 1000bp	1	D9	
		5.	rep33	300bp, 600bp	2	D4 and D6	
7.	Sheep	1.	rep17	300bp, 600bp, 700bp, 1000bp	6	SV2, SV3, SV4, SN3, SN4 and SN14	6
8.	Buffalo	1.	rep6	300bp, 700bp, 1000bp	1	B26	21
		2.	rep11	300bp, 400bp, 600bp, 700bp, 1000bp	1	B46	
		3.	rep12	300bp, 400bp, 600bp, 700bp, 1050bp	3	B5, B19 and B21	
		4.	rep19	300bp, 400bp, 700bp, 1000bp	1	B24	

		5.	rep21	300bp, 400bp, 600bp, 1000bp	7	B27, B28, B29, B30, B31, B34 and B36,	
		6.	rep22	300bp, 400bp, 600bp, 700bp	6	B10, B39, B42, B43, B55 and B57	
		7.	rep26	300bp, 600bp,700bp	1	B23	
		8.	rep36	300bp	1	B1	
9.	Goat	1.	rep23	300bp, 350bp, 600bp,700bp	2	AG8 and G29	28
		2.	rep24	300bp,350bp,600bp, 1000bp	1	G46	
		3.	rep30	300bp, 350bp, 600bp	25	AG5, AG6, AG10, AG13, AG15, AG17, G1, G2, G7, G9, G10, G11, G16, G21, G24, G35, G37, G39, G40, G41, G43, G45, G47, G49 and G55	
10.	Cattle	1.	rep23	300bp, 350bp, 600bp, 700bp	4	C3R, C8, C17 and C22	28
		2.	rep24	300bp,350bp,600bp, 1000bp	1	C15	
		3.	rep30	300bp, 350bp, 600bp	22	C2, C5R, C7, C9, C11, C12, C13, C20, C23, C26, C29, C34, C36, C37, C39, C40, C41, C43, C46, C47, C50 and C52	
		4.	rep33	300bp, 600bp	1	C2R	

8. Genotypic characterization of *S. aureus* in relation to following virulence factors and their associated genes:

A. Adherence Factor (*clfA, clfB, icaA, icaD, agr(agrI, agrII, agrIII and agrIV)* and *trap* gene):

Since, *Staphylococcus aureus* is a well-established important pathogen of human and animals because of its ability to adhere to a wide range of host tissues including host extracellular matrix proteins such as fibrinogen, fibronectin and collagen. Adhesion to host proteins is mediated by bacterial cell-wall-associated proteins and expression of these binding proteins governed by various molecules and their associated genes *viz.* clumping factors A and B(*clfA* and *clfB* gene), intercellular adhesion molecule(*icaA* and *icaD* gene), accessory gene regulator molecule *(*group of *agr* genes*)* and target of RNA III-activating molecule(*trap* gene).

Of the total 157 isolates, *clfA* and *clfB* genes were detected in 148 (94.3%) different isolates with single amplicon of 1000bp (Fig. 23) and 205bp (Fig. 24), respectively. Both *clfA* and *clfB* genes were absent in nine (5.7%) isolates namely Mt20, Mt22, J9, B30, G16, C2, C7, C12 and C15 and H44, J4, G29, G43, C11, C12, C13, C15 and C20, respectively. The *icaA* gene was detected in 150 (95.5%) isolates with 1315bp (Fig. 25) of single amplicon while absent in seven (4.5%) isolates namely Hrs3, D13, B26, G16, G29, C15 and C50. The *icaD* gene was present in 144 (91.7%) isolates with 381bp of single amplicon (Fig. 16) in duplex PCR with *blaZ* gene while absent in 13 (8.3%) isolates such as H30, H34, H46, Mt4, J2, J4, J10, D7, B43, G11, G16, G29 and C15. The *trap* gene was detected in 154 (98.0%) isolates with 504bp of single amplicon (Fig. 26) while absent in three (2.0%) isolates i.e. H16, J4 and B26 as mentioned in table 27.

Of the total 157 isolates, except one isolate (H24) all isolates were successfully typed with *agr* genes typing system. The *agrI* was detected in 63 (40.1%), *agrII* in 42 (26.8%), *agrIII* in 27 (17.2%) and *agrIV* was detected in 24 (15.3%) isolates with 441bp, 575bp, 323bp and 659bp of single amplicon in multiplex PCR (Fig. 27). In *agr* typing, maximum prevalence was recorded for *agrI* and all *agr* types were only present in human, camel, buffalo, goat and cattle while other sources deficient were at

least for one *agr* type as mentioned in table 27. For six adherence genes, only three isolates were found to be deficient for at least three genes namely *clfB, icaD* and *trap* were absent in J4 (camel), *clfA, clfB, icaA* and *icaD* in C15 (cattle) and *clfA, icaA* and *icaD* was absent in G16 (goat). Among all studied adherence genes *agr* was most prevalent (99.4%) followed by *trap, icaA, clfA, clfB* and *icaD* in decreasing order. Polymorphism was not detected in any of the studied adherence genes in the present investigation.

Our results are in agreement to reports of Tristan *et al.* (2003) who found high prevalence of *clfA* (99%) and *clfB* (100%) genes among 157 human clinical samples. Close to our results, Gilot *et al.* (2002) reported 100% isolates were positive for *trap* gene and similar to this study, *agrI* (69.0%) was found to be most prevalent followed by *agrII* in 23.9%, *agrIII* in 2.8% and *agrIV* was found in 1.4% isolates of cow mastitis samples.

Booth *et al.* (2001) also reported 96.2% (51/53) prevalence of *clf*A gene in isolates from human clinical samples and Stephan *et al*. (2001) reported 100% prevalence of *clf*A gene in isolates from cow mastitis samples.

Our results are in agreement to finding of Peerayeh *et al.* (2009) who also reported that majority of (55.1%) isolates belonged to *agr*-I, followed by *agr*-II (16.9%), *agr*-III (16.5%) and *agr*-IV (9.4%) group while 1.8% isolates were not typeable with *agr* groups among 212 *S. aureus* strains isolated from human clinical infections. Similarly, Jarraud *et al.* (2002) also investigated relationship between *agr* groups and human *S. aureus* isolateswith prevalence of 30.8% *agr*I, 24.7% *agr*II, 21.7% *agr*III and 22.7% *agr*IV among 198 *S. aureus* strains isolated from human with various clinical samples.

Almost similar observations were made by Khoramian *et al.* (2015) who reported 88.4% *icaD* and 87.9% *icaA* gene prevalence among 215 isolates from human and dairy cow's infections and Castelani *et al.* (2015) found 98% and 100% *icaA* and i*caD* genes respectively among 110 isolates from heifers and cows with mastitis.

Yazdani *et al*. (2006) and Vasudevan *et al.* (2003) found 100% isolates were positive for *icaA* and *icaD* genes from human clinical and bovine mastitis samples respectively and close to our results Ando *et al.* (2004) also reported 99.1% isolates positive for *icaD* and slightly lower prevalence for *clfA* (77.1%) gene among human samples. While Ando *et al.* (2004) also found presence among one (0.9%), 99 (90.8%), two (1.8%) and zero (0%) strains for *agr*I, *agr*II, *agr*III and *agr*IV gene respectively same human samples. El-Sayed *et al.* (2006) reported polymorphism in *clfA* gene with two different sized amplicon one is 1000bp similar to present study and other is 900bp in the samples of clinical and subclinical mastitic milk of cow with variable prevalence. They also conducted *agr* typing and reported that *agr*I was present in six (25%) and 12 (75%) strains *agr*II in 16 (66.6%) and four (25%) strains of the clinical and subclinical mastitic samples, respectively. Two (8.3%) *S. aureus* strains could not be characterized by the use of the oligonucleotide primers specific for *agr*I and II.

Reports of Xu *et al.* (2015) were also similar to our findings. They reported *clfA, clfB, icaD, agr-1* and *agr-2* genes with 89.3%, 85.7%, 71.4, 64.3% and 17.9% prevalence, respectively among 28 *S. aureus* isolates from mastitis milk samples of cow but contrary to our results they were not detected *icaA,agr-3, agr-4* genes inany of the studied isolates.

Similar to our results high prevalence percentage was reported by many researchers *i.e.* Atshan *et al.* (2012) reported 100% presence of *clfA, clfB icaA* and *icaD* genes among 60 human isolates, Bnyan (2013) reported 100% presence of *clfA,icaA* and *icaD* among six isolates of human, Tang *et al.* (2013) reported 100% presence of *clfB* gene, 87.50% of *icaA* and *icaD* and 25% presence of *clfA* gene among nosocomial infection and cross-contamination of foods samples and Barbieri *et al.* (2015) also reported that 100 % isolates carried *icaA, icaD* and *clfA* gene among thirteen *S. aureus* isolates from breast peri-implant infections but Li-li *et al.* (2012) reported slight lower prevalence in compare to present study, such as 55.5% presence of *clfA* gene, 36.4%of *clfB* and 31.3% presence of *icaAD* gene among 137 isolates of bovine mastitis.

Table 27: Detection of Adherence factor associated genes in *S. aureus* isolates from various sources.

S. No.	Source of Isolate	Total No. of isolate	Adherence factor associated genes (%)													
			clfA (1000bp)		*clfB* (205bp)		*icaA* (1315bp)		*icaD* (381bp)		*trap* (504bp)		*agr*[f]			
			P	N	P	N	P	N	P	N	P	N	*agrI* (441bp)	*agrII* (575bp)	*agrIII* (323bp)	*agrIV* (659bp)
1.	Human	35	35 (100.0)	0 (0.0)	34 (97.1)	1 (2.9)	35 (100.0)	0 (0.0)	32 (91.4)	3 (8.6)	34 (97.1)	1 (2.9)	10 (28.6)	5 (14.3)	14 (40.0)	5 (14.3)
2.	Meat piece	20	19 (90.0)	2 (10.0)	20 (100.0)	0 (0.0)	20 (100.0)	0 (0.0)	19 (95.0)	1 (5.0)	20 (100.0)	0 (0.0)	15 (75.0)	3 (15.0)	2 (10.0)	0 (0.0)
3.	Horse	3	3 (100.0)	0 (0.0)	3 (100.0)	0 (0.0)	2 (66.7)	1 (33.3)	3 (100.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)
4.	Pig	2	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)
5.	Camel	8	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	8 (100.0)	0 (0.0)	5 (62.5)	3 (37.5)	7 (87.5)	1 (12.5)	3 (37.5)	2 (25.0)	2 (25.0)	1 (12.5)
6.	Dog	6	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)	5 (83.3)	1 (16.7)	5 (83.3)	1 (16.7)	6 (100.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)	3 (50.0)
7.	Sheep	6	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	2 (33.3)	1 (16.7)	3 (50.0)
8.	Buffalo	21	20 (95.2)	1 (4.8)	21 (100.0)	0 (0.0)	20 (95.2)	1 (4.8)	20 (95.2)	1 (4.8)	20 (95.2)	1 (4.8)	11 (52.4)	3 (14.3)	2 (9.2)	5 (23.8)
9.	Goat	28	27 (96.4)	1 (3.6)	26 (92.9)	2 (7.1)	26 (92.9)	2 (7.1)	25 (89.3)	3 (10.7)	28 (100.0)	0 (0.0)	12 (42.8)	10 (35.7)	4 (14.3)	2 (7.1)
10.	Cattle	28	24 (85.7)	4 (14.3)	23 (82.1)	5 (17.9)	26 (92.9)	2 (7.1)	27 (96.4)	1 (3.6)	28 (100.0)	0 (0.0)	11 (39.3)	11 (39.3)	1 (3.6)	5 (17.9)
Total		**157**	**148 (94.3)**	**9 (5.7)[b]**	**148 (94.3)**	**9 (5.7)[b]**	**150 (95.5)**	**7 (4.5)[c]**	**144 (91.7)**	**13 (8.3)[d]**	**154 (98.0)**	**3 (2.0)[e]**	**63 (40.1)**	**42 (26.8)**	**27 (17.2)**	**24 (15.3)**

Abbreviations:- P- Positive, N- Negative

Superscript:- a- Isolates negative for *clfA* gene (Mt20, Mt22, J9, B30, G16, C2, C7, C12 and C15), b- Isolates negative for *clfB* gene (H44, J4, G29, G43, C11, C12, C13, C15 and C20), c- Isolates negative for *icaA* gene (Hrs3, D13, B26, G16, G29, C15 and C50), d- Isolates negative for *icaD* gene (H30, H34, H46, Mt4, J2, J4, J10, D7, B43, G11, G16, G29 and C15), e- Isolates negative for *trap* gene (H16, J4 and B26), f- Isolate negative for all type *agr* gene (H24)

Mohsenzadeh *et al.* (2015) reported *agr* typing for 31 isolates of *S. aureus* obtained from mastitis in dairy cattle. They also reported that most of the isolates belonged to *agr* group I (54.8%), followed by *agr*III (25.8%) and *agr*II (19.4%). But contrary to our study there was no isolates belonging to *agr* group IV. Havaei *et al.* (2014) reported *agr* typing of 50 isolates from human clinical specimens and *agr* typing results revealed that 45 (90%) isolates were *agr* type I, two (4%) were *agr* type III, and 3 (6%) were nontypeable. Fabres-Klein *et al.* (2015) also reported *agr* typing of 54 *S. aureus* isolates from cows with subclinical mastitis. The bacteria belonged to *agr* groups I (22.2%), II (62.9%), III (11.1%) and IV (3.7%) while none of the isolate was nontypeable.

Our results are in complete agreement to those of Xie *et al.* (2011) who reported *agr* typing of 108 isolates from human clinical infections. The results showed that *agr* group I was the most predominant, detected in 60.2% (65/108) of the strains. The groups of *agr* III, II, and IV were less common and found in 19.4%, 16.7%, and 2.8% of the isolates, respectively. One isolate repeatedly yielded negative result for any of the *agr* types tested.

B. Antiphagocytosis (Capsule) factor (*cap5K* and *cap8K* gene)

Capsule production is an another important virulence factor which does not cause direct damage to the host but it helps in survival of the organism in the host by evading the host-immune system especially phagocytosis. Among 11 capsular serotypes, capsular type 5 and 8 have been found to be most common. However, there seems to be great variation in capsular types depending on source of isolation (i.e. human, animal food and cross contaminating environment sources) and geographical distribution of organism. Thus this study has designed to find variability among *S. aureus* isolates regarding capsular genes especially *cap5K* and *cap8K*.

In the present study duplex PCR was developed to detect both capsular gene in a single reaction. In capsule associated genes, *cap5K* gene was found in 73 (46.5%) isolates with single amplicon of 361bp, *cap8K* in 59 (37.6%) isolates with 173bp amplicon, both genes were found in 22 (14.0%) isolates (Fig. 28). Three isolates (D9, B55 and C34) were nontypeable in capsular typing. The *cap5K* gene was more prevalent among all studied sources except meat piece, in which *cap8K* gene was more prevalent. None of the dog isolate carried *cap8K* gene. Both capsular type genes were detected in six (17.1%) human isolates, in five (17.9%) goat isolates, in four (20%) meat piece isolates and other sources were positive with less than two isolates while none of the pig, dog and sheep isolates carried both genes in single isolate (table 28).

Similar to present study Salasia *et al.* (2011) reported that most of the isolates from bovine origin harbored *cap5* (74%), while most of the isolates from humans harbored *cap8* (91%) and isolates from food sources were positive for *cap5* (100%) and *cap8* (64%). In complete agreement to present study, they also found that both *cap5* and *cap8* genes were present in seven of the 19 bovine isolate, two of the 11 human isolate and seven of the 11 food origin isolates with overall 39% prevalence among 21 isolates.

Similar to present study, Reinoso *et al.* (2008) also reported 21 (47%) isolates were positive for *cap5* (11 human, nine bovine and one food sample strain), seven (15%) isolates were for *cap8* gene (four human and three food sample strains) while 17 (38%) isolates were non-*cap5* or *cap8* among 45 isolates from humans, bovine subclinical mastitis and food samples. Our results were similar to those of Khichar and Kataria (2014) who reported that *cap5k* gene was more prevalent in the isolates of bovine mastitic samples. They found 26 isolates (92.86%) with *cap5k* gene and two isolates (7.14%) with *cap8k* gene.

Similar to results in the present study, Xu *et al.* (2015) reported that *cap5* and *cap8* genes were found in 46.4% and 39.3% isolates, respectively among 28 isolates from mastitic milk samples of cow and Yadav *et al.* (2015a) found 68.75% isolates were carry *cap5K* gene and 21.87% isolates were carry *cap8K* gene whereas three isolates (9.37%) were found nontypeable among 32 isolates from milk of cattle and buffalo with clinical mastitis. Similarly, Nathawat *et al.* (2015) reported 68.38% presence of *cap5K* gene and 34.61% presence of *cap8K* while one isolate was nontypeable among 27 isolates from milk samples of goat mastitis.

Table 28: Detection of Antiphagocytosis (Capsule) factor associated genes among *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Capsule associated genes (%)			
			cpa5k (361bp)	*cap8k* (173bp)	Both	NT
1.	Human	35	15 (42.9)	14 (40.0)	6 (17.1)	0 (0.0)
2.	Meat piece	20	4 (20.0)	12 (60.0)	4 (20.0)	0 (0.0)
3.	Horse	3	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)
4.	Pig	2	1 (50.0)	1 (50.0)	0 (0.0)	0(0.0)
5.	Camel	8	5 (62.5)	2 (25.0)	1 (12.5)	0 (0.0)
6.	Dog	6	5 (83.3)	0 (0.0)	0 (0.0)	1 (16.7)
7.	Sheep	6	5 (83.3)	1 (16.7)	0 (0.0)	0 (0.0)
8.	Buffalo	21	8 (38.0)	9 (42.9)	3 (14.9)	1 (4.2)
9.	Goat	28	18 (64.2)	5 (17.9)	5 (17.9)	0 (0.0)
10.	Cattle	28	12 (42.9)	13 (46.4)	2 (7.1)	1 (3.5)
Total		**157**	**73 (46.5)**	**59 (37.6)**	**22 (14.0)**	**3 (1.9)**

NT- Non type able (Negative for both *cap5k* and *cap8k* gene- D9, B55 and C34)

C. Exoenzymes (*coa* and *aur* gene)

Staphylococcus aureus secrete various exoenzyems, of which enzymes staphylocoagulase and aureolysin are important virulent exoenzyme to trigger staphylococcal infections. Coagulase is a collagen binding enzyme encoded by *coa* gene and aureolysin is a zinc dependent metalloprotease enzyme govern by *aur* gene. Both enzymes facilitate the spread of infection in the host system and destroys host defense molecules. Since, both enzymes have significant role in virulence and are encoded by polymorphic genes thus it is very important to study distinct molecular polymorphism patterns up to sequence level to established epidemiological tool for investigation of *S. aureus* from various infection sources and geographical regions.

coa gene typing

In present investigation, all the *S. aureus* isolates were found to be positive for *coa* gene with nine amplicon sizes varying from 500-900bp, which including four isolates of 500bp, seven of 550bp, 18 of 600bp, 21 of 650bp, 74 of 700bp (maximum), eight of 750bp, 12 of 800bp, two of 850bp (minimum) and 11 isolates of 900bp as mentioned in table 29 (Fig. 29) with 0.7353 discriminatory index. The *coa* gene amplification was found to be suitable for isolate differentiation with good discriminatory index value (D.I.= 0.7353). Maximum variations in regards to amplicon sizes were recorded in goat isolates with six different amplicon sizes (from 550-800bp) followed by cattle with five different amplicon sizes (from 500-900bp), human with four different amplicon sizes (from 500-850bp) and other sources showed variability with four or less than four different amplicon sizes. But horse and pig isolates showed single amplicon of 650bp and 600bp respectively as described in table 29.

To find the nucleotide polymorphism in the *coa* gene RFLP of *coa* gene was carried out (Fig. 30) with nucleotide site specific restriction endonuclease *Alu*I (5'…AGCT…3' and 3'…TCGA…5'), which revealed the 33 different coa-RFLP patterns among all isolates with 0.9301 discriminatory index. The D.I. value of *coa* PCR amplification was 0.7353 and the *Alu*I digestion of the PCR products increased this value to D.I.- 0.9301. This increased discriminatory index indicating that RFLP is a more precise differentiating technique instead of only PCR amplification. The discriminatory index of *coa*- RFLP typing revelled that it may considered as very good tool to differentiate various *S. aureus* isolates. On the basis of observed discriminatory index *coa*-RFLP method (D.I.-0.9301) is very good method to differentiate different strains of *S. aureus* in comparison of *rep*-PCR method (D.I.-0.8892). Out of the total 33 coa-RFLP types, coa30 and coa15 types are most common types including 25 (dog, sheep, buffalo goat and cattle) and 20 (buffalo, goat and cattle except four human isolates) isolates with most of animal origin isolates respectively followed by coa5 including 19 isolates and most of isolates were meat piece origin, coa19 type including 12 isolates and all from human origin (except two cattle isolates) other remaining *coa* types includes less than nine isolates as mentioned in table 30.

Table 29: GroupWise distribution of *coa* gene among *S. aureus* isolates in the present study including its RFLP patterns with *Alu*I enzyme.

S. No.	Source of isolate	S. No.	*coa* amplicon Size	*coa* gene RFLP Pattern	Isolate I.D.	No. of Isolates	Subtotal of Isolates	Total No. of Isolates
1.	Human	1.	850bp	300bp, 350bp	H13	1	2	35
				150bp, 200bp	H31	1		
		2.	700bp	200bp, 250bp	H1, H4, H15, H21, H24 and H29	6	24	
				180bp, 220bp, 300bp	H2, H3, H5 and H7	4		
				220bp, 480bp	H6, H8, H9, H10 and H11	5		
				60bp, 250bp, 350bp	H12 and H28	2		
				150bp, 200bp, 300bp	H14, H16, H22, H25 and H27	5		
				60bp, 200bp, 380bp	H30	1		
				700bp	H41	1		
		3.	650bp	200bp, 250bp	H33, H37, H39 and H40	4	8	
				60bp, 200bp, 380bp	H34, H44 and H45	3		
				80bp, 250bp, 300bp	H48	1		
		4.	500bp	220bp, 250bp	H46	1	1	
2.	Meat piece	1.	700bp	80bp, 110bp, 120bp, 220bp	Mt11, Mt12, Mt13 and Mt27	4	11	20
				100bp, 300bp	Mt15, Mt26 and Mt28	3		
				80bp, 250bp, 350bp	Mt4, Mt19	2		
				220bp, 250bp	Mt2 and Mt9	2		
		2.	650bp	80bp, 110bp, 120bp, 220bp	Mt1, Mt3, Mt10, Mt14, Mt20, Mt22 and Mt31	7	7	
		3.	600bp	110bp, 300bp, 350bp	Mt24 and Mt25	2	2	
3.	Horse	1.	650bp	80bp, 250bp, 300bp	Hrs1	1	3	3
				80bp, 110bp, 120bp, 220bp	Hrs2 and Hrs3	2		
4.	Pig	1.	600bp	80bp, 110bp, 120bp, 220bp	Pg2 and Pg4	2	2	2
5.	Camel	1.	700bp	80bp, 110bp, 120bp, 220bp	J4	1	4	8
				180bp, 200bp, 300bp	J9, J10 and J15	3		
		2.	600bp	100bp, 180bp, 300bp	J3	1	3	
				80bp, 110bp, 120bp, 220bp	J14 and J18	2		
		3.	500bp	220bp, 250bp	J2	1	1	
6.	Dog	1.	750bp	80bp, 110bp, 120bp, 220bp	D4	1	3	6

				180bp, 200bp, 300bp	D6 and D7	2		
		2.	700bp	180bp, 200bp, 300bp	D9	1	1	
		3.	600bp	300bp	D10 and D13	2	2	
7.	Sheep	1.	700bp	100bp, 300bp	SN3 and SN4	2	2	6
		3.	600bp	300bp	SV2, SV3, SV4 and SN14	4	4	
8.	Buffalo	1.	800bp	800bp	B27, B43 and B46	3	6	21
				100bp, 220bp, 480bp	B1, B10 and B23	3		
		2.	750bp	750bp	B34	1	3	
				220bp, 480bp	B31 and B36	2		
		3.	700bp	180bp, 220bp, 300bp	B24 B19, B39, B42 and B57	5	10	
				220bp, 250bp	B26, B28, B29 and B30	4		
				700bp	B21	1		
		4.	600bp	300bp	B5 and B55	2	2	
9.	Goat	1.	800bp	180bp, 200bp, 380bp	G21 and G16	2	2	28
		2.	750bp	260bp, 300bp	G9	1	1	
		3.	700bp	150bp, 180bp, 240bp	G1	1	12	
				180bp, 220bp, 300bp	G47 and G49	2		
				150bp, 250bp, 300bp	G24, G37 and G39	3		
				80bp, 250bp, 300bp	G55	1		
				150bp, 300bp	AG17	1		
				300bp, 400bp	G11	1		
				220bp, 400bp	G10	1		
				260bp, 300bp	G7	1		
				700bp	G46	1		
		4.	650bp	300bp, 350bp	AG13 and AG15	2	3	
				300bp	G45	1		
		5.	600bp	300bp	AG5, AG6 and AG8	3	3	
		6.	550bp	220bp, 300bp	G43	1	7	
				200bp, 300bp	G2	1		
				300bp	AG10, G29, G35, G40 and G41	5		

10.	Cattle	1.	900bp	180bp, 220bp, 300bp	C8, C9, C11, C12 and C22	5	11	28
				200bp, 300bp, 380bp	C36	1		
				250bp, 380bp	C40	1		
				300bp	C23,C26,C34 and C41	4		
		2.	800bp	180bp, 220bp, 300bp	C17	1	4	
				200bp, 250bp	C39	1		
				800bp	C2R and C5R	2		
		3.	750bp	250bp	C43	1	1	
		4.	700bp	180bp, 220bp, 300bp	C13, C46 and C47	3	10	
				80bp, 250bp, 300bp	C7 and C50	2		
				200bp, 250bp	C52	1		
				300bp	C2, C15, C20 and C29	4		
		5.	500bp	300bp, 200bp	C3R	1	2	
				250bp	C37	1		
Total No. of isolates						157	157	157

Table 30: *Staphylococcus aureus* typing on the basis of overall distribution of *coa* gene RFLP patterns among isolates in the present study.

S. No.	*coa* pattern I.D.	*coa*-RFLP pattern	Number of Isolates	Isolate I.D.
1.	coa1	60bp, 250bp, 350bp	2	H12 and H28
2.	coa2	60bp, 200bp, 380bp	4	H30, H34, H44 and H45
3.	coa3	80bp, 250bp, 300bp	5	H48, Hrs1, G55, C7 and C50
4.	coa4	80bp, 250bp, 350bp	2	Mt4, Mt19
5.	coa5	80bp, 110bp, 120bp, 220bp	19	Mt1, Mt3, Mt10, Mt11, Mt12, Mt13, Mt14, Mt20, Mt22, Mt27, Mt31, Hrs2, Hrs3, Pg2, Pg4, J4, J14, J18 and D4
6.	coa6	100bp, 300bp	5	Mt15, Mt26, Mt28, SN3 and SN4
7.	coa7	100bp, 180bp, 300bp	1	J3
8.	coa8	100bp, 220bp, 480bp	3	B1, B10 and B23
9.	coa9	110bp, 300bp, 350bp	2	Mt24 and Mt25
10.	coa10	150bp, 200bp, 300bp	5	H14, H16, H22, H25 and H27
11.	coa11	150bp, 250bp, 300bp	3	G24, G37 and G39
12.	coa12	150bp, 180bp, 240bp	1	G1

13.	coa13	150bp, 200bp	1	H31
14.	coa14	150bp, 300bp	1	AG17
15.	coa15	180bp, 220bp, 300bp	20	H2, H3, H5, H7, B24, B19, B39, B42, B57, G47, G49, C8, C9, C11, C12, C13, C17, C22, C46 and C47
16.	coa16	180bp, 200bp, 300bp	6	J9, J10, J15, D6, D7 and D9
17.	coa17	180bp, 200bp, 380bp	2	G21 and G16
18.	coa18	200bp, 300bp, 380bp	1	C36
19.	coa19	200bp, 250bp	12	H1, H4, H15, H21, H24, H29 H33, H37, H39, H40, C39 and C52
20.	coa20	200bp, 300bp	2	G2, C3R
21.	coa21	220bp, 300bp	1	G43
22.	coa22	220bp, 250bp	8	H46, Mt2, Mt9, J2, B26, B28, B29 and B30
23.	coa23	220bp, 400bp	1	G10
24.	coa24	220bp, 480bp	7	H6, H8, H9, H10, H11, B31 and B36
25.	coa25	250bp, 380bp	1	C40
26.	coa26	260bp, 300bp	2	G9 and G7
27.	coa27	300bp, 350bp	3	H13, AG13 and AG15
28.	coa28	300bp, 400bp	1	G11
29.	coa29	250bp	2	C37 and C43
30.	coa30	300bp	25	D10, D13, SV2, SV3, SV4, SN14, B5, B55, AG5, AG6 AG8, AG10, G29, G35, G40, G41, G45, C2, C15, C20 C23,C26, C29, C34 and C41
31.	coa31	700bp	3	H41, B21 and G46
32.	coa32	750bp	1	B34
33.	coa33	800bp	5	B27, B43, B46, C2R and C5R

Similar to *coa* size amplicon variations it was also observed during coa- RFLP analysis that maximum RFLP types were detected among goat isolates (13 RFLP types) followed by human (11 types), cattle (nine type), buffalo (seven types) and remaining sources were differentiated by less than four coa-RFLP types (table 29).

During phylogenetic cluster analysis of *S. aureus* coa-RFLP types in the present study, all 33 coa-RFLP types were divided into three clusters on the basis of 80% genetic similarity (Fig. 31) by Dice and UPGMA method. First cluster was made of 65 isolates (most of animal origin except few of human and meat piece isolates) and 12 coa- RFLP types (coa6, coa7, coa9, coa11, coa12, coa14, coa15, coa21, coa26, coa27, coa28 and coa30), second cluster comprised of 82 isolates (most of human and meat piece origin except few of animal origin isolates) and 17 coa-RFLP types (coa2, coa5, coa8, coa10, coa13, coa16, coa17, coa18, coa19, coa20, coa22, coa23, coa24, coa29, coa31, coa32 and coa33), third cluster included 10 isolates (five from human and meat piece and five of animal origin) and four coa-RFLP types (coa1, coa3, coa4 and coa25) as described in table 31.

Table 31 Cluster analysis of *S. aureus* on the basis of *coa* RFLP patterns by Dice and UPGMA method.

S. No.	Source of Isolate	Total No. of isolate	Clusters at 80% similarity level (%)		
			Cluster-I	Cluster-II	Cluster-III
1.	Human	35	5 (14.3)	27 (77.1)	3 (8.6)
2.	Meat piece	20	5 (25.0)	13 (65.0)	2 (10.0)
3.	Horse	3	0 (0.0)	2 (66.7)	1 (33.3)
4.	Pig	2	0 (0.0)	2 (100.0)	0 (0.0)
5.	Camel	8	1 (12.5)	7 (87.5)	0 (0.0)
6.	Dog	6	2 (33.3)	4 (66.7)	0 (0.0)
7.	Sheep	6	6 (100.0)	0 (0.0)	0 (0.0)
8.	Buffalo	21	7 (33.3)	14 (66.7)	0 (0.0)
9.	Goat	28	22 (78.6)	5 (17.9)	1 (4.5)
10.	Cattle	28	17 (60.7)	8 (28.6)	3 (10.7)
Total Isolates		157	65 (41.4)	82 (52.2)	10 (6.4)
Cluster- I includes coa types namely coa6, coa7, coa9, coa11, coa12, coa14, coa15, coa21, coa26, coa27 and coa28, cluster- II includes coa2, coa5, coa8, coa10, coa13, coa16, coa17, coa18, coa19, coa20, coa22, coa23, coa24, coa29, coa31, coa32 and coa33 and cluster- III includes coa1, coa3, coa4 and coa25					

In the present investigation with *Alu*I nine isolates remained undigested. Three isolates (H41, B21 and G46) of 700bp amplicon, one isolate (B34) of 750bp amplicon and five isolates (B27, B43, B46, C2R and C5R) of 800bp amplicon were not digested which indicated the absence of specific cutting site for *Alu*I. This observation is in agreement to findings of Lange *et al.* (1999) who also did not observe digestion of three PCR products from *S. aureus* of bovine mastitis origin. Similarly, da Silva and da Silva (2005) also observed non-digestion of some of the amplicons. Similar to our results, a wide range of *coa* gene amplicons were reported by various scientist from different sources *viz.* 579 to 1442bp (da Silva and da Silva, 2005); 730-1050bp (Aslantas *et al.,* 2007); 710 to 1456bp (Tiwari *et al.,* 2008); 490 to 850bp (Saei *et al.,* 2009), 610 to 960bp (Sindhu *et al.,* 2010) and 400 to 800bp (Abeer *et al.,* 2010).

Similar to our results, Lange *et al.* (1999) observed seven different sized PCR products as 580-1060bp among 66 isolates from bovine mastitic samples which were further classified into 14 different *Alu*I digestion types. They also reported that D.I. = 0.82 of *coa* PCR increased to D.I. = 0.88 in *Alu*I digestion as similar to present study. Su *et al*. (1999) also observed 40 genotypes during genotyping of 453 *S. aureus* isolates from bovine mastitic milk.

Our results were in agreement to those of Schlegelova *et al.* (2003) who were able to differentiate human and animal origin isolates on the basis of *coa* gene RFLP technique. They reported *coa* gene having variable product size of 650bp, 730bp, 810bp and 1050bp and molecular analyses identified 10 polymorphism types with prevalence of type II in isolates from cow's milk and type IV in isolates from people coming into contact with dairy cows on the farm (the cattlemen) and the other farm personnel. Seven further genotypes were identified among the isolates from the cattlemen.

Similar to present investigation, Goh *et al*. (1992) also obtained 440 to 950bp amplicons during *coa* gene amplification and they observed 10 distinct RFLP patterns by *Alu*I enzyme digestion among 30 different isolates from human clinical samples. Similarly, Hookey *et al.* (1998) also observed single amplicon of 875, 660, 603, or 547bp and 10 distinct RFLP patterns were found by enzyme *Alu*I among 85 isolates of methicillin-resistant *S. aureus* (MRSA) and 10 methicillin-sensitive *S. aureus* (MSSA) examined. Annemuller *et al.* (1999) also discriminated 25 *S. aureus* strains isolates from bovine subclinical mastitis and obtained six different types of RFLP patterns. Some of the restriction fragments obtained in the present investigation were similar to those in the study by Annemuller *et al*. (1999).

Our results corroborated the earlier observations of Gharib *et al.* (2013) who reported 648, 723, 812 and 913bp sizes amplicon of *coa* gene in coagulase positive *S. aureus.* The product of 812bp was the most frequent and accounted for 5/15 (33.3%) of the isolates followed by 648bp (26.6%), 723bp (13.3%) and 913bp (6.6%) from human and animal sources.

Bhati *et al.* (2014) reported single amplicon of either 400, 490, 510, 550, 600, 710, 760, 810 or 850bp while three isolates did not produce any *coa* amplicon from 38 isolates of Holstein-Friesian (H-F) crossbred and Rathi cattle with subclinical mastitis. Subsequently, the *coa* products were digested with *AluI*. The restriction digestion generated four and seven RFLP patterns with isolates from H-F crossbred and Rathi cattle respectively.

Yadav *et al.* (2015b) studied mastitc milk samples and reported four different *coa* PCR products (400bp, 510bp, 600bp and 650bp) from 16 cattle isolates and five different products (400bp, 510bp, 600bp, 650bp and 680bp) from 16 buffalo isolates. They obtained five and six RFLP patterns from cattle and buffalo isolates, respectively. The *coa* gene amplicon of 600bp was produced by the maximum number of isolates.

aur gene typing

In present investigation all the *S. aureus* isolates were subjected to *aur*(aureolysin) gene typing, *aur* gene was found in 152 (96.8%) isolates with single amplicon of 1526bp (Fig. 32) while five (3.2%) isolates *i.e.* Mt22, J4, B46, G16 and C15 were detected without this gene (Table. 32).

To find the nucleotide polymorphism in the *aur* gene RFLP of *aur* gene (Fig. 30) with nucleotide site specific restriction endonuclease *Hinf*I (5'…G ANTC…3' and 3'…CTNA G…5', Single Letter Codes (N)- A or C or G or T) was carried out. Three different aur-RFLP types *i.e.* A1 (50bp, 150bp, 200bp, 1000bp), A2 (50bp, 650bp, 800bp) and A3 (50bp, 100bp, 140bp, 480bp, 700bp) were found among studied isolates (Fig. 33). Among all aur-EFLP types, A3 type included in 119 isolates with highest prevalence (78.3%) followed by A1 type with 17 (11.2%) isolates and A2 type included 16 isolates with lowest prevalence (10.5%). A3 type included isolates belonging to all studied sources while A2 and A1 type only existed in buffalo, meat piece and human, goat isolates, respectively. Source-wise analysis revealed that human isolates belonged to all three aur-RFLP types while horse, pig, camel, dog, sheep and cattle isolates belonged to only A3 type. Meat piece and buffalo isolates belonged to A2 and A3 types and goat isolates belonged to A1 and A3 types as mentioned in table 32.

Three clusters were found during phylogenetic cluster analysis of *S. aureus* aur- RFLP types at the level of 80% genetic similarity (Fig. 34) by Dice and UPGMA method. First cluster was made of 17 isolates (most of human isolates except two of goat isolates), second cluster comprising of 16 isolates (most of human (11) while four meat piece and one buffalo isolate), third cluster included 119 isolates (most of animal origin (95) while nine human and 15 meat piece isolate).

Table 32: Detection and distribution of _aur_ gene and its RFLP patterns among _S. aureus_ isolates.

S. No.	Source of Isolate	aur type	*aur* RFLP patterns	Isolate I.D.	No. of isolates	Total No. of Isolates
1.	Human	A1	50bp, 150bp, 200bp, 1000bp	H1, H2, H6, H8, H10, H11, H12, H28, H30, H33, H34, H37, H39, H40 and H41	15	35
		A2	50bp, 650bp, 800bp	H3, H4, H5, H9, H15, H21, H24, H27, H29, H31 and H48	11	
		A3	50bp, 100bp, 140bp, 480bp, 700bp	H7, H13, H14, H16, H22, H25, H44, H45 and H46	9	
2.	Meat Piece	A2	50bp, 650bp, 800bp	Mt2, Mt9, Mt26 and Mt27	4	20
		A3	50bp, 100bp, 140bp, 480bp, 700bp	Mt1, Mt3, Mt4, Mt10, Mt11, Mt12, Mt13, Mt14, Mt15, Mt19, Mt20, Mt24, Mt25, Mt28 and Mt31	15	
			-	Mt22 (*aur* -ve)	1	

3.	Horse	A3	50bp, 100bp, 140bp, 480bp, 700bp	Hrs1, Hrs3 and Hrs4	3	3
4.	Pig	A3	50bp, 100bp, 140bp, 480bp, 700bp	Pg2 and Pg4	2	2
5.	Camel	A3	50bp, 100bp, 140bp, 480bp, 700bp	J2, J3, J9, J10, J14, J15 and J18	7	8
		-	-	J4 (*aur* -ve)	1	
6.	Dog	A3	50bp, 100bp, 140bp, 480bp, 700bp	D4, D6, D7, D9, D10 and D13	6	6
7.	Sheep	A3	50bp, 100bp, 140bp, 480bp, 700bp	SV2, SV3, SV4, SN3, SN4 and SN14	6	6
8.	Buffalo	A2	50bp, 650bp, 800bp	B26	1	21
		A3	50bp, 100bp, 140bp, 480bp, 700bp	B1, B5, B10, B19, B21, B23, B24, B27, B28, B29, B30, B31, B34, B36, B39, B42, B43, B55 and B57	19	
			-	B46 (*aur* -ve)	1	
9.	Goat	A1	50bp, 150bp, 200bp, 1000bp	AG13 and AG15	2	28
		A3	50bp, 100bp, 140bp, 480bp, 700bp	AG5, AG6, AG8, AG10, AG17, G1, G2, G7, G9, G10, G11, G21, G24, G29, G35, G37, G39, G40, G41, G43, G45, G46, G47, G49 and G55	25	
		-	-	G16 (*aur* -ve)	1	
10.	Cattle	A3	50bp, 100bp, 140bp, 480bp, 700bp	C2, C2R, C3R, C5R, C7, C8, C9, C11, C12, C13, C17, C20, C22, C23, C26, C29, C34, C36, C37, C39, C40, C41, C43, C46, C47, C50 and C52	27	28
		-	-	C15 (*aur* -ve)	1	
Total No. of isolate					157	157
All *aur* gene positive isolates were showed 1526bp size amplicon. Five isolates were negative for *aur* gene (Mt22, J4, B46, G16 and C15)						

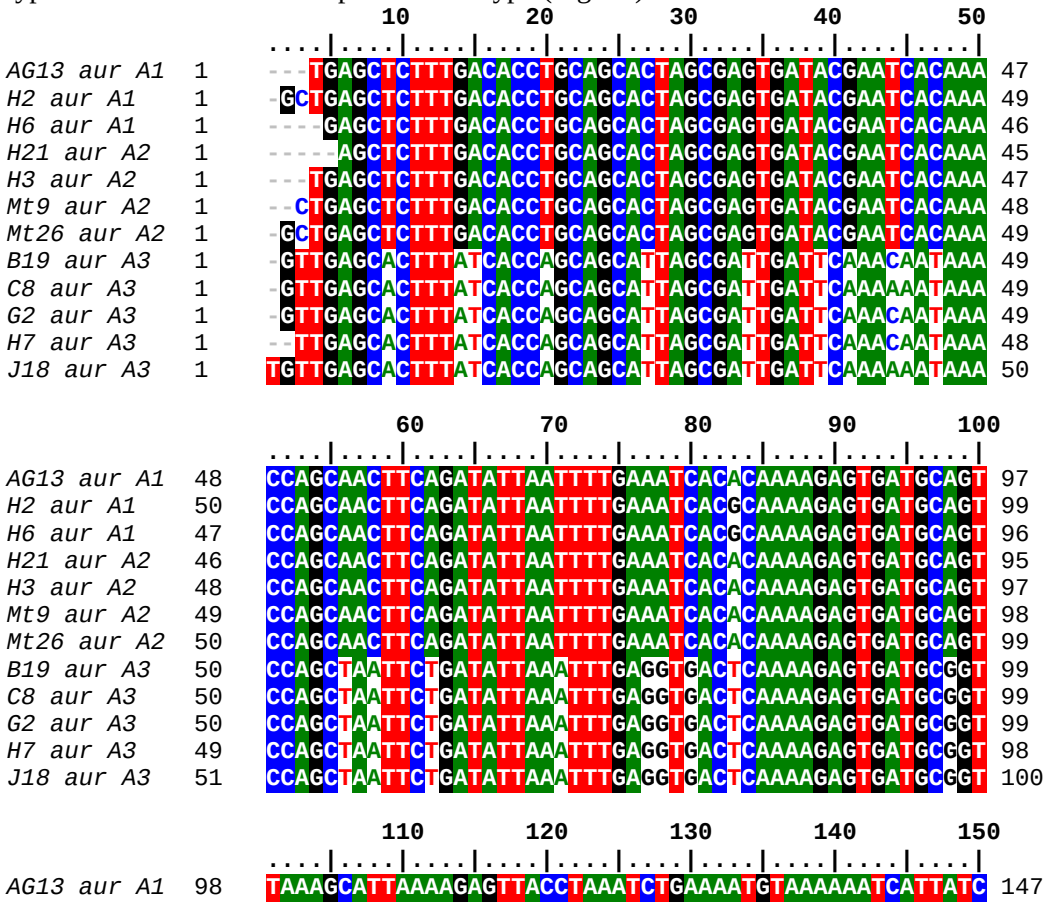
Similar to our results high prevalence of *aur* gene was reported by many researchers with similar size (1.5kb) single amplicon *i.e.* Zdzalik *et al.* (2012) reported 99% prevalence of this gene in 167 human clinical samples, Sabat *et al.* (2000) observed 100% presence in 53 *S. aureus* strains, isolated from healthy as well as diseased persons, Takeuchi *et al.* (2002) detected 100% presence of *aur* gene among 74 isolates from cows, pigs and chickens and similarly, Sabat *et al.* (2008) also reported 100% prevalence of *aur* gene among human MRSA and MSSA strains with same length of 1530bp as in present study.

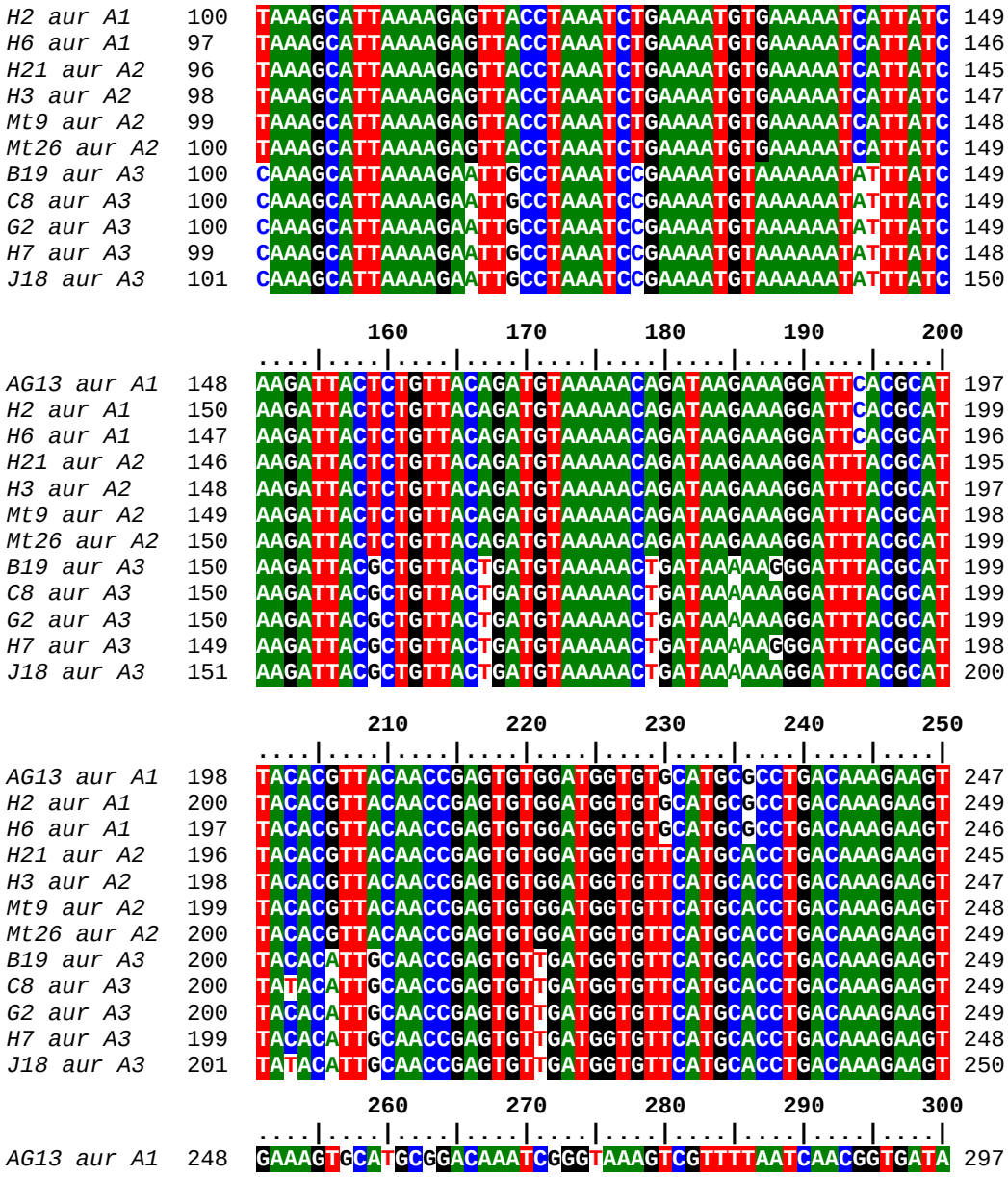
During RFLP analysis of *aur* gene many researchers demonstrated that *aur* gene occurred in two allelic forms (type I and II) among human and animal isolates of *S. aureus* (Sabat *et al.*, 2000; Takeuchi *et al.*, 2002; Zdzalik *et al.*, 2012) but we observed that *aur* gene existed with three allelic forms. Our results were based on substantial number of isolates and varied sources hence we opted for nucleotide sequencing of these three allelic forms to confirm and compare previous two allelic forms and three allelic form obtained in this study.

aur gene sequence analysis

For sequence analysis, 12 *aur* gene positive isolates from each aur-RFLP type were got sequenced *i.e.* the isolate AG13 (goat), H2 and H6 (human) from aur-RFLP typeA1, the isolates H3, H21 (human), Mt9 and Mt26 (meat piece) from aur-RFLP typeA2 and the isolates H7 (human), J18 (camel), B19 (buffalo), G2 (goat) and C8 (cattle) from aur-RFLP typeA3. First of all the sequences were BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed as *aur* gene sequences of *S. aureus* strains.

In sequence analysis of above 12 samples, overall more than 200 nucleotide and their corresponding amino acid variations were recorded at various positions. The big gaps or deletions were observed from 641 position to 679 position in all studied sequences. Except in isolate J18 sequence was without gap and in isolate G2 with only two gaps and both these sequences belonged to aur-RFLP typeA3. These number of nucleotide variability can be considered as massive variations among studied sequences. It was also found that, sequences from similar RFLP patterns had less difference in comparison to those from other patterns (Fig. 35). These sequence variations were non-significant (p>0.05) in within aur-RFLP type but variations were found significant (p≤0.05) with each other in between aur-RFLP type during analysis of codon based test of neutrality (Table 33). This test of neutrality analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 418 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 software. Three clusters were observed during phylogenetic tree analysis of *aur* gene sequences of present study and further revealed that aur-RFLP type A1, A2 and A3 classified in separate clusters and A1 and A2 types were more-closer compared to A3 type (Fig. 36).

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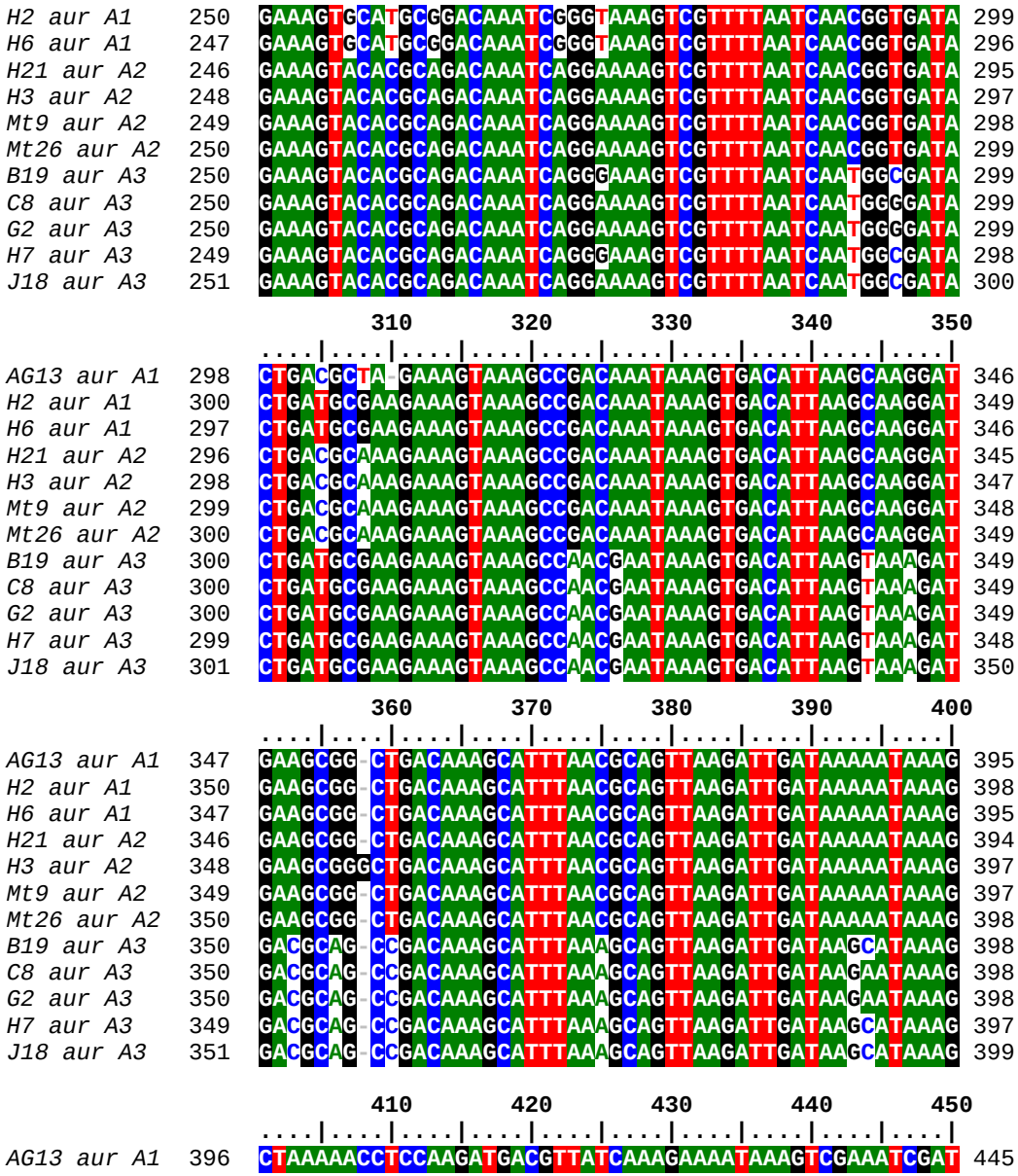
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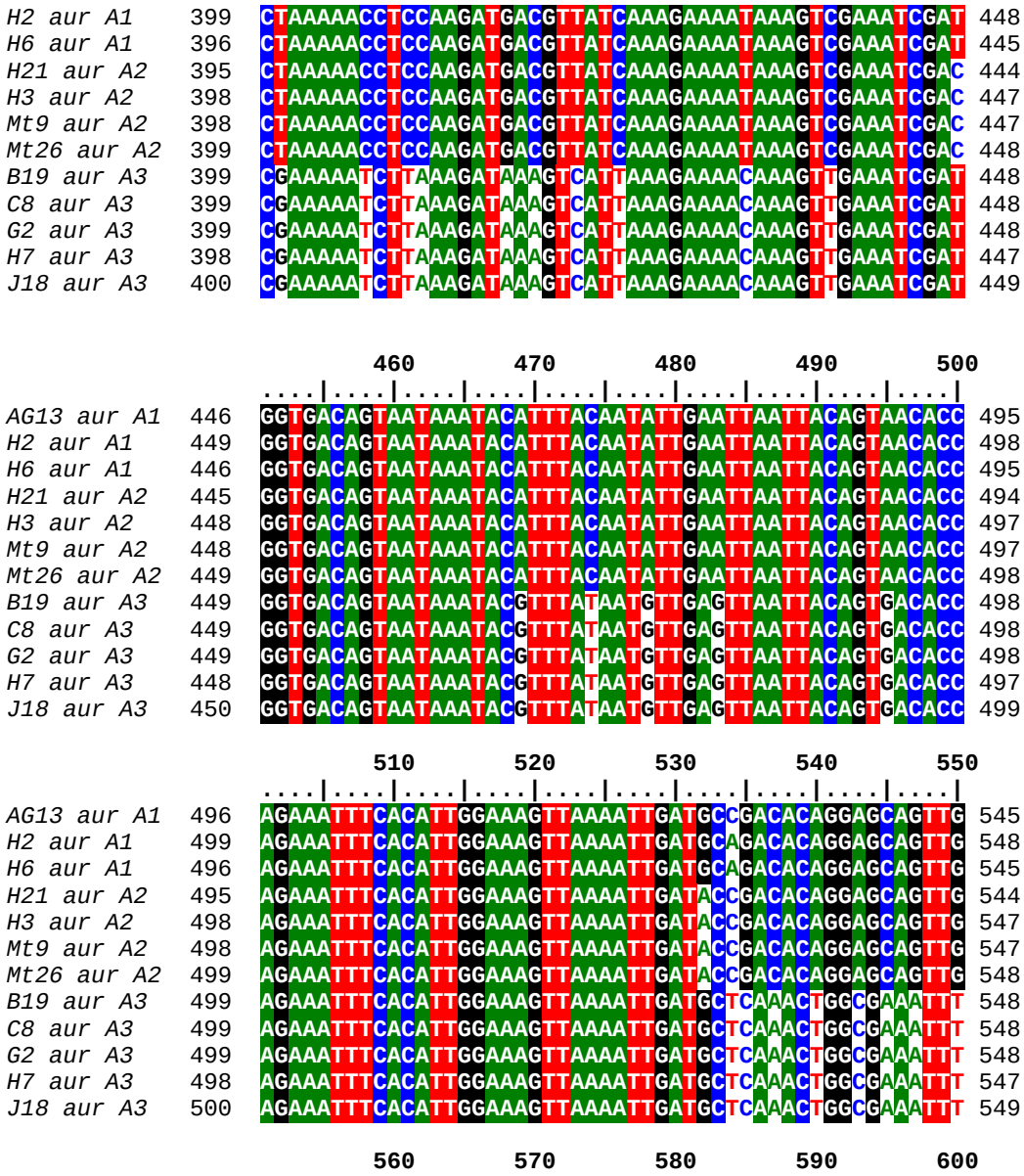
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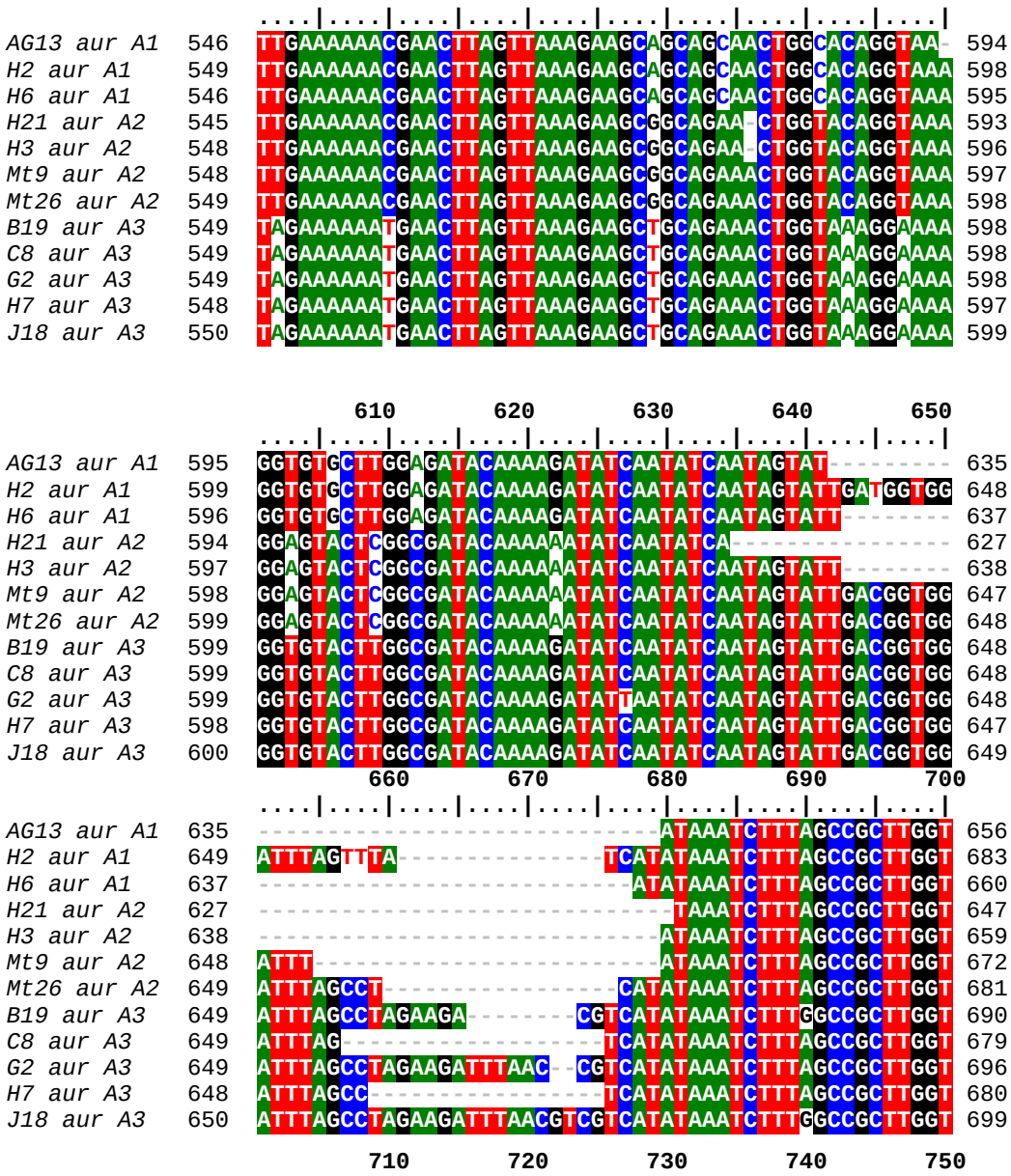
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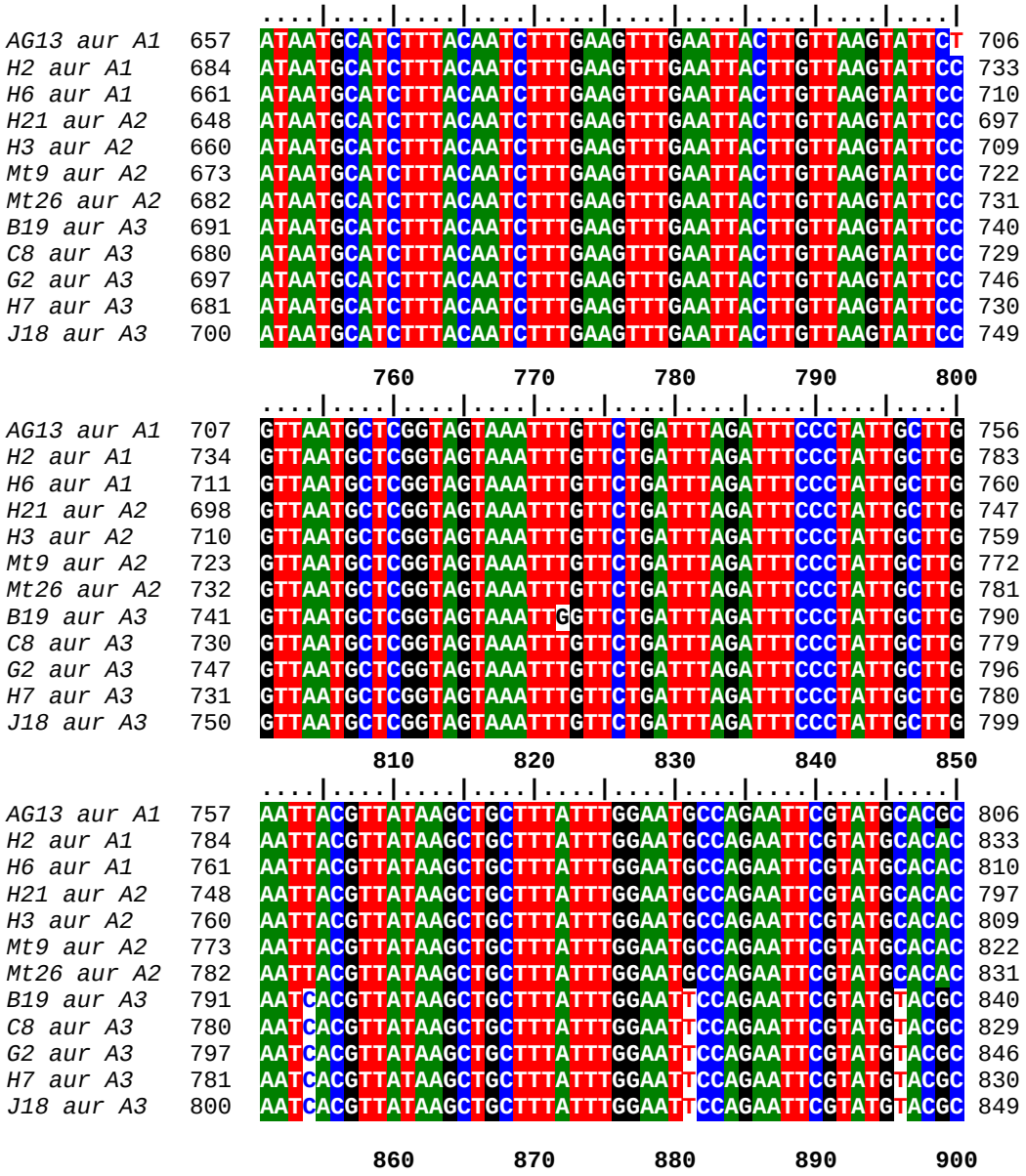
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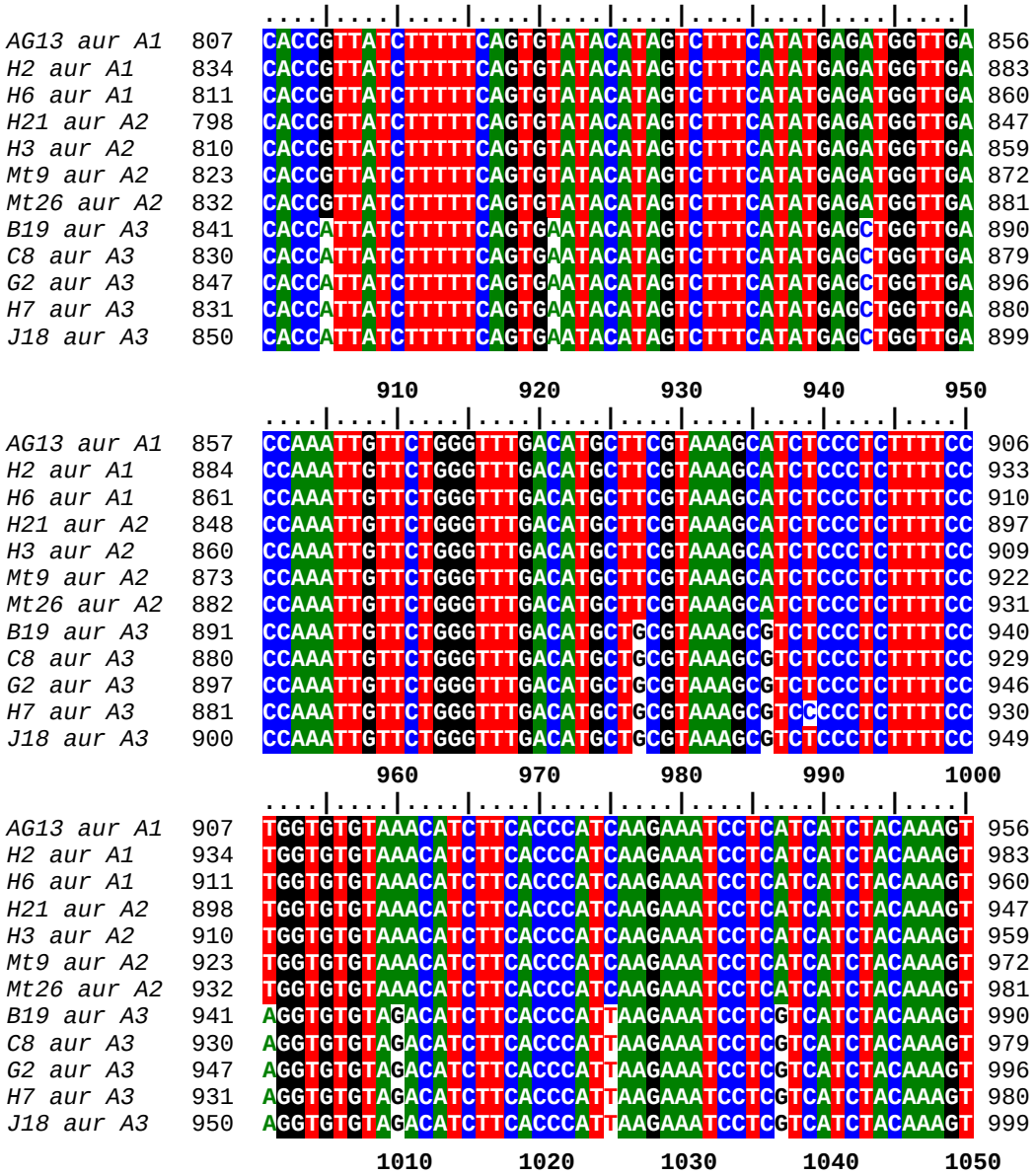
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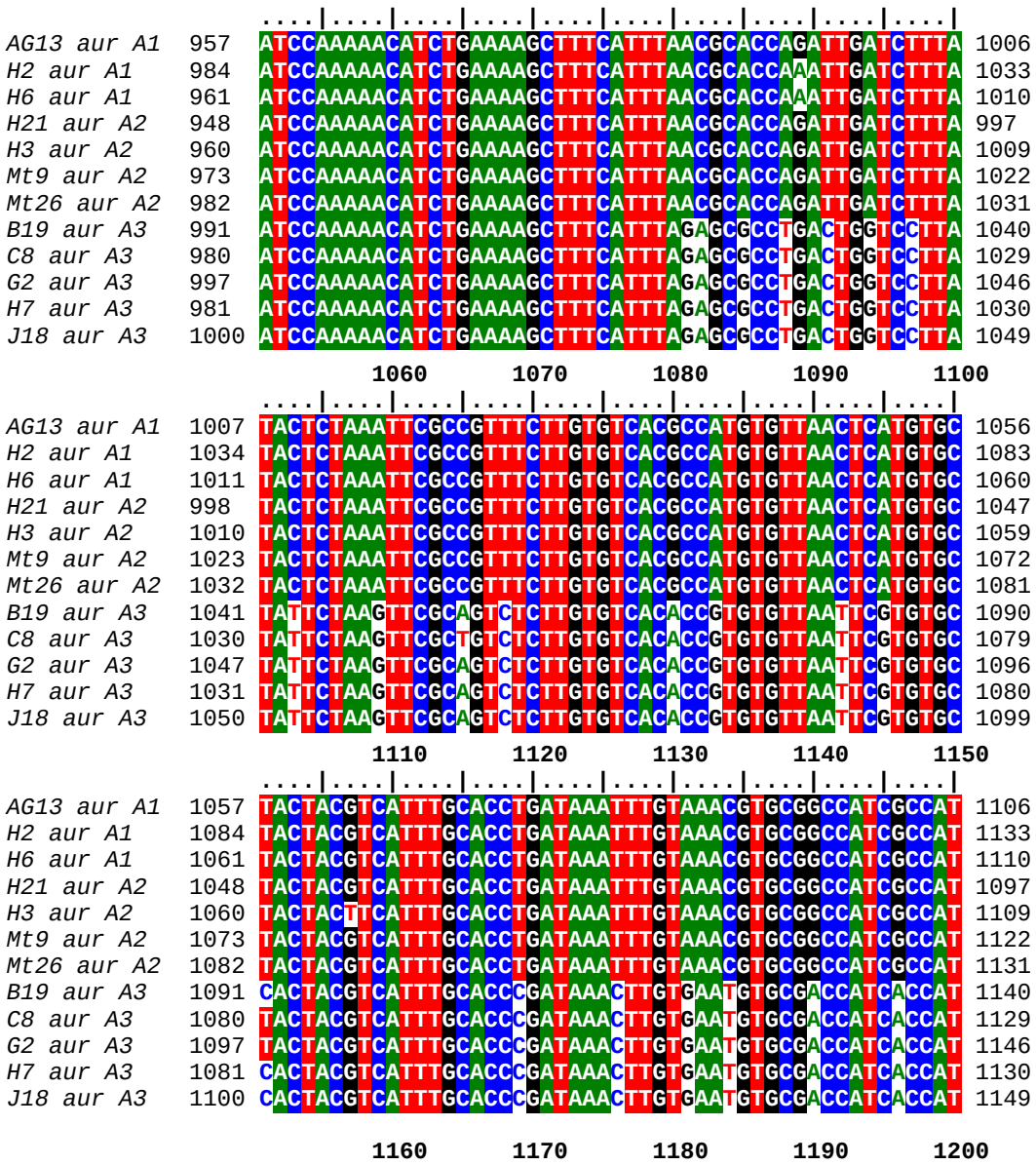
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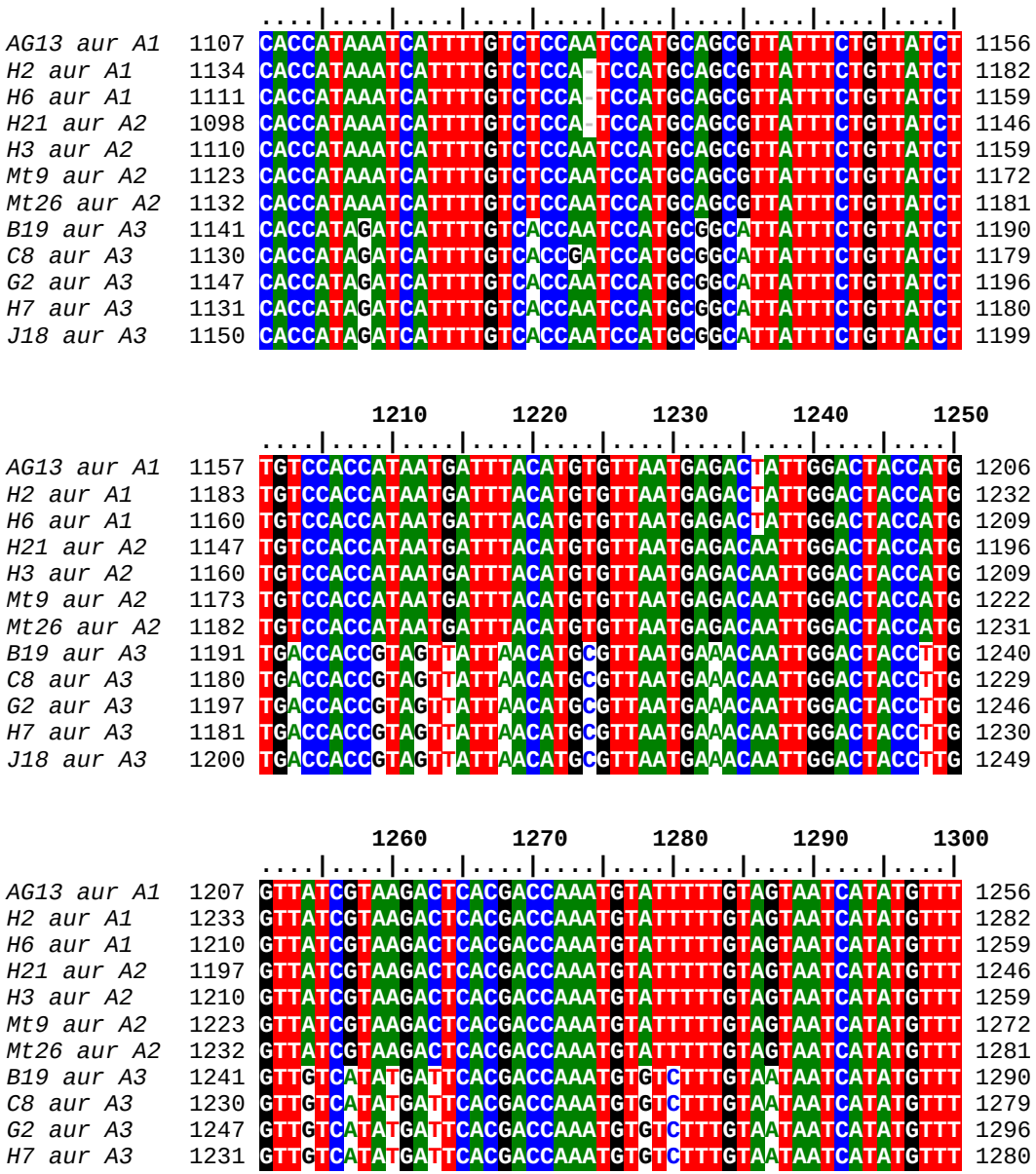
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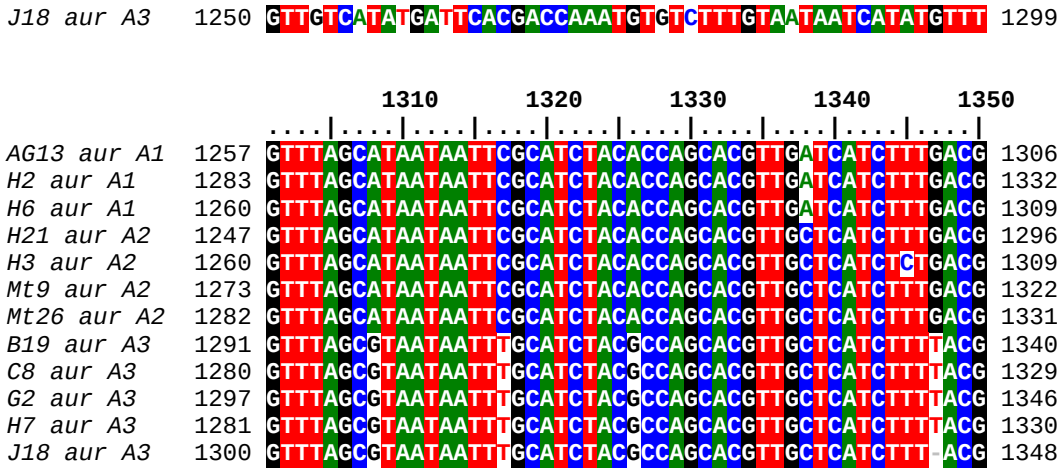
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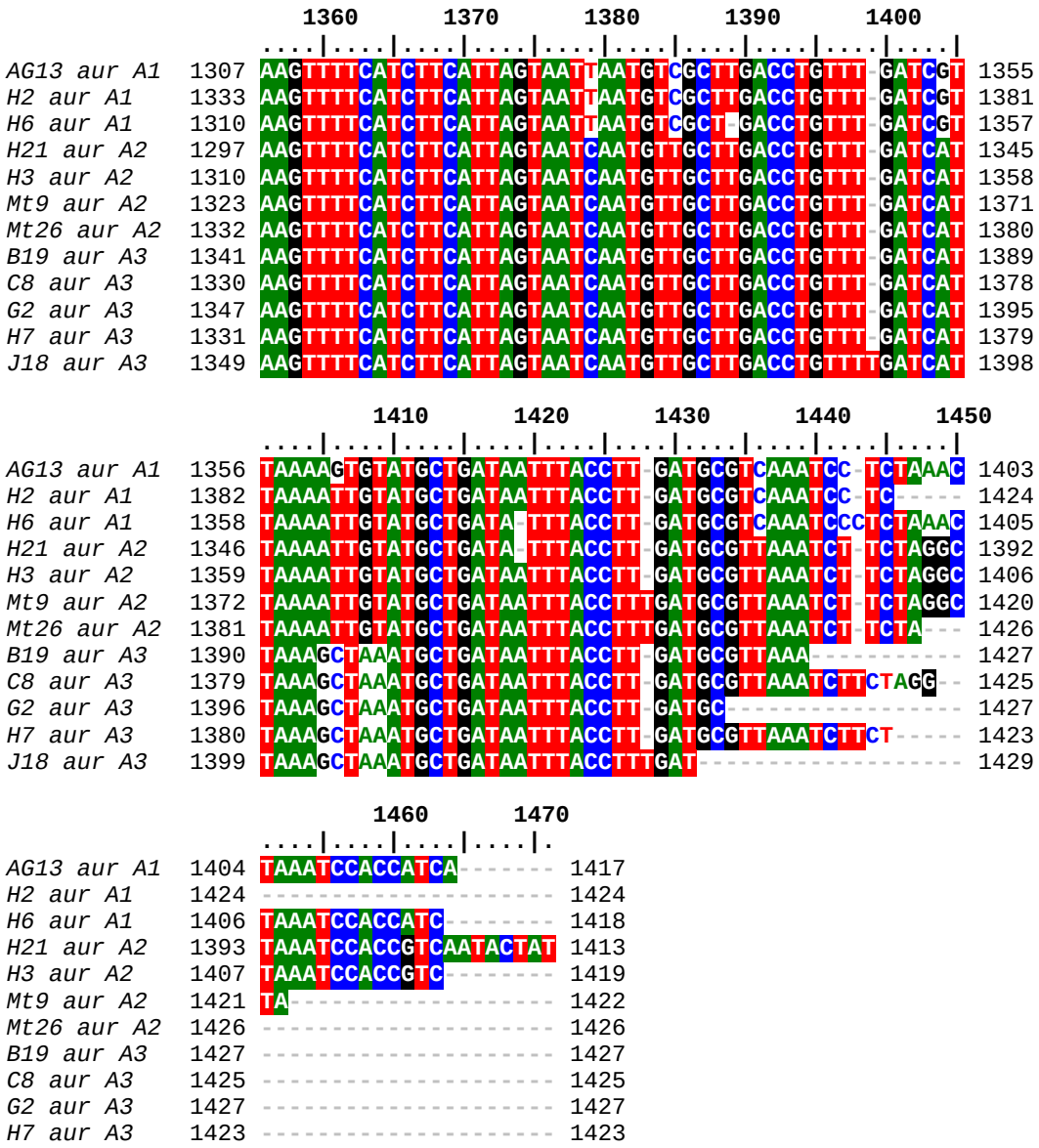
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H7 aur A3 1423 ---------------------- 1423

```
J18 aur A3    1429 ---------------------- 1429
```

Fig. 35: The sequence analysis of *aur* gene obtained in present study.

Table 33: Codon-based Test of Neutrality for analysis between *aur* sequences in present study.

M6: Z-Test of Neutral Evolution (C:\Users\SANDEE~1\AppData\Local\Temp\PhyloAnalysis-1.meg)

File Display Caption Help

	1	2	3	4	5	6	7	8	9	10	11	12
1. AG13 aur A1		-0.770	-2.331	-0.770	-2.134	-2.134	-2.134	-6.310	-6.247	-6.355	-6.359	-6.304
2. HU2 aur A1	0.443		-1.946	0.000	-1.731	-1.731	-1.731	-6.353	-6.290	-6.398	-6.401	-6.347
3. HU3 aur A2	0.021	0.054		-1.946	-1.002	-1.002	-1.002	-6.455	-6.436	-6.546	-6.504	-6.493
4. HU6 aur A1	0.443	1.000	0.054		-1.731	-1.731	-1.731	-6.353	-6.290	-6.398	-6.401	-6.347
5. HU21 aur A2	0.035	0.086	0.319	0.086		0.000	0.000	-6.353	-6.335	-6.445	-6.403	-6.391
6. Mt9 aur A2	0.035	0.086	0.319	0.086	1.000		0.000	-6.353	-6.335	-6.445	-6.403	-6.391
7. Mt26 aur A2	0.035	0.086	0.319	0.086	1.000	1.000		-6.353	-6.335	-6.445	-6.403	-6.391
8. B19 aur A3	0.000	0.000	0.000	0.000	0.000	0.000	0.000		-0.857	-0.783	-1.171	2.242
9. C8 aur A3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.393		-1.342	-1.012	-1.665
10. G2 aur A3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.435	0.182		-0.964	-0.967
11. HU7 aur A3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.244	0.313	0.337		-0.499
12. J18 aur A3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.027	0.098	0.335	0.619	

The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) (below diagonal) is shown. Values of *P* less than 0.05 are considered significant at the 5% level and are yellow highlighted. The test statistic ($d_N - d_S$) is shown above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. Analyses were conducted using the Nei-Gojobori method. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 418 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

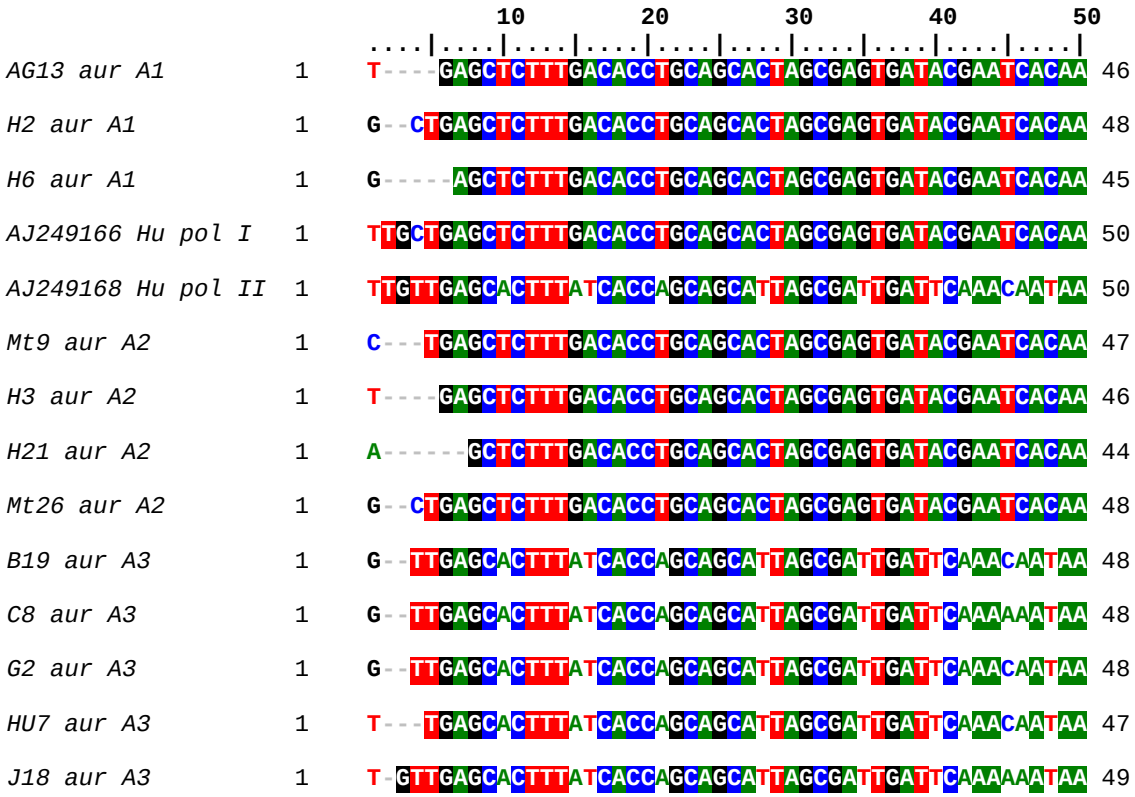
Multiple sequence alignment of present study sequences was conducted with reference sequences of NCBI database to find similarities and variations between our sequences and reference NCBI sequences. For this purpose, two sequences of NCBI database *viz.* Sequence ID- AJ249166 and AJ249168 from human strain of Poland belonging to aur-RFLP type I and II respectively (Sabat *et al.*, 2000) were chosen according to source of isolation and geographic variations to compare existed RFLP types with our findings. The multiple sequence alignment of all sequences revealed that both data base sequences existed without any gap or deletion, which was present in most of our sequences as described earlier. It was also observed that aur-RFLP type I sequence of data base (AJ249166) was more closer to A1 and A2 aur-RFLP types of our study while aur-RFLP type II sequence of data base (AJ249168) was more closer to A3 type of our study with similarity at nucleotide position 20th, 28th, 40th, 45th and at many more positions in the alignment (Fig. 37).

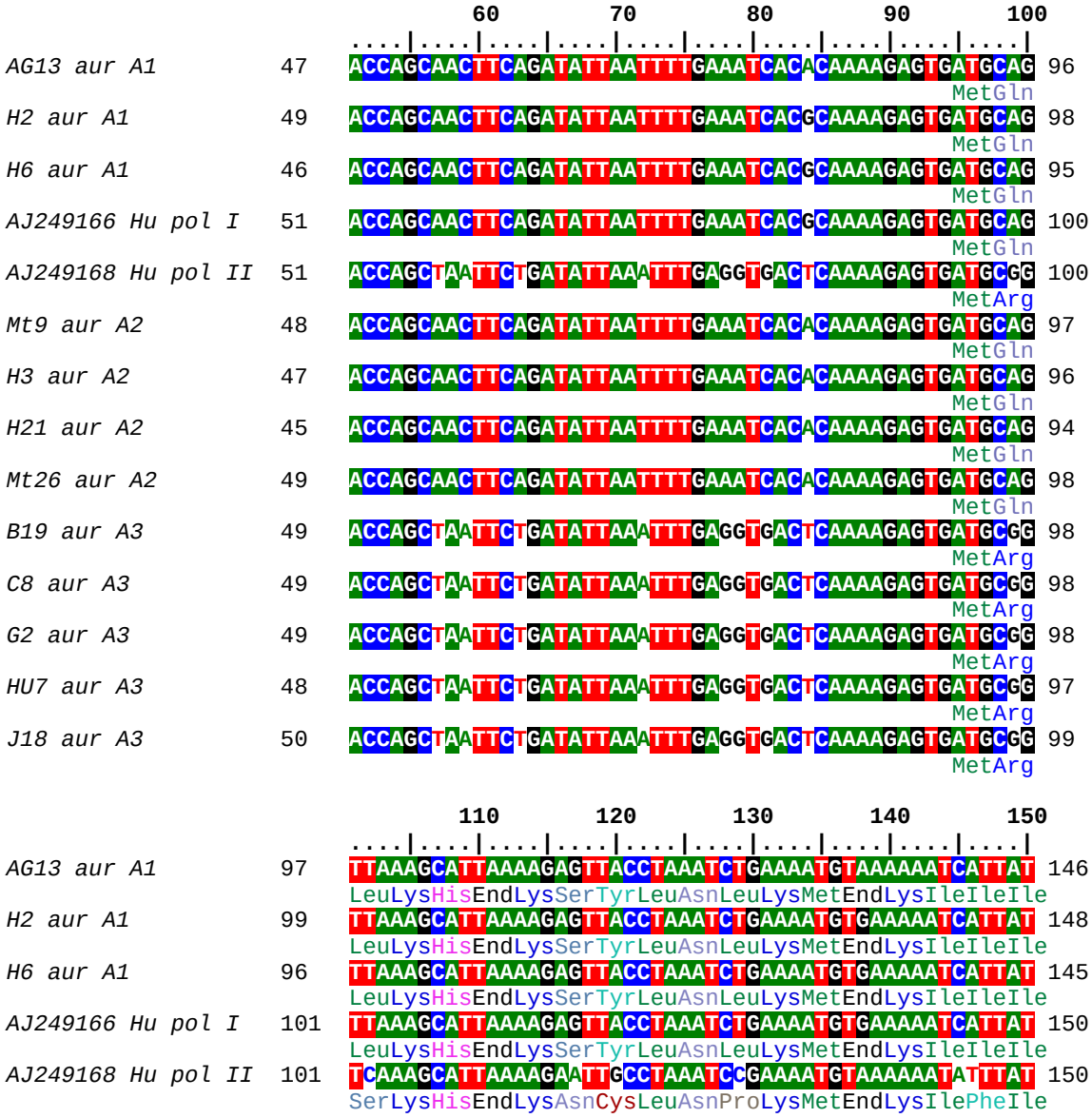
The statistic for codon-based test of neutrality between sequences conducted *i.e.* the probability of rejecting the null hypothesis of strict-neutrality $d_N = d_S$ (d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively) and significant ($p \leq 0.05$) variations were found between both aligned data base sequences and our sequences except G2 sequence of A3 type was non significantly ($p \leq 0.05$) related with aur-RFLP type II sequence of data base (AJ249168). The

analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 366 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 software (table 34).

Phylogenetic tree analysis of multiple align *aur* gene sequences revealed that separate cluster was observed for NCBI data bases sequences with most far 0.1659 scaled distance and having maximum differences from all other our align sequences. The aur-RFLP type A1 A2 and A3 classified into three clusters, A1 and A2 were separated with less scaled distance while A3 was separated with more scaled distance as depicting in figure 38.

The sequence analysis of *aur* gene revealed that sequence variations existed among aur- RFLP types rather than source of *S. aureus*. These less variations between animal and human origin sequences in the present study may indicates close interaction and exchange of bacterial genetic material between human, animals and cross infections (meat piece).

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MetGln
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HU7 aur A3 48 ACCAGCTAATTCTGATATTAAATTTGAGGTGACTCAAAAGAGTGATGCGG 97
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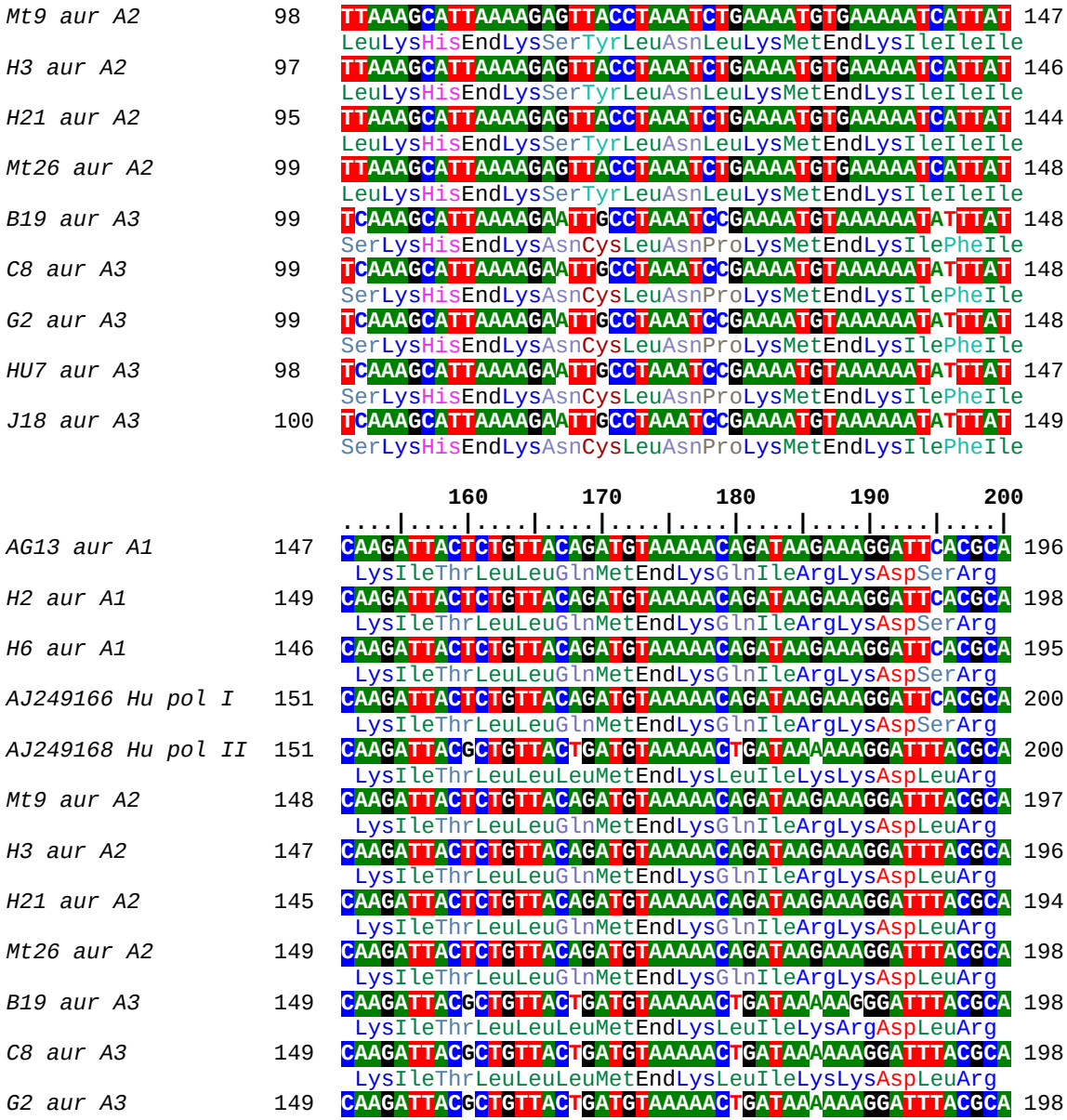
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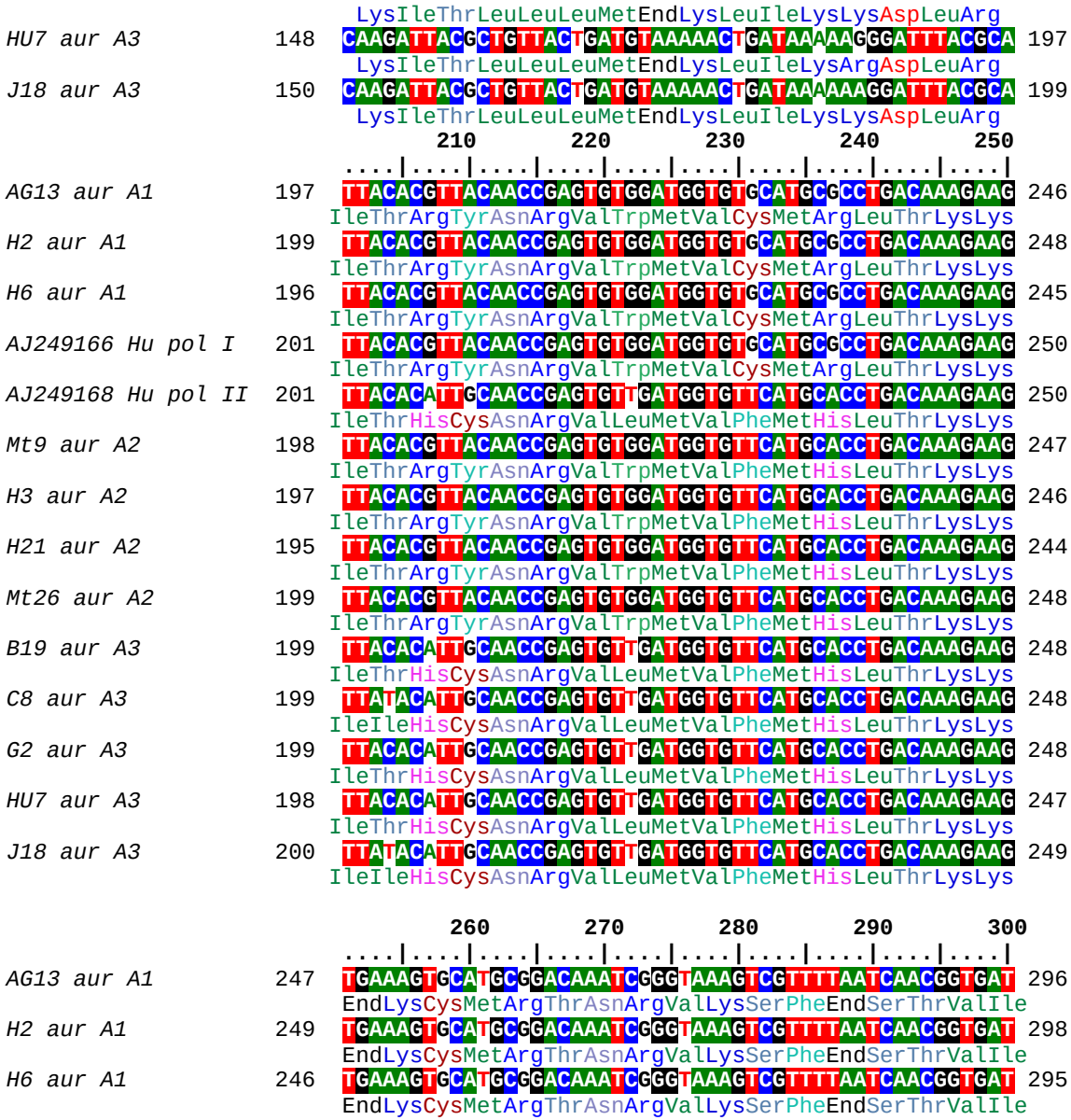
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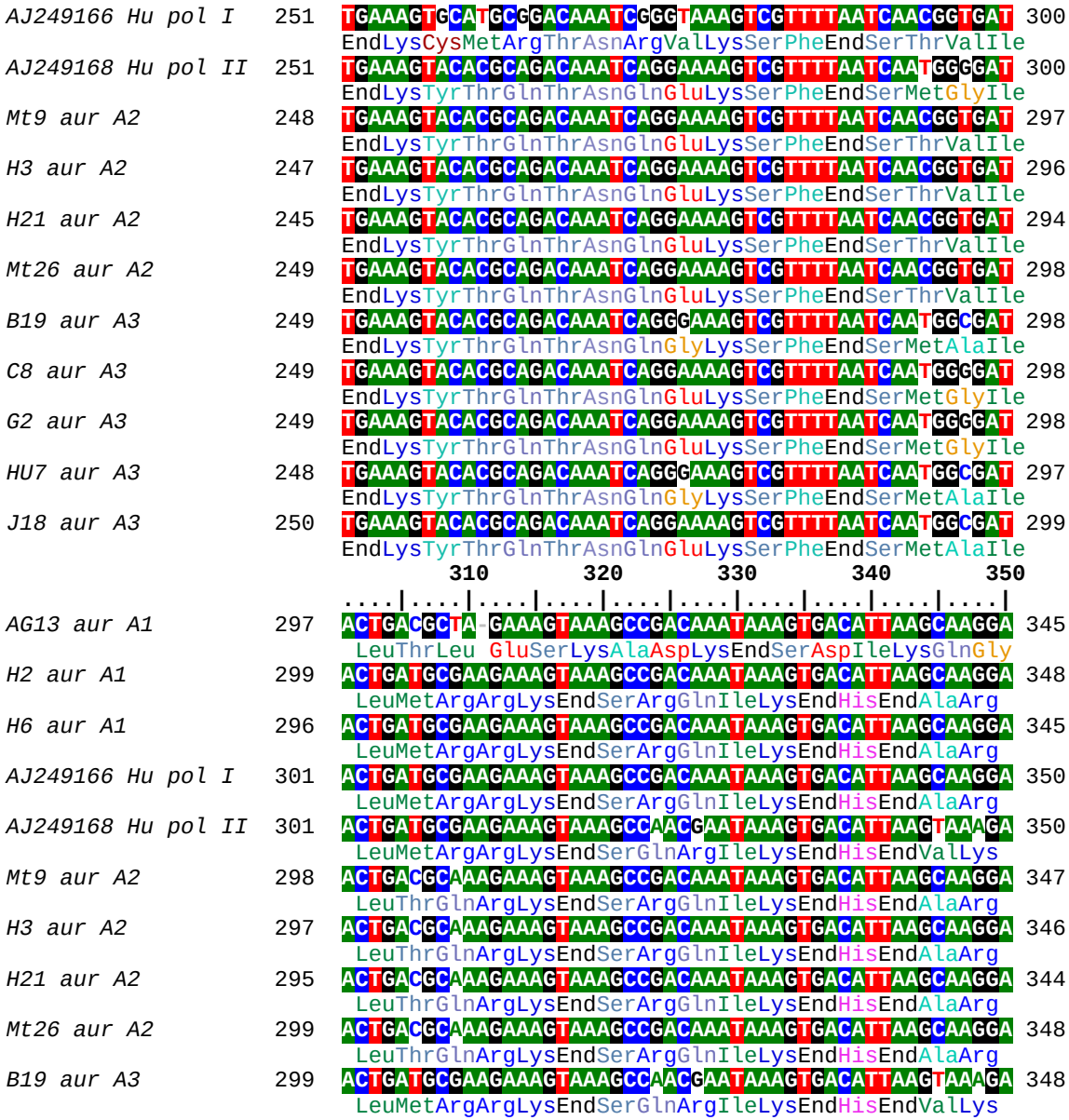
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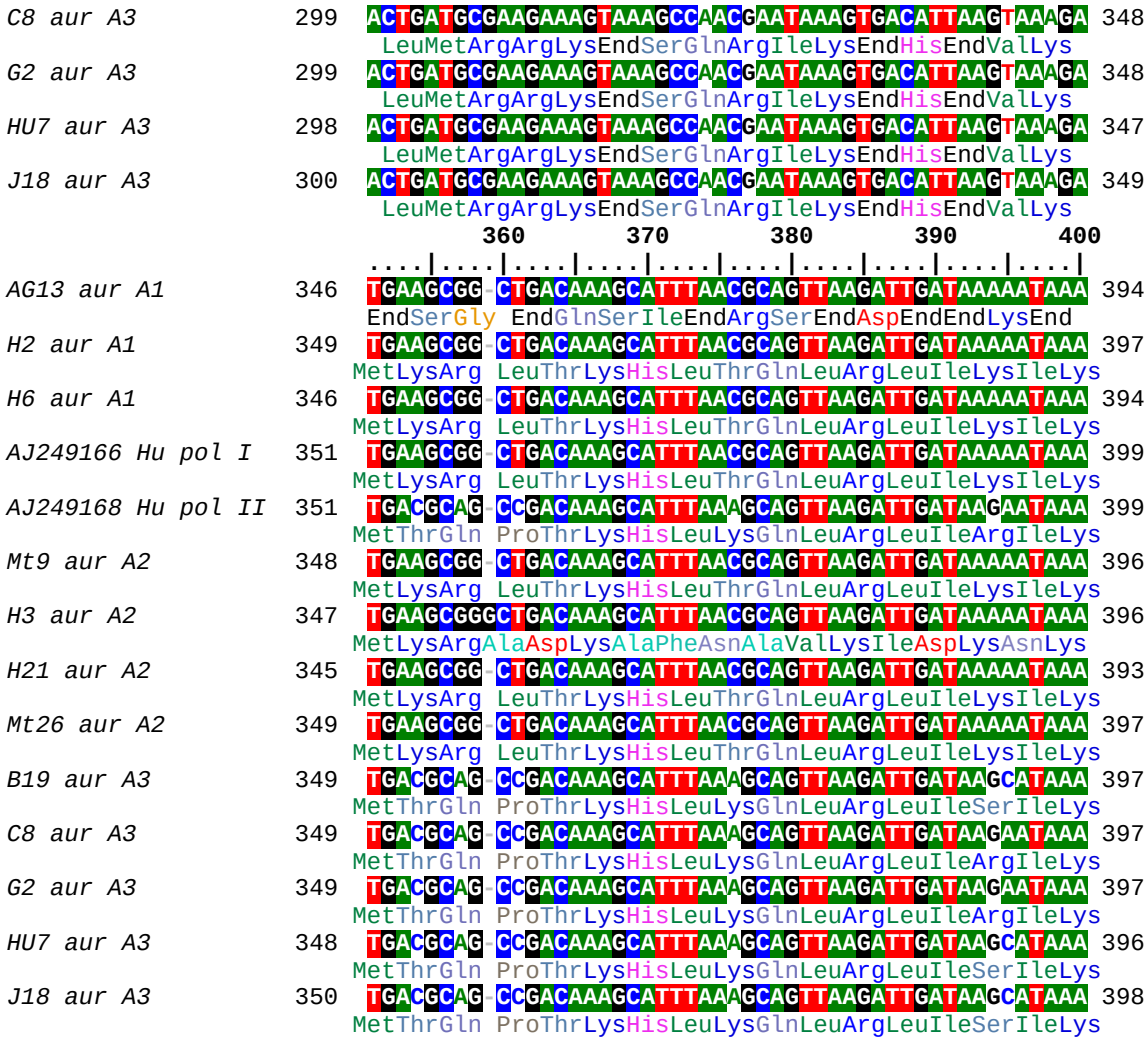
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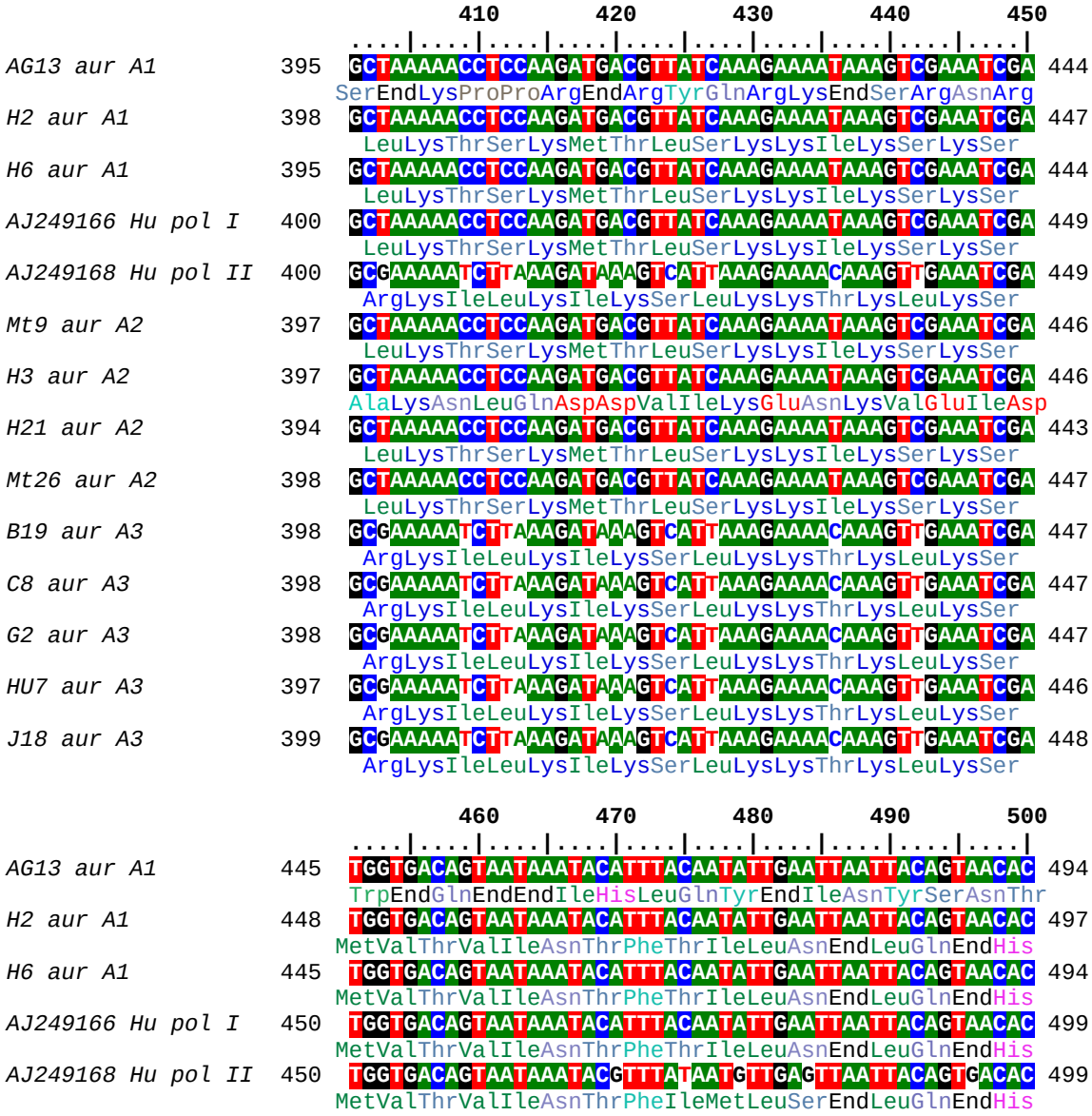
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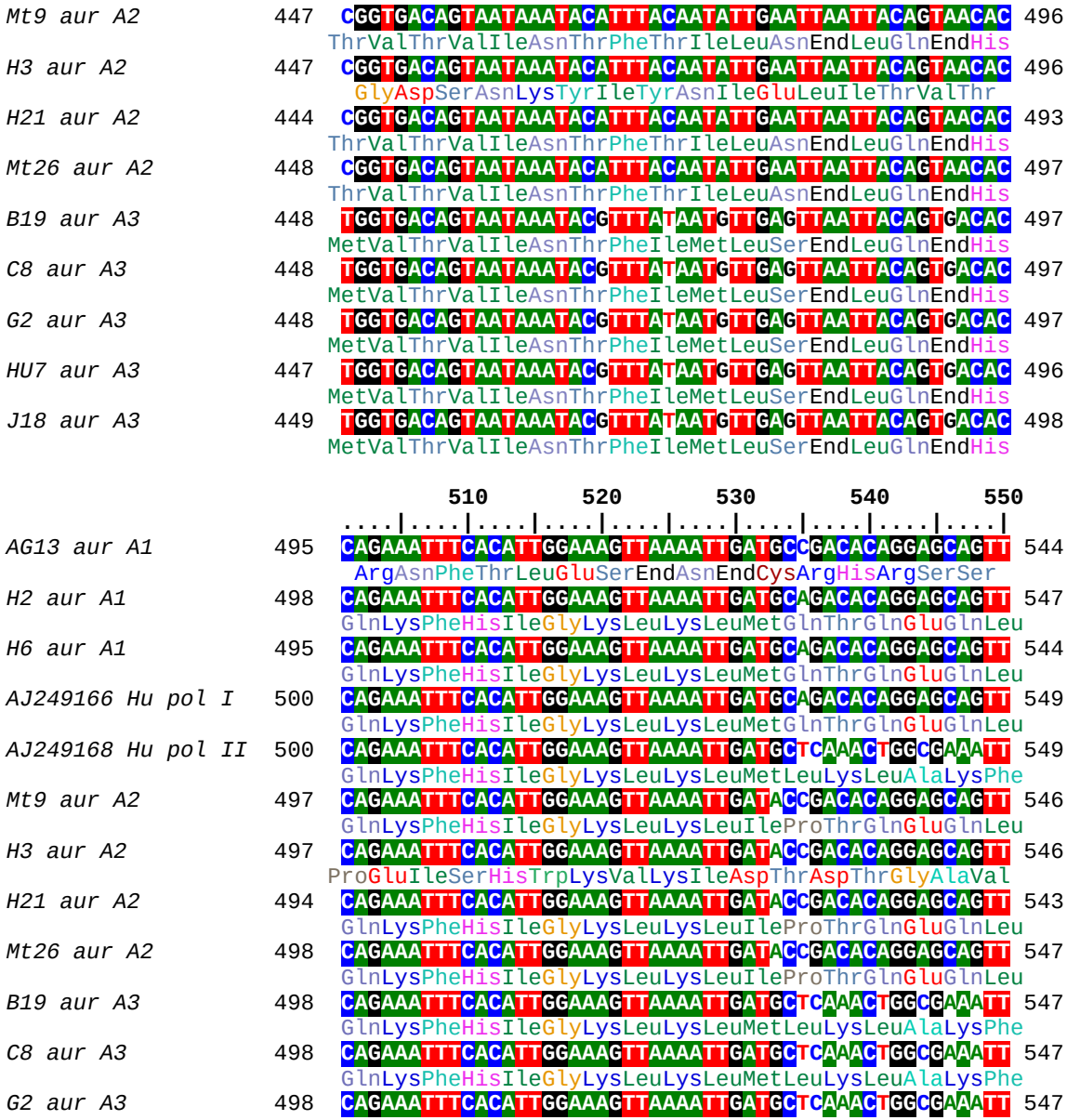
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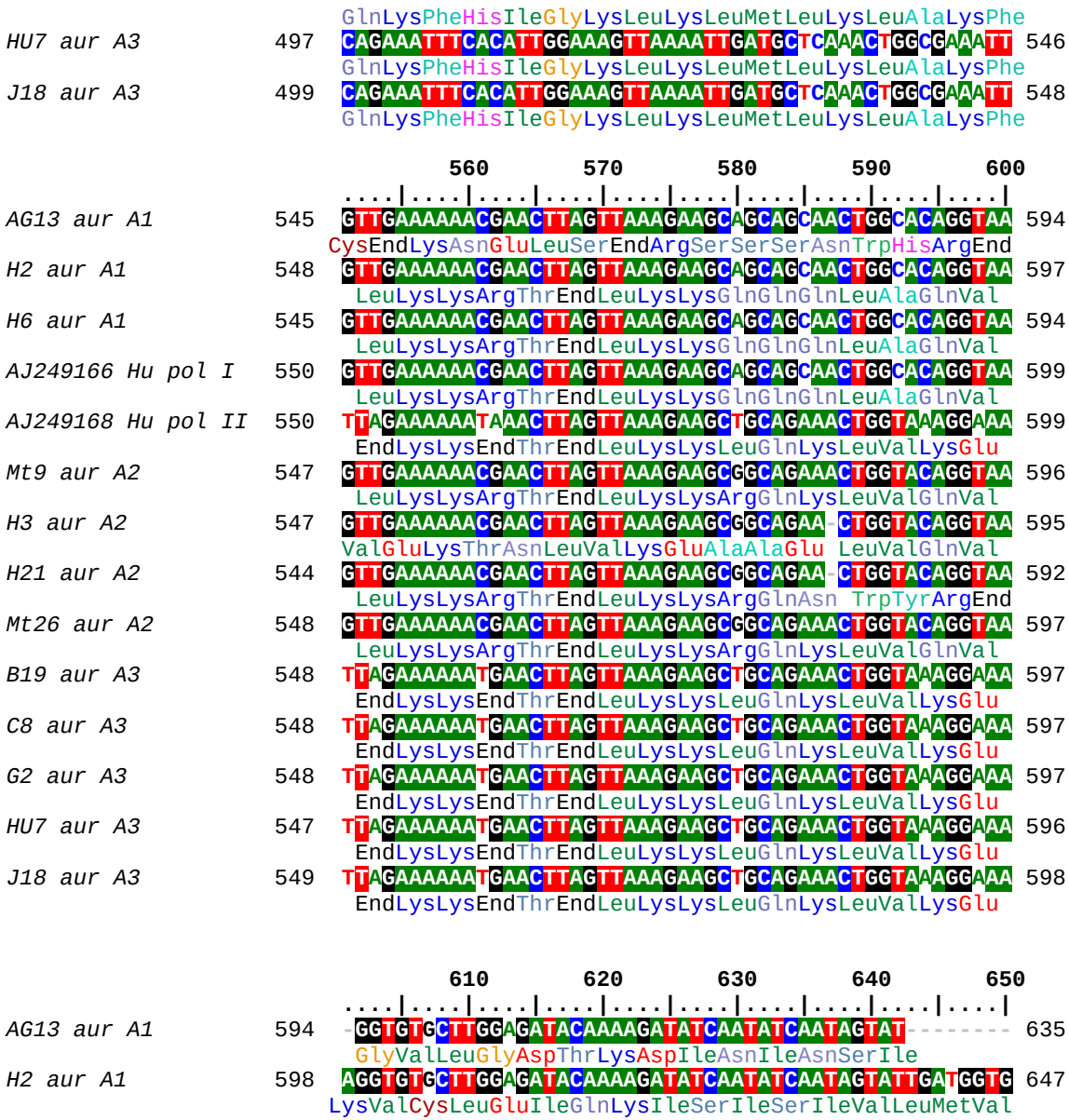
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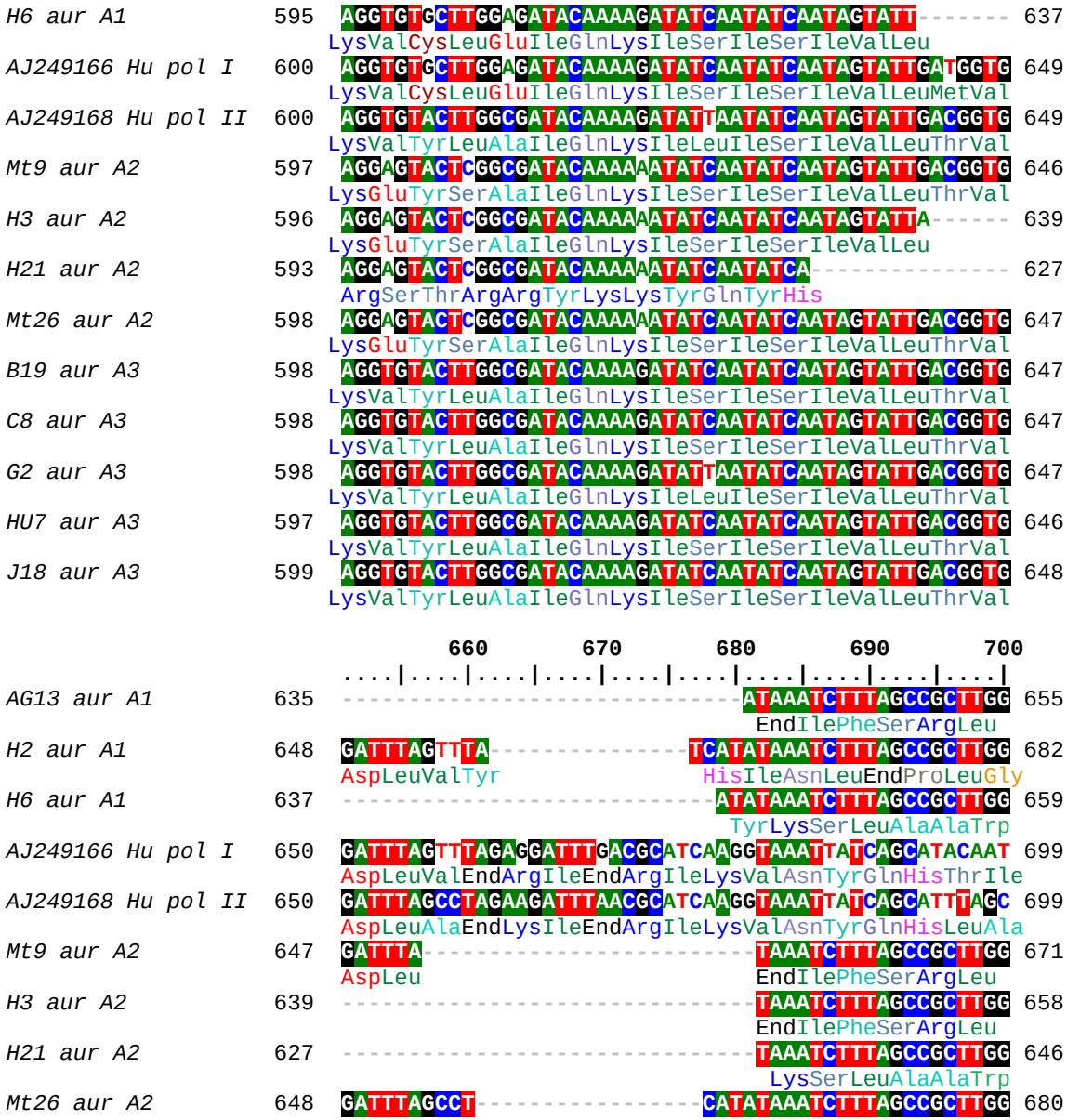
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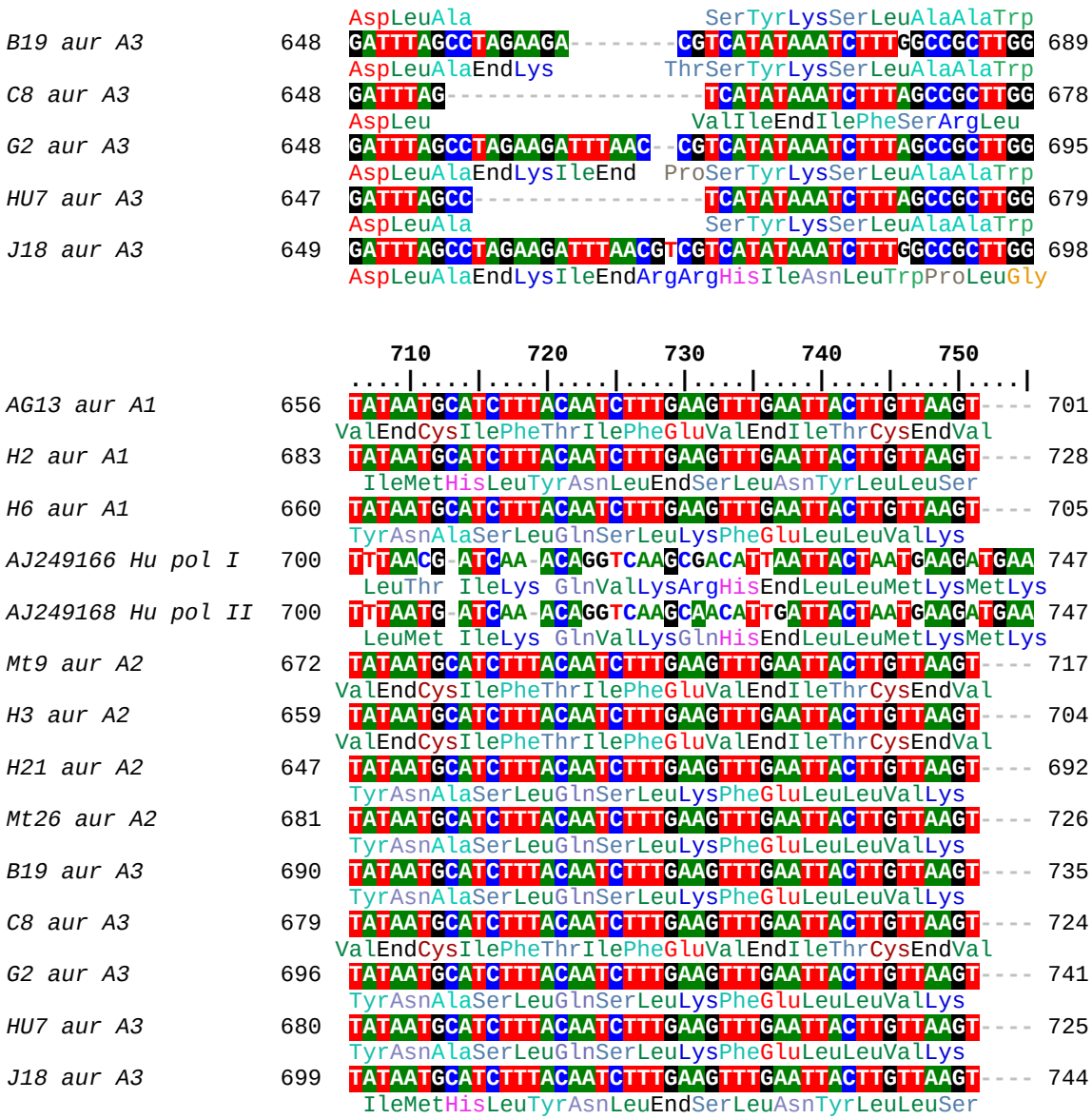
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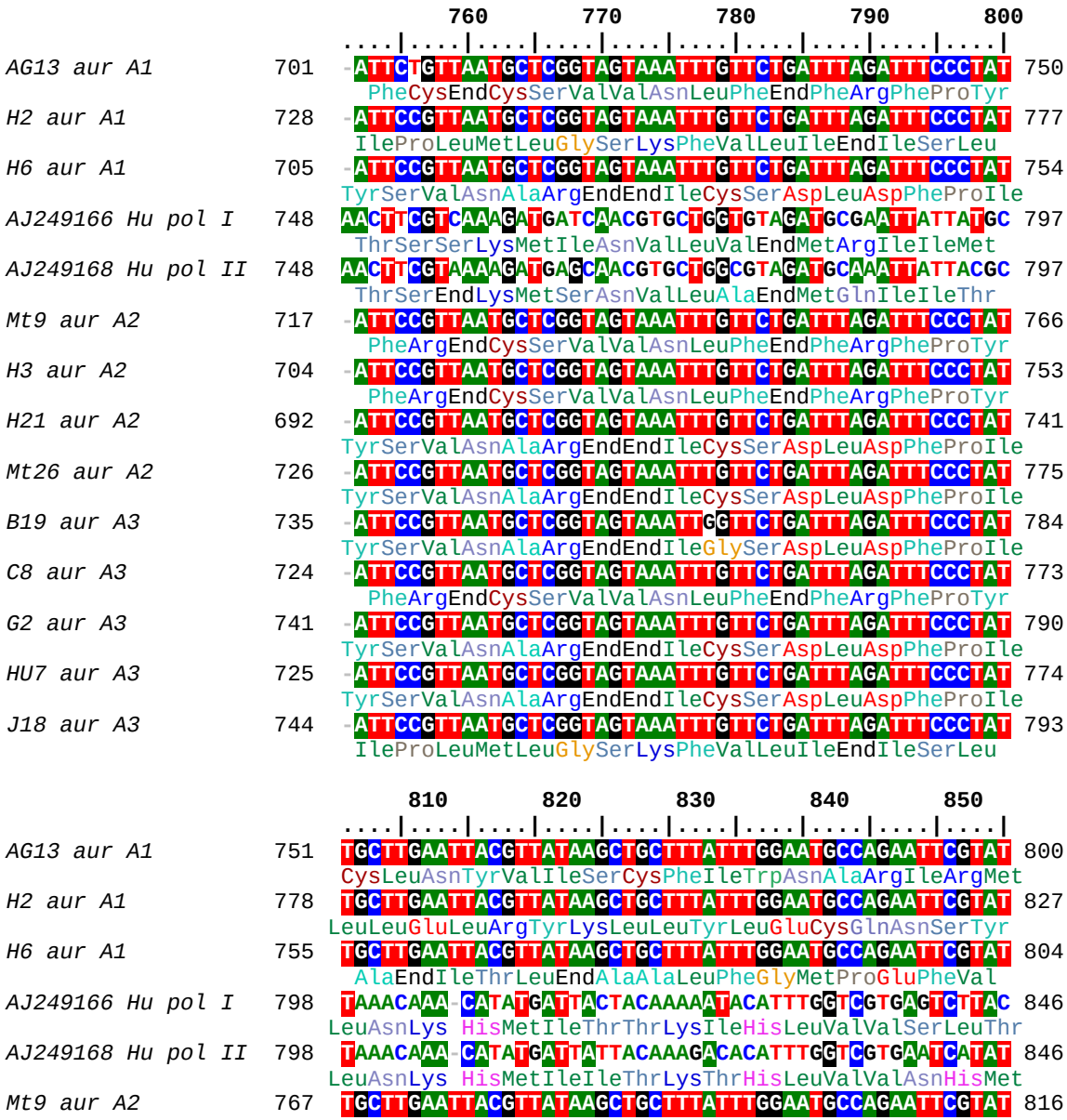
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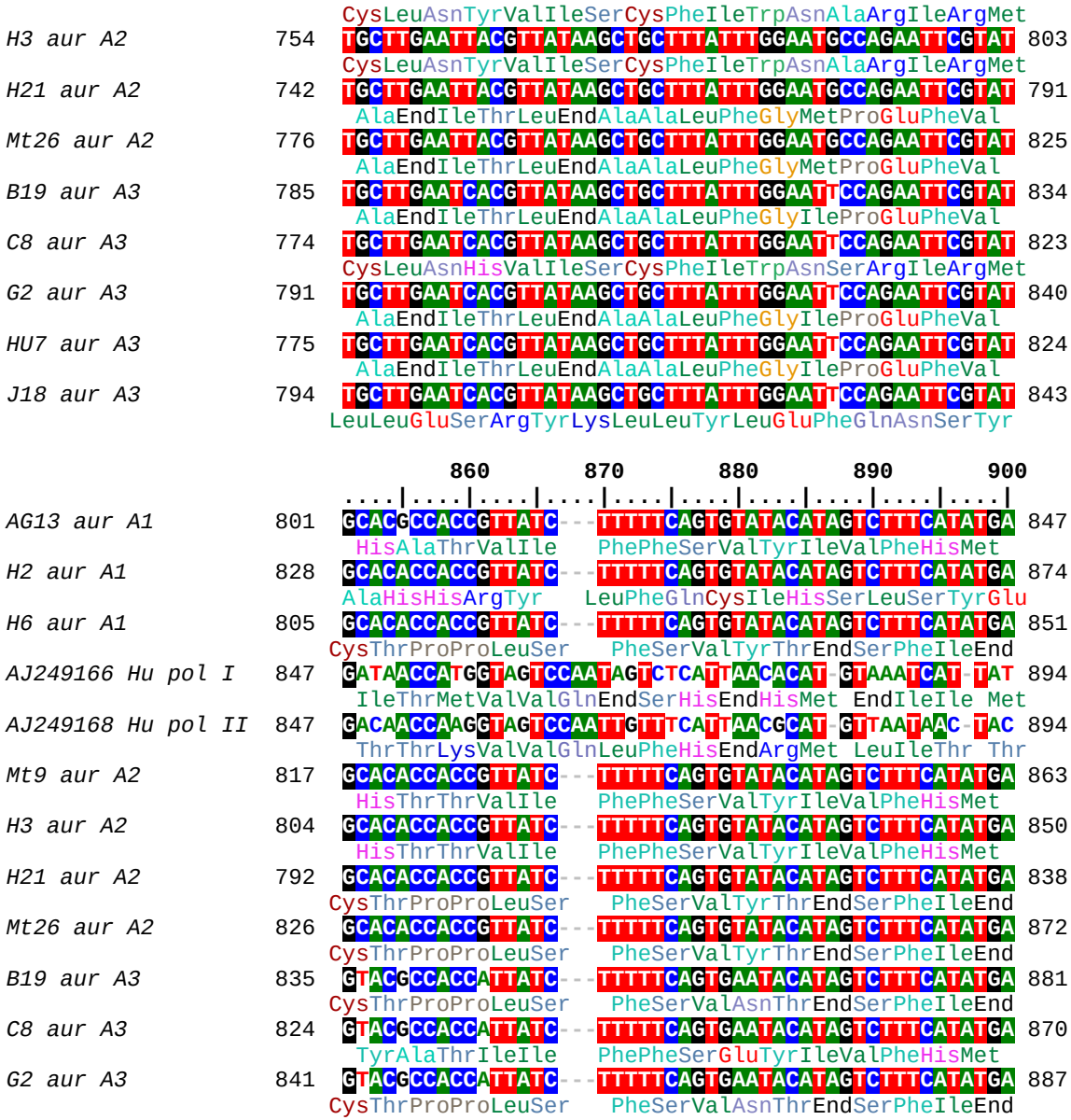
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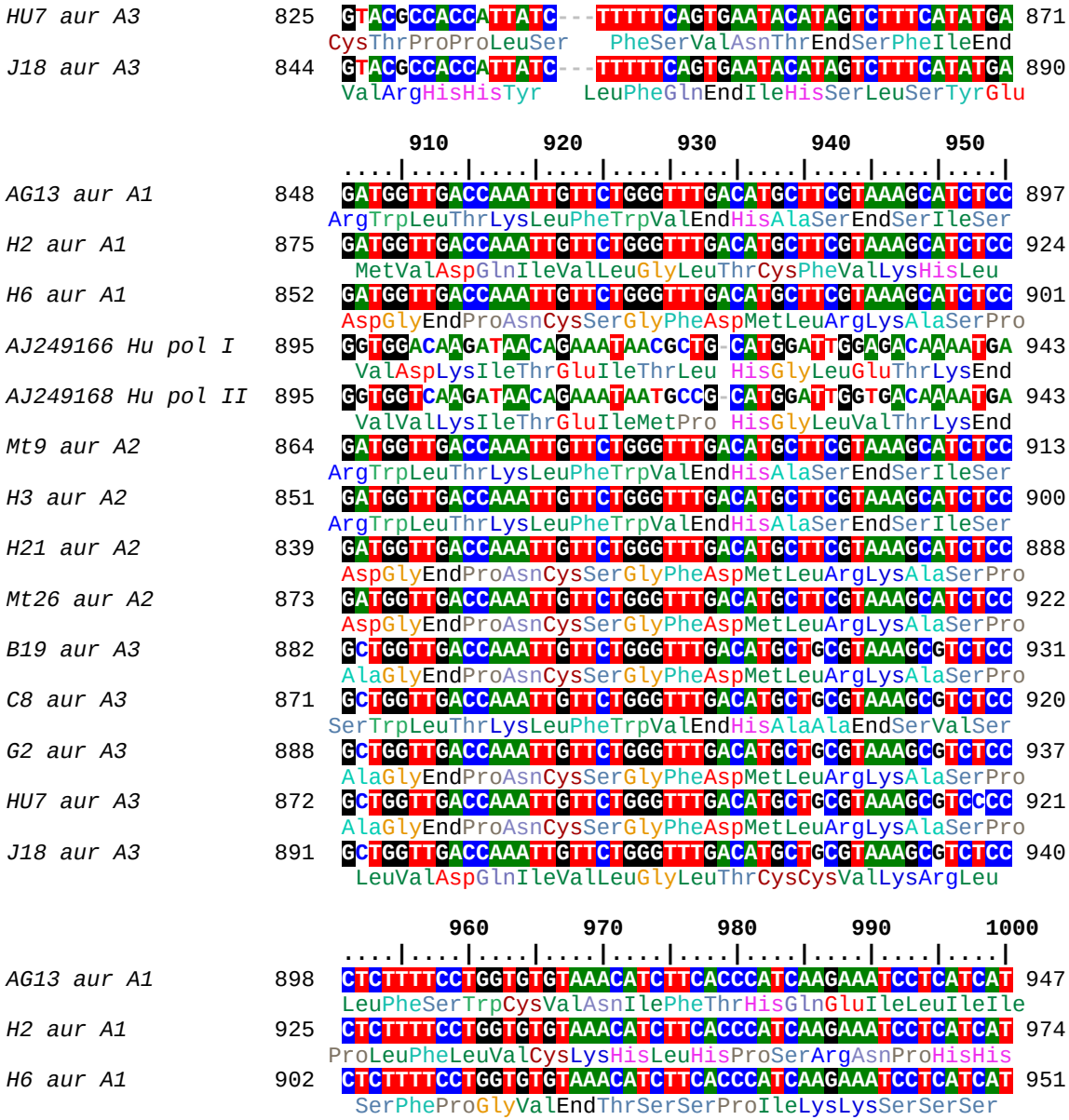
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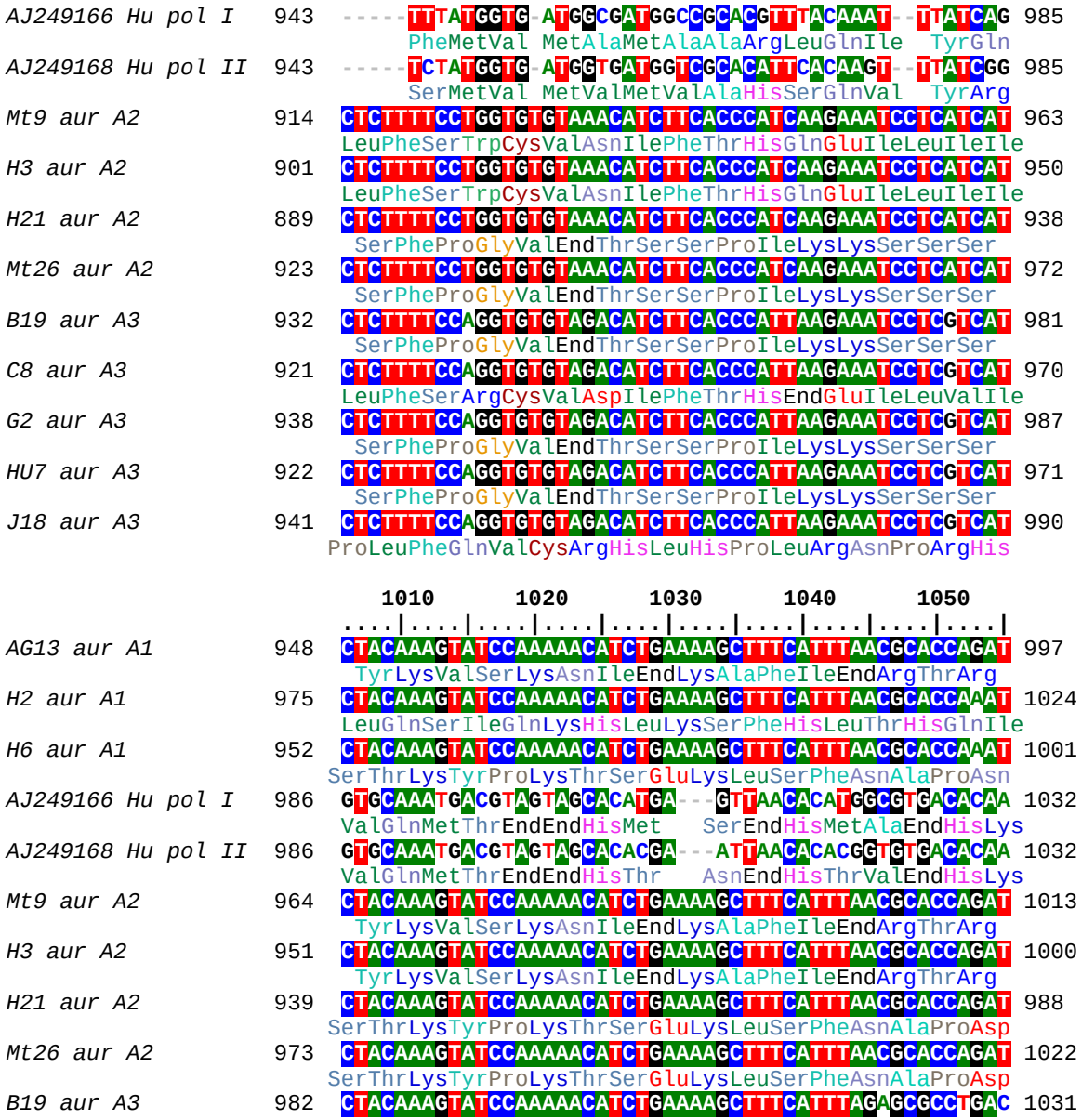
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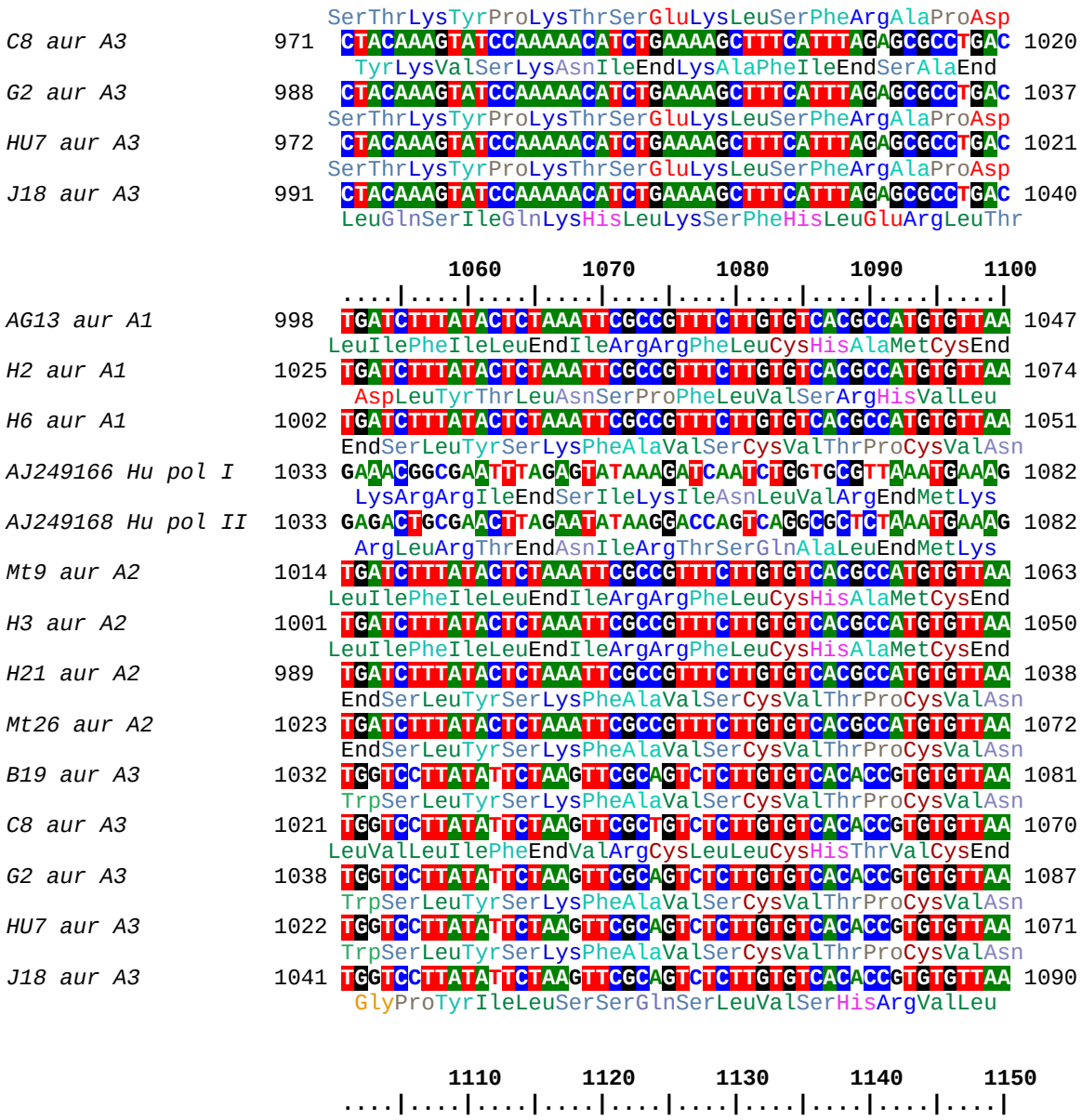
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AJ249168 Hu pol II 1033 GAGACTGCGAACTTAGAATATAAGGACCAGTCAGGCGCTCTAAATGAAAG 1082
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LeuIlePheIleLeuEndIleArgArgPheLeuCysHisAlaMetCysEnd
H3 aur A2 1001 TGATCTTTATACTCTAAATTCGCCGTTTCTTGTGTCACGCCATGTGTTAA 1050
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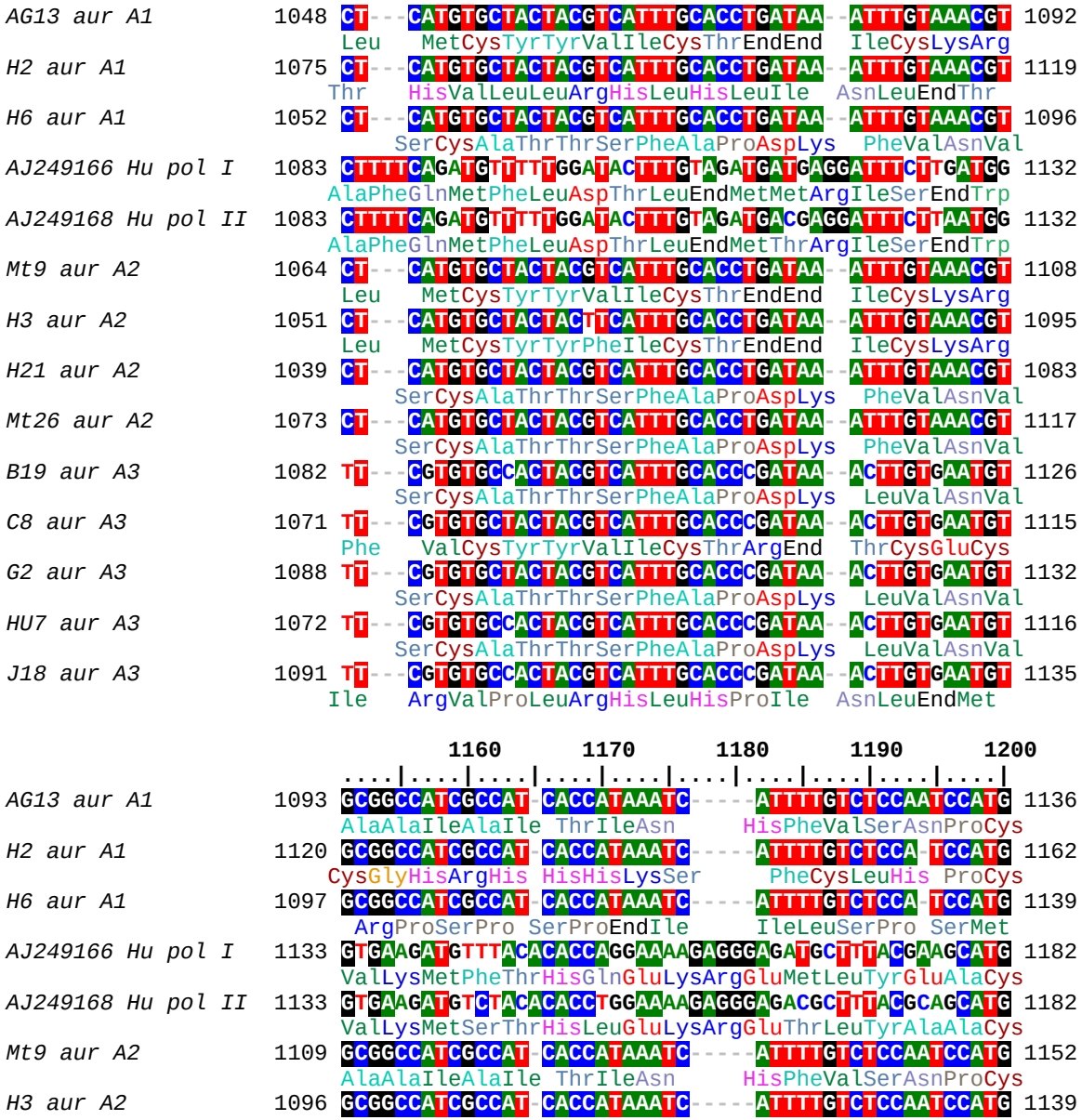
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Mt26 aur A2 1073 CT---CATGTGCTACTACGTCATTTGCACCTGATAA--ATTTGTAAACGT 1117
SerCysAlaThrThrSerPheAlaProAspLys PheValAsnVal
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SerCysAlaThrThrSerPheAlaProAspLys LeuValAsnVal
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J18 aur A3 1091 TT---CGTGTGCCACTACGTCATTTGCACCCGATAA--ACTTGTGAATGT 1135
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H6 aur A1 1097 GCGGCCATCGCCAT-CACCATAAATC-----ATTTTGTCTCCA-TCCATG 1139
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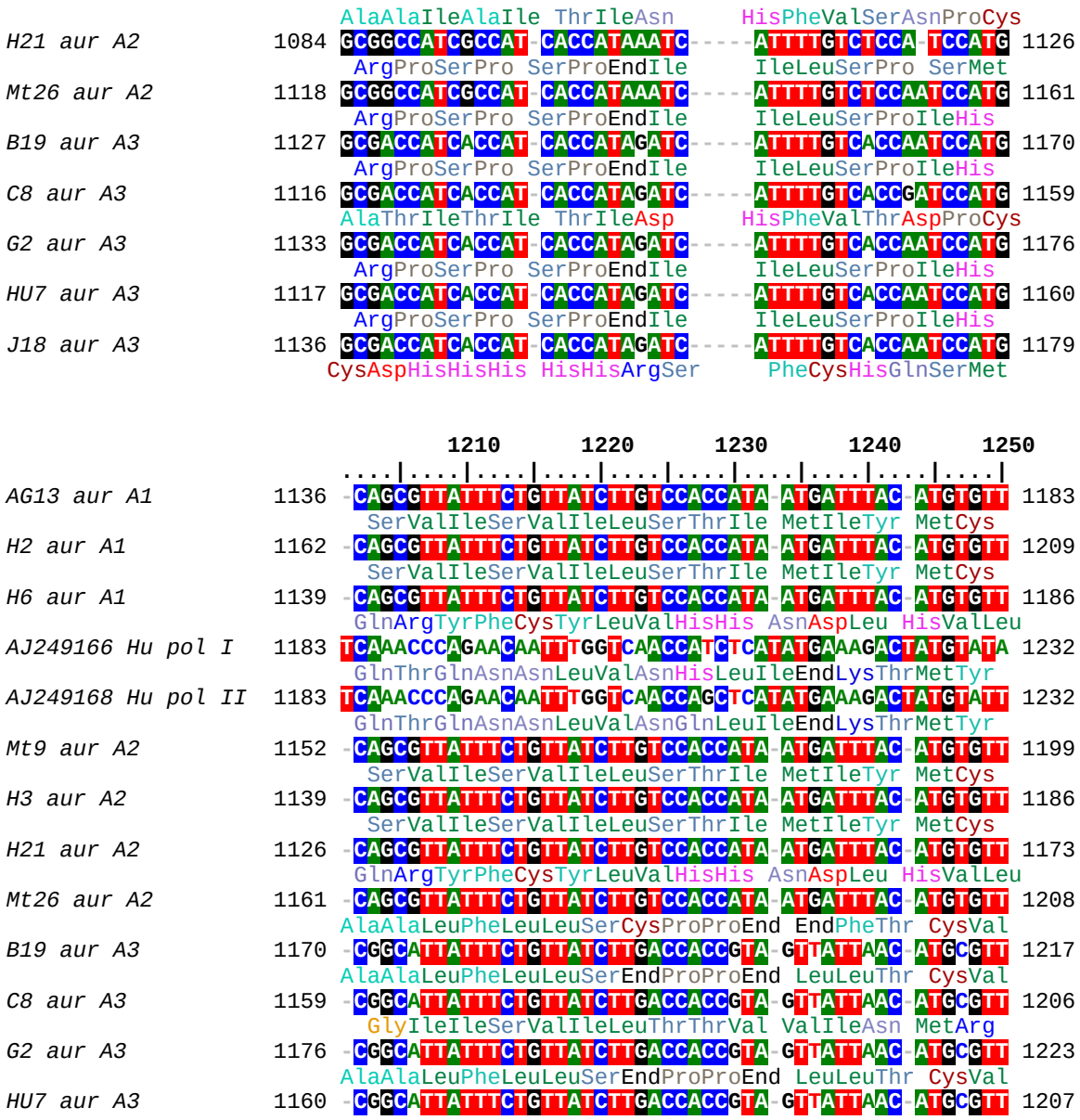
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G2 aur A3 1176 -CGGCATTATTTCTGTTATCTTGACCACCGTA-GTTATTAAC-ATGCGTT 1223
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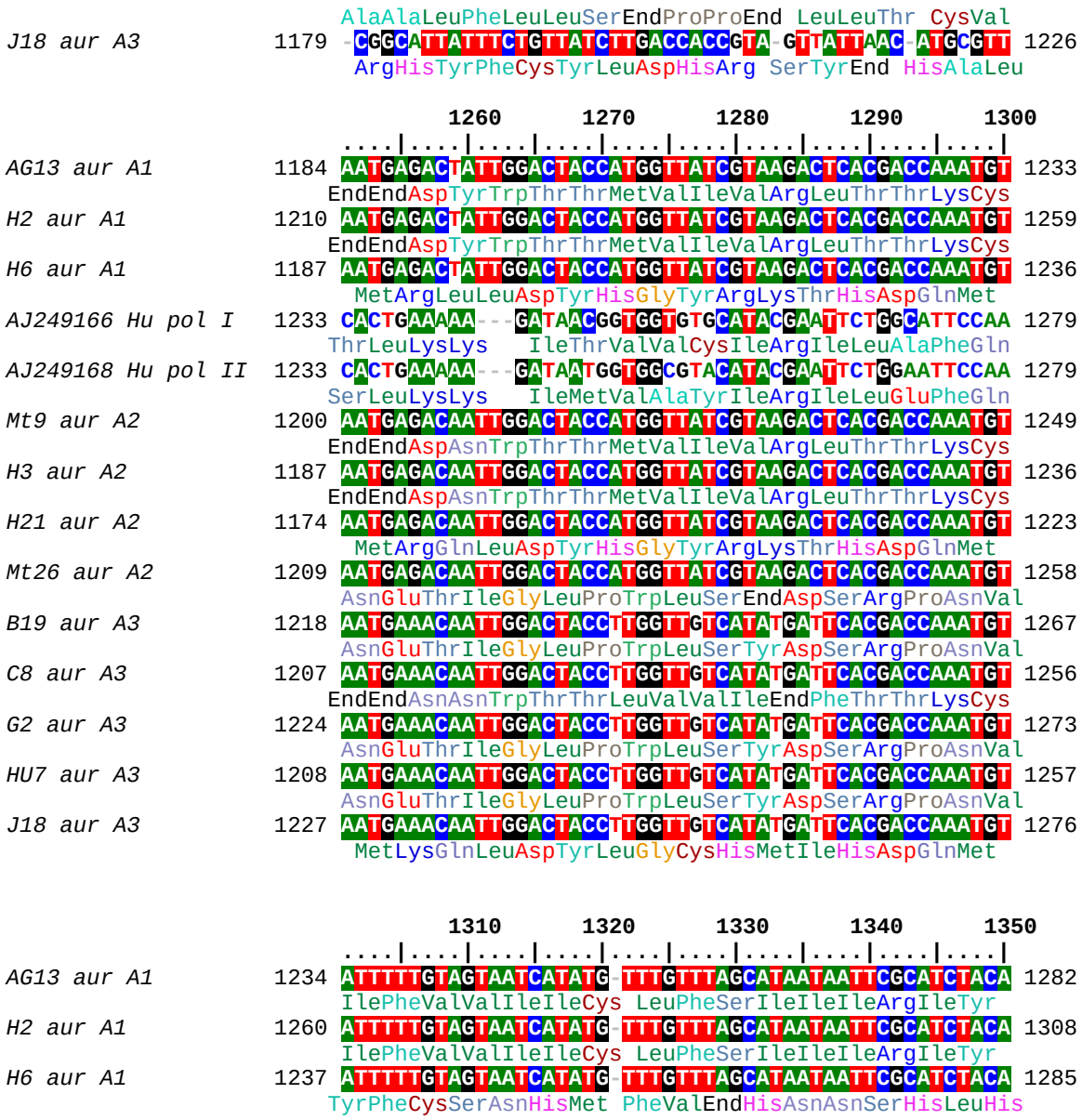
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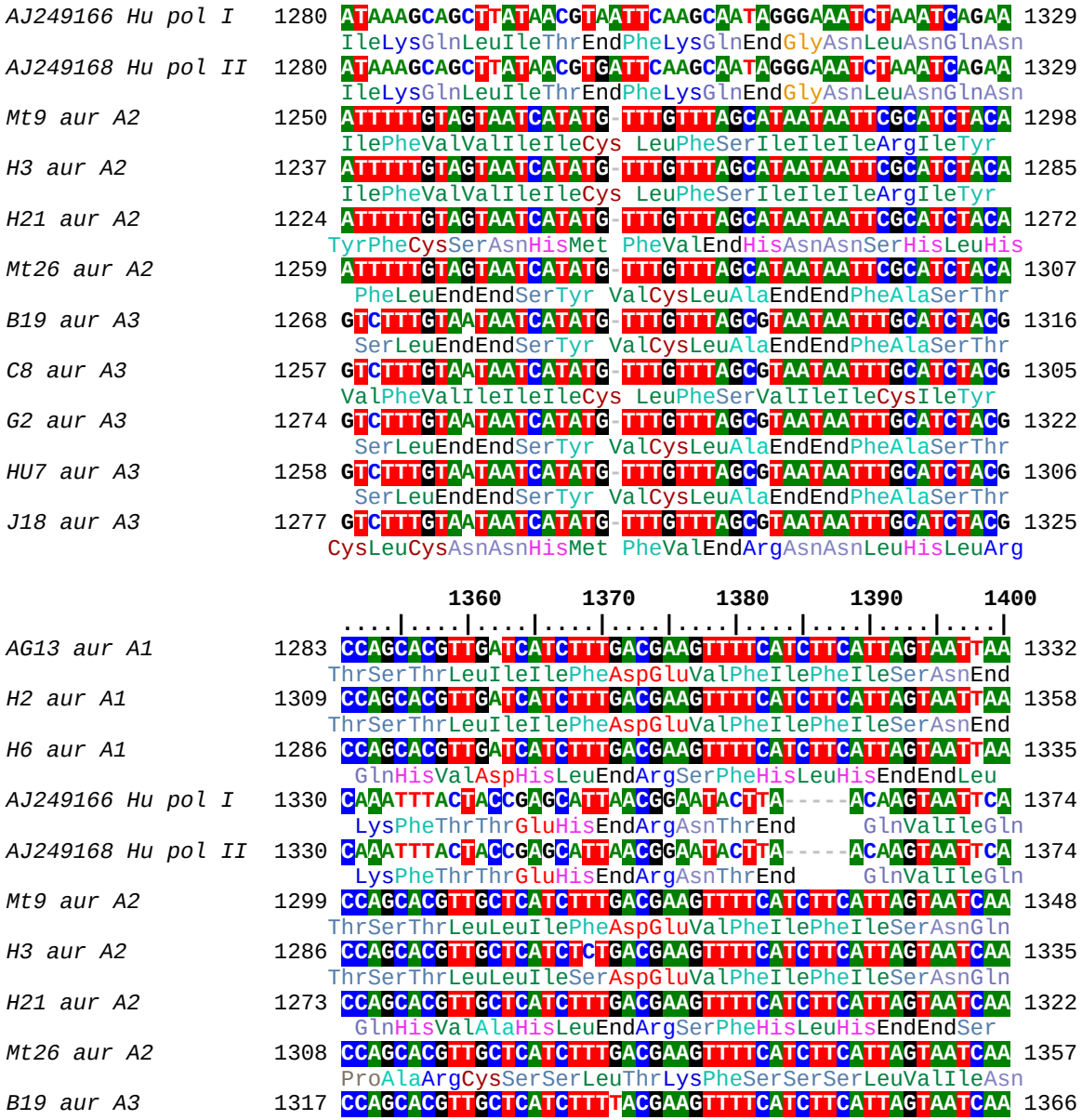
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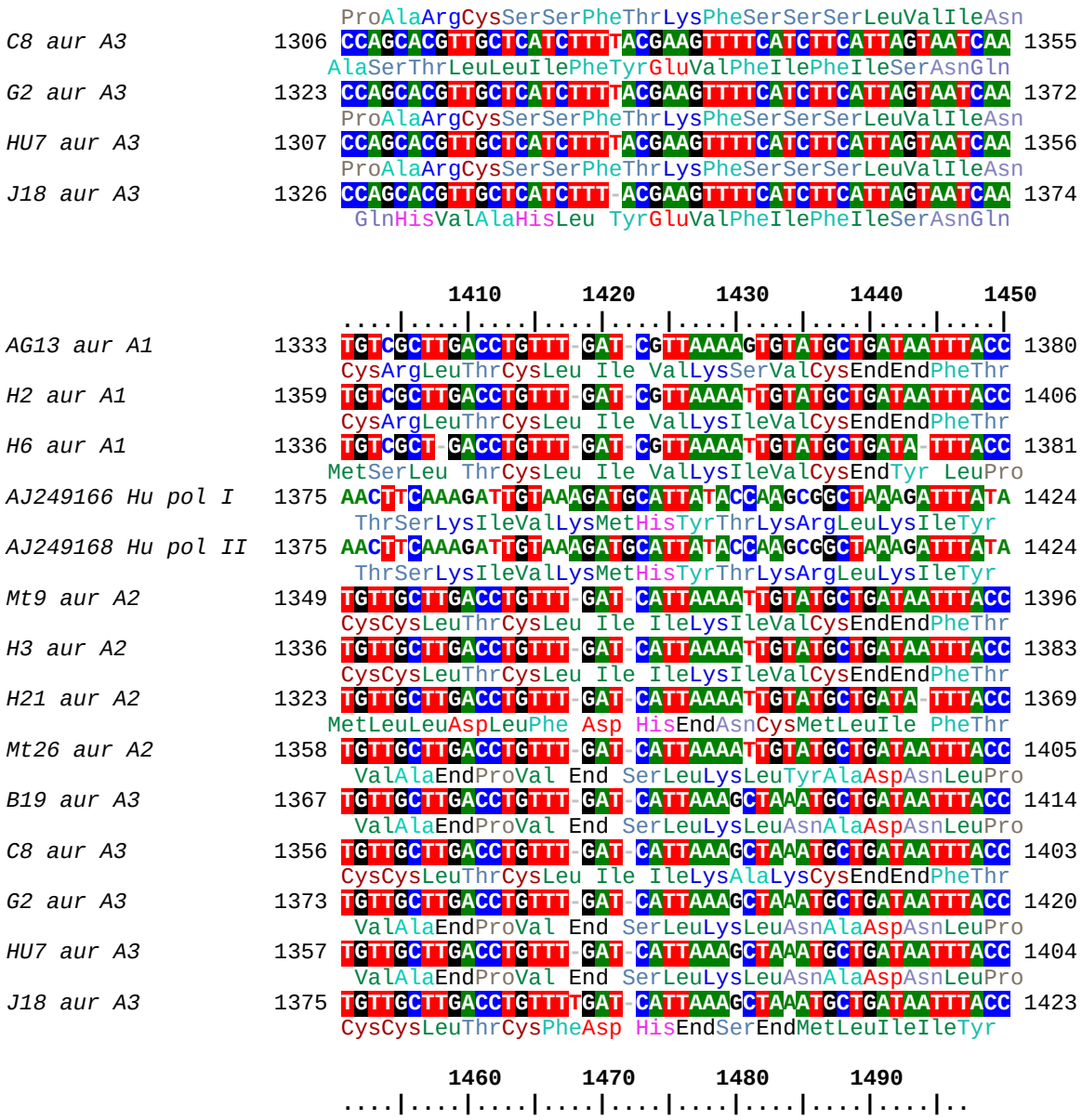
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HU7 aur A3 1357 TGTTGCTTGACCTGTTT-GAT-CATTAAAGCTAAATGCTGATAATTTACC 1404
ValAlaEndProVal End SerLeuLysLeuAsnAlaAspAsnLeuPro
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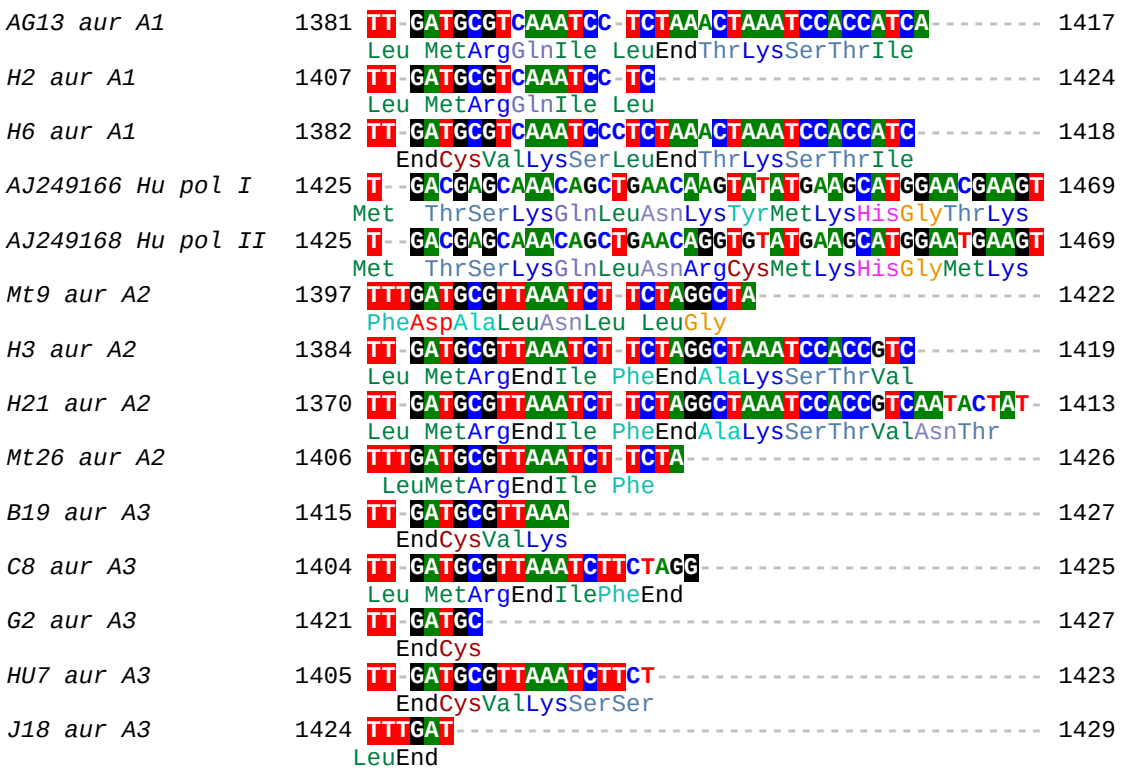

Fig. 37: Multiple sequence alignment of *aur* gene sequences of present study with reference sequences of NCBI database.

Table 34: Codon-based Test of Neutrality for multiple alignment analysis between sequences of present study with reference sequences of NCBI database.

M6: Z-Test of Neutral Evolution (C:\Users\SANDEE~1\AppData\Local\Temp\PhyloAnalysis-1.meg)

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. AG13 aur A1		-1.845	-2.405	-1.845	-2.290	-2.936	-6.088	-2.290	-2.290	-4.147	-4.156	-4.203	-4.235	-4.017
2. AHU2 aur A1	0.068		-3.012	0.000	-2.919	-2.584	-6.204	-2.919	-2.919	-4.196	-4.207	-4.253	-4.285	-4.067
3. AHU3 aur A2	0.018	0.003		-3.012	-0.692	-3.726	-5.152	-0.692	-0.692	-3.345	-3.223	-3.272	-3.438	-3.077
4. AHU6 aur A1	0.068	1.000	0.003		-2.919	-2.584	-6.204	-2.919	-2.919	-4.196	-4.207	-4.253	-4.285	-4.067
5. AHU21 aur A2	0.024	0.004	0.491	0.004		-3.669	-5.090	0.000	0.000	-3.255	-3.131	-3.181	-3.348	-2.984
6. AJ249166 hu pol I	0.004	0.011	0.000	0.011	0.000		-4.137	-3.669	-3.669	-5.592	-5.469	-5.540	-5.592	-5.387
7. AJ249168 hu pol II	0.000	0.000	0.000	0.000	0.000	0.000		-5.090	-5.090	-2.251	-2.123	-1.969	-2.251	-2.050
8. AMt9 aur A2	0.024	0.004	0.491	0.004	1.000	0.000	0.000		0.000	-3.255	-3.131	-3.181	-3.348	-2.984
9. AMt26 aur A2	0.024	0.004	0.491	0.004	1.000	0.000	0.000	1.000		-3.255	-3.131	-3.181	-3.348	-2.984
10. B19 aur A3	0.000	0.000	0.001	0.000	0.001	0.000	0.026	0.001	0.001		-1.093	-1.029	1.416	-1.376
11. C8 aur A3	0.000	0.000	0.002	0.000	0.002	0.000	0.036	0.002	0.002	0.276		-0.402	-1.389	0.120

D. **Immune evasion (*spa-X, spa-IgG, chp* and *scn* gene)**

Staphylococcus aureus secretes various proteins that can significantly suppress the innate and adaptive immune system. *i.e.* Staphylococcal protein A (SpaA), Staphylococcal complement inhibitor (SCIN) and Chemotaxis inhibitory protein of *S. aureus* (CHIPS) encoded by *spa*, *scn* and *chp* gene, respectively. The *spa* gene is composed of two distinct regions: IgG, Fc binding region (*spa-IgG*) and X region (*spa-X*) at C terminus, required for cell wall attachment and impairs opsonisation by serum complement and phagocytosis by leukocytes. It has also been proved that more than seven repeats in the X-region tended to be epidemic or more virulent while the presence of less than seven repeats is a non-epidemic or less virulent. SCIN is a C3 convertase inhibitor, inhibit complement activation and CHIPS has inhibition effect on neutrophils activation.

Out of the 157 isolates immune invasion genes, *chp* and *scn* genes were detected in 114 (72.6%) and 40 (25.5%) isolates, with single amplicon of 404bp (Fig. 39) and 320bp (Fig. 24) respectively. In the present investigation, *scn* gene was detected in multiplex PCR with *clfB* and *sak*. Most animal isolates were found to be negative for *chp* gene except horse, pig, camel, dog and cattle isolates. Similarly, most of animal isolates were negative for *scn* gene while most of human and meat piece isolates were positive for both *chp* and *scn* genes as mentioned in table 35.

Table 35: Detection of Immune evasion factor associated genes among *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Immune evasion associated genes (%)			
			chp (404bp)		*scn* (320bp)	
			P	N	P	N
1.	Human	35	31 (88.6)	4 (11.4)[a]	26 (74.3)	9 (25.7)[a]
2.	Meat piece	20	16 (80.0)	4 (20.0)	4 (20.0)	16 (80.0)
3.	Horse	3	2 (66.7)	1 (33.3)	0 (0.0)	3 (100.0)
4.	Pig	2	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)
5.	Camel	8	8 (100.0)	0 (0.0)	0 (0.0)	8 (100.0)
6.	Dog	6	5 (83.3)	1 (16.7)	2 (33.3)[b]	4 (66.7)
7.	Sheep	6	2 (33.3)	4 (66.7)	0 (0.0)	6 (100.0)
8.	Buffalo	21	10 (47.6)	11 (52.3)	3 (14.3)[b]	18 (85.7)
9.	Goat	28	13 (46.4)	15 (52.6)	2 (7.1)[b]	26 (92.9)
10.	Cattle	28	25 (89.3)	3 (10.7)	3 (10.7)[b]	25 (89.3)
Total		**157**	**114 (72.6)**	**43 (27.4)**	**40 (25.5)**	**117 (74.5)**

Abbreviations:- P- Positive, N- Negative
Superscript:- a- Human origin isolates negative for *chp* and *scn* gene respectively (H14, H21, H28, H46 and H10, H11, H15, H28, H31, H40, H41, H44, H48), b- animal origin isolates positive for *scn* gene (D9, D10, B39, B42, B57, AG13, AG15, C29, C37 and C43)

Similar to our results, Sung *et al.* (2008) compared 56 *S. aureus* isolates from infection in cows, horses, goats, sheep and a camel with 161 human *S. aureus* isolates from healthy carriers and community acquired infections in the UK for genes carried on mobile genetic elements (MGEs) such as *scn* and *chp*. The *scn* and *chp* gene was found in 154 (96 %) and 134 (83%) of human isolates and 12 (21 %) and 8 (14 %) of animal origin isolates, respectively. Interestingly, such genes carried on mobile genetic elements (MGEs) were less common in animal *S. aureus* isolates.

Similar to our result, Ikawaty *et al.* (2010) also reported very low prevalence of *scn* gene among animal isolates as they reported that only one isolate was positive for both *scn and chp* genes from 76 bovine mastitis isolates. Verkaik *et al.* (2011) also reported that 90% human isolates carried *scn* and *chp* genes while only 34% animal isolates were positive for both gens from 21 human and 77 animal isolates. van Wamel *et al.* (2006) also reported high prevalence of *scn* (85.7%) gene while comparatively low prevalence of *chp* gene (56.6%) among human clinical isolates. Gomez *et al.* (2014) studied 13 isolates and reported three isolates were positive for *scn* and one isolate was found to be positive for *chp* gene from faecal carriage of wild small mammals.

Similar to present study, Puacz *et al.* (2015) reported 42.85% isolates to be positive for *chp* gene and 28.57% positive for *scn* gene in 21 isolates of mastitic milk of cows.

In the present study,100% isolates were found to be positive for *spa-IgG* gene with three polymorphic (600bp, 750bp and 950bp) band patterns (Fig. 40). Out of total 157 isolates, 144 (91.7%) isolates were positive with 950bp, nine (5.7%) isolates (H1, H12, H13, H41,B21, B34, B43, B46 and AG13) with 650bp and four (2.5%) isolate (Hrs4, G10, G16 and G47) with 600bp amplicon size. Meat piece, pig, camel, dog, sheep and cattle were positive with only single band pattern (950bp) while human, buffalo and horse isolates were showed two band patterns *i.e.* 750bp and 950bp and 600bp and 950bp, respectively and goat isolates were showed three band patterns (600bp, 750bp and 950bp) as mentioned in table 36.

Table 36: Detection of *spa-Ig* gene (Immune evasion) variability among *S. aureus* isolates in the present study.

S. No.	Source of Isolates	*Spa-IgG* gene amplicon	Isolate ID	No. of isolates	Total No. of Isolates
1	Human	750bp	H1, H12, H13 and H41	4	35
		950bp	H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H14, H15, H16, H21, H22, H24, H25, H27, H28, H29, H30, H31, H33, H34, H37, H39, H40, H44, H45, H46 and H48	31	
2	Meat	950bp	Mt1, Mt2, Mt3, Mt4, Mt9, Mt10, Mt11, Mt12, Mt13, Mt14, Mt15, Mt19, Mt20, Mt22, Mt24, Mt25, Mt26, Mt27, Mt28 and Mt31	20	20
3	Horse	600bp	Hrs4	1	3
		950bp	Hrs1 and Hrs3	2	
4	Pig	950bp	Pg2 and Pg4	2	2
5	Camel	950bp	J2, J3, J4, J9, J10, J14, J15 and J18	8	8
6	Dog	950bp	D4, D6, D7, D9, D10 and D13	6	6
7	Sheep	950bp	SV2, SV3, SV4, SN3, SN4 and SN14	6	6
8	Buffalo	750bp	B21, B34, B43 and B46	4	21
		950bp	B1, B5, B10, B19, B23, B24, B26, B27, B28, B29, B30, B31, B36, B39, B42, B55 and B57	17	
9	Goat	600bp	G10, G16 and G47	3	28
		750bp	AG6	1	
		950bp	AG5, AG8, AG10, AG13, AG15, AG17, G1, G2, G7, G9, G11, G21, G24, G29, G35, G37, G39, G40, G41, G43, G45, G46, G49 and G55	24	
10	Cattle	950bp	C2, C2R, C3R, C5R, C7, C8, C9, C11, C12, C13, C15, C17, C20, C22, C23, C26, C29, C34, C36, C37, C39, C40, C41, C43, C46, C47, C50 and C52	28	28
Total No. of Isolates				157	157

In genes associated with immune invasion, *spa-X* gene was detected in 100% isolates with nine polymorphic bands ranging from 150bp (4 repeats) to 380bp (14 repeats), which included one isolate of the 150bp, two of 170bp, five of 180bp, 15 of 200bp, 53 of 250bp, nine of 280bp, 56 of 300bp, 15 of 350bp and one isolate of 380bp as mentioned in table 38 (Fig. 41). The *spa-X* gene amplification was found to be suitable for isolate differentiation with good discriminatory index value (D.I. =0.7407).

Of the 157 isolates, 134 (85.3%) isolates were positive with more than 7 repeats while only 23 (14.6%) isolates were positive with less than seven repeats *viz.* one isolate (B57) with four repeats, two isolate (Mt15 and B39) with five repeats and 20 isolates were positive with six repeats (table 38). Maximum variations in regards to amplicon sizes and number of repeats were recorded in buffalo and goat isolates while less variations were found in dog, human and camel as mentioned in Table 37.

On the basis of discriminatory index value,*spa-X* gene amplification (D.I.- 0.7407) is a more suitable method to differentiate various *S. aureus* isolates in comparison to *coa* gene PCR amplification (D.I.- 0.7353) but *coa*-RFLP method (D.I.-0.9301) is more precise and good method to differentiate different strains of *S. aureus* in comparison of *rep*-PCR method (D.I.-0.8892) and *spa-X* typing (D.I.- 0.7407).

Similar to present study, many researchers reported size and repeats variability in *spa-X* and *spa-IgG* region of protein A gene. Annemuller *et al*. (1999) obtained amplified *spa-X* gene fragments of 120, 150, 170, 250 and 300bp with calculated number of repeats of 3, 4, 5, 8 and 10, respectively and for *spa-IgG* gene obtained

products of 620bp for 20 of the isolates and 280bp for four isolates from bovine mastitis samples. Stephan *et al*. (2001) recorded *spa-X* region amplicons of 100, 200, 280 and 300bp with repeats of 2, 6, 10 and 11, respectively and similar to present study *spa- IgG* with 920bp amplicon size for most of the isolates while three isolates were positive with 750bp from bovine mastitis samples. Salasia *et al*. (2004) reported immense variations in *spa-X* gene with amplicon sizes 100, 150, 200, 230, 240, 250, 270, 290, 320 and 340bp and *spa-IgG* with two band patterns such as most of isolate with 900bp while three isolate with 780bp amplicon among bovine mastitis samples.

Indrajulianto (2000) reported polymorphism of *spa* gene from human and bovine isolates with a size of 100, 150, 200, 250, 280, 300 and 330bp corresponding to 3, 4, 6, 8, 9, 10 and 11 number of repeats, respectively. Similar to this study, it was also recorded that most of the human isolates were longer than those from bovine isolates.

In agreement to our results, Reinoso *et al.* (2008) reported *spa-IgG* with 900bp size for 42 isolates and 700bp amplicon size for three isolates and spa-X with typical size polymorphisms ranging from 100 to 315bp (2–10 repeats) from 45 isolates of humans, bovine subclinical mastitis and food samples while Momtaz *et al.* (2010) reported 80.2% isolates were positive for *spa-IgG* with single amplicon of 920bp and only 25.5% isolates were positive for *spa-X* gene with single amplicon of 320bp without any polymorphism from 86 bovine mastitis milk isolates.

Our finding were close to those of Karahan *et al*. (2011) who reported two polymorphic band pattern for *spa-IgG* gene where 72.8% isolates with 920bp and 27.2% isolates with 750bp amplicon size were obtained. Similarly polymorphic patterns were also recorded for spa-X gene i.e. 110, 140, 170, 190, 220, 240, 270, 290 and 320 bp with 3, 4, 5, 6, 7, 8, 9, 10 and 11 repeat units, respectively from 92 isolates from subclinical mastitis affected bovines. In the study by Nathawat (2013), the variable amplicon sizes of 200, 240, 250, 290, 300, 320, 330, 350, 380, 400 and 650 with calculated number of repeats of 7, 9, 9, 11, 11, 12, 12, 13, 14, 15, and 26, respectively for X-region *spa* gene in *S. aureus* isolates from caprine mastitic milk isolates were recorded.

Singh *et al*. (2011) carried out the *spa* typing of *S. aureus* isolates from intra-mammary infections of cattle and buffalo. They recorded 3-12 repeats, the most common number of repeat was eight (57.9%) in Sahiwal cattle and seven (53.2%) in Murrah buffalo. We also observed similar results for cattle isolates with most common repeats of eight (53.5%) whereas in buffalo isolates we observed that most common number of repeat was eleven (47.1%).

The present study suggested that there was great variability in studied source of *S. aureus* isolates in regards to *spa* typing. Similar results were recorded by many workers (Lange *et al*., 1999; Stephan *et al*., 2001; Khichar *et al*., 2012 and Yadav *et al*., 2015c) and used this typing to study the epidemiological spread of the isolates.

In the present study 134 isolates out of 157 were considered to be pathogenic since they possessed more than seven repeats. This is based on reports by Frenay *et al.* (1996) who suggested that the isolates possessing seven or more tandem repeats are pathogenic isolates. On the other hand, no correlation was reported between tandem repeats and pathogenicity of the isolates by Nashev *et al*. (2004) from humans; Kuzma *et al*. (2005) and Jakubczak *et al*. (2007) in isolates from mastitc cows and Kurlenda *et al*. (2010) in human isolates.

This study further extend to *spa-X* gene sequence analysis, to find variations in relation to source of isolation, amplicon size, number of repeats and virulence propertied of *S. aureus* isolates.

Table 37: Detection of *spa-X* gene (Immune evasion) variability among *S. aureus* isolates in the present study.

S. No.	Source of isolates	*spa*-X amplicon size	No. of repeats	Isolate ID	No. of isolates	Total no. of Isolates
1.	Human	200bp	6	H46 and H30	2	35
		250bp	8	H1, H2, H7, H8, H9, H12, H16, H24, H27, H29, H33, H44 and H45	13	
		280bp	10	H5, H6, H10, H11, H14 and H21	6	
		300bp	11	H4, H15 and H41	3	
		350bp	13	H3, H13, H22, H25, H28, H31, H34, H37, H39, H40 and H48	11	
2.	Meat piece	170bp	5	Mt15	1	20

		200bp	6	Mt28	1	
		250bp	8	Mt3, Mt4 and Mt9	3	
		300bp	11	Mt1, Mt2, Mt10, Mt11, Mt12, Mt13, Mt14, Mt19, Mt22, Mt24, Mt25, Mt26, Mt27 and Mt31	14	
		350bp	13	Mt20	1	
3.	Horse	250bp	8	Hrs1	1	3
		300bp	11	Hrs3 and Hrs4	2	
4.	Pig	300bp	11	Pg2 and Pg4	2	2
5.	Camel	180bp	6	J2	1	8
		250bp	8	J15	1	
		300bp	11	J3, J4, J9, J10, J14 and J18	6	
6.	Dog	250bp	8	D4, D7, D9 and D10	4	6
		300bp	11	D13	1	
		350bp	13	D6	1	
7.	Sheep	200bp	6	SV2, SV3, SV4 and SN14	4	6
		250bp	8	SN3 and SN4	2	
8.	Buffalo	150bp	4	B57	1	21
		170bp	5	B39	1	
		180bp	6	B26 and B28	2	
		200bp	6	B27 and B42	2	
		250bp	8	B1, B5, B29 and B55	4	
		280bp	10	B10	1	
		300bp	11	B19, B21, B23, B24, B30, B31, B34, B36, B43 and B46	10	
9.	Goat	180bp	6	G29	1	28
		200bp	6	AG10 and G16	2	
		250bp	8	G2, G9, G10, G11, G35, G40, G41, G43, G45 and G55	10	
		280bp	10	G46	1	
		300bp	11	AG5, AG6, AG8, AG17, G1, G7, G21, G24, G37, G39, G47 and G49	12	
		350bp	13	AG15	1	
		380bp	14	AG13	1	
10.	Cattle	180bp	6	C46	1	28
		200bp	6	C2, C3R, C7 and C26	4	
		250bp	8	C2R C13, C15, C20, C22, C23, C29, C34, C36, C37, C39, C40, C47, C50 and C52	15	
		280bp	10	C12	1	
		300bp	11	C5R, C8, C9, C11, C41 and C43	6	
		350bp	13	C17	1	
Total No. of Isolates					157	157

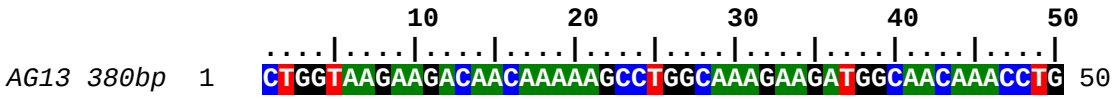
Table 38: *Staphylococcus aureus* typing on the basis of overall distribution of *spa- X* gene patterns among isolates in the present study.

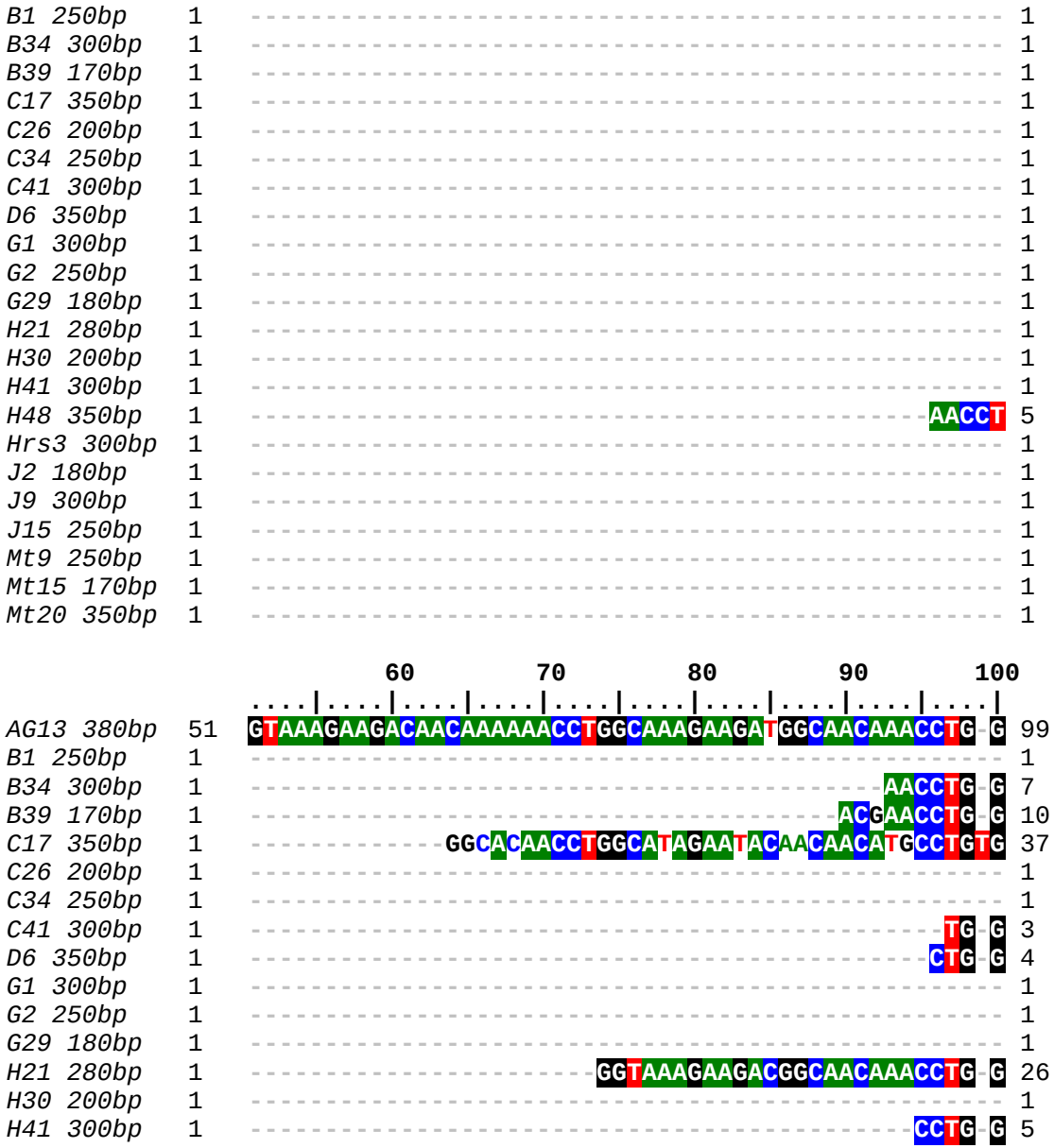
S. No.	*spa-X* amplicon Size	No. of repeats	Isolate I.D.	No. of Isolates	Significance
1.	150bp	4	B57	1	Strains with seven or less than seven repeats consider as non-virulent strains
2.	170bp	5	Mt15, B39	2	
3.	180bp	6	J2, B26, B28, G29 and C46	5	
4.	200bp	6	H46, H30, Mt28, SV2, SV3, SV4, SN14, B27, B42, AG10, G16, C2, C3R, C7 and C26	15	
5.	250bp	8	H1, H2, H7, H8, H9, H12, H16, H24, H27, H29, H33, H44, H45, Mt3, Mt4, Mt9, Hrs1, J15, D4, D7, D9, D10, SN3, SN4, B1, B5, B29, B55, G2, G9, G10, G11, G35, G40, G41, G43, G45, G55, C2R C13, C15, C20, C22, C23, C29, C34, C36, C37, C39, C40, C47, C50 and C52	53	Strains having more than seven repeats would be consider as more virulent strains
6.	280bp	10	H5, H6, H10, H11, H14, H21, B10, G46 and C12	9	
7.	300bp	11	H4, H15, H41, Mt1, Mt2, Mt10, Mt11, Mt12, Mt13, Mt14, Mt19, Mt22, Mt24, Mt25, Mt26, Mt27, Mt31, Hrs3, Hrs4, Pg2, Pg4, J3, J4, J9, J10, J14, J18, D13, B19, B21, B23, B24, B30, B31, B34, B36, B43, B46, AG5, AG6, AG8, AG17, G1, G7, G21, G24, G37, G39, G47, G49, C5R, C8, C9, C11, C41 and C43	56	
8.	350bp	13	H3, H13, H22, H25, H28, H31, H34, H37, H39, H40, H48, Mt20, D6, AG15 and C17	15	
9.	380bp	14	AG13	1	
Total No. of Isolates				**157**	

spa-X gene sequence analysis

For sequence analysis, 23 *spa-X* gene positive isolates from various sources and of different amplicon sizes were got sequenced *i.e.* the isolate H21 (280bp), H30 (200bp), H41 (300bp) and H48 (350) were included from human, Mt9 (250bp), Mt15 (170bp) and Mt20 (350bp) from meat piece, Hrs3 (300bp) from horse, J2 (180bp), J9 (300bp), and J15 (250bp) from camel, D6 (350bp) from dog, B1 (250bp), B34 (300bp) and B39 (170bp) from buffalo, AG13 (380bp), G1 (300bp), G2 (250bp) and G29 (180bp) from goat and C17 (350bp), C26 (200bp), C34 (250bp) and C41 (300bp) isolates were included from cattle isolates. First of all the sequences were BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed as *spa-X* gene sequences of *S. aureus* strains.

In sequence analysis of above 23 representative isolates, only 37 nucleotide from 245 nucleotide position to 350 nucleotide position were found to be similar in all studied sequences (Fig. 42). Hence, massive variations were detected at from one position to 390 aligned position among all studied sequences in our results. Large overhanging flanks and big gap were found at starting and end sides among sequences indicates more or less repeats in sequences according to their bigger and smaller amplicon sizes. One big insertion of 24bp was observed between 192 and 216 nucleotide position in B39 isolate and other big insertion of 24bp was detected in between 264 and 288 nucleotide position in the G29 isolate. This 24bp insertion of different nucleotide sequence indicate one different repeat in corresponding sequence.

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B34 300bp 1 1
B39 170bp 1 1
C17 350bp 1 1
C26 200bp 1 1
C34 250bp 1 1
C41 300bp 1 1
D6 350bp 1 1
G1 300bp 1 1
G2 250bp 1 1
G29 180bp 1 1
H21 280bp 1 1
H30 200bp 1 1
H41 300bp 1 1
H48 350bp 1 AACCT 5
Hrs3 300bp 1 1
J2 180bp 1 1
J9 300bp 1 1
J15 250bp 1 1
Mt9 250bp 1 1
Mt15 170bp 1 1
Mt20 350bp 1 1
60
70
80
90
100
AG13 380bp 51 GTAAAGAAGACAACAAAAAACCTGGCAAAGAAGATGGCAACAAACCTG G 99
B1 250bp 1 1
B34 300bp 1 AACCTG G 7
B39 170bp 1 ACGAACCTG G 10
C17 350bp 1 GGCACAACCTGGCATAGAATACAACAACATGCCTGTG 37
C26 200bp 1 1
C34 250bp 1 1
C41 300bp 1 TG G 3
D6 350bp 1 CTG G 4
G1 300bp 1 1
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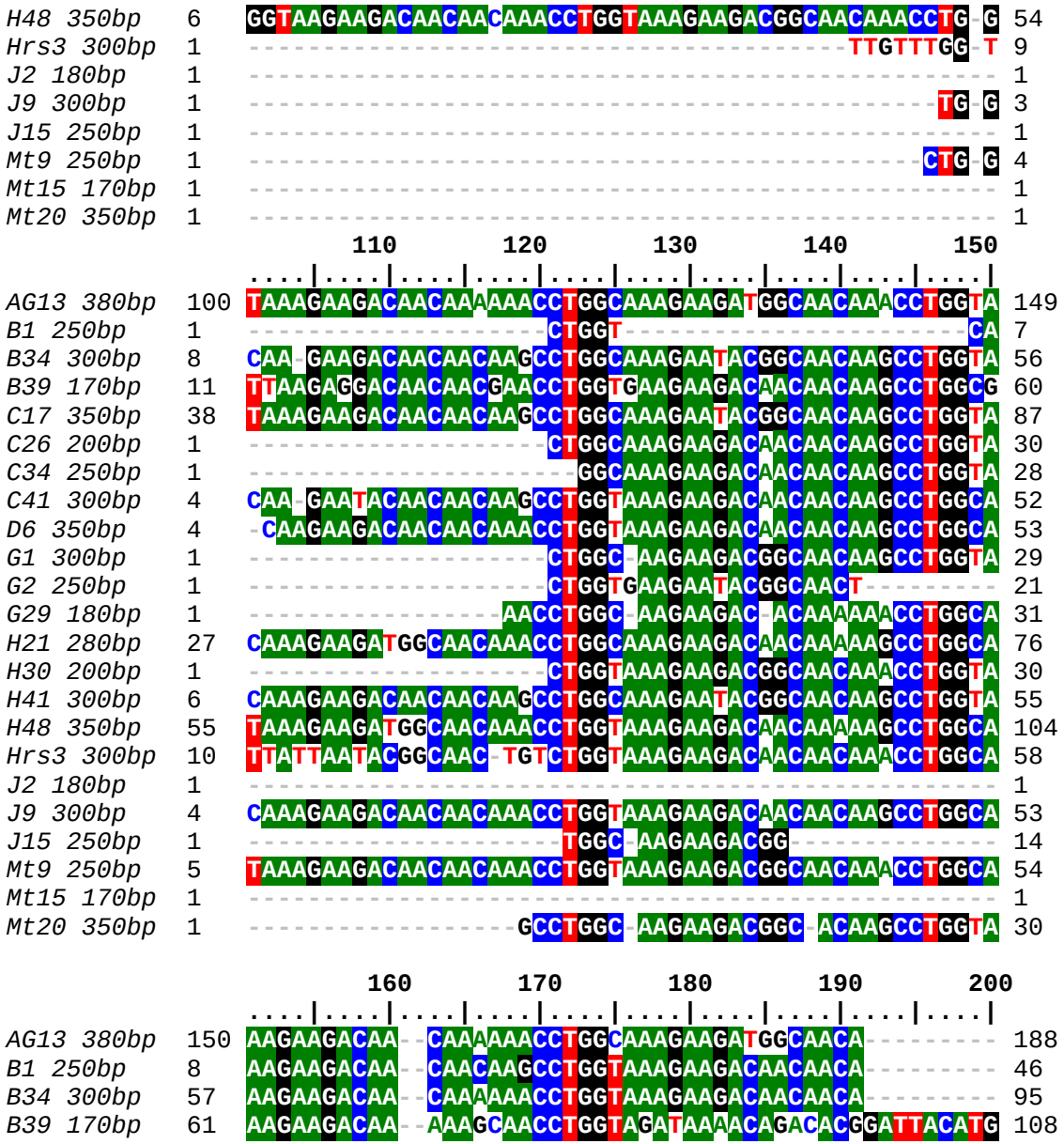
H30 200bp 1 1
H41 300bp 1 CCTG G 5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Hrs3 300bp 1 TTGTTTGG T 9
J2 180bp 1 1
J9 300bp 1 TG G 3
J15 250bp 1 1
Mt9 250bp 1 CTG G 4
Mt15 170bp 1 1
Mt20 350bp 1 1
110 120 130 140 150
AG13 380bp 100 TAAAGAAGACAACAAAAAACCTGGCAAAGAAGATGGCAACAAACCTGGTA 149
B1 250bp 1 CTGGT CA 7
B34 300bp 8 CAA GAAGACAACAACAAGCCTGGCAAAGAATACGGCAACAAGCCTGGTA 56
B39 170bp 11 TTAAGAGGACAACAACGAACCTGGTGAAGAAGACAACAACAAGCCTGGCG 60
C17 350bp 38 TAAAGAAGACAACAACAAGCCTGGCAAAGAATACGGCAACAAGCCTGGTA 87
C26 200bp 1 CTGGCAAAGAAGACAACAACAAGCCTGGTA 30
C34 250bp 1 GGCAAAGAAGACAACAACAAGCCTGGTA 28
C41 300bp 4 CAA GAATACAACAACAAGCCTGGTAAAGAAGACAACAACAAGCCTGGCA 52
D6 350bp 4 CAAGAAGACAACAACAAACCTGGTAAAGAAGACAACAACAAGCCTGGCA 53
G1 300bp 1 CTGGC AAGAAGACGGCAACAAGCCTGGTA 29
G2 250bp 1 CTGGTGAAGAATACGGCAACT 21
G29 180bp 1 AACCTGGC AAGAAGAC ACAAAAAACCTGGCA 31
H21 280bp 27 CAAAGAAGATGGCAACAAACCTGGCAAAGAAGACAACAAAAGCCTGGCA 76
H30 200bp 1 CTGGTAAAGAAGACGGCAACAAACCTGGTA 30
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H48 350bp 55 TAAAGAAGATGGCAACAAACCTGGTAAAGAAGACAACAAAAGCCTGGCA 104
Hrs3 300bp 10 TTATTAATACGGCAAC TGTCTGGTAAAGAAGACAACAACAAACCTGGCA 58
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B1 250bp 8 AAGAAGACAA CAACAAGCCTGGTAAAGAAGACAACAACA 46
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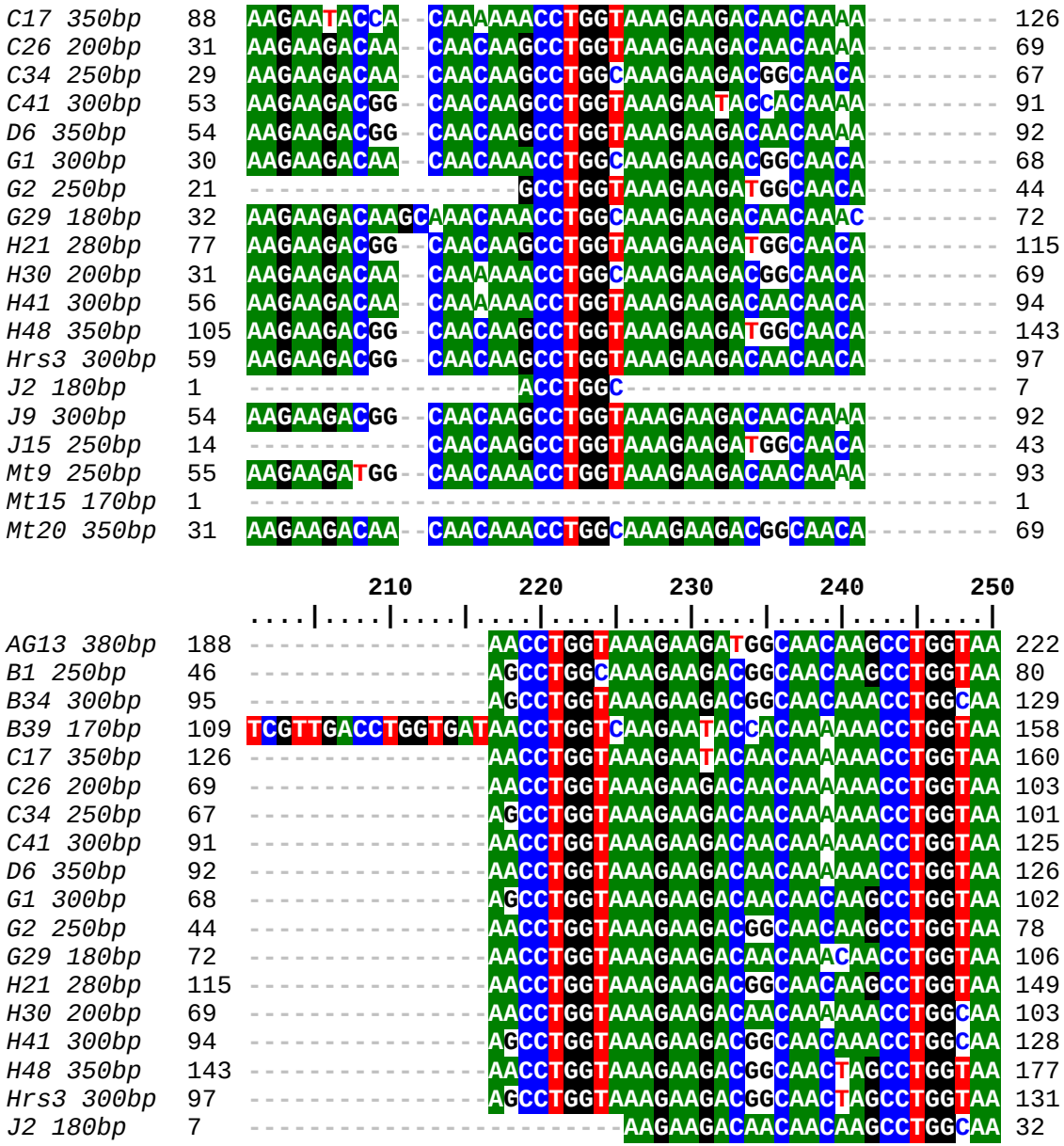
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Mt15 170bp 1 ---------- 1
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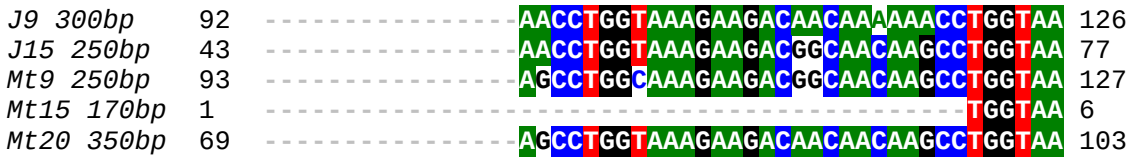
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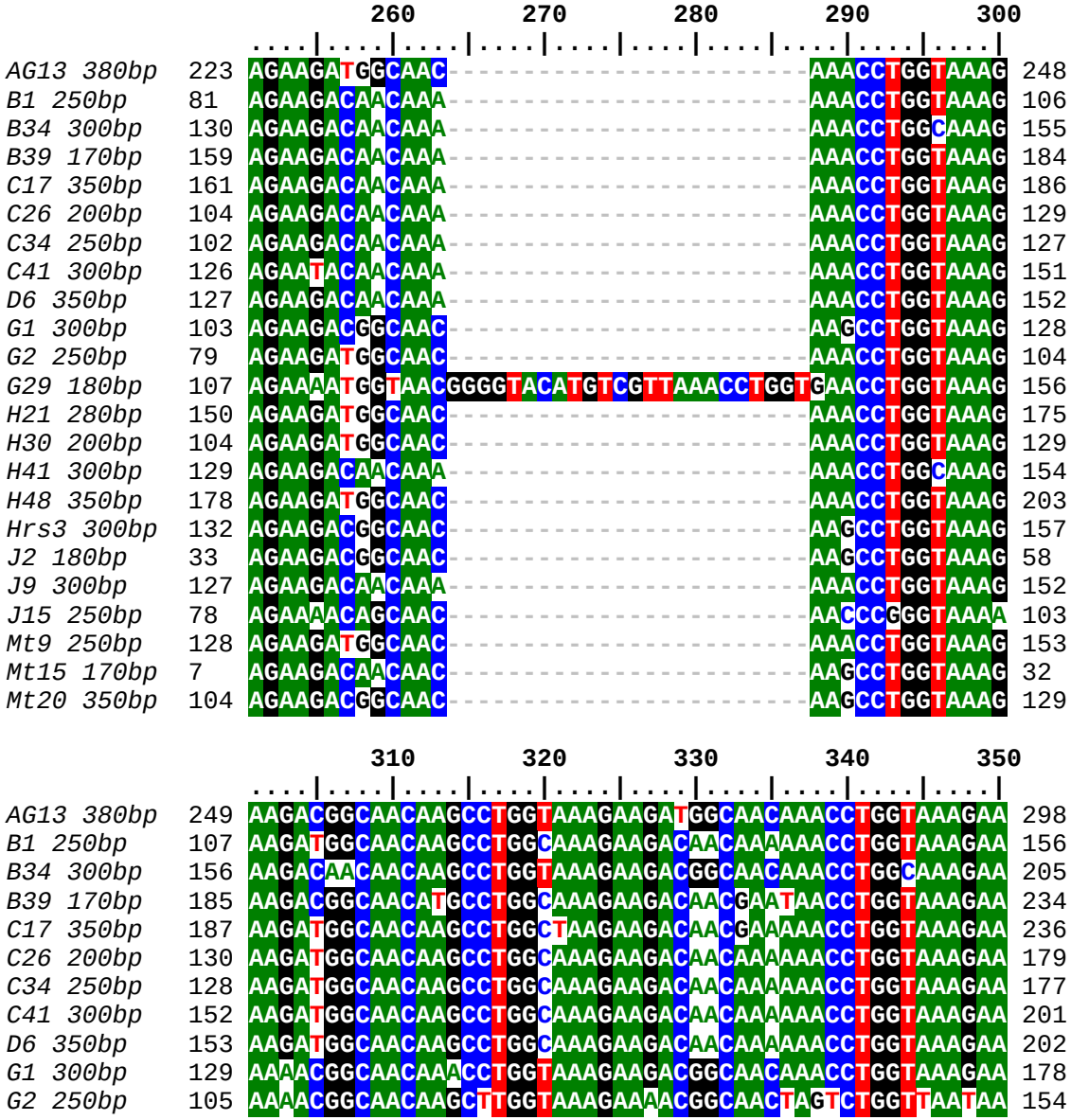
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H48 350bp 178 AGAAGATGGCAAC AAACCTGGTAAAG 203
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J2 180bp 33 AGAAGACGGCAAC AAGCCTGGTAAAG 58
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J15 250bp 78 AGAAAACAGCAAC AACCCGGGTAAAA 103
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Mt20 350bp 104 AGAAGACGGCAAC AAGCCTGGTAAAG 129
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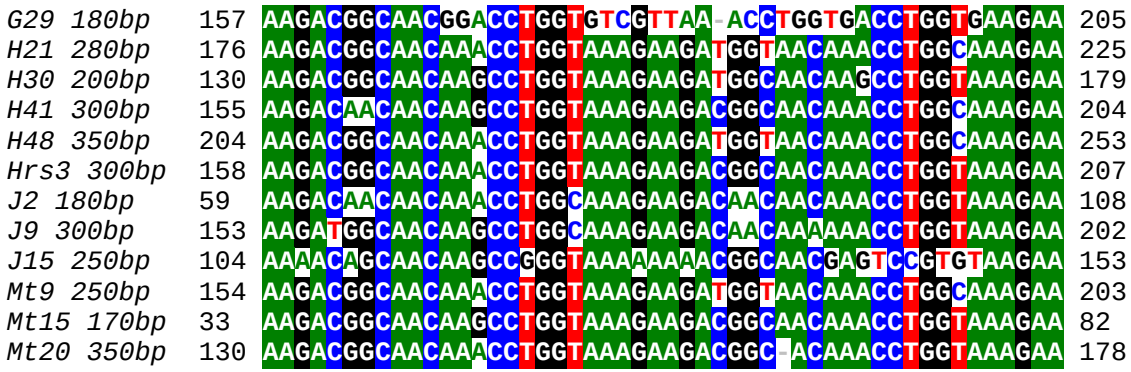
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J15 250bp 104 AAAACAGCAACAAGCCGGGTAAAAAAAACGGCAACGAGTCCGTGTAAGAA 153
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Mt15 170bp 33 AAGACGGCAACAAGCCTGGTAAAGAAGACGGCAACAAACCTGGTAAAGAA 82
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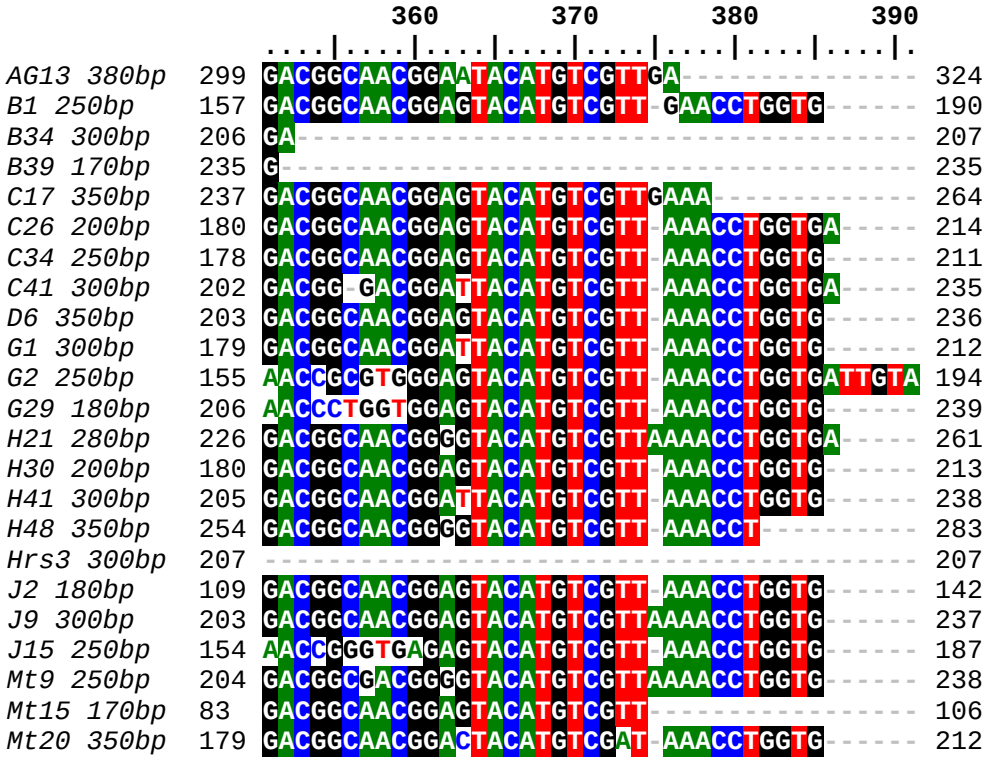
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B34 300bp 206 GA---------- 207
B39 170bp 235 G---------- 235
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H41 300bp 205 GACGGCAACGGATTACATGTCGTT-AAACCTGGTG------ 238
H48 350bp 254 GACGGCAACGGGGTACATGTCGTT-AAACCT---------- 283
Hrs3 300bp 207 ---------- 207
J2 180bp 109 GACGGCAACGGAGTACATGTCGTT-AAACCTGGTG------ 142
J9 300bp 203 GACGGCAACGGAGTACATGTCGTTAAAACCTGGTG------ 237
J15 250bp 154 AACCGGGTGAGAGTACATGTCGTT-AAACCTGGTG------ 187
Mt9 250bp 204 GACGGCGACGGGGTACATGTCGTTAAAACCTGGTG------ 238
Mt15 170bp 83 GACGGCAACGGAGTACATGTCGTT---------- 106
Mt20 350bp 179 GACGGCAACGGACTACATGTCGAT-AAACCTGGTG------ 212

Fig. 42: The sequence analysis*spa-X* gene obtained from present study.

On analysis of codon based test of neutrality, most of *spa-X* sequences were significantly ($p \leq 0.05$) different from each other with some of non- significantly ($p > 0.05$) associated sequences as mentioned in table 39. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 18 positions in the final data set. Evolutionary analyses were conducted in MEGA6 software. Phylogenetic tree analysis of *spa-X* gene sequences of this study revealed five separate clusters among studied sequences, first cluster included 12 isolates namely H48, Mt9, H21, AG13, H30, Mt15, G1, Hrs3, Mt20, J2, B34 and H41 with 0.0101 scaled distance, second cluster including eight isolates namely B39, C17, C41, B1, C26, C34, D6 and J9 with 0.0555 scaled distance. Third, fourth and fifth each cluster included single isolate namely G2, J15 and G29 with 0.1089, 0.1844 and 0.2219 scaled distances, respectively as depicting in figure 43.

Table 39: Codon-based Test of Neutrality for analysis between *spa-X* sequences of present study.

M6: Z-Test of Neutral Evolution (C:\Users\SANDEE~1\AppData\Local\Temp\PhyloAnalysis-1.meg)

File Display Caption Help

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. AG13 380bp		1.799	0.015	0.721	-0.059	1.799	1.799	1.799	1.799	-0.301	-2.855	-0.562	1.451	1.013	0.015	1.451	1.451	0.307	1.799	-2.657	1.451	1.013	1.451
2. B1 250bp	0.075		1.517	1.798	-1.044	0.000	0.000	0.000	0.000	0.481	-2.051	-0.034	2.386	2.105	1.517	2.386	2.386	1.250	0.000	-1.992	2.386	2.105	2.386
3. B34 300bp	0.988	0.132		0.467	0.386	1.517	1.517	1.517	1.517	-0.206	-2.751	-0.500	0.015	0.307	0.000	0.015	0.581	2.389	1.517	-1.944	0.015	0.307	0.581
4. B39 170bp	0.472	0.075	0.641		-0.082	1.798	1.798	1.798	1.798	0.234	-1.808	-0.041	1.318	1.030	0.467	1.318	1.318	0.234	1.798	-1.827	1.318	1.030	1.318
5. C17 350bp	0.953	0.298	0.700	0.935		-1.044	-1.044	-1.044	-1.044	-0.303	-2.570	-0.034	0.481	0.219	0.386	0.481	0.481	0.160	-1.044	-2.572	0.481	0.219	0.481
6. C26 200bp	0.075	1.000	0.132	0.075	0.298		0.000	0.000	0.000	0.481	-2.051	-0.034	2.386	2.105	1.517	2.386	2.386	1.250	0.000	-1.992	2.386	2.105	2.386
7. C34 250bp	0.075	1.000	0.132	0.075	0.298	1.000		0.000	0.000	0.481	-2.051	-0.034	2.386	2.105	1.517	2.386	2.386	1.250	0.000	-1.992	2.386	2.105	2.386
8. C41 300bp	0.075	1.000	0.132	0.075	0.298	1.000	1.000		0.000	0.481	-2.051	-0.034	2.386	2.105	1.517	2.386	2.386	1.250	0.000	-1.992	2.386	2.105	2.386
9. D6 350bp	0.075	1.000	0.132	0.075	0.298	1.000	1.000	1.000		0.481	-2.051	-0.034	2.386	2.105	1.517	2.386	2.386	1.250	0.000	-1.992	2.386	2.105	2.386
10. G1 300bp	0.764	0.631	0.837	0.815	0.762	0.631	0.631	0.631	0.631		-1.968	-1.025	-0.301	0.015	-0.206	-0.301	-1.039	-0.953	0.481	-1.955	-0.301	-0.649	-1.039
11. G2 250bp	0.005	0.042	0.007	0.073	0.011	0.042	0.042	0.042	0.042	0.051		-1.061	-2.442	-3.074	-2.751	-2.442	-2.442	-2.557	-2.051	-1.084	-2.442	-2.644	-2.442
12. G29 180bp	0.575	0.973	0.618	0.967	0.973	0.973	0.973	0.973	0.973	0.307	0.291		-0.562	-0.381	-0.500	-0.562	-0.562	-0.672	-0.034	-1.142	-0.562	-0.381	-0.562
13. H21 280 BP	0.149	0.019	0.988	0.190	0.631	0.019	0.019	0.019	0.019	0.764	0.016	0.575		1.800	0.015	0.000	1.451	0.307	2.386	-2.651	0.000	1.800	1.451
14. H30 200bp	0.313	0.037	0.759	0.305	0.827	0.037	0.037	0.037	0.037	0.988	0.003	0.704	0.074		0.307	1.800	1.800	0.581	2.105	-2.852	1.800	1.451	1.800
15. H41 300bp	0.988	0.132	1.000	0.641	0.700	0.132	0.132	0.132	0.132	0.837	0.007	0.618	0.988	0.759		0.015	0.581	2.389	1.517	-1.944	0.015	0.307	0.581
16. H48 350bp	0.149	0.019	0.988	0.190	0.631	0.019	0.019	0.019	0.019	0.764	0.016	0.575	1.000	0.074	0.988		1.451	0.307	2.386	-2.651	0.000	1.800	1.451
17. Hrs3 300bp	0.149	0.019	0.563	0.190	0.631	0.019	0.019	0.019	0.019	0.301	0.016	0.575	0.149	0.074	0.563	0.149		-0.301	2.386	-2.467	1.451	1.013	0.000
18. J2 180bp	0.759	0.214	0.018	0.815	0.873	0.214	0.214	0.214	0.214	0.343	0.012	0.503	0.759	0.563	0.018	0.759	0.764		1.250	-1.601	0.307	0.015	-0.301
19. J9 300bp	0.075	1.000	0.132	0.075	0.298	1.000	1.000	1.000	1.000	0.631	0.042	0.973	0.019	0.037	0.132	0.019	0.019	0.214		-1.992	2.386	2.105	2.386
20. J15 250bp	0.009	0.049	0.054	0.070	0.011	0.049	0.049	0.049	0.049	0.053	0.281	0.256	0.009	0.005	0.054	0.009	0.015	0.112	0.049		-2.651	-2.657	-2.467
21. Mt9 250bp	0.149	0.019	0.988	0.190	0.631	0.019	0.019	0.019	0.019	0.764	0.016	0.575	1.000	0.074	0.988	1.000	0.149	0.759	0.019	0.009		1.800	1.451
22. Mt15 170bp	0.313	0.037	0.759	0.305	0.827	0.037	0.037	0.037	0.037	0.518	0.009	0.704	0.074	0.149	0.759	0.074	0.313	0.988	0.037	0.009	0.074		1.013
23. Mt20 350bp	0.149	0.019	0.563	0.190	0.631	0.019	0.019	0.019	0.019	0.301	0.016	0.575	0.149	0.074	0.563	0.149	1.000	0.764	0.019	0.015	0.149	0.313	

The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) (below diagonal) is shown. Values of P less than 0.05 are considered significant at the 5% level and are yellow highlighted. The test statistic ($d_N - d_S$) is shown above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. Analyses were conducted using the Nei-Gojobori method. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 18 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

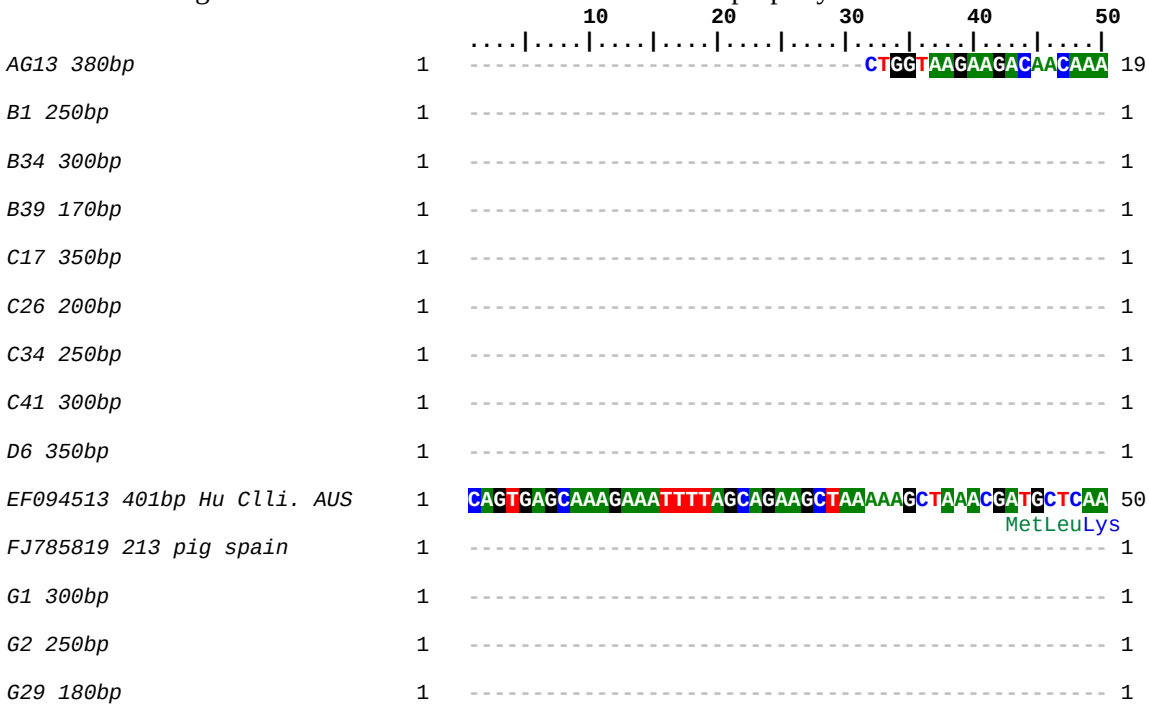
Multiple sequence alignment of present study sequences was conducted with reference sequences of NCBI database to find similarities and variations between our sequences and reference NCBI sequences. For this purpose, four different sequences of NCBI database such as Sequence ID- EF094513 401bp from human clinical isolate of Australia, Sequence ID- FJ785819 (Porrero *et al.*, 2012) 213bp from pig strain of Spain, Sequence ID- HM215122 (Unpublished) 421bp from small ruminants strain of Spain and Sequence ID- KJ000057 (Alreshidi *et al.*, 2013) 298bp from human clinical isolate of Saudi Arabia were chosen according to source of isolation and

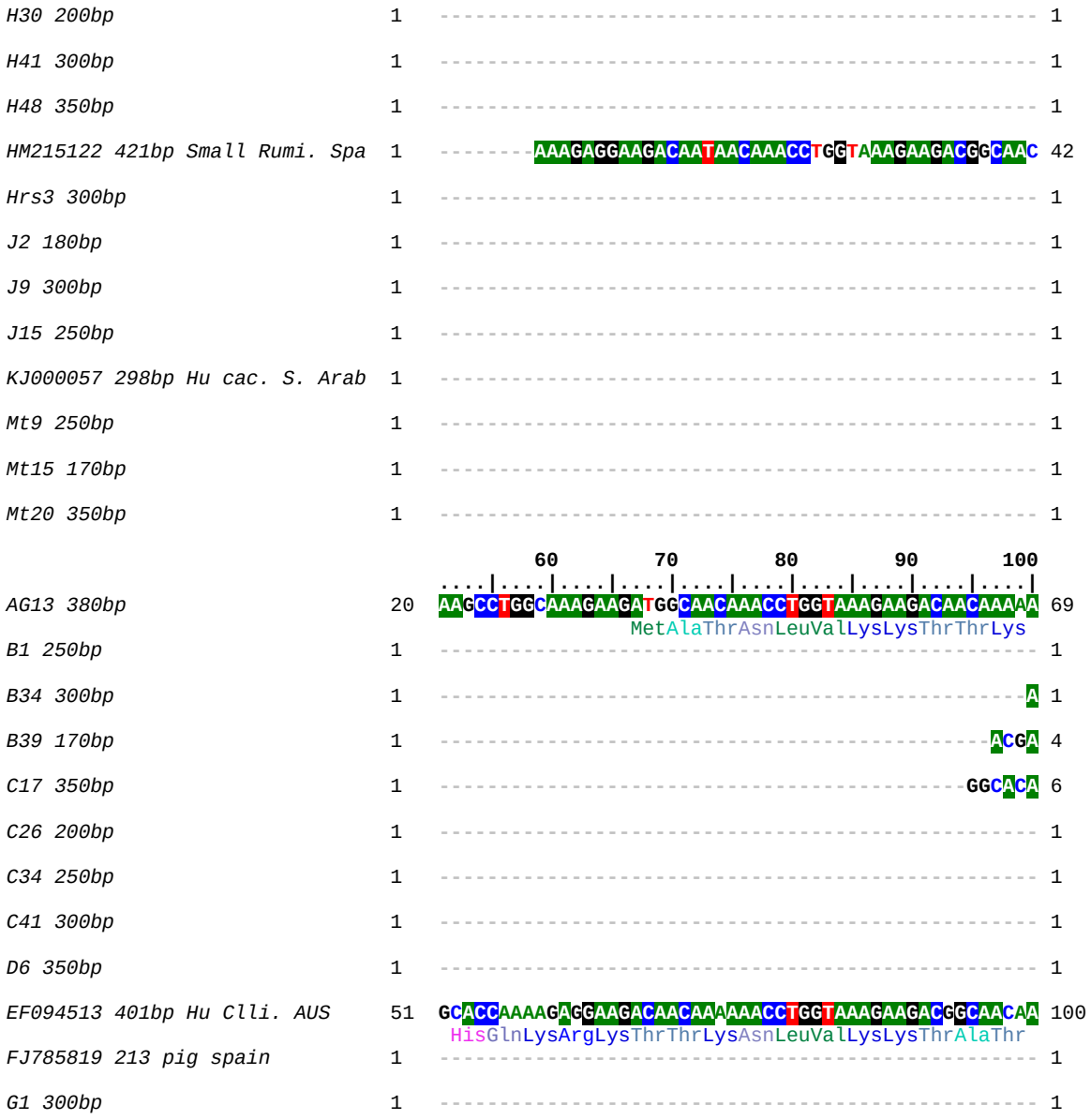
geographic variations to compare existed variations with our findings. The multiple sequence alignment of all sequences revealed massive variations among aligned sequences, only 39 nucleotide positions were completely similar from one to 490 aligned length. Large overhanging flanks, big insertions among sequences and big gap were found at starting, in between and at end sides among sequences indicates many variations and more or less repeats in sequences according to their bigger and smaller amplicon sizes. Multiple sequence alignment of sequences suggested to determine spa types among present study sequence so that it will be possible to decide that these *Spa-X* gene variations are newer or existed in a data base.

The statistic for codon-based test of neutrality between sequences conducted *i.e.* the probability of rejecting the null hypothesis of strict-neutrality $d_N = d_S$ (where d_S and d_N are the number of synonymous and nonsynonymous substitutions per site, respectively). Both significant ($p \leq 0.05$) and non-significant ($p > 0.05$) associations were found among aligned sequences as mentioned in Table 40. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 16 positions in the final data set. Evolutionary analyses were conducted in MEGA6.

Phylogenetic tree analysis of multiple align *spa-X* gene sequences revealed similar five separate clusters among our sequences and NCBI data base sequence. All four data base sequence were classified into single cluster, which was the first cluster as described earlier other four clusters existed similarly and more significantly ($p \leq 0.05$) far from database sequence as depicted in figure 45.

These substantial variation among present study sequences and multiple align *spa-X* gene sequences suggested that *spa-X* gene is an important candidate to study variations among *S. aureus* isolates and to see their virulence property.

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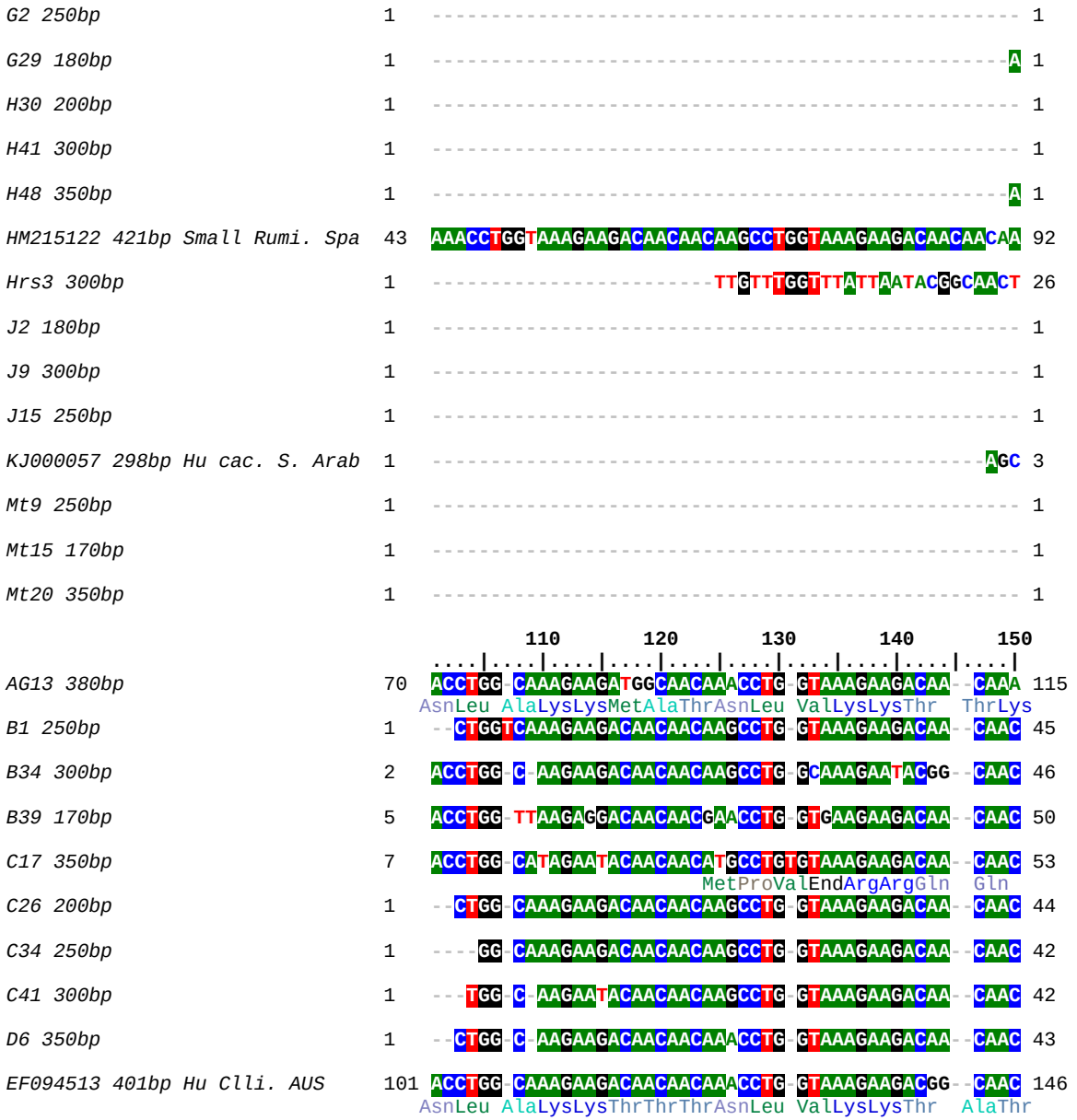
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B39 170bp 5 50
C17 350bp 7 53
MetProValEndArgArgGln Gln
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C34 250bp 1 42
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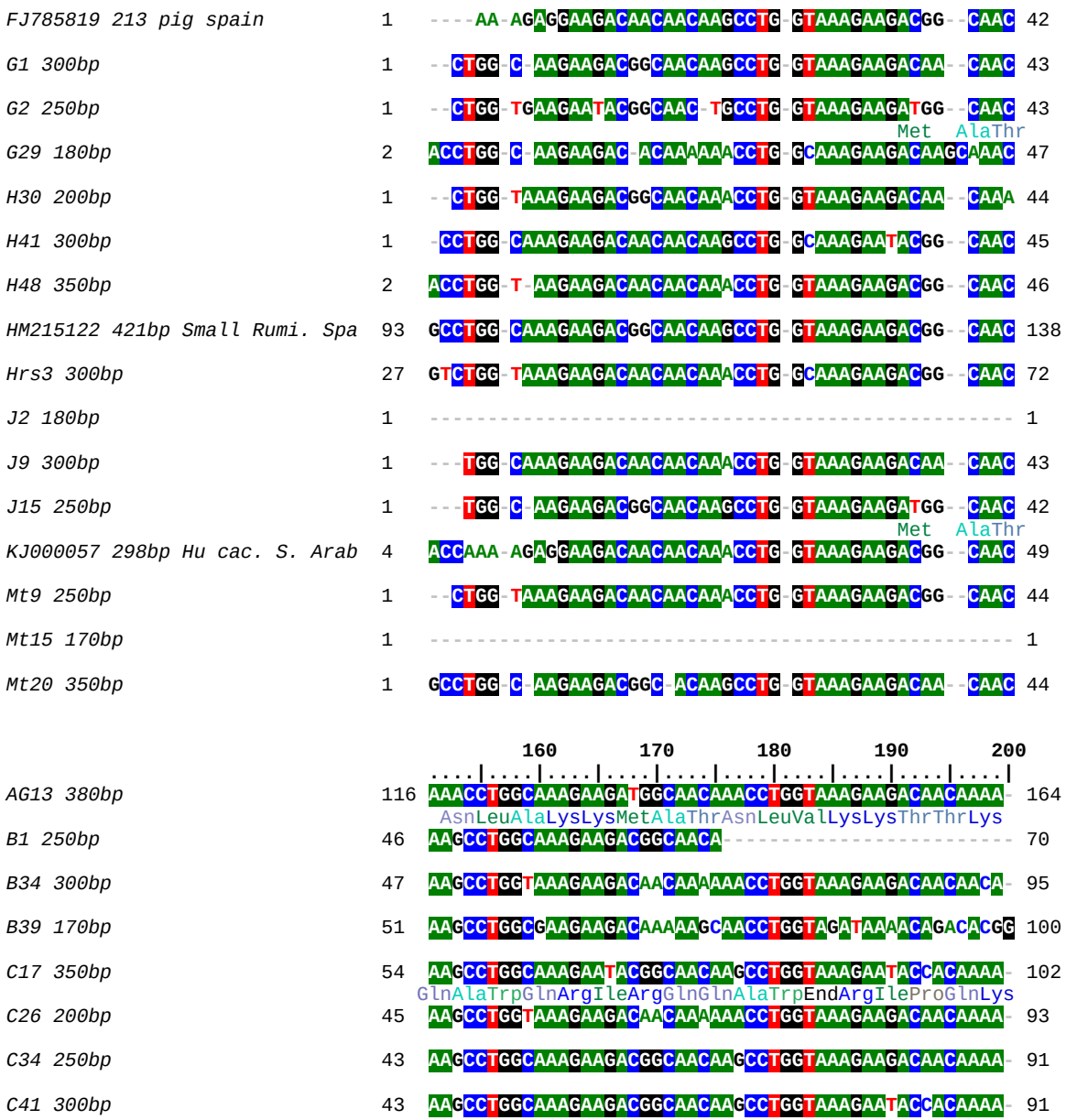
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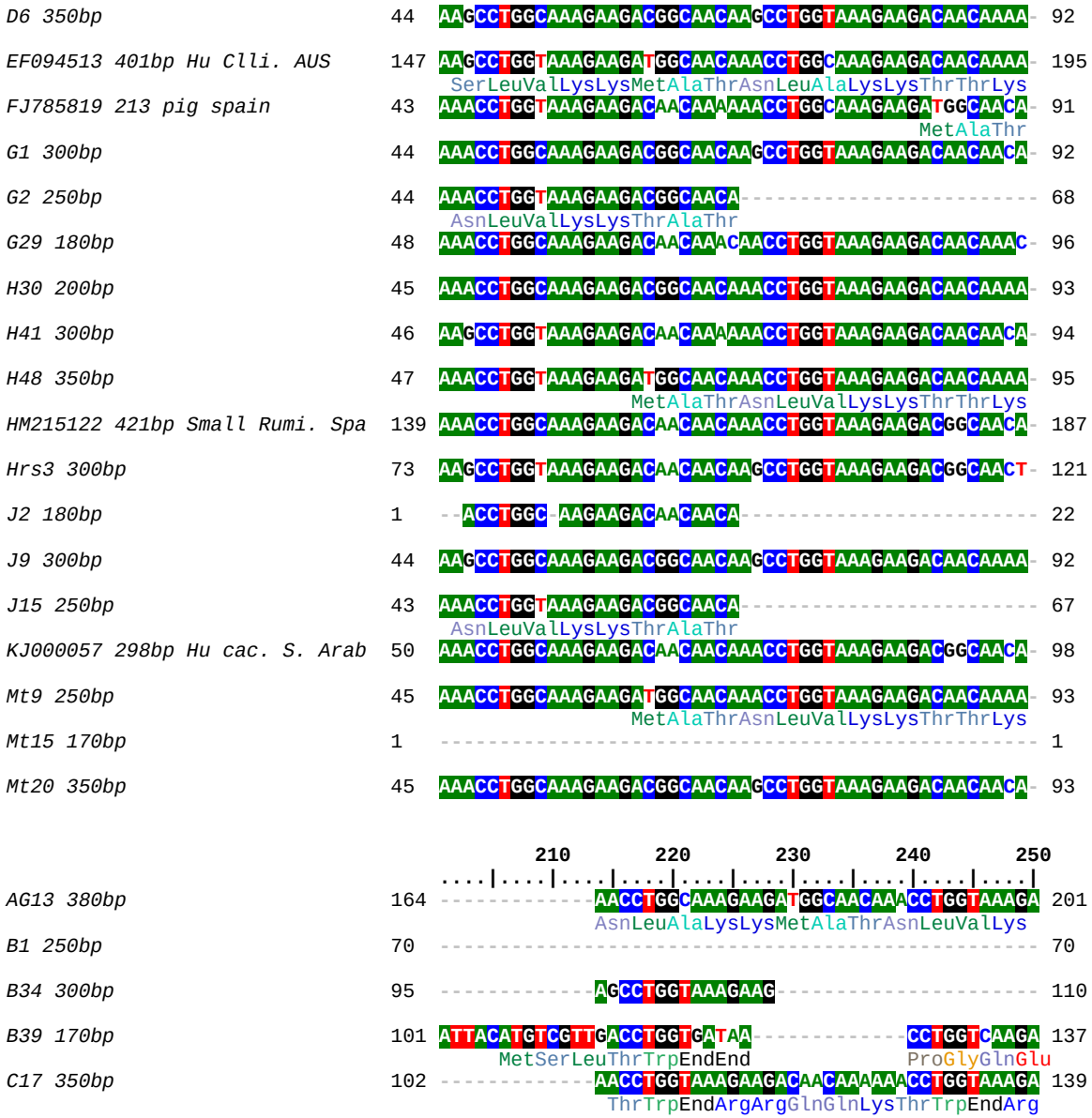
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H41 300bp 46 94
H48 350bp 47 95
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Hrs3 300bp 73 121
J2 180bp 1 22
J9 300bp 44 92
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Mt15 170bp 1 1
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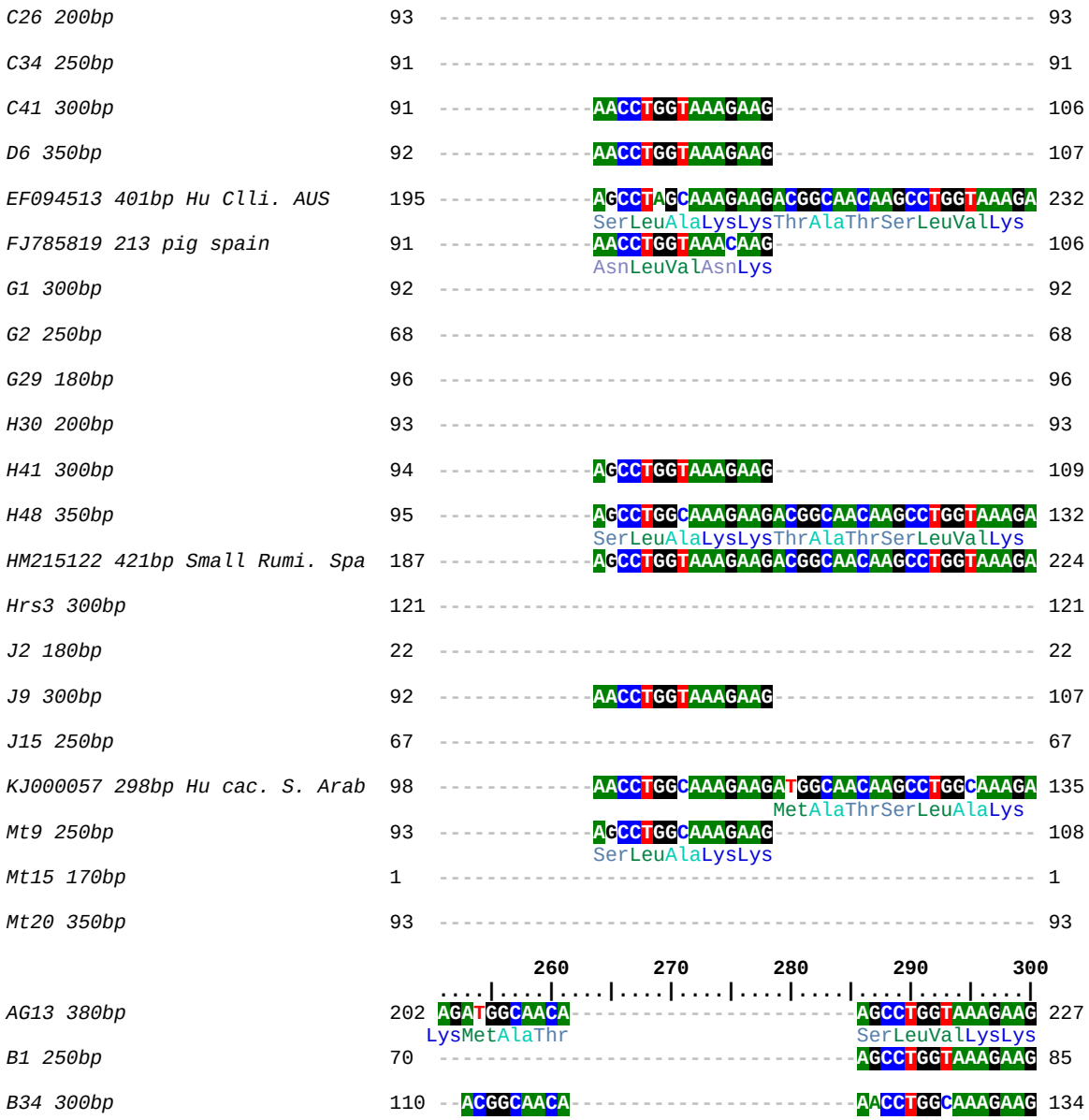
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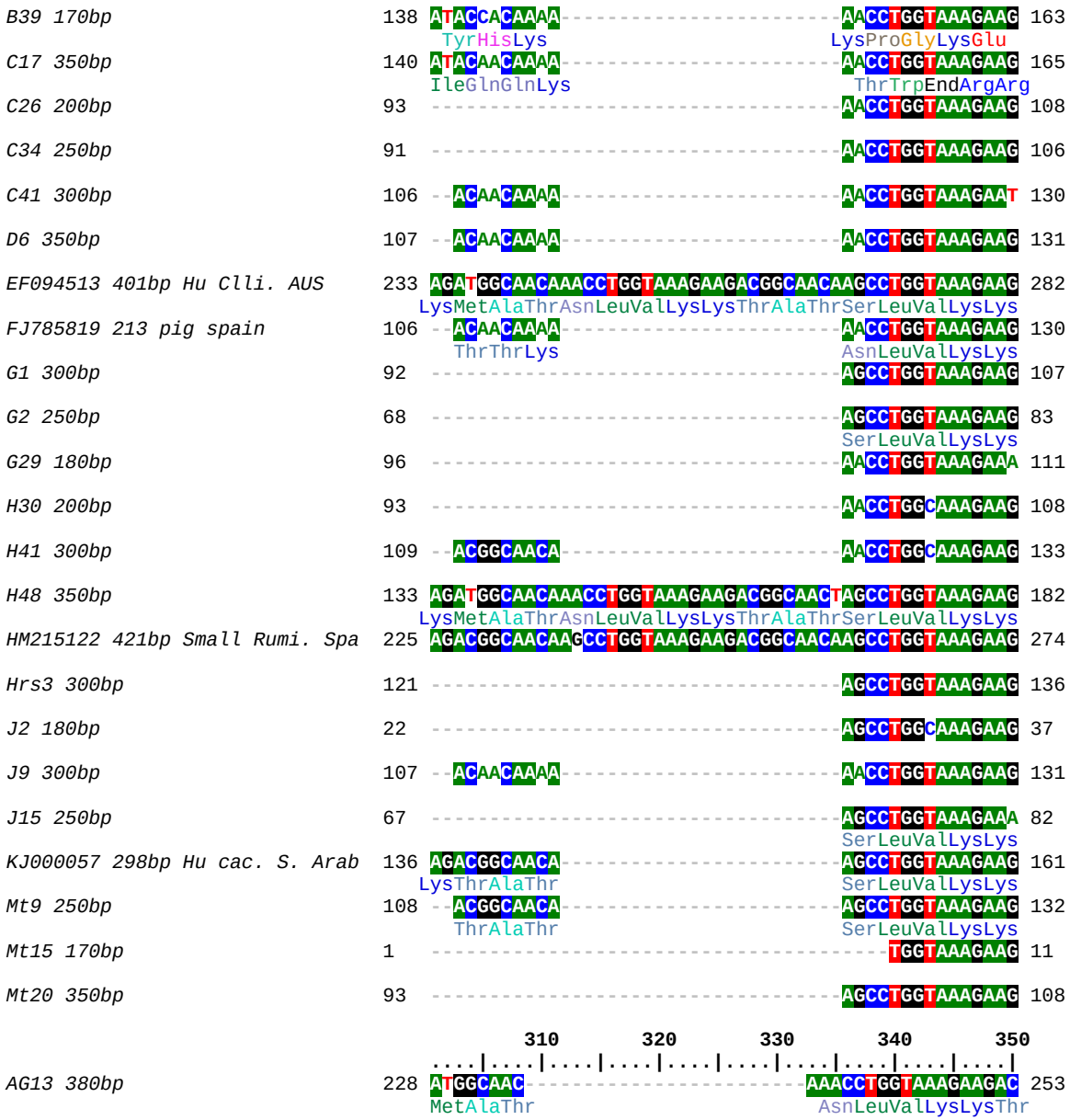
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H48 350bp 133 AGATGGCAACAAACCTGGTAAAGAAGACGGCAACTAGCCTGGTAAAGAAG 182
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Hrs3 300bp 121 AGCCTGGTAAAGAAG 136
J2 180bp 22 AGCCTGGCAAAGAAG 37
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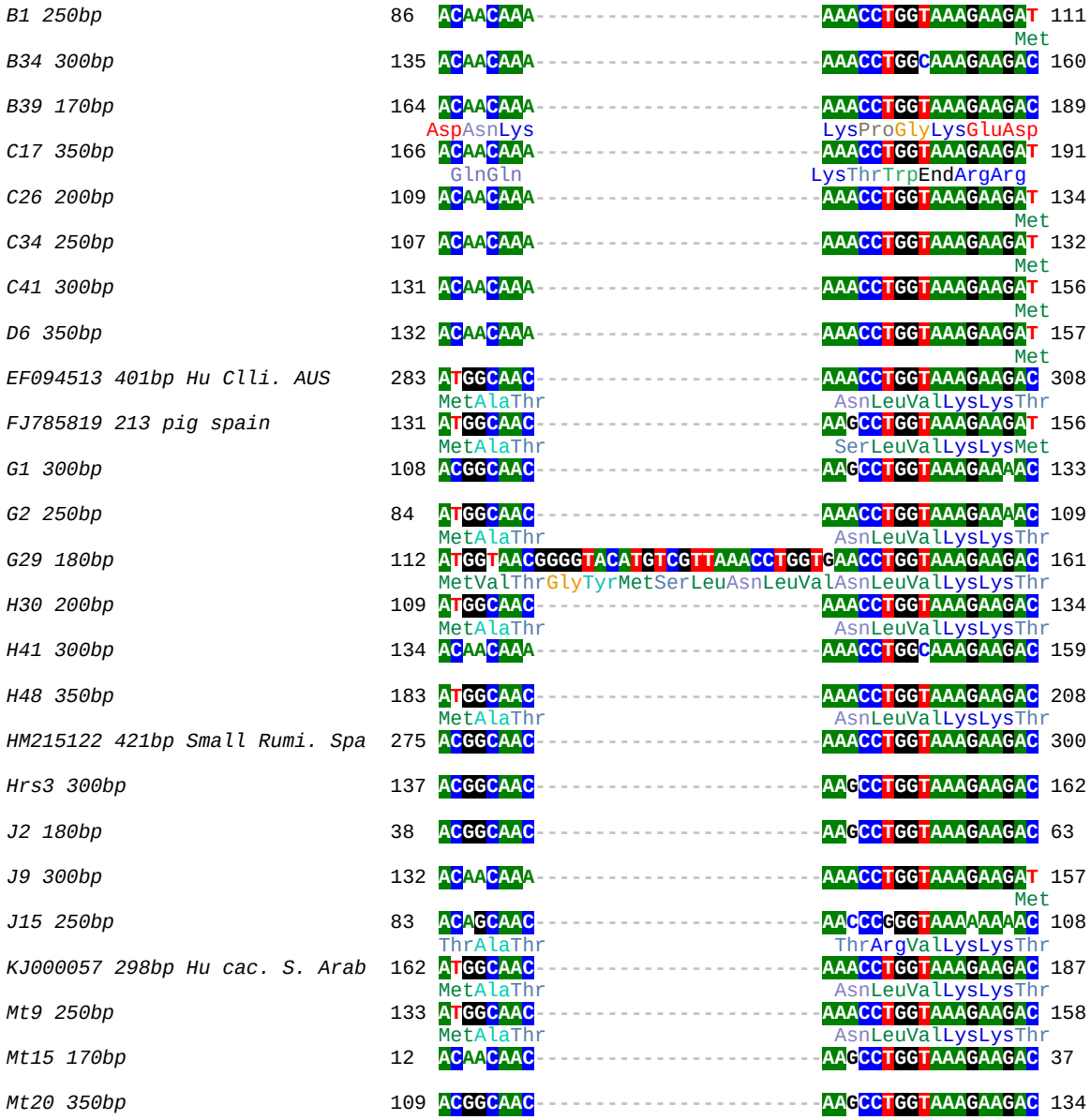
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Met
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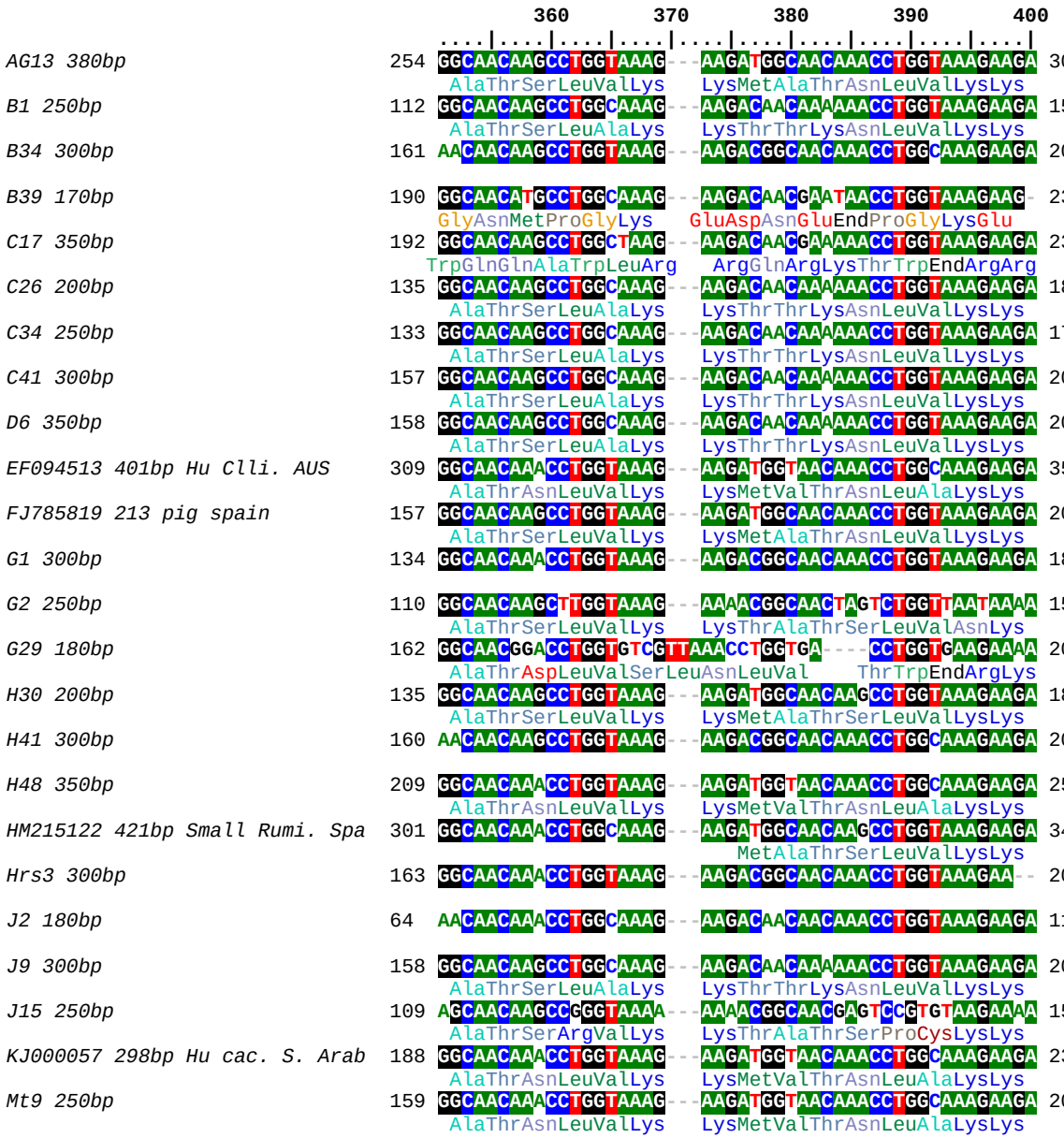
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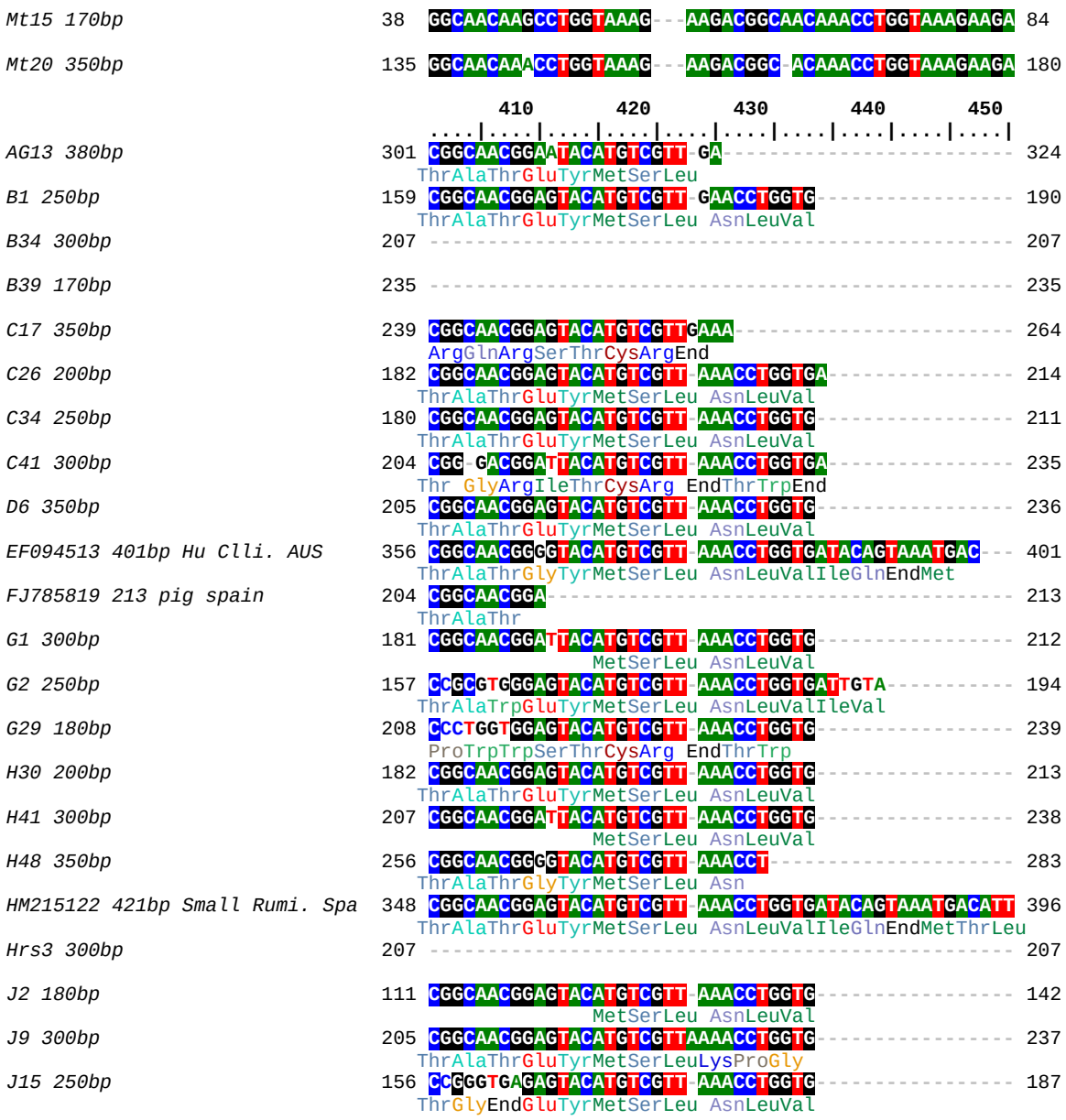
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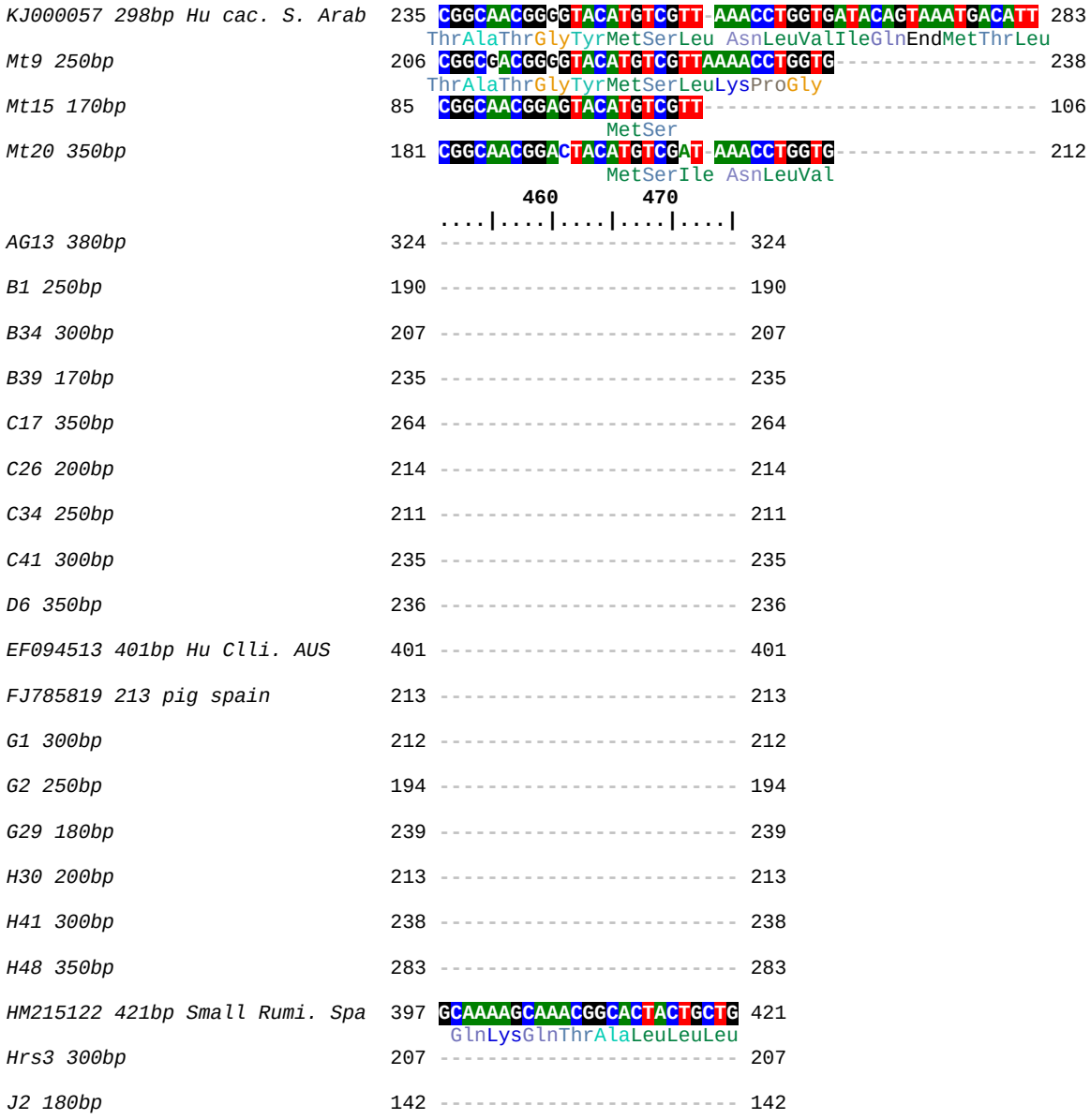
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Mt20 350bp 181 CGGCAACGGACTACATGTCGAT-AAACCTGGTG---------- 212
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B34 300bp 207 ---------- 207
B39 170bp 235 ---------- 235
C17 350bp 264 ---------- 264
C26 200bp 214 ---------- 214
C34 250bp 211 ---------- 211
C41 300bp 235 ---------- 235
D6 350bp 236 ---------- 236
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FJ785819 213 pig spain 213 ---------- 213
G1 300bp 212 ---------- 212
G2 250bp 194 ---------- 194
G29 180bp 239 ---------- 239
H30 200bp 213 ---------- 213
H41 300bp 238 ---------- 238
H48 350bp 283 ---------- 283
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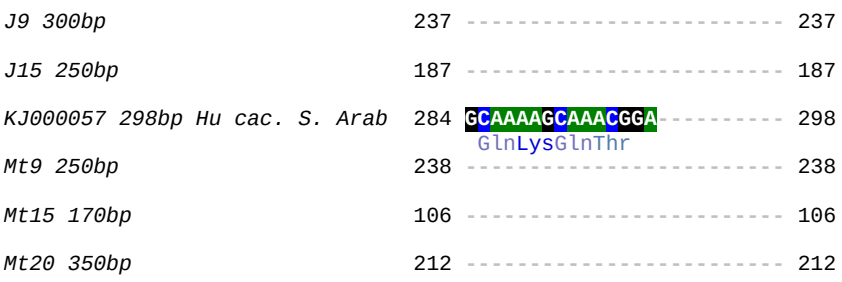
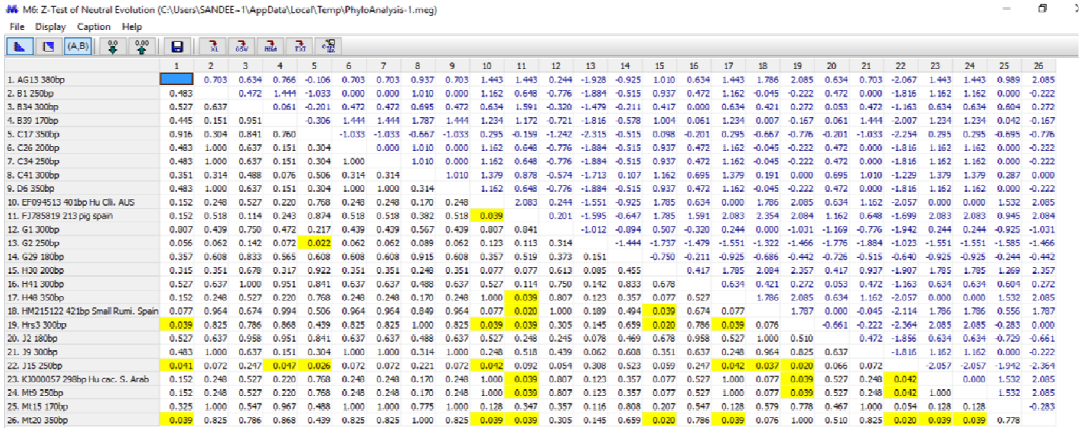


Fig. 44: Multiple sequence alignment of *spa-X*gene sequences of present study with reference sequences of NCBI database.

Table 40: Codon-based Test of Neutrality for multiple alignment analysis between sequences of present study with reference sequences of NCBI database.



M6: Z-Test of Neutral Evolution (C:\Users\SANDEE~1\AppData\Local\Temp\PhyloAnalysis-1.meg)

File Display Caption Help

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1. AG13 380bp		0.703	0.634	0.766	-0.106	0.703	0.703	0.937	0.703	1.443	1.443	0.244	-1.928	-0.925	1.010	0.634	1.443	1.786	2.085	0.634	0.703	-2.067	1.443	1.443	0.989	2.085
2. B1 250bp	0.483		0.472	1.444	-1.033	0.000	0.000	1.010	0.000	1.162	0.648	-0.776	-1.884	-0.515	0.937	0.472	1.162	-0.045	-0.222	0.472	0.000	-1.816	1.162	1.162	0.000	-0.222
3. B34 300bp	0.527	0.637		0.061	-0.201	0.472	0.472	0.695	0.472	0.634	1.591	-0.320	-1.479	-0.211	0.417	0.000	0.634	0.421	0.272	0.053	0.472	-1.163	0.634	0.634	0.604	0.272
4. B39 170bp	0.445	0.151	0.951		-0.306	1.444	1.444	1.787	1.444	1.234	1.172	-0.721	-1.816	-0.578	1.004	0.061	1.234	0.007	-0.167	0.061	1.444	-2.007	1.234	1.234	0.042	-0.167
5. C17 350bp	0.916	0.304	0.841	0.760		-1.033	-1.033	-0.667	-1.033	0.795	-0.159	-1.242	-2.315	-0.515	0.098	-0.201	0.795	-0.667	-0.776	-0.201	-1.033	-2.254	0.795	0.795	-0.695	-0.776
6. C26 200bp	0.483	1.000	0.637	0.151	0.304		0.000	1.010	0.000	1.162	0.648	-0.776	-1.884	-0.515	0.937	0.472	1.162	-0.045	-0.222	0.472	0.000	-1.816	1.162	1.162	0.000	-0.222
7. C34 250bp	0.483	1.000	0.637	0.151	0.304	1.000		1.010	0.000	1.162	0.648	-0.776	-1.884	-0.515	0.937	0.472	1.162	-0.045	-0.222	0.472	0.000	-1.816	1.162	1.162	0.000	-0.222
8. C41 300bp	0.351	0.314	0.488	0.076	0.506	0.314	0.314		1.010	1.379	0.878	-0.574	-1.713	0.107	1.162	0.695	1.379	0.191	0.000	0.695	1.010	-1.229	1.379	1.379	0.287	0.000
9. D6 350bp	0.483	1.000	0.637	0.151	0.304	1.000	1.000	0.314		1.162	0.648	-0.776	-1.884	-0.515	0.937	0.472	1.162	-0.045	-0.222	0.472	0.000	-1.816	1.162	1.162	0.000	-0.222
10. EF094513 401bp Hu Cli. AUS	0.152	0.248	0.527	0.220	0.768	0.248	0.248	0.170	0.248		2.083	0.244	-1.551	-0.925	1.785	0.634	0.000	1.786	2.085	0.634	1.162	-2.057	0.000	0.000	1.532	2.085
11. FJ785819 213 pig spain	0.152	0.518	0.114	0.243	0.874	0.518	0.518	0.382	0.518	0.039		0.201	-1.595	-0.647	1.785	1.591	2.083	2.354	2.084	1.162	0.648	-1.699	2.083	2.083	0.945	2.084
12. G1 300bp	0.807	0.409	0.750	0.472	0.217	0.409	0.409	0.567	0.409	0.807	0.841		-1.012	-0.894	0.507	-0.320	0.244	0.000	-1.031	-1.169	-0.776	-1.942	0.244	0.244	-0.925	-1.031
13. G2 250bp	0.056	0.062	0.142	0.072	0.022	0.062	0.062	0.089	0.062	0.123	0.113	0.314		-1.444	-1.737	-1.479	-1.551	-1.322	-1.466	-1.776	-1.884	-1.023	-1.551	-1.551	-1.585	-1.466
14. G29 180bp	0.357	0.608	0.833	0.565	0.608	0.608	0.608	0.915	0.608	0.357	0.519	0.373	0.151		-0.750	-0.211	-0.925	-0.686	-0.442	-0.726	-0.515	-0.640	-0.925	-0.925	-0.244	-0.442
15. H20 200bp	0.245	0.351	0.678	0.217	0.922	0.351	0.351	0.248	0.351	0.077	0.077	0.613	0.085	0.455		0.417	1.785	2.084	2.357	0.417	0.927	-1.907	1.785	1.785	1.269	2.357
16. H41 300bp	0.527	0.637	1.000	0.951	0.841	0.637	0.637	0.488	0.637	0.527	0.114	0.750	0.142	0.833	0.678		0.634	0.421	0.272	0.053	0.472	-1.163	0.634	0.634	0.604	0.272
17. H48 350bp	0.152	0.248	0.527	0.220	0.768	0.248	0.248	0.170	0.248	1.000	0.038	0.807	0.123	0.357	0.077	0.527		1.786	2.085	0.634	1.162	-2.057	0.000	0.000	1.532	2.085
18. HM215122 421bp Small Rumi. Spain	0.077	0.964	0.674	0.994	0.506	0.964	0.964	0.849	0.964	0.077	0.020	1.000	0.189	0.494	0.039	0.674	0.077		1.787	0.000	-0.045	-2.114	1.786	1.786	0.556	1.787
19. Hrs3 300bp	0.039	0.825	0.786	0.868	0.439	0.825	0.825	1.000	0.825	0.039	0.038	0.305	0.145	0.659	0.020	0.786	0.039	0.076		-0.661	-0.222	-2.364	2.085	2.085	-0.283	0.000
20. J2 180bp	0.527	0.637	0.958	0.951	0.841	0.637	0.637	0.488	0.637	0.527	0.248	0.245	0.078	0.469	0.678	0.958	0.527	1.000	0.510		0.472	-1.896	0.634	0.634	-0.729	-0.661
21. J9 300bp	0.483	1.000	0.637	0.151	0.304	1.000	1.000	0.314	1.000	0.248	0.518	0.439	0.062	0.608	0.351	0.637	0.248	0.964	0.825	0.637		-1.816	1.162	1.162	0.000	-0.222
22. J15 250bp	0.041	0.072	0.247	0.047	0.026	0.072	0.072	0.221	0.072	0.042	0.092	0.054	0.308	0.523	0.059	0.247	0.042	0.037	0.020	0.066	0.072		-2.057	-2.057	-1.942	-2.364
23. KJ000057 298bp Hu cac. S. Arab	0.152	0.248	0.527	0.220	0.768	0.248	0.248	0.170	0.248	1.000	0.038	0.807	0.123	0.357	0.077	0.527	1.000	0.077	0.039	0.527	0.248	0.042		0.000	1.532	2.085
24. Mt9 250bp	0.152	0.248	0.527	0.220	0.768	0.248	0.248	0.170	0.248	1.000	0.038	0.807	0.123	0.357	0.077	0.527	1.000	0.077	0.039	0.527	0.248	0.042	1.000		1.532	2.085
25. Mt15 170bp	0.325	1.000	0.547	0.967	0.488	1.000	1.000	0.775	1.000	0.128	0.547	0.357	0.116	0.808	0.207	0.547	0.128	0.579	0.778	0.467	1.000	0.054	0.128	0.128		-0.283
26. Mt20 350bp	0.039	0.825	0.786	0.868	0.439	0.825	0.825	1.000	0.825	0.039	0.039	0.305	0.145	0.659	0.020	0.786	0.039	0.076	1.000	0.510	0.825	0.020	0.039	0.039	0.778	

The probability of rejecting the null hypothesis of strict-neutrality ($d_N - d_S$) (below diagonal) is shown. Values of *P* less than 0.05 are considered significant at the 5% level and are yellow highlighted. The test statistic ($d_N - d_S$) is shown above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. Analyses were conducted using the Nei-Gojobori method. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 16 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

E. Plasminogen activator/ Staphylokinase (*sak* gene)

The staphylokinase (SAK) is a one of the important complement inhibitory and enterotoxins proteins. Staphylokinase not only functions to prevent host bactericidal activities but also has role in activation of host plasminogen thus known as plasminogen activator. Expression of staphylokinase governed by the *sak* gene. Therefore, it is important to characterize *S. aureus* isolates in regards to *sak* gene from various sources.

Out of 157 isolates, 79 (50.3%) isolates were found to be positive for *sak* gene with single amplicon of 403bp (Fig. 24). Most of the human (80.0%) and all (100.0%) meat piece isolates were positive while most of animal origin isolates were negative for *sak* gene as mentioned in Table 41.

The present study supported earlier reports by many researchers who also observed that veterinary sources commonly lacked SAK production and *sak* gene was less common in animal origin isolates while majority of human disease associated isolates carried this gene (Jin *et al.*, 2003; Sung *et al.*, 2008; Stastkova *et al.*, 2011; Gomez *et al.*, 2014; Sarrou *et al.*, 2015).

Similar to our results, Monecke *et al.* (2007) reported 89.1% isolates without *sak* gene from 128 bovine mastitis isolates. Sung *et al.* (2008) also observed that 134 (83%) human and 11 (20 %) animal isolates were positive for *sak* gene. Similarly, Ikawaty *et al.* (2010) reported that only one isolate was found to be positive for *sak* genes from 76 isolates of bovine mastitis.

Our results are in complete agreement to those of Stastkova *et al.* (2011) who reported that 100% isolates positive for *sak* gene from retail meat products from year 2005 to 2008. Contrary to our results, Cuny *et al.* (2013) reported none of the human and animal origin isolate positive for *sak* gene while Sarrou *et al.* (2015) reported that all livestock associated isolates were negative while three isolates from human origin were positive for *sak* genes from nine livestock-associated and eight human isolates, respectively.

Table 41: Detection of Staphylokinase factor associated gene among *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Plasminogen activator associated gene (%)	
			sak (403bp)	
			P	N
1.	Human	35	28 (80.0)	7 (20.0)[a]
2.	Meat piece	20	20 (100.0)	0 (0.0)
3.	Horse	3	2 (66.7)	1 (33.3)
4.	Pig	2	1 (50.0)	1 (50.0)
5.	Camel	8	2 (25.0)	6 (75.0)
6.	Dog	6	3 (50.0)	3 (50.0)
7.	Sheep	6	1 (16.7)	5 (83.3)
8.	Buffalo	21	9 (42.9)	12 (57.1)
9.	Goat	28	8 (28.6)	20 (71.4)
10.	Cattle	28	5 (17.9)	23 (82.1)
Total		**157**	**79 (50.3)**	**78 (49.7)**

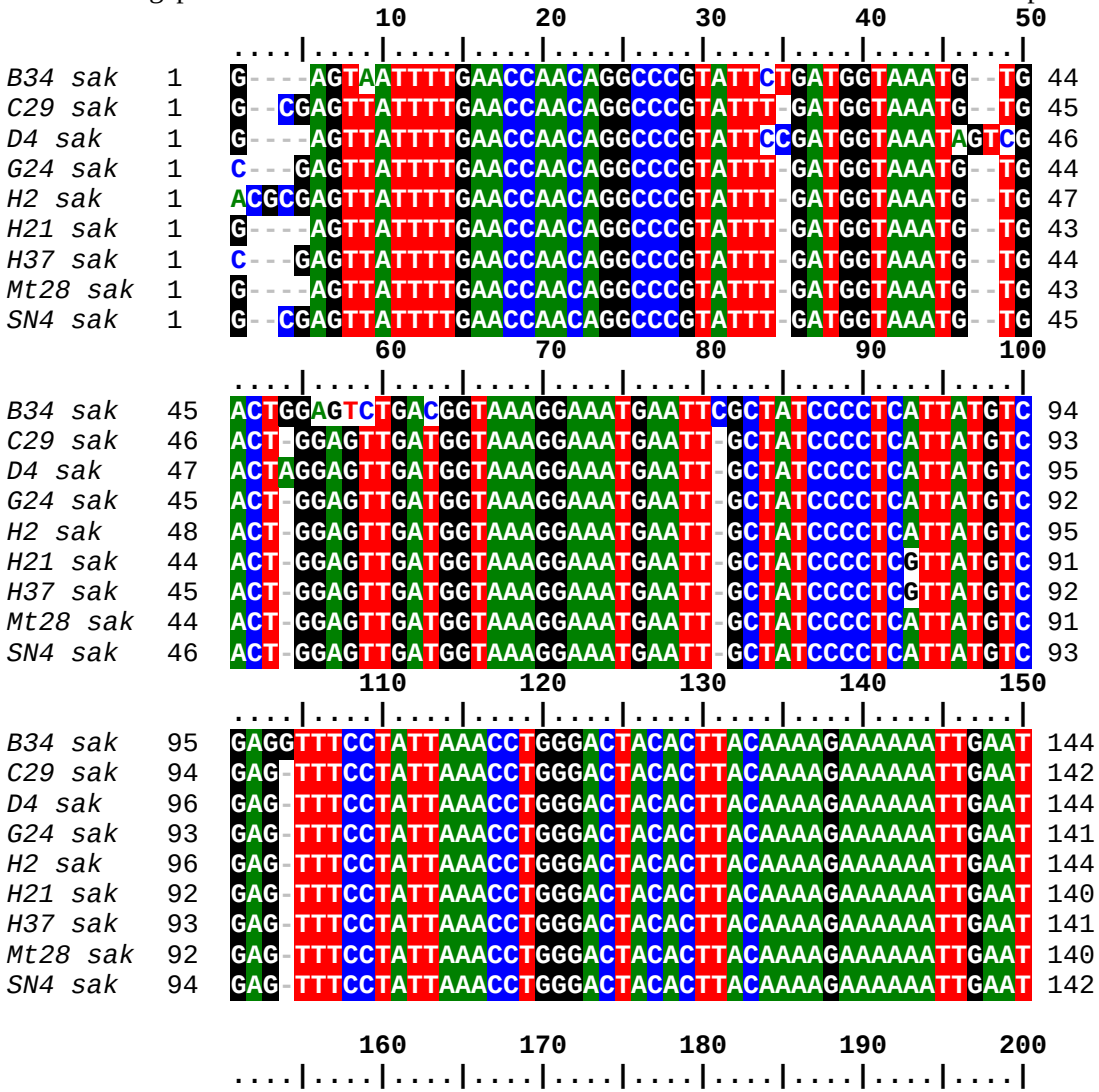
Abbreviations:- P- Positive, N- Negative
Superscript:- a- Human origin isolates negative for *sak* gene (H5, H7, H14, H16, H22, H25 and H44)

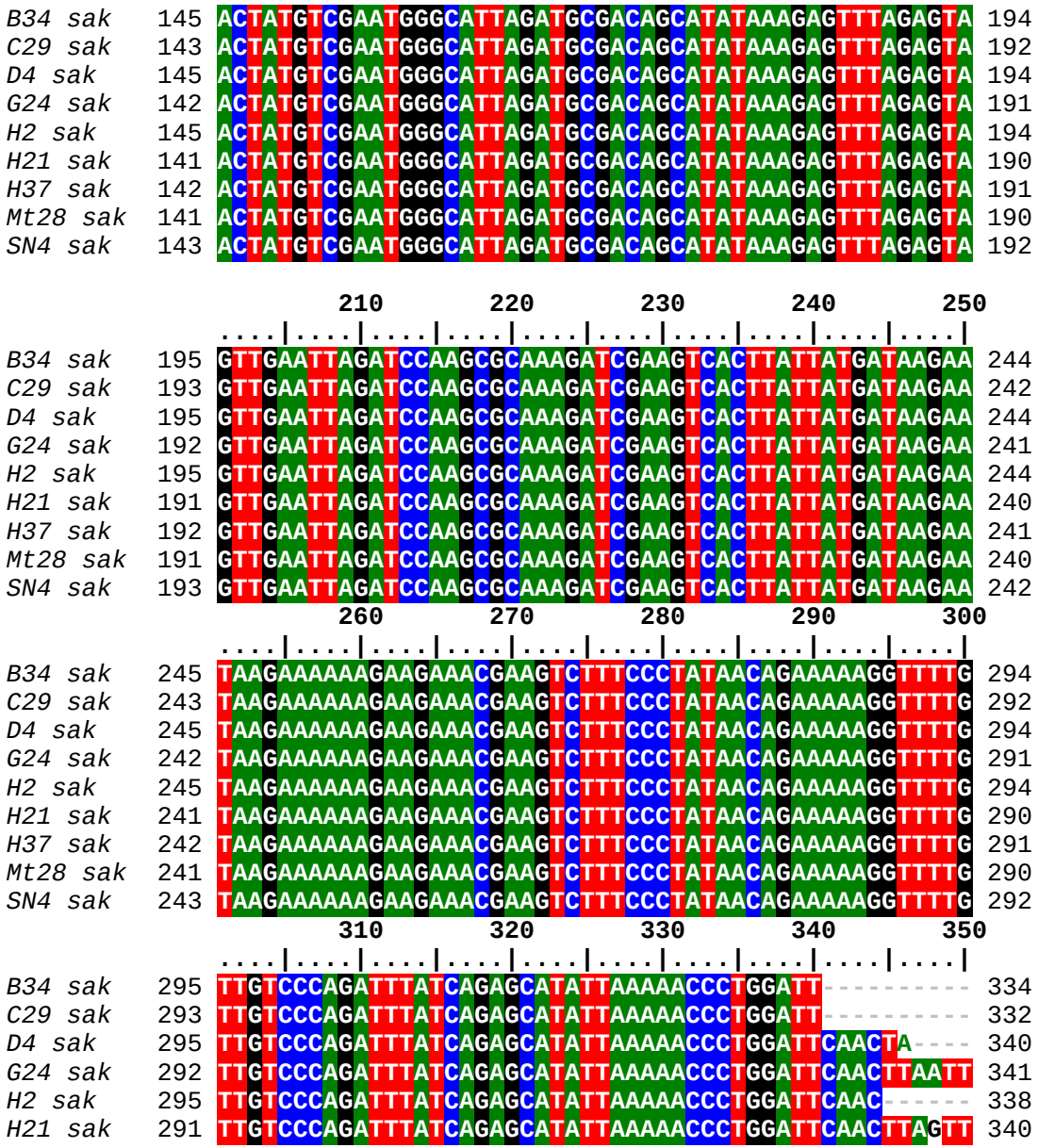
***sak* gene sequence analysis**

The nine *sak* gene positive isolate from this study, namely H2, H21 and H37 from human, Mt28 from meat piece, D4 from dog, SN4 from sheep, B34 from buffalo, G24 from goat and C29 from cattle were sequenced. First of all the sequences were BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed as *sak* gene sequences of *S. aureus* strains.

The sequence analysis of *sak* gene revealed that total 21 nucleotide variations, were observed in *sak* gene sequences of present study. The sequences of B34 (buffalo) and D4 (dog) were found more closer in compare to others. However, these two sequences (B34 and D4) were carried most of variations from other studied sequences, only one nucleotide variation adenine replaced by guanine at 93th aligned nucleotide position was found in H21 and H37 human isolate. In analysis, it was found that, B34 isolate carried A, G, T, C, C, C and G while all other sequences carried G, A, G, T, T and two gaps at 56th, 57th, 58th, 59th, 64th, 81th and 104th nucleotide

positions respectively. The D4 isolate carried G, T and C while all others carried two gaps and T at 47th, 48th and 49 nucleotide positions respectively. Four nucleotide positions (9th, 34th, 35th and 46th) were found variable in B34 (A, C, T and G) isolate, D4 (T, C, C and A) isolate and all other (T, T, and two gaps) isolates respectively. Some other gaps and insertions with nucleotide variations were found from nucleotide position at 341 to 365 as depicting in figure 46.

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C29 sak 143 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 192
D4 sak 145 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 194
G24 sak 142 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 191
H2 sak 145 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 194
H21 sak 141 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 190
H37 sak 142 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 191
Mt28 sak 141 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 190
SN4 sak 143 ACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAGTA 192
210 220 230 240 250
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G24 sak 192 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 241
H2 sak 195 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 244
H21 sak 191 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 240
H37 sak 192 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 241
Mt28 sak 191 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 240
SN4 sak 193 GTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAGAA 242
260 270 280 290 300
B34 sak 245 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 294
C29 sak 243 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 292
D4 sak 245 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 294
G24 sak 242 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 291
H2 sak 245 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 294
H21 sak 241 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 290
H37 sak 242 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 291
Mt28 sak 241 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 290
SN4 sak 243 TAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTTTG 292
310 320 330 340 350
B34 sak 295 TTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATT---------- 334
C29 sak 293 TTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATT---------- 332
D4 sak 295 TTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATTCAACTA---- 340
G24 sak 292 TTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATTCAACTTAATT 341
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H21 sak 291 TTGTCCCAGATTTATCAGAGCATATTAAAAACCCTGGATTCAACTTAGTT 340

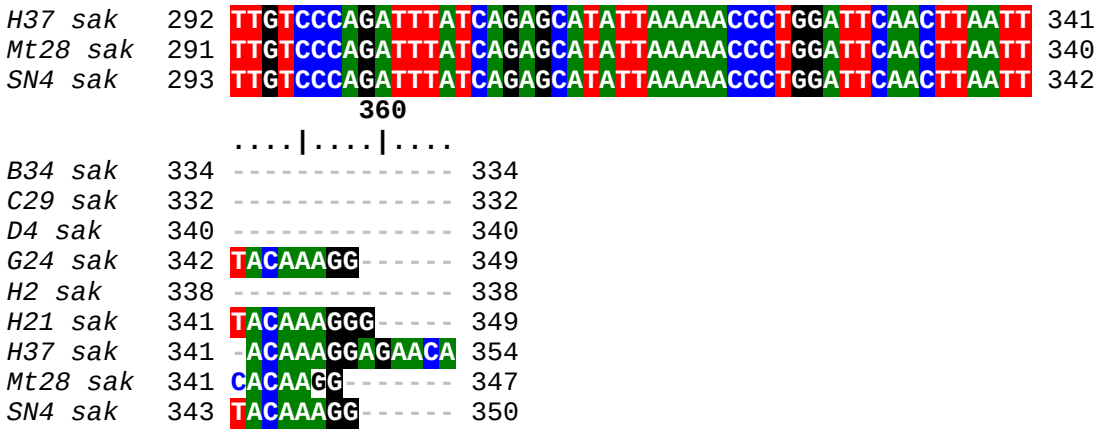


Fig. 46:The sequence analysis *sak* gene obtained in present study.

These variations were found to be non-significant ($p > 0.05$) during analysis of codon based test of neutrality. Three distinct clusters were found in phylogenetic tree analysis of *sak* gene sequences of the present study, first cluster was including sequence of buffalo (B34) isolate separated with 0.012 scaled distance, second cluster comprising dog (D4) isolate separated with 0.006 scaled distance and third cluster was made up by remaining other seven isolates (Mt28, SN4, C29, H21, H2, G24 and H37) as depicting in figure 47.

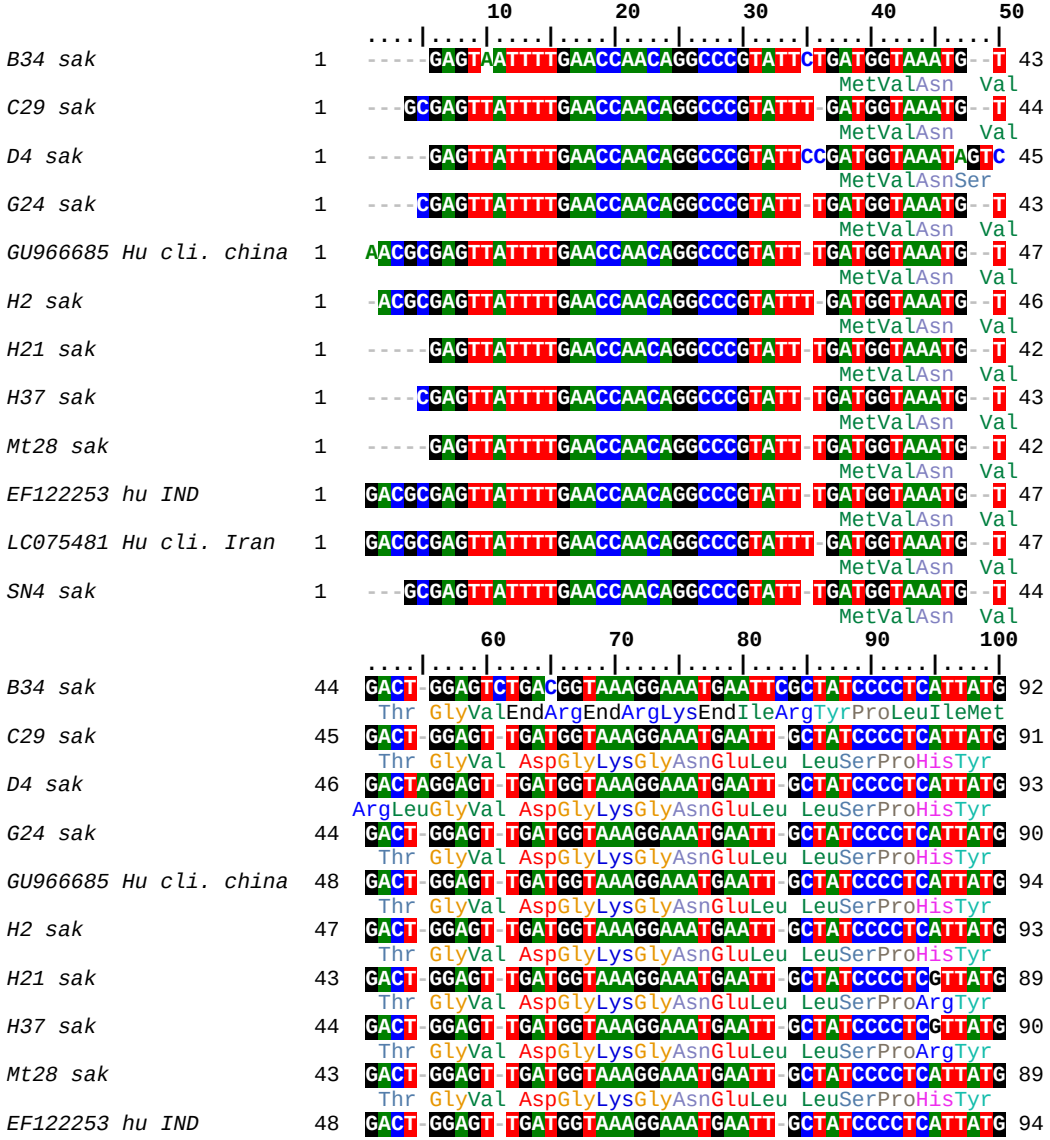
Multiple sequence alignment of present study sequences was conducted with reference sequences of NCBI database to find similarities and variations between our sequences and reference NCBI sequences. For this purpose, three sequences of NCBI database namely Sequence ID- EF122253 from human isolate of India, Sequence ID- GU966685 from human clinical isolate from China and Sequence ID- LC075481 from human isolate of Iran were choose according to availability in the database and geographic variations of bacterial isolation.

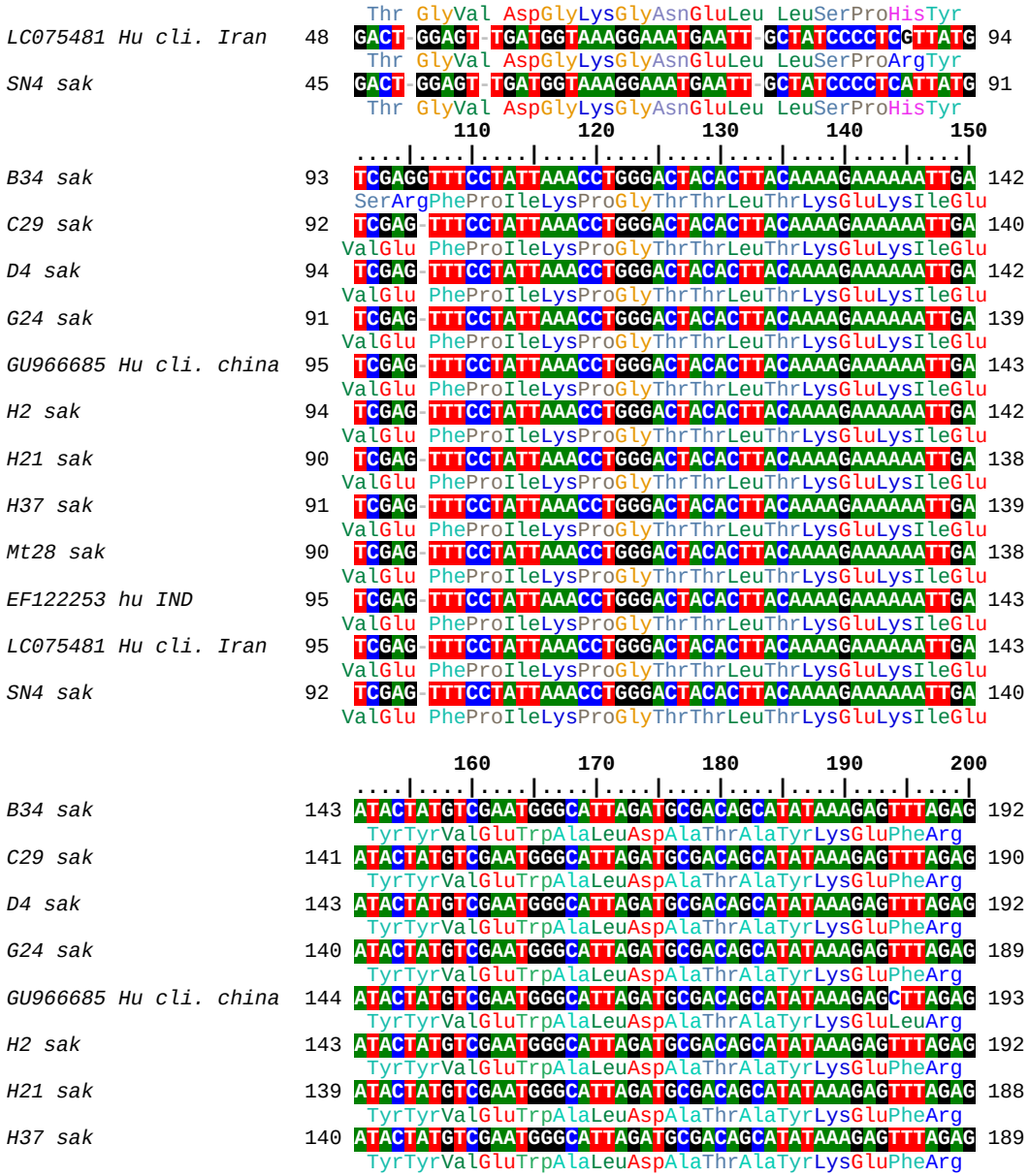
The multiple sequence alignment of all sequences revealed very close similarities (except B34 and D4) with all aligned sequences. Only very few variations were detected *i.e.* ten nucleotide variations were found in the database sequence LC075481 when compared with our sequences present study sequences except that for H21 and H37 sequence. Less than four variation were recorded in GU966685 database sequence and negligible differences were recorded in EF122253 strain of India when compared with our sequences (Fig. 48). The statistics for codon-based test of neutrality between sequences *i.e.* the probability of rejecting the null hypothesis of strict-neutrality $d_N = d_S$ (d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively) and non-significant ($p > 0.05$) variations were found between all aligned sequences.

Phylogenetic tree analysis of multiple sequence alignment of *sak* gene sequences revealed three clusters as described earlier. All three database sequences were comprised in third cluster, this third cluster can sub classify with minor differences such as H21, H37 and LC075481 database sequences are closer with 0.0018 scaled distance in compare to other sequences and GU966685 database sequence separated with 0.0015 scaled distance. The database sequence EF122253 strain of India was closer with our remaining sequences (SN4, Mt28, H2, G24 and C29) with scaled distance 0.0015 as depicted in figure 49.

Multiple sequence alignment and phylogenetic tree analysis indicated that nucleotide variations not only depend on source of isolation but also on geographic variations. The close similarities between animal and human isolates sequences in the present study may indicates close interaction and exchange of bacterial genetic

material between human, animals and other sources (meat piece). Though, previous studies and our results reported the less prevalence of *sak* gene in animal isolates but in sequence analysis, in our study did not detect significant nucleotide variations between human and animal isolates in *sak* gene sequences.

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LC075481 Hu cli. Iran 48 GACT-GGAGT-TGATGGTAAAGGAAATGAATT-GCTATCCCCTCGTTATG 94
Thr GlyVal AspGlyLysGlyAsnGluLeu LeuSerProArgTyr
SN4 sak 45 GACT-GGAGT-TGATGGTAAAGGAAATGAATT-GCTATCCCCTCATTATG 91
Thr GlyVal AspGlyLysGlyAsnGluLeu LeuSerProHisTyr
110 120 130 140 150
B34 sak 93 TCGAGGTTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 142
SerArgPheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
C29 sak 92 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 140
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
D4 sak 94 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 142
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
G24 sak 91 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 139
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
GU966685 Hu cli. china 95 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 143
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H2 sak 94 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 142
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H37 sak 91 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 139
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EF122253 hu IND 95 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 143
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
LC075481 Hu cli. Iran 95 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 143
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
SN4 sak 92 TCGAG-TTTCCTATTAAACCTGGGACTACACTTACAAAAGAAAAAATTGA 140
ValGlu PheProIleLysProGlyThrThrLeuThrLysGluLysIleGlu
160 170 180 190 200
B34 sak 143 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 192
TyrTyrValGluTrpAlaLeuAspAlaThrAlaTyrLysGluPheArg
C29 sak 141 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 190
TyrTyrValGluTrpAlaLeuAspAlaThrAlaTyrLysGluPheArg
D4 sak 143 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 192
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G24 sak 140 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 189
TyrTyrValGluTrpAlaLeuAspAlaThrAlaTyrLysGluPheArg
GU966685 Hu cli. china 144 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGCTTAGAG 193
TyrTyrValGluTrpAlaLeuAspAlaThrAlaTyrLysGluLeuArg
H2 sak 143 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 192
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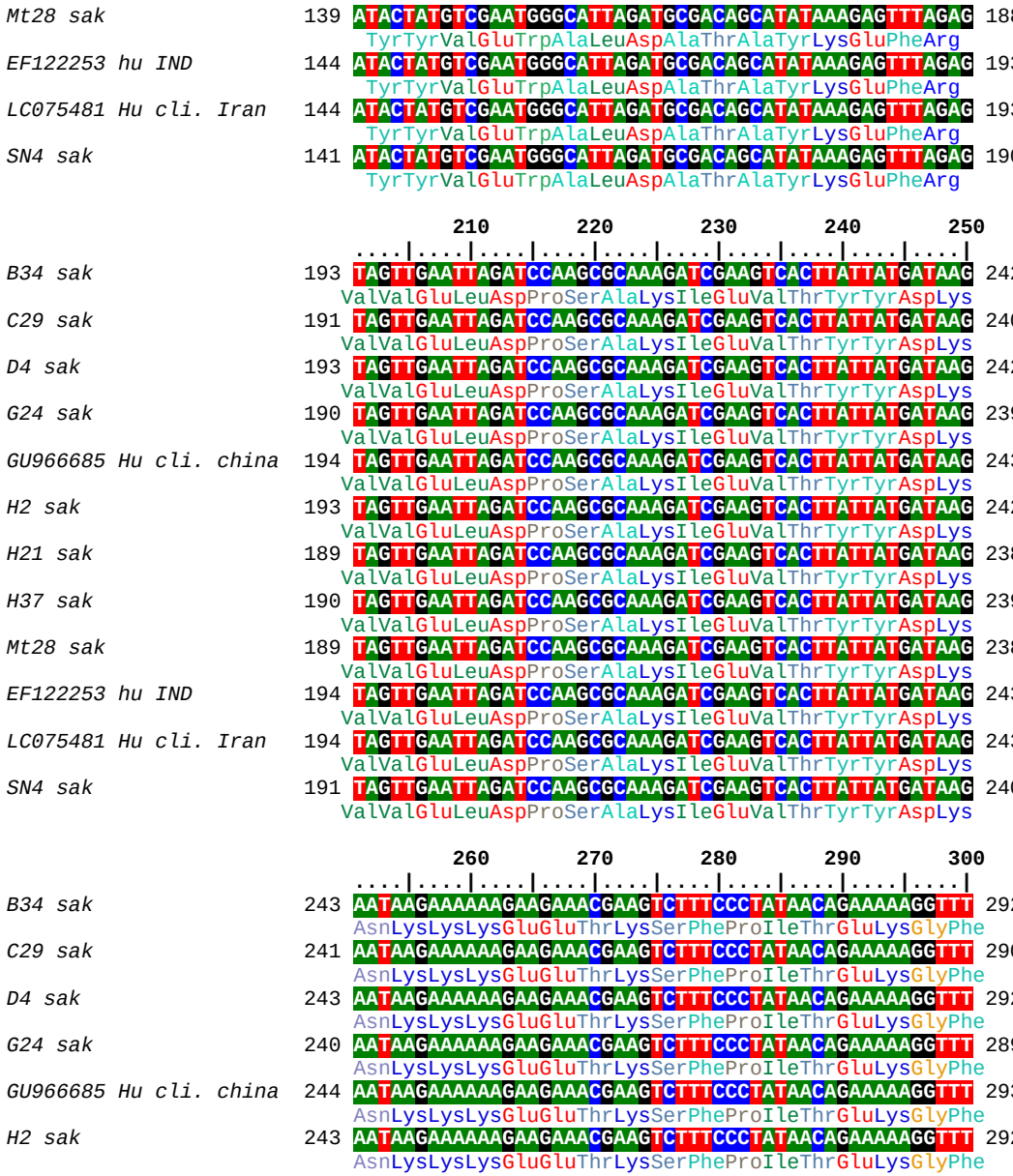
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LC075481 Hu cli. Iran 144 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 193
TyrTyrValGluTrpAlaLeuAspAlaThrAlaTyrLysGluPheArg
SN4 sak 141 ATACTATGTCGAATGGGCATTAGATGCGACAGCATATAAAGAGTTTAGAG 190
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210 220 230 240 250
B34 sak 193 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 242
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D4 sak 193 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 242
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
G24 sak 190 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 239
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
GU966685 Hu cli. china 194 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 243
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
H2 sak 193 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 242
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H21 sak 189 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 238
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H37 sak 190 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 239
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Mt28 sak 189 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 238
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
EF122253 hu IND 194 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 243
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
LC075481 Hu cli. Iran 194 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 243
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
SN4 sak 191 TAGTTGAATTAGATCCAAGCGCAAAGATCGAAGTCACTTATTATGATAAG 240
ValValGluLeuAspProSerAlaLysIleGluValThrTyrTyrAspLys
260 270 280 290 300
B34 sak 243 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 292
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
C29 sak 241 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 290
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
D4 sak 243 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 292
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
G24 sak 240 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 289
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GU966685 Hu cli. china 244 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 293
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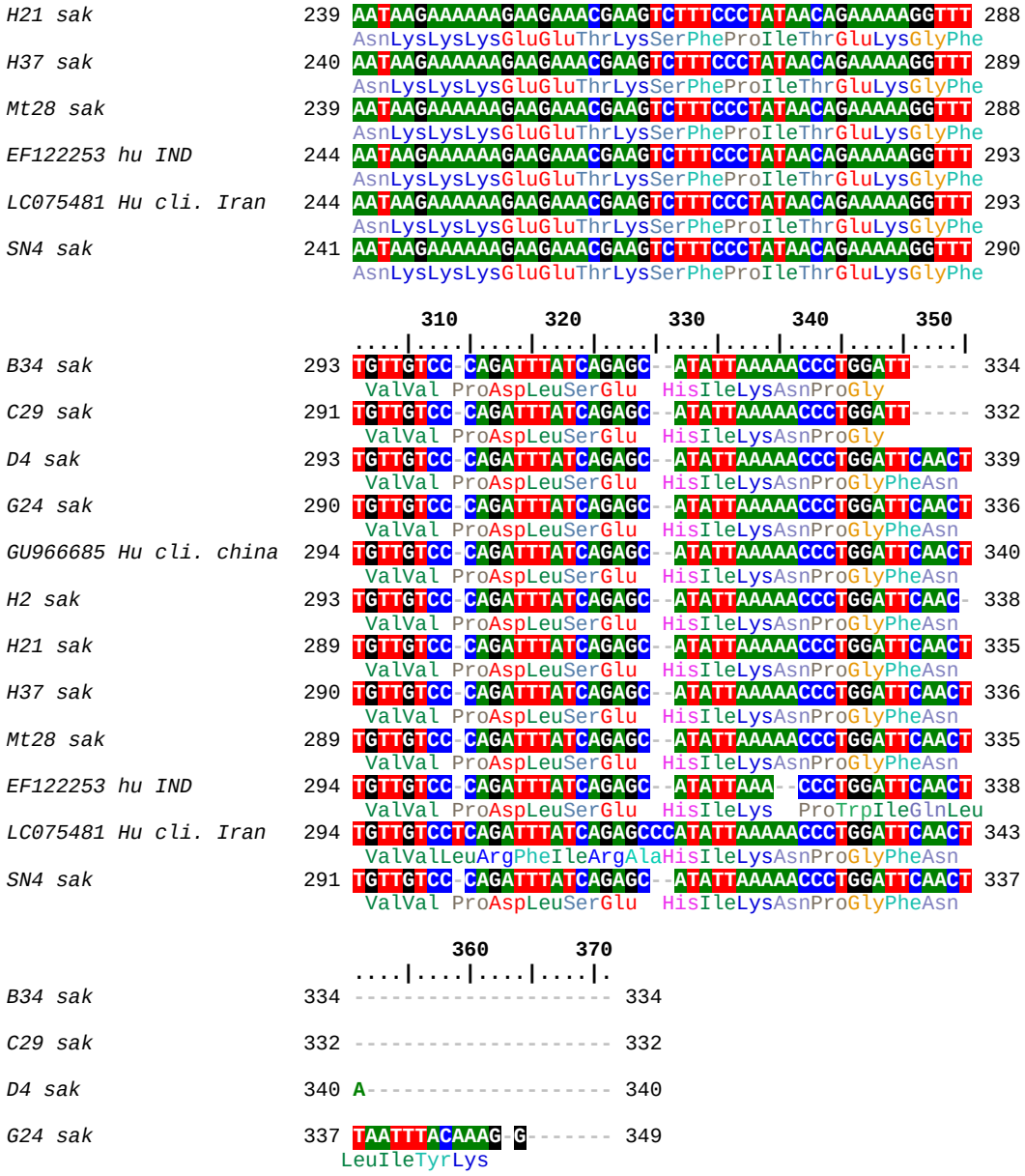
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AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
Mt28 sak 239 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 288
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
EF122253 hu IND 244 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 293
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
LC075481 Hu cli. Iran 244 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 293
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
SN4 sak 241 AATAAGAAAAAAGAAGAAACGAAGTCTTTCCCTATAACAGAAAAAGGTTT 290
AsnLysLysLysGluGluThrLysSerPheProIleThrGluLysGlyPhe
310 320 330 340 350
B34 sak 293 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATT----- 334
ValVal ProAspLeuSerGlu HisIleLysAsnProGly
C29 sak 291 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATT----- 332
ValVal ProAspLeuSerGlu HisIleLysAsnProGly
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ValVal ProAspLeuSerGlu HisIleLysAsnProGlyPheAsn
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GU966685 Hu cli. china 294 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATTCAACT 340
ValVal ProAspLeuSerGlu HisIleLysAsnProGlyPheAsn
H2 sak 293 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATTCAAC 338
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H37 sak 290 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATTCAACT 336
ValVal ProAspLeuSerGlu HisIleLysAsnProGlyPheAsn
Mt28 sak 289 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATTCAACT 335
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LC075481 Hu cli. Iran 294 TGTTGTCCTCAGATTTATCAGAGCCCATATTAAAAACCCTGGATTCAACT 343
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SN4 sak 291 TGTTGTCC-CAGATTTATCAGAGC--ATATTAAAAACCCTGGATTCAACT 337
ValVal ProAspLeuSerGlu HisIleLysAsnProGlyPheAsn
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C29 sak 332 ---------- 332
D4 sak 340 A---------- 340
G24 sak 337 TAATTTACAAAG-G------- 349
LeuIleTyrLys

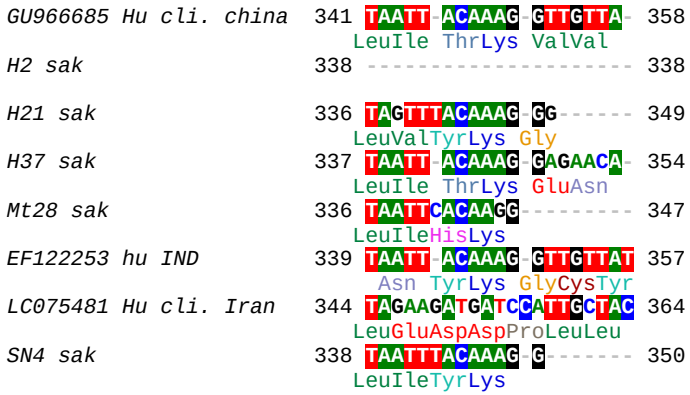


Fig. 48: Multiple sequence alignment of *sak* gene sequences of present study with reference sequences of NCBI database.

F. Toxins (*hla, hlb, hld* and *tst* gene)

Staphylococcus aureus isolates secretes various exotoxins such as haemolysins (α, β, γ, δ), enterotoxins, superantigens (toxic shock syndrome toxin-1/ TSST-1) and leukocidins. Among these, haemolysins (alpha, beta and delta governed by *hla,hlb* and *hld* genes, respectively) are cytolytic pore forming toxins to a variety of host cells which not only lyse cells but depress host immune response also. Toxic shock toxin (*tst* gene) is a pyrogenic toxin superantigens of *S. aureus* that can cause nonspecific T-lymphocyte activation and massive cytokine release characterized by fever, rash, hypotension, multiple-organ-system dysfunction, lethal shock (food poisoning) and desquamation in the host. The immense clinical implications of toxins make it important to study *S. aureus* isolates from various sources in regards todifferenttoxin genes.

Out of the 157 isolates toxin associated genes were reported with variable percentage as mentioned in table 42. The *hla* gene was detected in 155 (98.7%) isolates with single amplicon of 534bp (Fig. 50) while only two isolates (G16 and G45) were found to be negative for *hla* gene.

The *hlb* gene was observed in 94 (59.9%) isolates with single amplicon of 833bp (Fig. 51). Maximum prevalence of *hlb* gene was recorded in camel (87.5%) isolates while minimum presence was found in meat piece (35.0%) isolates.

The *hld* gene was detected in 150 (95.5%) isolates with single amplicon of 111bp (Fig. 52) while seven isolates (H27, H44, Mt11, Mt19, 2J, B10 and C13) were found negative for this gene.

The *tst* gene was detected in only 19 (12.1%) isolates with single amplicon of 350bp (Fig. 53) Maximum eight isolates from human were positive while most of animal isolates were and none isolate from meat piece, horse, pig and dog was positive for this gene. In the toxin associated genes, *hla* gene was detected with maximum prevalence followed by *hld, hlb* and *tst* genes in decreasing order.

Table 42: Detection of Toxin factor associated genes in *S. aureus* isolates.

S. No.	Source of Isolate	Total No. of isolate	Toxins associated genes (%)							
			hla (534bp)		*hlb* (833bp)		*hld* (111bp)		*tst* (350bp)	
			P	N	P	N	P	N	P	N
1.	Human	35	35 (100.0)	0 (0.0)	15 (42.9)	20 (57.1)	33 (94.3)	2 (5.7)	8 (22.9)	27 (77.1)
2.	Meat piece	20	20 (100.0)	0 (0.0)	7 (35.0)	13 (65.0)	18 (90.0)	2 (10.0)	0 (0.0)	20 (100.0)
3.	Horse	3	3 (100.0)	0 (0.0)	2 (66.7)	1 (33.3)	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)
4.	Pig	2	2 (100.0)	0 (0.0)	1 (50.0)	1 (50.0)	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)
5.	Camel	8	8 (100.0)	0 (0.0)	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	1 (12.5)	7 (87.5)
6.	Dog	6	6 (100.0)	0 (0.0)	3 (50.0)	3 (50.0)	6 (100.0)	0 (0.0)	0 (0.0)	6 (100.0)
7.	Sheep	6	6 (100.0)	0 (0.0)	6 (50.0)	0 (0.0)	6 (100.0)	0 (0.0)	3 (50.0)	3 (50.0)
8.	Buffalo	21	21 (100.0)	0 (0.0)	16 (76.2)	5 (23.8)	20 (95.2)	1 (4.8)	4 (19.0)	17 (81.0)
9.	Goat	28	26 (92.9)	2 (7.1)	19 (67.9)	9 (32.1)	28 (100.0)	0 (0.0)	2 (7.1)	26 (92.9)
10.	Cattle	28	28 (100.0)	0 (0.0)	18 (64.3)	10 (35.7)	27 (96.4)	1 (3.6)	1 (3.6)	27 (96.4)
Total		**157**	**155 (98.7)**	**2 (1.3)[a]**	**94 (59.9)**	**63 (40.1)**	**150 (95.5)**	**7 (4.5)[b]**	**19 (12.1)[c]**	**138 (87.9)**

Abbreviations:- P- Positive, N- Negative
Superscript:- a- Isolates negative for *hla* gene (G16 and G45), b- Isolates negative for *hld* gene (H27, H44, Mt11, Mt19, 2J, B10 and C13),
c- Isolates positive for *tst* gene (H1, H2, H10, H12, H15, H21, H25, H28, 15J, SV2, SV3, SV4, B24, B29, B30, B55, AG10, G2 and C2)

Our results corroborated earlier observations of Booth *et al.* (2001) who reported that the *hla, hlb and tst* genes in 100% (128/128), 38% (77/200) and 28.6% (45/157), respectively of the *S. aureus* isolates obtained human clinical infections. Similarly, Jarraud *et al.* (2002) also reported that 100 % isolates carried *hla* and *hld* gene while only 27% and 10% isolates were positive for *tst* and *hlb* gene, respectively among 198 human clinical isolates. Tkalec *et al.* (2015) also reported that 100% strains were positive for *hla* and *hld* genes followed by 74.5 % presence of *hlb* gene and 21.3 % for *tst* gene from 47 *S. aureus* strains from mastitic milk of cows. Similarly, Yang *et al.* (2015) also reported 5.4%, 70.3%, 73.0%, and 70.3% frequency of *tst, hla, hlb,* and *hld* genes, respectively in 37 *S. aureus* isolates from bovine mastitis.

Ohkura *et al.* (2009) reported similar prevalence for *hld* (97.6%) and *hla* (97.1%) genes but contrary finding for *hlb* (84.6%)and *tst* (80.3%) genes in 208 MRSA strains of human origin.

Our result were similar to observations of many researchers who reported high prevalence of *hla* and *hld* gene such as Kim and Lee (2015) and Alfatemi *et al.* (2014) found 98% and 100%, and 93.15% and 84.24% detection of *hla* and *hld* in human isolates, respectively. Argudin *et al.* (2011) and Gharsa *et al.* (2012) reported 100% occurrence of each gene in human clinical isolates and nasal swabs of donkey, respectively. Similarly, Ote *et al.* (2011) also detected 98.7% and 100% *hla* and *hld* genes, respectively in *S. aureus* isolates associated with bovine mastitis. In contrary to our results, Kumar *et al*. (2011) reported more than 90% presence of *hlb* gene while none of the isolate was found positive for *tsst-1* gene in isolates from milk samples.

Many researchers reported lower prevalence of *hlb* and *tst* genes as recorded in the present study. Gharsa *et al.* (2012) detected 70% *hlb* and 12% *tst* gene in *S. aureus* isolates from nasal swabs of donkey, Delgado *et al.* (2011) observed 50% and 13.3% presence of *hlb* and *tst* genes, respectively from human and bovine mastitic milk *S. aureus* isolates. Similarly, Argudin *et al.* (2011) also reported 64.5% and 29.0% occurrence of *hlb* and *tst* gene from human isolates. Likewise, Wang *et al.* (2011), Ariyanti *et al.* (2011) and Coelho *et al.* (2011) also detected 42.6%, 18.18% and 16% prevalence of *hlb,* respectively in isolates from various sources.

Variable results for prevalence of toxin associated genes were also described in previous literature. El-Sayed *et al.* (2006) and Ikawaty *et al.* (2010) recorded 100% occurrence of *hlb* gene in 24 and 76 isolates of *S. aureus* from bovine mastitis, Ando *et al.* (2004) detected 72.5% presence of *tst* gene from 109 human isolates while Kateete *et al.* (2011) reported 10% presence of *tst* gene in 41 human clinical infections.

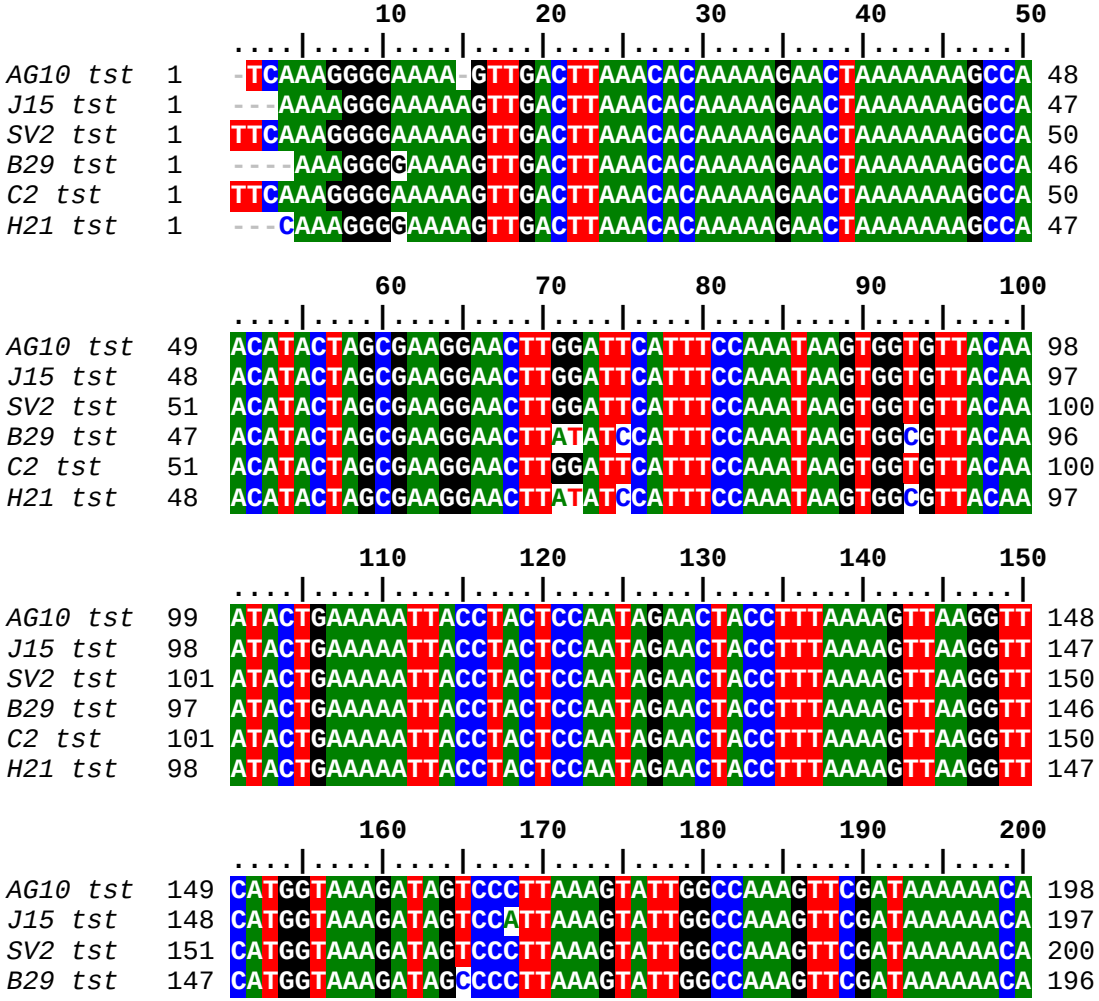
***tst* gene sequence analysis**

Six *tst* gene positive isolates from this study, namely H21 from human, J2 from camel, SV2 from sheep, B29 from buffalo, AG10 from goat and C2 from cattle were subjected to sequencing. First of all the sequences were BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were confirmed as *tst* gene sequences of *S. aureus* strains.

The sequence analysis of *tst* gene revealed that there were 12 nucleotide variations in *tst* gene sequences. The sequences of B29 (buffalo) and H21 (human) were found more closer compared to others. These two sequences (B29 and H21) were carried most of variations from other studied sequences. In other, sequences variations were at only two nucleotide positions *i.e.* one gap at 15^{th} position in AG10 isolate while other sequences having adenine and second variations at 168^{th} position in J15 isolate which having adenine while other have cytosine. In analysis, it was found that, H21 and B29 isolates carried A, G, A T, C, C, C and G while all other sequences

carried G, A, G, G, T, T, T and T at 7th, 11th, 71th, 72th, 76th, 93th, 165th and 288th nucleotide positions respectively. Some other gaps and insertions with nucleotide variations were found from nucleotide position at starting and after 281 nucleotide position as depicting in figure 54.

These variations were found to be non-significant ($p > 0.05$) during analysis of codon based test of neutrality. Two distinct clusters were found in phylogenetic tree analysis of *tst* gene sequences of the present study, first cluster included sequence of buffalo (B29) and human (H21) isolate separated with 0.0128 scaled distance from others and second cluster comprised of two subdivisions of which first included cattle (C2), sheep (SV2) and goat (AG10) separated from second which included camel (J15) with negligible 0.0055 distance as depicting in the figure 55.



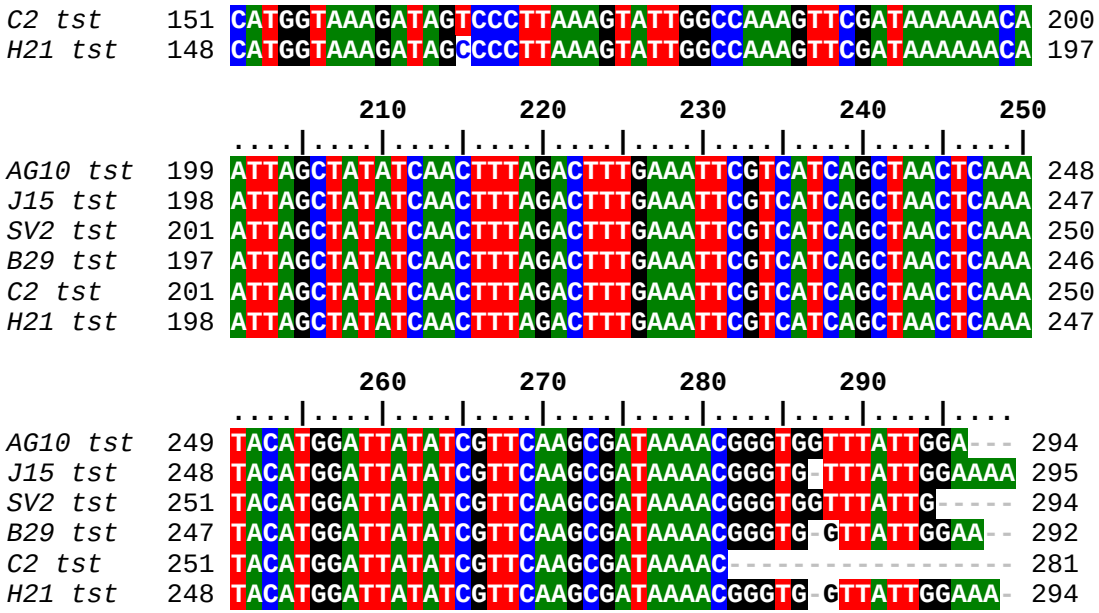


Fig. 54: The sequence analysis *tst* gene obtained from present study.

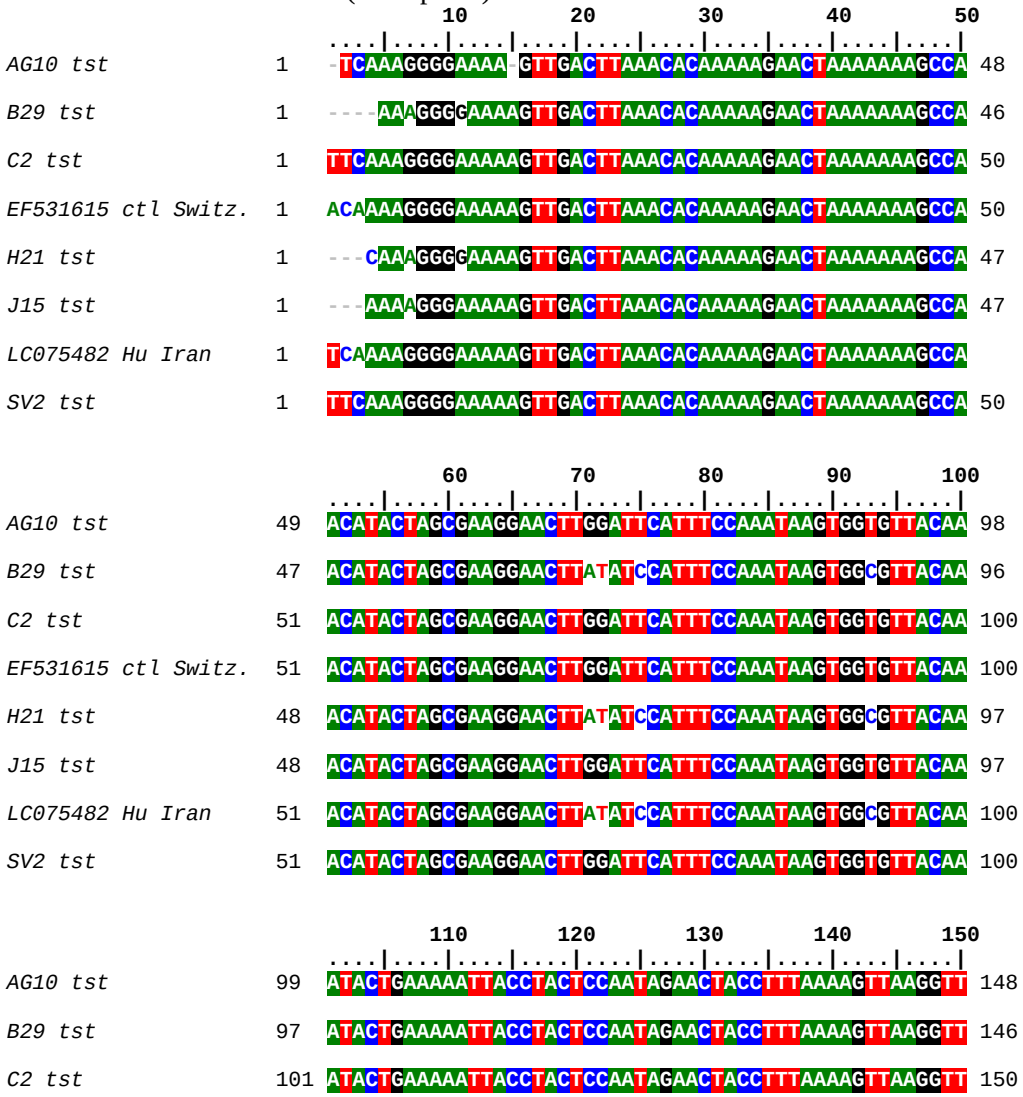
Multiple sequence alignment of present study sequences was conducted with reference sequences of NCBI database to find similarities and variations between our sequences and reference NCBI sequences. For this purpose, two sequences of NCBI database namely Sequence ID- EF531615 (Monecke *et al.*, 2007) cattle isolate sequence from mastitis sample of Switzerland and Sequence ID- LC075482 from human isolate of Iran were chosen according to source of isolation and geographic variations.

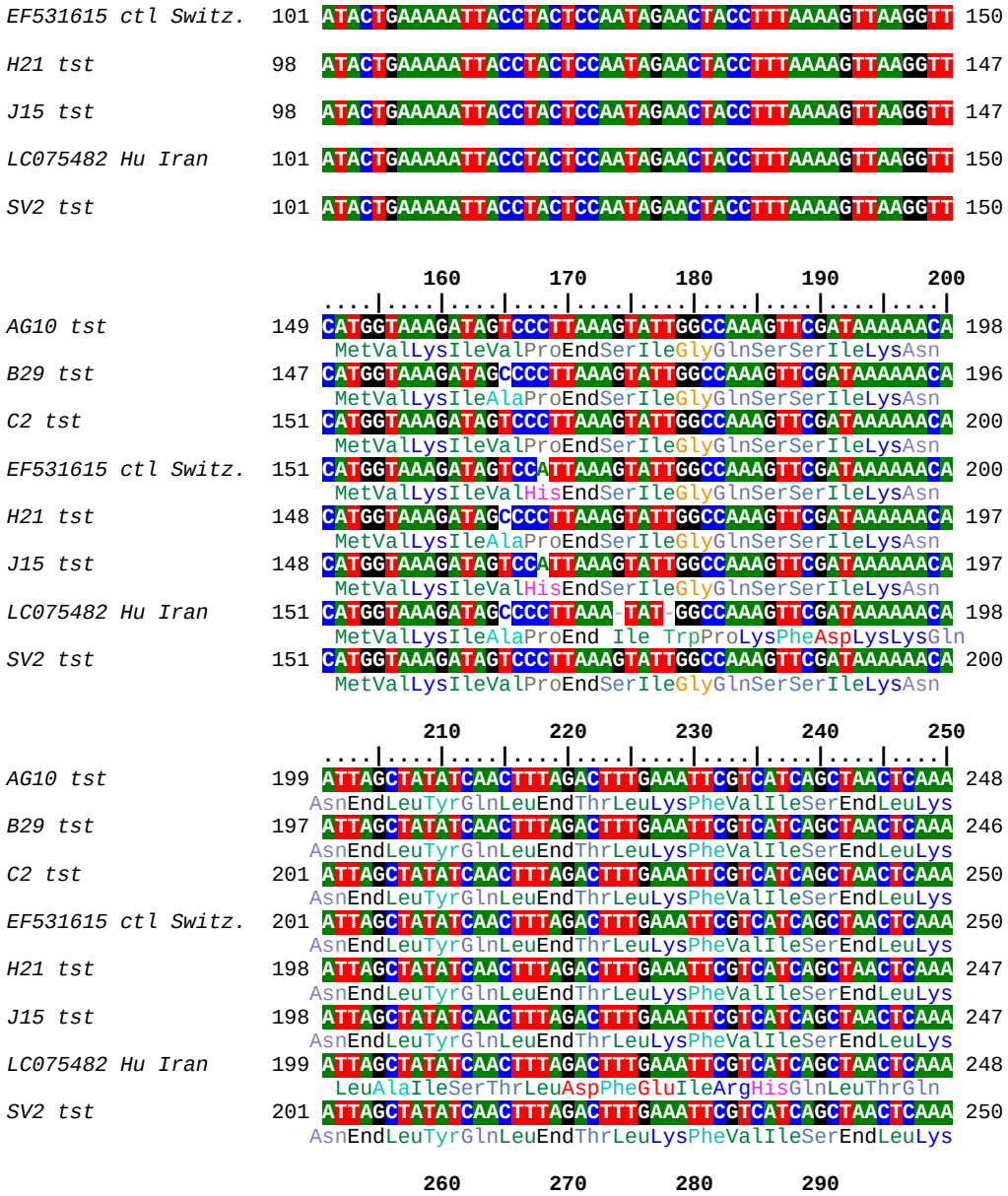
The multiple sequence alignment of all sequences revealed very close similarities among all aligned sequences. Only very few variations were detected *i.e.* five nucleotide variations were found in the database sequence LC075482 with present study sequences except H21 and B29 sequence of this study. While the sequence EF531615 had negligible difference with C2, SV2 J15 and AG10 but sharing similar variations with B29 and H21 sequences as describe earlier (Fig. 56). The statistics for codon-based test of neutrality between sequences conducted *i.e.* the probability of rejecting the null hypothesis of strict-neutrality $d_N = d_S$ (d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively) and non-significant (p>0.05) variations were found between all aligned sequences.

Phylogenetic tree analysis of multiple sequence alignment of *tst* gene sequences revealed two clusters as described earlier. One of the database (LC075482) human sequence included into first cluster with sequences of buffalo (B29) and human (H21) isolate and second cluster comprised two subdivisions first included cattle (C2), sheep (SV2) and goat (AG10) isolate which separated from second subdivision which included camel (J15) and data base sequence (EF531615) of cattle mastitic isolate as depicting in the figure 57.

Multiple sequence alignment and phylogenetic tree analysis indicated that nucleotide variations depending on source of isolation and geographic variations. The sequence of human isolates of present study were more closes to human isolate sequence of database. Similarly, sequences of cattle and other animal isolates were more

close to sequence of cattle isolate from database except buffalo isolate which was more closer to human isolates sequence. The non-significant difference between animal and human origin sequences in the present study or database sequences also indicated close interaction and exchange of bacterial genetic material between human, animals and cross infections (meat piece).

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J15 tst 98 ATACTGAAAAATTACCTACTCCAATAGAACTACCTTTAAAAGTTAAGGTT 147
LC075482 Hu Iran 101 ATACTGAAAAATTACCTACTCCAATAGAACTACCTTTAAAAGTTAAGGTT 150
SV2 tst 101 ATACTGAAAAATTACCTACTCCAATAGAACTACCTTTAAAAGTTAAGGTT 150
160 170 180 190 200
AG10 tst 149 CATGGTAAAGATAGTCCCTTAAAGTATTGGCCAAAGTTCGATAAAAAACA 198
MetValLysIleValProEndSerIleGlyGlnSerSerIleLysAsn
B29 tst 147 CATGGTAAAGATAGCCCCTTAAAGTATTGGCCAAAGTTCGATAAAAAACA 196
MetValLysIleAlaProEndSerIleGlyGlnSerSerIleLysAsn
C2 tst 151 CATGGTAAAGATAGTCCCTTAAAGTATTGGCCAAAGTTCGATAAAAAACA 200
MetValLysIleValProEndSerIleGlyGlnSerSerIleLysAsn
EF531615 ctl Switz. 151 CATGGTAAAGATAGTCCATTAAAGTATTGGCCAAAGTTCGATAAAAAACA 200
MetValLysIleValHisEndSerIleGlyGlnSerSerIleLysAsn
H21 tst 148 CATGGTAAAGATAGCCCCTTAAAGTATTGGCCAAAGTTCGATAAAAAACA 197
MetValLysIleAlaProEndSerIleGlyGlnSerSerIleLysAsn
J15 tst 148 CATGGTAAAGATAGTCCATTAAAGTATTGGCCAAAGTTCGATAAAAAACA 197
MetValLysIleValHisEndSerIleGlyGlnSerSerIleLysAsn
LC075482 Hu Iran 151 CATGGTAAAGATAGCCCCTTAAA-TAT-GGCCAAAGTTCGATAAAAAACA 198
MetValLysIleAlaProEnd_Ile_TrpProLysPheAspLysLysGln
SV2 tst 151 CATGGTAAAGATAGTCCCTTAAAGTATTGGCCAAAGTTCGATAAAAAACA 200
MetValLysIleValProEndSerIleGlyGlnSerSerIleLysAsn
210 220 230 240 250
AG10 tst 199 ATTAGCTATATCAACTTTAGACTTTGAAATTCGTCATCAGCTAACTCAAA 248
AsnEndLeuTyrGlnLeuEndThrLeuLysPheValIleSerEndLeuLys
B29 tst 197 ATTAGCTATATCAACTTTAGACTTTGAAATTCGTCATCAGCTAACTCAAA 246
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C2 tst 201 ATTAGCTATATCAACTTTAGACTTTGAAATTCGTCATCAGCTAACTCAAA 250
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J15 tst 198 ATTAGCTATATCAACTTTAGACTTTGAAATTCGTCATCAGCTAACTCAAA 247
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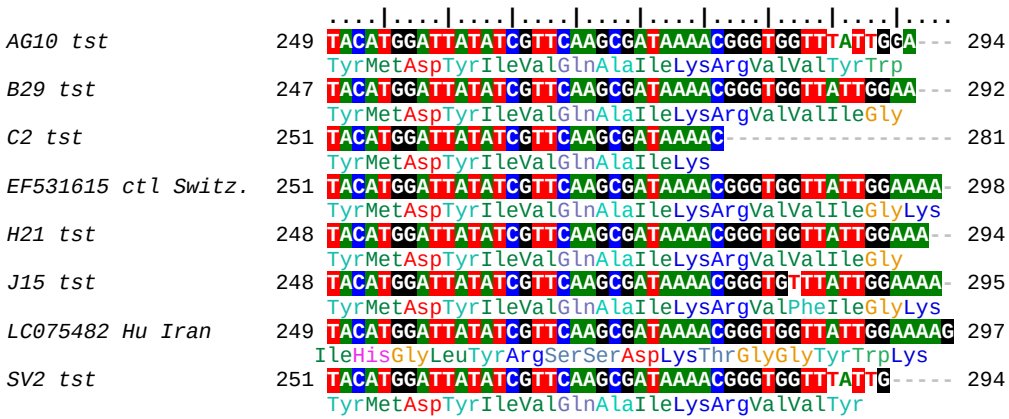

Fig. 56: Multiple sequence alignment of *tst* gene sequences of present study with reference sequences of NCBI database

9. Virulotyping of *Staphylococcus aureus*

Staphylococcus aureus produces a variety of extracellular, intracellular and cell wall associated virulence factors *viz.* adherence factors, capsule, exoenzyems, immune evasion factor, staphylokinase and toxins governed by corresponding genes. Hence, virulence of the organism depends on presence or absence of these virulence factors associated genes. In the present study virulotyping was done based on presence or absence of the virulent genes in the isolates.

In the present study, all 157 isolates were subjected to detection of 19 various virulence factors associated genes (*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*).

Total 83 virulotypes were detected among 157 isolates in the present study where all 19 genes were present in virulotype1 (V1) including one human (H3) isolate followed by virulotypes namely V2, V3 and V4 having 18 genes with four human isolates (H1, H8, H12 and H15), virulotypes V5 to V9 having 17 genes with 14 isolates and virulotypes V10 to V28 and virulotypes V29 to V50 having 16 and 15 genes with 35 and 56 isolates, respectively. Virulotyping revealed that out of 157 isolates, more than 110 isolates were positive with more than 15 genes while three virulotypes V81, V82 and V83 had 11, nine and eight genes which included J4, C15 and G16 isolate, respectively as mentioned in table 43.

Table 43: Virulotypes of the *Staphylococcus aureus* isolates.

S. No.	Virulot-ypes	isolates	No. of gene present	Gene Pattern	No. of Isolates	Subtotal of isolates
1	V1	H3	*19*	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	1
2	V2	H1	18	*clfA, clfB, icaA, icaD, trap, cap8K, chp, scn, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	4
3	V3	H8	18	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
4	V4	H12, H15	18	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	2	
5	V5	H2	*17*	*clfA, clfB, icaA, icaD, trap, cap8K, chp, scn, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	14
6	V6	H29, H39, Mt12, D10	17	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	4	
7	V7	H33, H37, H45, Mt2	17	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	4	
8	V8	H41, Mt10, J18, B34	17	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	4	
9	V9	B24	17	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
10	V10	H21	16	*clfA, clfB, icaA, icaD, trap, cap5K, scn, sak, hla, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	35
11	V11	C43	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
12	V12	H25	16	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, hla, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
13	V13	H27	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, coa, spa-X, spa-IgG, agr, aur*	1	
14	V14	H28, B29	16	*clfA, clfB, icaA, icaD, trap, cap8K, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	2	
15	V15	H4	*16*	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
16	V16	H5	*16*	*clfA, clfB, icaA, icaD, trap, cap8K, chp, scn, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
17	V17	H6, H9, H13, Mt9, C29	16	*clfA, clfB, icaA, icaD, trap, cap8K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	5	
18	V18	H10	16	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
19	V19	H11, H40, Mt15, SN4, G40	16	*clfA, clfB, icaA, icaD, trap, cap5K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	5	
20	V20	H31, Mt14, Mt31, J14	16	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	4	
21	V21	Mt27, G39	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	2	
22	V22	Hrs3	16	*clfA, clfB, icaD, trap, cap5K, cap8K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
23	V23	J15	16	*clfA, clfB, icaA, icaD, trap, cap8K, chp, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
24	V24	SV3	16	*clfA, clfB, icaA, icaD, trap, cap5K, chp, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
25	V25	B31	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
26	V26	B39, B42, AG13, C37	16	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	4	
27	V27	G2	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
28	V28	C26	16	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
29	V29	H7, H22, AG15	15	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, hla, hld, coa, spa-X, spa-IgG, agr, aur*	3	56
30	V30	C34	*15*	*clfA, clfB, icaA, icaD, trap, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
31	V31	H30, H34	15	*clfA, clfB, icaA, trap, cap8K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	2	
32	V32	H46	*15*	*clfA, clfB, icaA, trap, cap8K, scn, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
33	V33	H48, D4, G46	*15*	*clfA, clfB, icaA, icaD, trap, cap5K, chp, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	3	
34	V34	Mt1, B23	*15*	*clfA, clfB, icaA, icaD, trap, cap8K, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	2	
35	V35	Mt3, Mt13, Mt24, Mt25, Hrs1, Pg2, B36, G37, C2R	*15*	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	9	
36	V36	Mt 11	15	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hlb, coa, spa-X, spa-IgG, agr, aur*	1	
37	V37	Mt20	15	*clfB, icaA, icaD, trap, cap5K, cap8K, chp, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
38	V38	AG5, AG8	15	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	2	
39	V39	Pg4, J3, B28, G1, G21, G35, C8, C9, C23, C39, C40, C41	15	*clfA, clfB, icaA, icaD, trap, cap5K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	12	
40	V40	B21, C3R, C17, C22, C36, C52	15	*clfA, clfB, icaA, icaD, trap, cap8K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	6	
41	V41	D13	15	*clfA, clfB, icaD, trap, cap5K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	

S. No.	Virulot-ypes	isolates	No. of gene present	Gene Pattern	No. of Isolates	Subtotal of isolates
42	V42	SV2, SV4, AG10	15	*clfA, clfB, icaA, icaD, trap, cap5K, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	3	
43	V43	B57	*15*	*clfA, clfB, icaA, icaD, trap, cap5K, scn, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
44	V44	G9, G41	15	*clfA, clfB, icaA, icaD, trap, cap5K, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	2	
45	V45	C20	15	*clfA, icaA, icaD, trap, cap5K, cap8K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
46	V46	H24	15	*clfA, clfB, icaA, icaD, trap, cap5K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, aur*	1	
47	V47	B46	15	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr*	1	
48	V48	G49	15	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
49	V49	G55	15	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
50	V50	C2	*15*	*clfB, icaA, icaD, trap, cap5K, cap8K, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
51	V51	H14	14	*clfA, clfB, icaA, icaD, trap, cap5K, scn, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	29
52	V52	Mt19	*14*	*clfA, clfB, icaA, icaD, trap, cap8K, chp, sak, hla, coa, spa-X, spa-IgG, agr, aur*	1	
53	V53	Mt22	*14*	*clfB, icaA, icaD, trap, cap8K, chp, scn, sak, hla, hld, coa, spa-X, spa-IgG, agr*	1	
54	V54	Mt26, Mt28, G24	14	*clfA, clfB, icaA, icaD, trap, cap5K, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	3	
55	V55	J10, D7	14	*clfA, clfB, icaA, trap, cap5K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	2	
56	V56	D6	14	*clfA, clfB, icaA, icaD, trap, cap5K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
57	V57	Hrs4, SN3, AG6	14	*clfA, clfB, icaA, icaD, trap, cap8K, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	3	
58	V58	SN14, B5, B19, AG17, G7, G10	14	*clfA, clfB, icaA, icaD, trap, cap5K, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	6	
59	V59	B10	14	*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, hla, hlb, coa, spa-X, spa-IgG, agr, aur*	1	
60	V60	B26	14	*clfA, clfB, icaD, cap5K, chp, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
61	V61	B27, C47	14	*clfA, clfB, icaA, icaD, trap, cap8K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	2	
62	V62	B43	14	*clfA, clfB, icaA, trap, cap8K, sak, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
63	V63	B55	14	*clfA, clfB, icaA, icaD, trap, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
64	V64	C11, G43	14	*clfA, icaA, icaD, trap, cap8K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	2	
65	V65	G45	14	*clfA, clfB, icaA, icaD, trap, cap5K, chp, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
66	V66	C5R	14	*clfA, clfB, icaA, icaD, trap, cap8K, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
67	V67	C7	14	*clfB, icaA, icaD, trap, cap8K, chp, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
68	V68	H16	13	*clfA, clfB, icaA, icaD, cap5K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	13
69	V69	B30	13	*clfB, icaA, icaD, trap, cap8K, hla, hld, tst, coa, spa-X, spa-IgG, agr, aur*	1	
70	V70	Mt 4	*13*	*clfA, clfB, icaA, trap, cap8K, sak, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
71	V71	J2	*13*	*clfA, clfB, icaA, trap, cap5K, chp, hla, hlb, coa, spa-X, spa-IgG, agr, aur*	1	
72	V72	J9	13	*clfB, icaA, icaD, trap, cap5K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
73	V73	D9	*13*	*clfA, clfB, icaA, icaD, trap, scn, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
74	V74	B1, G47, C46	13	*clfA, clfB, icaA, icaD, trap, cap5K, hla, hld, coa, spa-X, spa-IgG, agr, aur*	3	
75	V75	C13	13	*clfA, icaA, icaD, trap, cap5K, chp, hla, hlb, coa, spa-X, spa-IgG, agr, aur*	1	
76	V76	G11	13	*clfA, clfB, icaA, trap, cap5K, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
77	V77	G29	13	*clfA, trap, cap5K, cap8K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr, aur*	1	
78	V78	C50	13	*clfA, clfB, icaD, trap, cap5K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
79	V79	H44	12	*clfA, icaA, icaD, trap, cap8K, chp, hla, coa, spa-X, spa-IgG, agr, aur*	1	2
80	V80	C12	12	*icaA, icaD, trap, cap8K, chp, hla, hld, coa, spa-X, spa-IgG, agr, aur*	1	
81	V81	J4	11	*clfA, icaA, cap5K, chp, hla, hlb, hld, coa, spa-X, spa-IgG, agr*	1	1
82	V82	C15	*9*	*trap, cap5K, chp, hla, hld, coa, spa-X, spa-IgG, agr*	1	1
83	V83	G16	8	*clfB, trap, cap5K, hld, coa, spa-X, spa-IgG, agr*	1	1
Total no. of isolate						157

5. SUMMARY

Staphylococcus aureus is an important pathogen causing heavy economical losses to dairy industry mainly because of mastitis. In humans it causes severe infections including superficial skin lesions, deep abscesses and more serious infections such as pneumonia, mastitis, meningitis, urinary tract infections, corneal infections, arthritis, osteomyelitis, food poisoning, toxic shock syndrome, endocarditis and toxinosis. The organism is endowed with many virulence properties and is also showing increasing trend towards antibiotic resistance. The organism shows variations in phenotypic and genotypic properties and a vast variations have been recorded in their virulence over the passage of time.

The present study was undertaken with objective to study phenotypic properties of *S. aureus* including growth on different media, colony characteristics, haemolysis on blood agar, production of coagulase in tube test, slime production, production, typing and titration of haemolysins, antibiotic resistance pattern with determination of minimum inhibitory concentration (MIC) of some antibiotics including screening of MRSA, VRSA, beta-lactamase and ESBL activity. The *S. aureus* were also characterized for *mecA* and *blaZ* gene including sequence analysis of *mecA* gene.

Molecular typing of *S. aureus* isolates was done with *rep* PCR. Genetic differentiation of isolates was conducted on the basis of genes associated with virulence such as adherence factors (*clfA, clfB, icaA, icaD, agr*I, *agr*II, *agr*III, *agr*IV and *trap* gene), antiphagocytic factor (*cap5K* and *cap8K* gene), exoenzymes (*coa* and *aur* gene), immune evasion factor (*spa-X, spa-IgG, chp* and *scn* gene), staphylokinase as plasminogen activator factor (*sak* gene) and toxin secretory system (*hla, hlb, hld* and *tst* gene). Variations among the strains were detected by restriction fragment length polymorphism (RFLP) patterns obtained from *Alu*I digests of *coa* amplicons and *Hinf*I digest of *aur* amplicons. Sequence analysis was also conducted for *spa-X, sak, aur* and *tst* genes from some representative isolates.

In the present investigation, 517 samples were collected from various clinical and non-clinical sources *viz.* clinical infections of human, meat pieces, horse wounds, pig nasal cavity, camel wounds, dog skin infections, clinical infections of sheep and mastitic milk of buffalos, goats and cattle. The overall prevalence of *S. aureus* from various human and animal origin was 30.3% (157/517) with highest prevalence in human 43.75% (35/80) samples and lowest in sheep 17.1% (6/35) and pig 17.1% (2/11) samples. The genotypic confirmation of *S. aureus* was done by polymerase chain reaction using species-specific 23S rRNA based primers with 1250bp size amplicon.

The colonies of all the 157 *S. aureus* isolates were round, smooth and glistening but the color was variable as pale yellow 73 (46.5%), whitish 50 (31.8%), golden yellow 25 (15.9%), and mustard yellow 9 (5.7%) on nutrient agar for after 24 h incubation at 37˚C. In mannitol fermentation reaction, 153 (97.5%) isolates were mannitol fermenter while four isolates (H1, SV4 B34 and G2) did not ferment mannitol.

Coagulation production was shown by 153 (97.4%) isolates while four (2.5%) isolates did not produce coagulase at 24 hour recording. Human plasma showed the best coagulation reaction followed by plasma from camel, poultry, sheep, goat and cattle in decreasing order. The analysis of coagulase reactions suggested that coagulation activity not only depended on source of bacterial isolate but on source of plasma as well.

Slime production was exhibited by 145 (92.4%) isolates while 12 (7.6%) isolates did not produce it. Haemolysis was shown by 94.90% isolates of which 68.8% exhibited complete haemolysis, 24.8% incomplete / partial haemolysis and 1.3% isolates showed both complete and partial hemolysis while 5.1% isolates were ahaemolytic. In qualitative toxin assay, 75 (47.8%) isolates were detected as beta-toxin producers and 121 (77.1%) isolates were delta-toxin producers. The titration for β toxin revealed titre between 1:5 and 1:320 and for delta **(δ)** hemolysin titre ranged between 1:5 and 1:40.

The antibiogram revealed that all isolates were sensitive to meropenem, chloramphenicol and nitrofurantoin, more than 85% isolates were sensitive to nine antibiotic namely ampicillin+ sulbactum (96.2%), imipenem (95.5%), cefalothin (91.7%), piperacillin+tazobactam (91.7%), tobramycin (89.8%), doxycycline hydrochlorid (89.2%), ceftazidime+clavulanic Acid (88.5%), oxacillin (87.3%) and polymxin-B (86.0%) while more than 95.0% isolates were resistant to ampicillin and penicillin-G. The isolates from different sources showed highly significant ($p \leq 0.01$) variation in their resistance patterns for 39 antibiotics, significant variation ($p \leq 0.05$) for levofloxacin and nitrofurantoin, and no significant variation ($p > 0.05$) for clindamycin. In the duncan multiple range test (DMRT) analysis maximum five subsets were found for five antibiotic namely cefaclor, cefixime, cefixime+ clavulanic acid, imipenem and ticarcillin and four and less than four subset were found for remaining antibiotics. In Bonferroni correction, *S. aureus* isolates from human sources were significantly variable with $p<0.0001$ probability level of variance with other meat piece and animal origin sources for most of antibiotic. Isolates from horse, camel and pig sources were non-significantly variable with each other for most of antibiotics and the isolates of sheep, goat, buffalo and cattle were non-significantly variable with each other for most of the antibiotics.

On the basis of hierarchical ascendant cluster analysis, the isolates were grouped into three cluster, one cluster comprising of buffalo, cattle, sheep, dog and goat, second cluster included horse, camel, meat piece and pig while third cluster included human isolates. All three clusters had significant variation ($p \leq 0.05$) among each other. Overall lower resistance was recorded in animal isolates in comparison to human isolates. The *S. aureus* isolates from human sources (0.40) had highest multiple antibiotic resistance (MAR) index and sources from camel (0.25), meat pieces (0.24), pig (0.23) and horse (0.21) had more than 0.20 MAR in decreasing order. Other groups of *S. aureus* isolates of animal sources such as cattle, dog, buffalo, goat and sheep had less than 0.20 MAR as described. In the individual isolate MAR index, 66 (42%) isolates had 0.2 or more than 0.2 MAR index value and were comprised mostly of human and meat piece isolates while 91 (58%) isolates had less than 0.2 MAR index value with less risk source of MDR which were mostly animal origin isolates.

All isolates were subjected to minimum inhibitory concentration (MIC) determination for eight antibiotics in which average MIC for azithromycin was 73.40 mcg/ml, vancomycin was 1.22 mcg/ml, oxacillin was 4.92 mcg/ml, gentamicin was 12.35 mcg/ml, ciprofloxacin was 18.38 mcg/ml, chloramphenicol was 5.85 mcg/ml, ceftriaxone was 18.55 mcg/ml and average MIC for penicillin was 16.35 mcg/ml. The isolates from different sources showed highly significant ($p \leq 0.01$) variation in their MIC values for three antibiotics namely azithromycin, ciprofloxacin and penicillin, significant variation ($p \leq 0.05$) for vancomycin, and no significant variation ($p > 0.05$) for ceftriaxone, chloramphenicol, gentamicin and oxacillin. In the DMRT analysis maximum three subsets were detected for ciprofloxacin and two subsets for azithromycin, penicillin and vancomycin. In Bonferroni correction, *S. aureus* isolates from human and meat piece sources were significantly variable with $p<0.0001$ probability level of variance with other animal origin sources for azithromycin, ciprofloxacin and penicillin. Hierarchical ascendant cluster analysis of MICs values revealed three clusters with significant variation ($p \leq 0.05$) among each other, one cluster comprising of buffalo, cattle, sheep, dog, pig and goat isolates, second cluster

included horse, camel and meat piece isolates while third cluster included human isolates. Overall lower MICs for studied antibiotics were recorded among animal origin isolates in comparison to human origin isolates.

Phenotypically, 85 (54.1%) isolates were detected as methicillin resistant *S. aureus* (MRSA) by MeReSa agar base method and 19 (12.1%) isolates by methicillin disk methods. Vancomycin resistant *S. aureus* (VRSA) activity was detected in 52 (33.1%) isolates. Maximum VRSA activity (100%) was found in sheep isolates followed by horse 66.6 % (2/3), buffalo 57.1% (12/21) and human 51.4 (18/35) isolates in decreasing order but none of the isolates from pig, camel and dog was found to be positive for VRSA activity. Beta-lactamase activity was present in 142 (90.4%) isolates. The activity was shown by 100% isolates from human, meat piece, horse, pig and dog. Extended-spectrum beta-lactamase (ESBL) activity was exhibited by 108 (68.8%) isolates, with more than 85% isolates from human, pig and camel.

The *mecA* gene was detected in 37 (23.6%) isolates with single amplicon of 533bp with highest prevalence in human (51.4%) followed by horse (66.6%) meat piece (40.0%) and camel (37.5%) while none of the isolates from pig, dog and goat had *mecA gene*. The *blaZ* gene was found in 137 (83.3%) isolates with single amplicon of 517bp. Isolates from human, horse, pig were 100% positive. The sequence analysis of *mecA* gene revealed non-significant (p>0.05) variations, one variation at 316 (cytosine replaced by adenine) position, another at 378 (adenine replaced by thymine) position and additional 16 nucleotide insertion (GGTACTGGCAGAAACA) from 467 to 481 position in cattle (C12) sequence. Two distinct clusters with 0.0022 scaled distance were found in phylogenetic tree analysis of *mecA* gene sequences, first cluster included sequence of cattle (C12) isolate and second cluster included sequences of remaining other isolates.

In the present investigation, 36 different *rep* patterns comprising in the range size from 300 to 1400bp with different arrangements of band sizes were recorded among all 157 isolates by *rep*-PCR with 0.8892 discriminatory index (D.I.). This discriminatory index indicated good differentiations of *S. aureus* isolates by *rep* PCR. One pattern (rep33) was most common with 47 cattle and goat isolates. In the source-wise analysis of rep-patterns, human isolates were differentiated into maximum rep-profiles (16 rep-profiles) indicating more variations while sheep isolates were grouped only into one rep-pattern (rep17) indicating no variations. Exceptionally one isolate (J9) from camel was non-typeable with rep PCR. In phylogenetic cluster analysis, all 36 rep-patterns were divided into five clusters on the basis of 80% genetic similarity. First cluster was made up of seven isolates (mostly from buffalo and dog), second cluster comprised of six human isolates, third cluster included 29 isolates (mostly from human and meat piece, and one each from horse, pig and camel) fourth cluster comprised of 41 isolates (human, meat piece and few of sheep and buffalo) and fifth cluster included 73 isolates (mostly from cattle, goat, buffalo, and few from dog, camel and meat piece). The cluster analysis indicated that most of the animal isolates were separately clustered (I^{st} and V^{th} cluster) in comparison to human and meat piece isolates.

Of the total 157 isolates, *clfA* and *clfB* genes were detected in 148 (94.3%) isolates with single amplicon of 1000bp and 205bp, respectively. Both *clfA* and *clfB* genes were absent in nine (5.7%) isolates.

The *icaA* gene was detected in 150 (95.5%) isolates with 1315bp of single amplicon while absent in seven (4.5%) isolates. The *icaD* gene was present in 144 (91.7%) isolates in duplex PCR with *blaZ* gene while absent in 13 (8.3%) isolates. The *trap* gene was detected in 154 (98.0%) isolates with 504bp of single amplicon while absent in three (2.0%) isolates.

Except one isolate (H24) all other 156 isolates were successfully typed with *agr* genes typing system. The *agr*I was detected in 63 (40.1%), *agr*II in 42 (26.8%), *agr*III in 27 (17.2%) and *agr*IV was detected in 24 (15.3%) isolates with 441bp, 575bp, 323bp and 659bp of single amplicon in multiplex PCR.

Among all studied adherence genes *agr* was most prevalent (99.4%) followed by *trap*, *icaA*, *clfA*, *clfB* and *icaD* in decreasing order. Polymorphism was not detected in any of the studied adherence genes in the present investigation.

In the present study duplex PCR was developed to detect both capsular gene (*cap5K* and *cap8K*) in a single reaction. The *cap5K* gene was found in 73 (46.5%) isolates with single amplicon of 361bp, *cap8K* in 59 (37.6%) isolates with 173bp amplicon and both genes were found in 22 (14.0%) isolates. Three isolates (D9, B55 and C34) were non-typeable in capsular typing. The *cap5K* gene was more prevalent among all studied sources except meat piece, in which *cap8K* gene was more prevalent. None of the dog isolate carried *cap8K* gene.

All *S. aureus* isolates were found to be positive for *coa* gene with nine amplicon sizes varying from 500 to 900bp, which included four isolates of 500bp, seven of 550bp, 18 of 600bp, 21 of 650bp, 74 of 700bp, eight of 750bp, 12 of 800bp, two of 850bp and 11 isolates of 900bp with 0.7353 discriminatory index. The *coa* gene amplification was found to be suitable for isolate differentiation with good discriminatory index. All isolates could be digested with *Alu*I except nine isolates namely three isolates (H41, B21 and G46) of 700bp amplicon, one isolate (B34) of 750bp amplicon and five isolates (B27, B43, B46, C2R and C5R) of 800bp amplicon which indicated the absence of specific cutting site for *Alu*I. The RFLP of *coa* gene with *Alu*I revealed 33 different coa-RFLP patterns with 0.9301 discriminatory index. The *coa*-RFLP was found better method to differentiate strains of *S. aureus* in comparison to *rep*-PCR method (D.I.-0.8892). In phylogenetic cluster analysis, all 33 coa-RFLP types were divided into three clusters on the basis of 80% genetic similarity First cluster was made of 65 isolates (mostly from animal isolates except few from human and meat piece), second cluster comprised of 82 isolates (mostly from human and meat piece except few from other animal sources), third cluster included 10 isolates (five from human and meat piece and five of animal origin).

In present investigation *aur* gene was found in 152 (96.8%) isolates with single amplicon of 1526bp while five (3.2%) isolates *i.e.* Mt22, J4, B46, G16 and C15 were detected without this gene. Three different aur-RFLP types *i.e.* A1 (50bp, 150bp, 200bp, 1000bp), A2 (50bp, 650bp, 800bp) and A3 (50bp, 100bp, 140bp, 480bp, 700bp) were found among studied isolates with *Hinf*I digestion. Among all aur-RFLP types, A3 type included 119 isolates with highest prevalence (78.3%) followed by A1 type with 17 (11.2%) isolates and A2 type included 16 isolates with lowest prevalence (10.5%). Source-wise analysis revealed that human isolates belonged to all three aur types while horse, pig, camel, dog, sheep and cattle isolates belonged to only A3 type. Meat piece and buffalo isolates belonged to A2 and A3 types and goat isolates belonged to A1 and A3 types. Three clusters were found during phylogenetic cluster analysis of *S. aureus* aur-RFLP types at the level of 80% genetic similarity. First cluster was made of 17 isolates (mostly from human except two from goat), second cluster comprised of 16 isolates (11 human, four meat piece and one buffalo isolate) and third cluster included 119 isolates (95 animal origin, nine human and 15 meat piece isolates). In sequence analysis of above 12 *aur* gene samples, overall more than 200 nucleotides and their corresponding amino acid variations were recorded at various positions. The big gaps or deletions were observed from 641 position to 679 position in all studied sequences. Except in one isolate (J18), sequence was without gap and in isolate G2 with only two gaps and both these sequences belonged to aur-RFLP typeA3. These number of nucleotide variability could be considered as massive variations among studied sequences. It was also found that, sequences from similar RFLP patterns had less difference in comparison to those from other patterns. These sequence variations were non-significant (p>0.05) in

within aur-RFLP type but variations were found significant ($p \leq 0.05$) with each other in between aur-RFLP type during analysis of codon based test of neutrality. Three clusters were observed during phylogenetic tree analysis of *aur* gene sequences of present study and further revealed that aur-RFLP type A1, A2 and A3 classified in separate clusters and A1 and A2 types were more-closer as compared to A3 type.

The immune invasion genes namely *chp* and *scn* were detected in 114 (72.6%) and 40 (25.5%) isolates, with single amplicon of 404bp and 320bp, respectively. Most animal isolates were found to be negative for *chp* gene except horse, pig, camel, dog and cattle isolates. Similarly, most of animal isolates were negative for *scn* gene while most of human and meat piece isolates were positive for both *chp* and *scn* genes.

All isolates were found to be positive for *spa-IgG* gene with three polymorphic (600bp, 750bp and 950bp) band patterns. Out of total 157 isolates, 144 (91.7%) were positive with 950bp, nine (5.7%) with 650bp and four (2.5%) isolate with 600bp amplicon size. Isolates from meat piece, pig, camel, dog, sheep and cattle were positive with only single band pattern (950bp) while human, buffalo and horse isolates had two band patterns *i.e.* 750bp and 950bp and 600bp and 950bp, respectively and goat isolates showed three band patterns (600bp, 750bp and 950bp).

The *spa-X* gene was detected in 100% isolates with nine polymorphic bands ranging from 150bp (4 repeats) to 380bp (14 repeats), which included one isolate of the 150bp, two of 170bp, five of 180bp, 15 of 200bp, 53 of 250bp, nine of 280bp, 56 of 300bp, 15 of 350bp and one isolate of 380bp. The *spa-X* gene amplification was found to be suitable for isolate differentiation with good discriminatory index value (D.I. =0.7407). Of the 157 isolates, 134 (85.3%) isolates had more than 7 repeats while only 23 (14.6%) isolates had less than seven repeats. In sequence analysis of 23 representative isolates for *spa-X* gene, only 37 nucleotides from 245 nucleotide position to 350 nucleotide position were found to be similar. Massive variations were detected from position 1 to 390 aligned position among all studied sequences. Large overhanging flanks and big gap were found at starting and end sides in sequences indicated more or less repeats in sequences according to their bigger and smaller amplicon sizes, respectively. One big insertion of 24bp was observed between 192 and 216 nucleotide position in B39 isolate and other big insertion of 24bp was detected in between 264 and 288 nucleotide position in the G29 isolate. This 24bp insertion of different nucleotide sequence indicated one different repeat in corresponding sequence. Most of *spa-X* sequences were significantly ($p \leq 0.05$) different from each other with some of non-significantly ($p > 0.05$) associated sequences. Phylogenetic tree analysis of *spa-X* gene sequences revealed five separate clusters among studied sequences, first cluster included 12 isolates namely H48, Mt9, H21, AG13, H30, Mt15, G1, Hrs3, Mt20, J2, B34 and H41 with 0.0101 scaled distance, second cluster included eight isolates namely B39, C17, C41, B1, C26, C34, D6 and J9 with 0.0555 scaled distance. Third, fourth and fifth each cluster included single isolate namely G2, J15 and G29 with 0.1089, 0.1844 and 0.2219 scaled distances, respectively. On the basis of discriminatory index value, *spa-X* gene amplification (D.I.- 0.7407) was found more suitable method to differentiate various *S. aureus* isolates in comparison to *coa* gene PCR amplification (D.I.- 0.7353) but *coa*-RFLP method (D.I.-0.9301) was more precise to differentiate different strains of *S. aureus* in comparison to *rep*-PCR method (D.I.-0.8892) and *spa-X* typing (D.I.- 0.7407).

Out of 157 isolates, 79 (50.3%) isolates were found to be positive for *sak* gene with single amplicon of 403bp. Most of the human (80.0%) and all (100.0%) meat piece isolates were positive while most of animal isolates were negative. The sequence analysis of *sak* gene revealed 21 non-significant ($p > 0.05$) nucleotide variations. The sequences of B34 (buffalo) and D4 (dog) were found more closer as compared to others. However, these two sequences (B34 and D4) carried most of the variations from other studied sequences while in others only one nucleotide variation (adenine replaced by guanine at 93th aligned nucleotide position) was found in

H21 and H37 human isolate. Three distinct clusters were found in phylogenetic tree analysis, first cluster included sequence of buffalo (B34) isolate separated with 0.012 scaled distance, second cluster comprising of dog (D4) isolate separated with 0.006 scaled distance and third cluster was made up of remaining other seven isolates (Mt28, SN4, C29, H21, H2, G24 and H37).

Out of the toxin associated genes, *hla* gene was detected in 155 (98.7%) isolates with single amplicon of 534bp while only two isolates (G16 and G45) were found to be negative for *hla* gene. The *hlb* gene was observed in 94 (59.9%) isolates with single amplicon of 833bp. Maximum prevalence of *hlb* gene was recorded in camel (87.5%) isolates while minimum presence was found in meat piece (35.0%) isolates. The *hld* gene was detected in 150 (95.5%) isolates with single amplicon of 111bp (Fig. 52) while seven isolates were negative. The *tst* gene was detected only in 19 (12.1%) isolates with single amplicon of 350bp. In the toxin associated genes, *hla* gene was detected with maximum prevalence followed by *hld, hlb* and *tst* genes in decreasing order.

The sequence analysis of six *tst* gene isolates revealed 12 non-significant ($p > 0.05$) nucleotide variations in isolates. The sequences of B29 (buffalo) and H21 (human) were found more closer compared to others. In analysis, it was detected that H21 and B29 isolates carried A, G, A T, C, C, C and G while all other sequences carried G, A, G, G, T, T, T and T at 7^{th}, 11^{th}, 71^{th}, 72^{th}, 76^{th}, 93^{th}, 165^{th} and 288^{th} nucleotide positions, respectively. In other, sequences variations were at only two nucleotide positions *i.e.* one gap at 15^{th} position in AG10 isolate while other sequences having adenine and second variations at 168^{th} position in J15 isolate which had adenine while other had cytosine. Two distinct clusters were formed in phylogenetic tree analysis first cluster included sequence of buffalo (B29) and human (H21) isolate separated with 0.0128 scaled distance from others and second cluster comprised of two subdivisions of which first included cattle (C2), sheep (SV2) and goat (AG10) separated from second which included camel (J15) with negligible 0.0055 distance.

Total 83 virulotypes were detected among 157 isolates in the present study on the basis of presence or absence of 19 various virulence factor associated genes (*clfA, clfB, icaA, icaD, trap, cap5K, cap8K, chp, scn, sak, hla, hlb, hld, tst, coa, spa-X, spa-IgG, agr, aur*). All 19 genes were present in virulotype1 (V1) which included one human (H3) isolate. Virulotyping revealed that out of 157 isolates, more than 110 isolates were positive with more than 15 genes while three virulotypes V81, V82 and V83 had 11, nine and eight genes which included J4, C15 and G16 isolate, respectively.

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Agarose solution (1.2% and 0.8%)

To preapare 1.2% agarose, 1.8 gm of agarose was dissolved in 150 ml of 1 × TBE.
To prepare 0.8% agarose solution, 1.2 gm of agarose was dissolved in 150 ml of 1 × TBE.

Alcohol (70%)

In 70 ml of absolute alcohol 30 ml of double glass distilled water was added to prepare 70% alcohol.

Alsever's solution

Dextrose 20.50 g, sodium citrate 8.0 g, citric acid 0.55 g, and sodium chloride 4.20 g were dissolved in 800 ml of glass distilled water. The pH of the solution was adjusted to 7.1 and final volume of the solution was made to 1 litre then, autoclaved at 121^{o}C for 15 minutes and stored in refrigerator until use.

Ammonium chloride (0.17 M solution)

The required solution was prepared by dissolving 4.55 gm of ammonium chloride solution in 500 ml of distilled water. It was then autoclaved.

Assay Buffer for Taq DNA polymerase (10X)

1000 mM Tris HCl (pH, 9.0)
15 mM $MgCl_2$
500 mM KCl
0.5 % Gelatin

Cetyltrimethylammonium bromide (CTAB, 10%)

Cetyltrimethylammonium bromide (25 g) was dissolved in 200 ml of distilled water and the final volume was made up to 250 ml by distilled water.

Chloroform : Isoamyl alcohol (24:1)

For preparing 500 ml, an amount of 480 ml of chloroform was mixed with 20 ml of isoamyl alcohol was mixed.

DNA Extraction Buffer (DEB)

Chemical	Stock	Working	Quantity of stock required to make 100 ml of working DNA extraction buffer
Tris HCl pH-8.0	1 M	100 mM	10 ml
EDTA pH-8.0	5 M	20 mM	4 ml

NaCl	4 M	14 mM	35 ml
CTAB	10%	2%	20 ml
Water			31 ml
β-mercaptoethanol		0.2%	200µl added @ 2µl/ml to the DEB just before use

EDTA (0.5 M), pH- 8.0

To 800 ml of distilled water 186.1 g of disodium ethylene diaminetetra acetate.$2H_2O$ was added and shake vigorously on a magnetic stirrer for several hours. The pH was adjusted to 8.0 with 1.0 N NaOH, dispensed into aliquots and sterilized by autoclaving.

Ethidium bromide solution

Stock solution was prepared by using 5 mg ethidium bromide per ml of TBE working solution. Working solution was prepared in the concentration range of 0.5-1 mg/ml using TBE working solution.

Gel loading dye (10 x)

Bromophenol Blue 0.25 g
Xylene cyanol 0.25 g
Glycerol (50%) to make 100 ml

Lysozyme solution (3 mg/ml)

15 mg of lysozyme was dissolved in 5 ml of distilled water and stored at –20^{o}C use.

McFarland 0.5 Turbidity Standard

Solution A (0.048 M Barium chloride): 1.75 g of Barium Chloride ($BaCl_2 .2H_2O$) is dissolved in 100 ml distilled water.
Solution B (0.36 N Sulphuric acid) : 1.0 ml H_2SO_4 is diluted in 100 ml of distilled water.
Working solution is made by adding 0.5 ml of solution A and 99.5 ml of solution B. Store in dark at room temperature and replace 3 months after preparation. The turbidity standard should always be agitated before use.

β-Mercaptoethanol (BME)

It was obtained as 14.4 M solution and stored in a dark bottle at 4°C.

Nitrate test reagent

Solution A : Suphanilic acid soluction 0.8% w/v (Hi-media)
Solution B : ∝ - Naphthylamine solution (Hi-media)

Oxidase reagent

Tetramethylene-p-phenylene diamine dihydrochloride-100 mg was dissolved in 10 ml glass distilled water.

Phosphate buffer saline (1 %)

Solution A : Sodium diphosphate 1.4 gm
Distilled water 1000 ml
Solution B : Sodium dihydrogen 1.4 gm orthophosphate
Distilled water 1000 ml

An amount of 84.1 ml of solution A and 15.9 ml of solution B were mixed nad 8.5 g sodium chloride was added. The volume was made to 1000 ml with distilled water and autoclave it at 15 lb (121^{o}C) for 15 min.

Proteinase K solution

In 1 ml of distilled water 10 mg proteinase K was dissolved and stored at –20^{o}C.

Tris (0.5M)

In 800 ml of distilled water 4.44 g of Tris Hcl and 2.65 g of Tris base/ buffer was dissolved and the pH was adjusted to desired value. The volume was then adjusted to 1000 ml, dispensed in aliquots and sterilised by autoclaving.

TBE (Tris Borate EDTA) Buffer, pH 8.2

Stock solution (5X)

Tris base	54.0 gm
Boric acid	27.5 gm
Disodium EDTA (0.5M)	20.0 ml
Triple distilled water	1000ml

Working solution (0.5X)

One hundred ml stock solution of TBE buffer was dissolved in 900ml of triple distilled water prepare the working solution of 0.5x TBE buffer.

Working solution (1X)

One hundred ml stock solution of TBE buffer was dissolved in 400ml of triple distilled water prepare the working solution of 1x TBE buffer.

TE Buffer

20ml of 0.5 M Tris (pH,8.0) was added to 200 µl of 0.5 M EDTA pH, 8.0 and the volume was made up to 100 ml.

Detection of genetic variability among *Staphylococcus aureus* isolates from various clinical and non-clinical animal and human settings in relation to some virulence factors

Ph.D. Thesis

Department of Veterinary Microbiology and Biotechnology
College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences
Bikaner-334001 (Rajasthan)

Submitted by : Sandeep Kumar Sharma

Major Advisor : Prof. A.K. Kataria

ABSTRACT

The present study was aimed to find genetic variability among *Staphylococcus aureus* isolates from various clinical and non-clinical animal and human settings in relation to some virulence factors and antibiotic resistance pattern including their associated genes. Of the 517 samples processed from, various clinical and non-clinical sources *viz.* clinical infections of human, meat pieces, horse wounds, pig nasal cavity, camel wounds, dog skin infections, clinical infections of sheep and mastitic milk of buffalos, goats and cattle, overall prevalence of *S. aureus* was 30.3% (157/517) with highest prevalence in human 43.75% (35/80) samples using species-specific 23S rRNA based primers with 1250bp size amplicon. In phenotypic characterization, four types of colonies *viz.* pale yellow (46.5%), whitish (31.8%), golden yellow (15.9%), mustard yellow (5.7%) were obtained on nutrient agar, 97.5% isolates were mannitol fermenter while 2.5% isolates were non-fermenters, 92.4% isolates produced slime while 7.6% did not. Free coagulase was produced by 94.2% isolates produced while 5.7% isolates did not produce it. Human plasma showed the best coagulation reaction. Haemolysis was shown by 94.90% isolates of which 68.8% exhibited complete haemolysis, 24.8% incomplete / partial haemolysis and 1.3% isolates showed both complete and partial hemolysis while 5.1% isolates were ahaemolytic. In qualitative toxin assay, 75 (47.8%) isolates were beta-toxin produce and 121 (77.1%) isolates were delta-toxin producers. The β toxin titres were recorded between 1:5 and 1:320 and delta **(δ)** toxin titres between 1:5 and 1:40. In antibiogram studies more than 95.0% isolates were recorded resistant to ampicillin and penicillin-G while approximately 100% isolates were sensitive to chloramphenicol, meropenem and nitrofurantoin, more than 85% isolates were sensitive to ampicillin+ sulbactum, cefalothin, ceftazidime+ clavulanic acid, doxycycline hydrochloride, imipenem, oxacillin, piperacillin+ tazobactam, polymxin-B and tobramycin. Isolates were detected with highly significant ($p \leq 0.01$) variation in their resistance patterns for 39 antibiotics, significant variation ($p \leq 0.05$) for levofloxacin and nitrofurantoin, and no significant variation ($p > 0.05$) for clindamycin. The isolates were grouped in to three hierarchical ascendant clusters on the basis of antibiogram, one cluster comprising of buffalo, cattle, sheep, dog and goat, second cluster included horse, camel, meat piece and pig while third cluster included human isolates. Human isolates had highest (0.40) MAR index. Sixty six (42%) isolates had 0.2 or more than 0.2 MAR index while 91 (58%) isolates had less than 0.2 MAR index. The MIC for azithromycin was 73.40 mcg/ml, for vancomycin 1.22 mcg/ml, for oxacillin 4.92 mcg/ml, for gentamicin 12.35 mcg/ml, for ciprofloxacin 18.38 mcg/ml, for chloramphenicol 5.85 mcg/ml, for ceftriaxone 18.55 mcg/ml and for penicillin 16.35 mcg/ml, with highly significant ($p \leq 0.01$) variation for three antibiotics namely azithromycin, ciprofloxacin and penicillin, significant variation ($p \leq 0.05$) for vancomycin, and no significant variation ($p > 0.05$) for ceftriaxone, chloramphenicol, gentamicin and oxacillin in all isolates. Phenotypically, 54.1% and 12.1% isolates were identified as MRSA by MeReSa agar base method and methicillin disk methods, respectively. The Beta-lactamase production, ESBL and VRSA activity was detected in 90.4%, 68.8% and 33.1% isolates, respectively. The *mecA* and *blaZ* genes were found in 23.6% and 83.3% isolates with single amplicon of 533bp and 517bp, respectively. Non-significant variations were recorded in the sequences analysis of *mecA* gene.

Thirty six different *rep* patterns were obtained which, comprising of 300 to 1400bp band sizes with different arrangements and 0.8892 discriminatory index. The patterns were, grouped into five clusters at 80% genetic similarity level and most of the animal isolates were separately clustered in Ist and Vth clusters in contrast to human and meat piece isolates. In adherence genes, *clfA* (1000bp) and *clfB* (205bp) were found in 94.3% isolates, *icaA* (1315bp) in 95.5%, *icaD* (381bp) in 91.7% and *trap* (504bp) was found in 98.0% isolates. Except one isolate (H24) all were typeable with *agr* typing system, in which 40.1%, 26.8%, 17.2% and 15.3% were typed as *agr*I, *agr*II, *agr*III and *agr*IV with 441bp, 575bp, 323bp and 659bp of single amplicon in multiplex PCR, respectively. In capsule genes, 46.5% 37.6% and 14.0% isolates carried *cap5K* (361bp), *cap8K* (173bp) and both genes while three isolates (D9, B55 and C34) were negative for any of these genes. In exoenzyme genes, *coa* gene was detected in 100% isolates with nine amplicon sizes varying from 500 to 900bp. Thirty three 33 different coa-RFLP patterns were obtained with 0.9301 discriminatory index. All coa-RFLP types were grouped into three clusters at 80% genetic similarity. The *aur* gene (1526bp) was found in 96.8% which, comprised of three different aur-RFLP types (A1, A2 and A3). In sequence analysis of above 12 *aur* gene samples, overall more than 200 nucleotide and their corresponding amino acid variations were recorded at various positions. Significant ($p \leq 0.05$) variations were recorded in

sequence analysis between aur-RFLP types with nucleotide variations at more than 200 positions and aur-RFLP types were classified in separate clusters in phylogenetic tree analysis of *aur* gene sequences. The immune invasion genes, 72.6% and 25.5% isolates carried *chp* (404bp) and *scn* (320bp) genes, respectively. The *spa-IgG* gene was found in all isolates with three polymorphic (600bp, 750bp and 950bp) band patterns and *spa-X* gene was detected in 100% isolates with nine polymorphic bands (D.I.- 0.7407) ranging from 150bp (4 repeats) to 380bp (14 repeats). The 85.3% isolates had more than 7 repeats while only 14.6% isolates had less than seven repeats. Most of *spa-X* sequences were significantly ($p \leq 0.05$) different from each other with large overhanging flanks, 24bp big insertion and big nucleotide gaps. Five separate clusters were detected in *spa-X* gene sequences in phylogenetic tree analysis. The *sak* (403bp) gene was found in 50.3% isolates. Twenty one non-significant ($p > 0.05$) nucleotide variations were recorded in *sak* gene sequence analysis which, revealed three distinct clusters in phylogenetic tree analysis. Most of isolates of animal origin were deficient in *sak* and *scn* genes. In genes for toxins, 98.7%, 59.9%, 95.5% and 12.1% isolates carried *hla* (534bp), *hlb* (833bp), *hld* (111bp) and *tst* (350bp) genes respectively. The *tst* gene sequence analysis revealed 12 non-significant ($p > 0.05$) nucleotide variations in isolates with two distinct clusters in phylogenetic tree analysis. Total 83 virulotypes were detected among 157 isolates on the basis of presence or absence of studied 19 various virulence associated genes. All 19 genes were present in virulotype1 (V1) which included one human (H3) isolate while three virulotypes V81, V82 and V83 had 11, nine and eight genes which included J4, C15 and G16 isolate, respectively.

पशु एवं मनुष्य के नैदानिक और गैर नैदानिक परिस्थितियों से वियोजित *स्टेफायलोकॉकस ऑरियस* में कुछ डाह कारकों के संबंध में आनुवांशिक विभिन्नता का पता लगाना

विद्यावाचस्पति शोध ग्रन्थ

पशु सूक्ष्मजीव विज्ञान एवम बायोटेक्नोलॉजी विभाग,

पशु चिकित्सा एवम पशु विज्ञान महाविद्यालय,

राजस्थान पशु चिकित्सा एवम पशु विज्ञान विश्वविद्यालय

बीकानेर (334001) राजस्थान

शोध कर्ता : सन्दीप कुमार शर्मा

मुख्य उपादेष्टा : प्रोफेसर. ऐ. के. कटारिया

अनुशेपण

वर्तमान अध्यन को पशुओं एवं मनुष्य के नैदानिक और गैर नैदानिक परिस्थितियों से वियोजित *स्टेफायलोकॉकस ऑरियस* में कुछ डाह कारकों और उनसे सम्बंधित जीन सहित एंटीबायोटिक प्रतिरोध में अनुवांसिक विभिन्नता का पता लगाने के उद्देश्य से किया गया | 517 संसाधित नैदानिक और गैर नैदानिक नमूनों जेसे कि मनुष्य के नैदानिक, मांस के तुकडे, सूअर नक् गुहा के, ऊंट के घाव, कुतो के त्वचा संकर्मण, भेड़ के नैदानिक एवं गाय, भेंस और बकरी के थनेला ग्रसित दूध के नमूनों से कुल 30.3% (157/517) *एस. ऑरियस* प्रथक किये गये जोकि मनुष्य के नमूनों में सर्वाधिक 43.75% (35/80) पाये गये, ये सब आनुवांशिक रूप से 23S-rRNA जीन आधार पर 1250bp के साथ सुनिशचित किये गये | लाक्षणिक चरित्रांकन में, पोषक तत्व अगार पर चार तरह के जैसे कि हल्की पिली (46.5 %), सफ़ेद जैसी (31.8 %), सुनहरी पिली (15.9%) एवं सरंसो पिली (5.7%) लक्षण प्ररूप प्राप्त हुए, जिनमे से 97.5% वियोजक मेनिटोल किणवक जबकि 2.5 % वियोजक मेनिटोल किणवक नहीं थे | 92.4 % वियोजको ने स्वतंत्र कोगुलेज उत्पादन किया बल्कि 5.7% ने नहीं किया | कोगुलेज उत्पादन अभिक्रिया मनुष्य प्लाज्मा द्वारा सर्वोत्तम दिखायी गई | 94.9% वियोजको द्वारा हेमोलिसिस दिखाया गया, जिसमे से 68.8% वियोजको द्वारा पूर्ण हेमोलिसिस, 24.8% द्वारा आंशिक एवं 1.3% वियोजको द्वारा पूर्ण व आंशिक दोनों हेमोलिसिस दिखाए गये, जबकि 5.1% वियोजको ने किसी तरह का हेमोलिसिस नहीं दिखाया | गुणात्मक टोक्सिन जांच में 75 (47.8%) वियोजक बीटा टोक्सिन उत्पादक जबकि 121 (77.1%) वियोजक डेल्टा टोक्सिन उत्पादक थे | बीटा टोक्सिन का 1:5 एवं 1:320 टाईटर तथा डेल्टा टोक्सिन 1:5 एवं 1:40 के बीच टाईटर पाया गया | एंटीबायोटिक प्रतिरोध स्वरूप में, 95% से ज्यादा वियोजक एम्पीसिलीन एवं पेनिसिलिन-G के लिए प्रतिरोधी थे जबकि 100% वियोजक क्लोर्मफेनिकोल, मेरोपेनेम तथा नाइट्रोफ्यूरन्टाइन के लिए संवेदनशील साथ ही 85% से ज्यादा वियोजक एम्पीसिलीन+सल्बक्टम, सफ्लोथिन, सेफ्टजीडीम+काल्वुलिनिक एसिड, डॉक्सक्यिक्लन हाइड्रोक्लोराइड, इमिपेनम, ओक्सासिल्लिन, पिप्रसिललिन+ टेजोबकटम, पोल्यमिक्सिन-B तथा टोबराम्यिसन के लिए संवेदनशील थे | 39 एंटीबायोटिकस के लिए वियोजको में अत्याधिक अभिप्राय पुर्ण (*p≤0.05*) प्रतिरोध स्वरूप विभिन्नता, लेवोफ्लोक्सासिन एवं नाइट्रोफ्यूरन्टाइन में अभिप्राय पुर्ण (*p≤0.05*) विभिन्नता जबकि क्लिंडाम्यसिन के लिए अअभिप्राय पुर्ण (*p>0.05*) प्रतिरोध स्वरूप विभिन्नता पायी गयी | प्रतिरोध स्वरूपो के श्रेंणीबध्ह लग्न समूहीकरण में तीन समूह पाये गये, पहले समूह में भेंस, गाय, भेड़, कुत्ता एवं बकरी, दुसरे समूह में घोडा, ऊंट, मांस के टुकड़े एवं सूअर जबकि तीसरे समूह में मनुष्य वियोजक विभाजित हुए | मानव वियोजको में सर्वाधिक (0.40) ऍमएआर सूची मिली | 62 (42%) वियोजक 0.2 या उससे जयादा ऍमएआर सूची जबकि 91 (58%) वियोजक 0.2 से कम ऍमएआर सूची लिये हुए थे | नुन्यतम निषेधात्मक सान्द्रता, अज़ीथ्रोम्यिसन कि 73.40 ऍमसीजी/ऍमएल, वेन्कोम्यिसन कि 1.22 ऍमसीजी/ ऍमएल, ओक्सासिल्लिन कि 4.92 ऍमसीजी/ऍमएल, जेंटामिसिन कि 12.35 ऍमसीजी/ ऍमएल, सिप्रोफ्लोक्सासिन कि 18.38 ऍमसीजी/ऍमएल, क्लोर्मफेनिकोल कि 5.85 ऍमसीजी/ऍमएल, सेफ्टरिओक्सान कि 18.55 ऍमसीजी/ऍमएल तथा पेनिसिलिन कि 16.35 ऍमसीजी/ऍमएल पायी गयी | ऍमआईसी में तीन एंटीबायोटिकस: अज़ीथ्रोम्यिसन, सिप्रोफ्लोक्सासिन तथा पेनिसिलिन के लिए वियोजको में अत्याधिक अभिप्राय पुर्ण (*p≤0.05*) विभिन्नता जबकि अन्य एंटीबायोटिकस के लिए अअभिप्राय पुर्ण (*p>0.05*) विभिन्नता पायी गयी | लाक्षणिक रूप से, 54.1% एवं 12.1% वियोजक मेरेसा अगार बेस तरीके से तथा मेंथीसिलीन डिस्क तरीके से क्रमश: ऍमएआरएसए के रूप में पहचाने गये | 90.4%, 68.8% एवं 33.1% वियोजक बीटा लक्टामेसे उत्पादक, इएसबीएल एवं वीआरएसए गतिविधि वाले क्रमश: पाये गये | 26.6% एवं 83.3% वियोजको में *मेक***ए** (533बीपी) एवं *ब्ला***जेड** (517 बीपी) जीन क्रमश: पायी गयी | *मेक***ए** जीन के नुक्लियोटाईड क्रम जाँच में अअभिप्राय पुर्ण (*p>0.05*) विभिन्नता मिली |

36 विभिन्न रेप स्वरुप 300-1400बीपी के विभिन्न आकारों तथा 0.8892 विभेदक सूचकांक के साथ पाये गये | इन रेप स्वरूपों को 80% आनुवांशिक समानता के स्तर पर 5 समूहों में बाँटा गया | पशुओ के अधिकांश वियोजक 1वे व 5वे समूहों में थे जबकि मनुष्य एवं मांस टुकडों के वियोजक इनसे भिन्न अलग समूहों में थे | अवलम्बन संबंधित जीनों में 94.3% वियोजकों में *सीएलएफ***ए** (1000 बीपी) तथा *सीएलएफ***बी** (205 बीपी), 95.5% में *आइसीए***ए** (1315 बीपी), 91.7% में *आइसीए***डी** (381 बीपी) तथा 98.0% वियोजकों में ट्रैप (504 बीपी) जीन पायी गई | एक वियोजक (एच-24) को छोड़कर, सभी *एजीआर* टंकन प्रणाली से टंकित हुए जिसमें से 40.1%, 26.8%, 17.2% तथा 15.3% *एजीआर*1, *एजीआर*2, *एजीआर*3 तथा *एजीआर*4 क्रमशः 441बीपी, 575बीपी, 323बीपी तथा 659बीपी के एकल एम्पलीकोन के साथ बहुभागी पीसीआर में उपस्थित पाए गए | सम्पुट जीनों में 46.5%, 37.6% तथा 14.0% वियोजकों में *सीएपी*5K (361बीपी), *सीएपी*8K (173बीपी) तथा दोनों जीन क्रमशः पाए गए जबकि तीन वियोजकों में (डी9, बी55 एवं सी34) कोई सम्पुट जीन नहीं थी | बाहय एंजाइम सम्बंधित जीनों में *सीओए* जीन 500 से 900बीपी आकार के साथ सभी वियोजकों में पायी गई | 33 *सीओए*-आरअफएलपी स्वरुप 0.9301 विभेदक सूचकांक के साथ पाए गये

जो कि 80% आनुवांशिक समानता पर तीन समूहों में बांटे गए | 96.8% वियोजकों में *एयुआर* जीन (1526बीपी) तीन विभिन्न *एयुआर*-आरअफएलपी स्वरुप (ए1, ए2 तथा ए3) के साथ पायी गई | 12 नमूनों में *एयुआर* जीन के न्यूक्लियोटाइड क्रम जांच में कुलमिलाकर 200 न्यूक्लियोटाइड से ज्यादा विभिन्नताएँ पायी गई | अलग–अलग *एयुआर*- आरअफएलपी स्वरूपों में अभिप्राय पूर्ण (*P≤0.05*) विभिन्नता पायी गई | प्रतिरक्षा लंघन जीनों में 76.6% तथा 25.5% वियोजकों में *सीएचपी* (404बीपी) तथा *एससीएन* (320बीपी) जीन क्रमशः पायी गई | *स्पाIgG* जीन सभी वियोजकों में तीन बहुरूपों (600बीपी, 750बीपी तथा 950बीपी) के साथ पायी गई | स्पा X जीन सभी वियोजकों में बहुरूपी स्वरुप 150बीपी (4 रिपीट) से 380बीपी (14 रिपीट) तथा 0.7407 विभेदक सूचकांक के साथ पायी गई | 85.3% वियोजक 7 से ज्यादा रिपीट तथा 14.6% वियोजक 7 से कम रिपीट लिए हुए थे | *स्पाX* जीन के न्यूक्लियोटाइड क्रम जांच में अधिकांश नमूनों में अभिप्राय पूर्ण (*P≤0.05*) विभिन्नताएँ बड़े प्रलंबन पक्ष, 24बीपी निवेशन तथा बड़े न्यूक्लियोटाइड रिक्ति के साथ पायी गई | *स्पाX* जीन के जातिव्रतीय वृक्ष जांच में पांच अलग समूह पाए गए | 50.3% वियोजकों में *एसएके* जीन (403बीपी) पायी गई | इसके न्यूक्लियोटाइड क्रम जांच में 21 न्यूक्लियोटाइड के साथ अअभिप्राय पूर्ण (*P>0.05*) विभिन्नता तथा तीन अलग समूह जातिव्रतीय वृक्ष जांच में पाए गए | अधिकांश पशु वियोजकों में *एसएके* एवं *एससीएन* जीन नहीं थी | टोक्सिन जीनों में 98.7%, 59.9%, 95.5% तथा 12.1% वियोजकों में *एचएलए* (534बीपी), *एचएलबी* (833बीपी), *एचएलडी* (111बीपी) तथा *टीएसटी* (350बीपी) जीन क्रमशः पायी गई | *टीएसटी* जीन के न्यूक्लियोटाइड क्रम जांच में 12 न्यूक्लियोटाइड के साथ अअभिप्राय पूर्ण (*P>0.05*) विभिन्नताएँ, दो जातिव्रतीय वृक्ष जांच समूहों के साथ पायी गई | सभी 157 विभाजको को अध्यनित 19 जीनो की उपस्थिति के आधार पर 83 डाह समूहों में बांटा गया जिसमे से वी1 डाह समूह में सभी जीन उपस्थित थी जो कि मनुष्य के (अच3) वियोजक से बना था जबकि वी81, वी82 एवं वी83 डाह समूह में 11, 9 एवं 8 जीन उपस्थित थी तथा जो कि जे4, सी15 एवं जी16 वियोजक से क्रमशः बने थे |

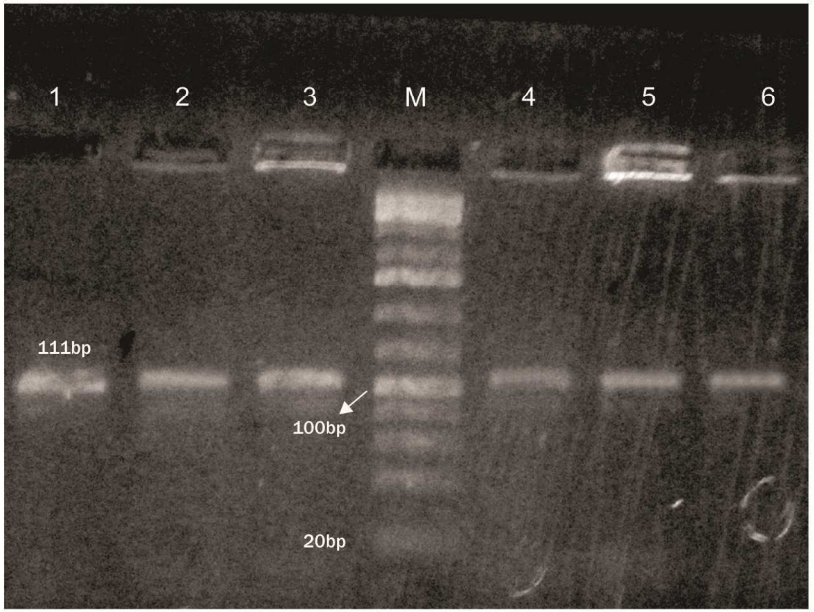
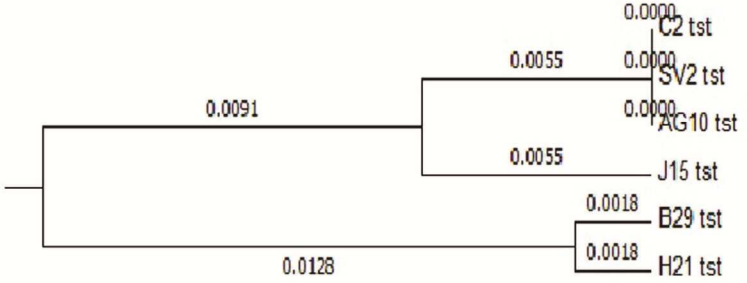


Fig. 52: Detection of *hld* gene among *S. aureus* isolates

M- 20bp Molecular Marker
Well No. 1-6: Positive for *hld* gene



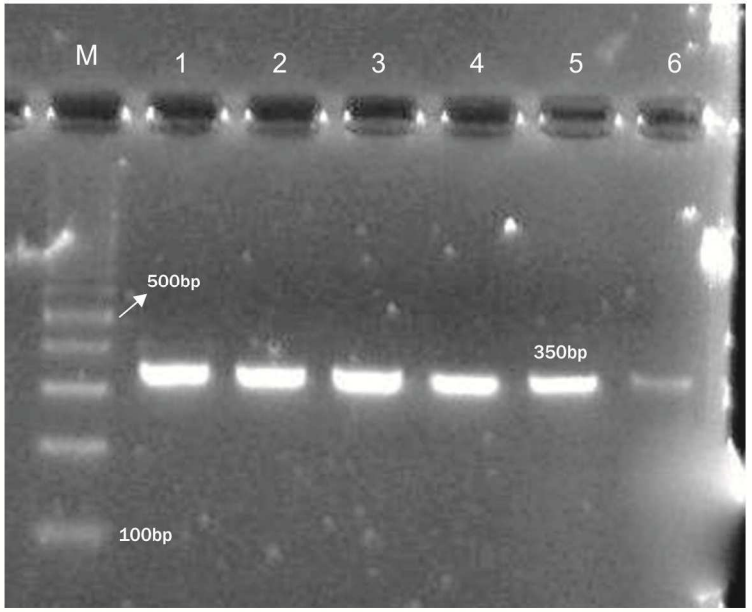
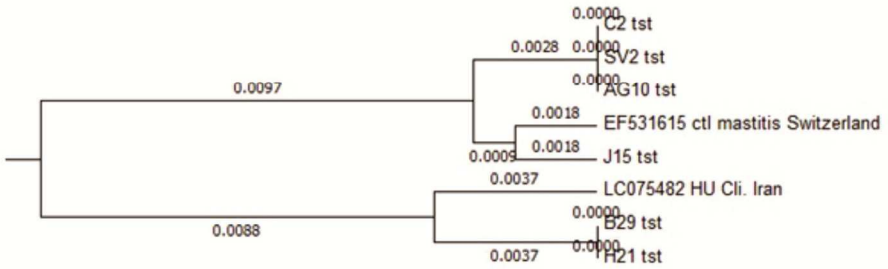


Fig. 53: Detection of *tst* gene among *S. aureus* isolates

M- 100bp Molecular Marker
Well No. 1-6: Positive for *tst* gene



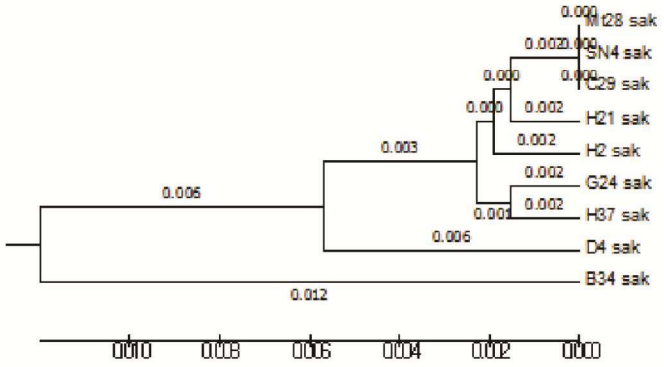
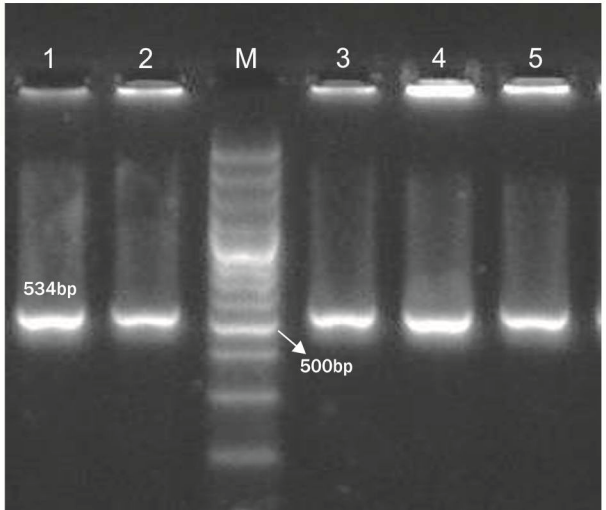


Fig. 47: Phylogenetic tree analysis of *sak* gene sequences of the present study



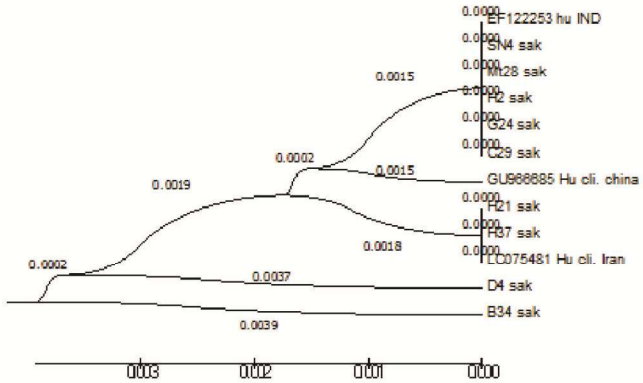
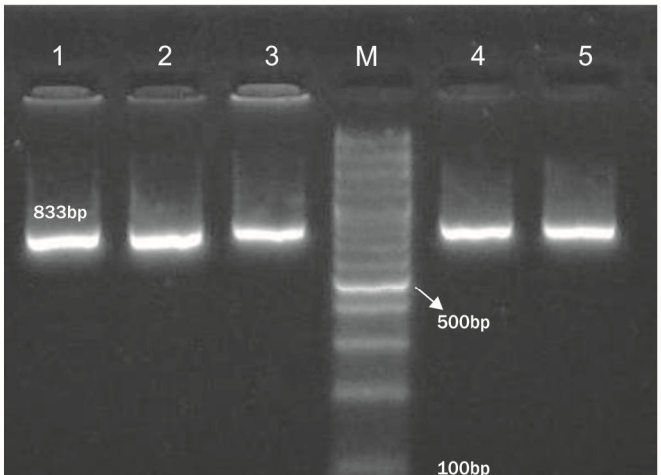
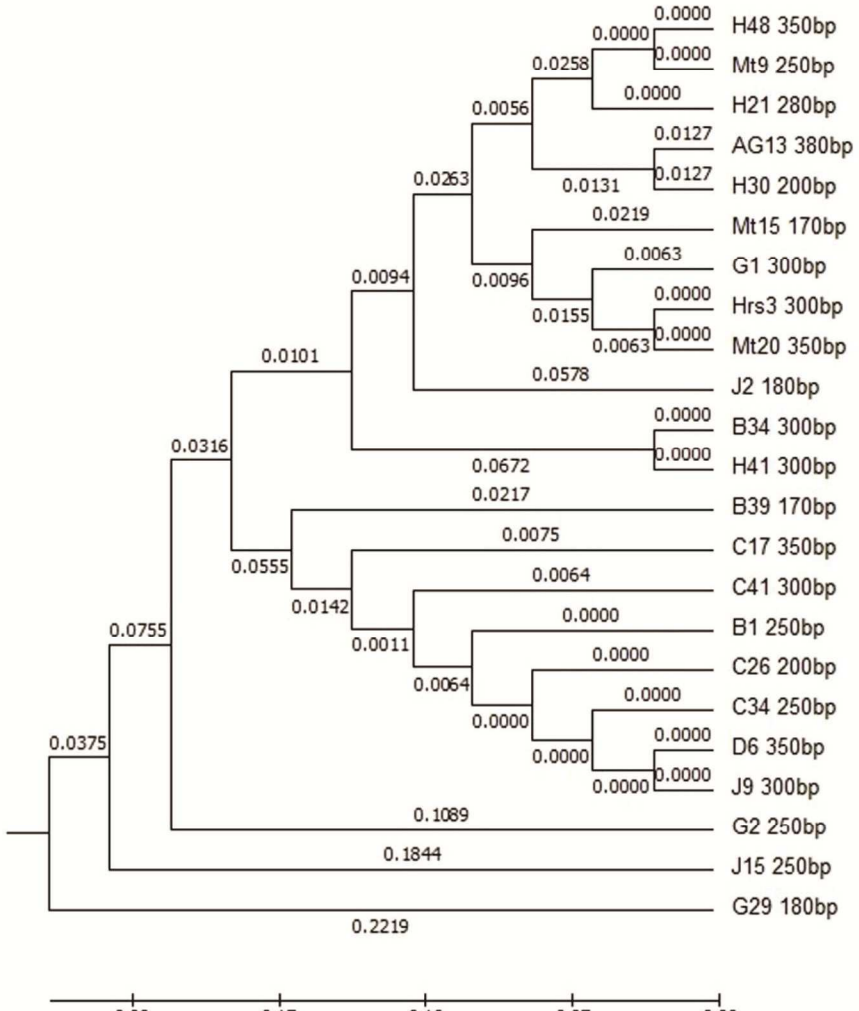
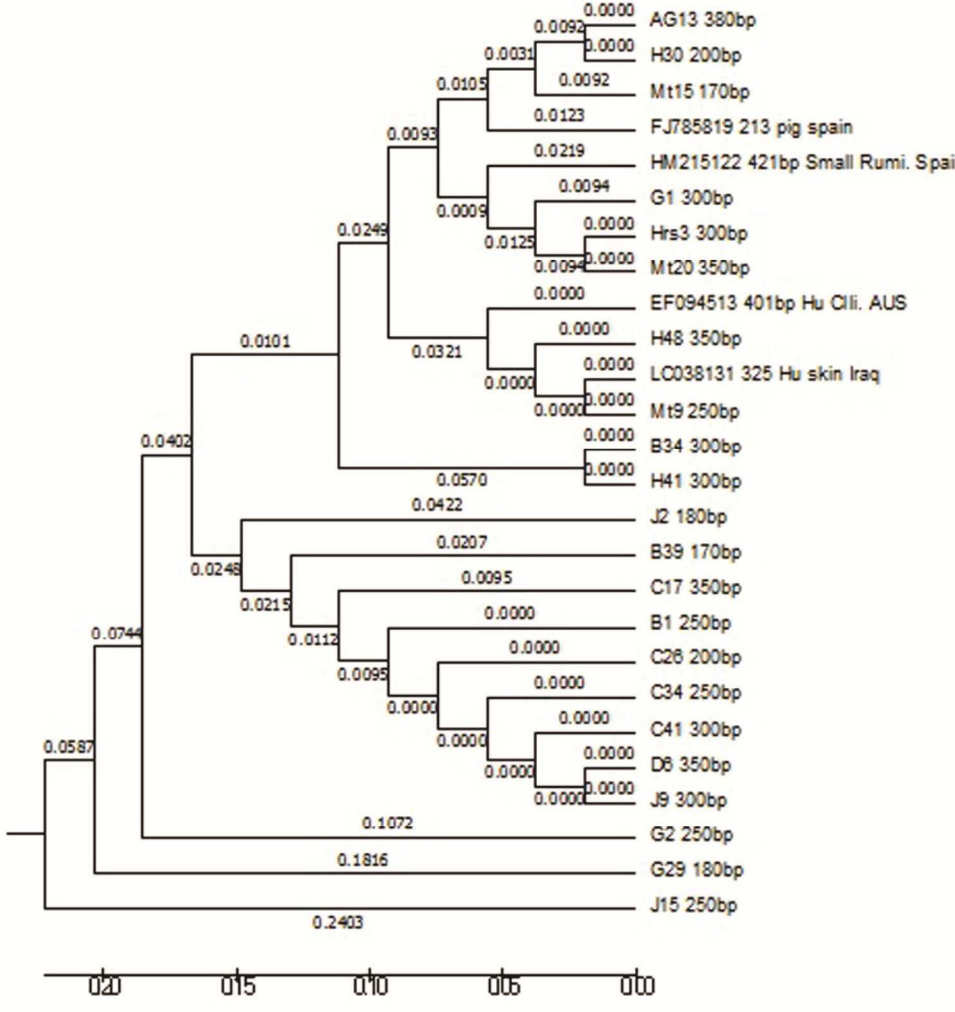


Fig. 49: Phylogenetic tree analysis of *sak* gene sequences of present study with reference sequences of NCBI database




H48 350bp
Mt9 250bp
H21 280bp
AG13 380bp
H30 200bp
Mt15 170bp
G1 300bp
Hrs3 300bp
Mt20 350bp
J2 180bp
B34 300bp
H41 300bp
B39 170bp
C17 350bp
C41 300bp
B1 250bp
C26 200bp
C34 250bp
D6 350bp
J9 300bp
G2 250bp
J15 250bp
G29 180bp


AG13 380bp
H30 200bp
Mt15 170bp
FJ785819 213 pig spain
HM215122 421bp Small Rumi. Spai
G1 300bp
Hrs3 300bp
Mt20 350bp
EF094513 401bp Hu Clli. AUS
H48 350bp
LC038131 325 Hu skin Iraq
Mt9 250bp
B34 300bp
H41 300bp
J2 180bp
B39 170bp
C17 350bp
B1 250bp
C26 200bp
C34 250bp
C41 300bp
D6 350bp
J9 300bp
G2 250bp
G29 180bp
J15 250bp

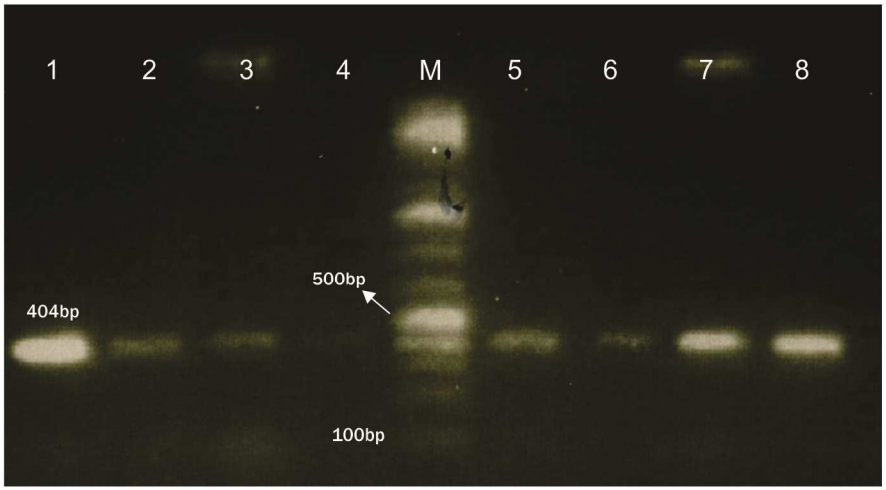


Fig. 39: Detection of *chp* gene among *S. aureus* isolates

M- 100bp Molecular Marker
Well No. 1-8: Positive for *chp* gene

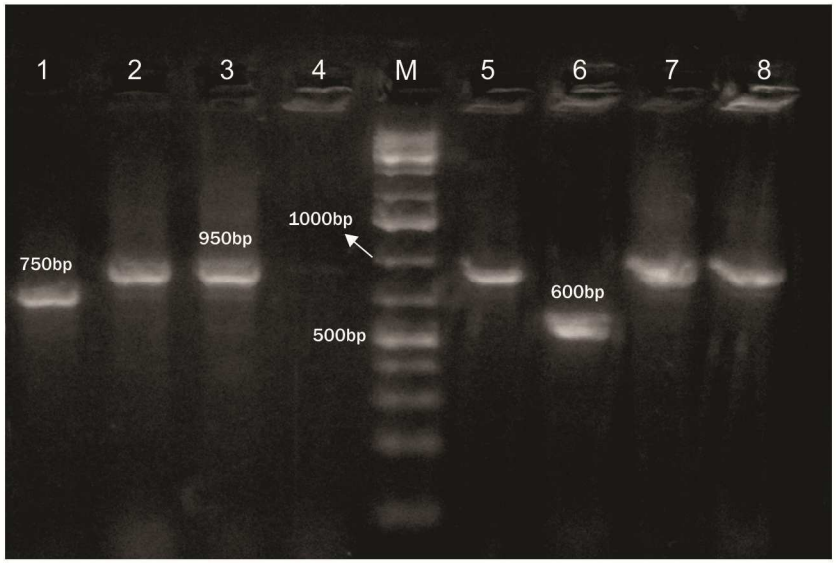


Fig. 40: Detection of *spa-IgG* gene among *S. aureus* isolates

M- 1kb Molecular Marker
Well No. 1: *spa-IgG* gene Positive with 750bp size
Well No. 5: *spa-IgG* gene Positive with 600bp size
Well No. 2, 3, 4, 6, 7: *spa-IgG* gene Positive with 950bp size

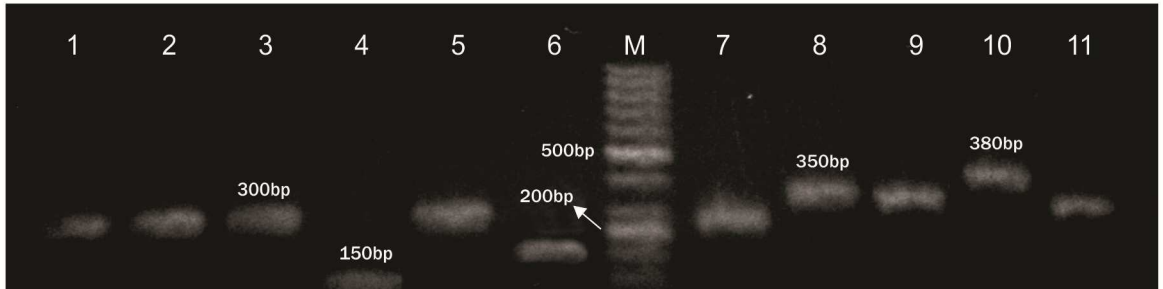
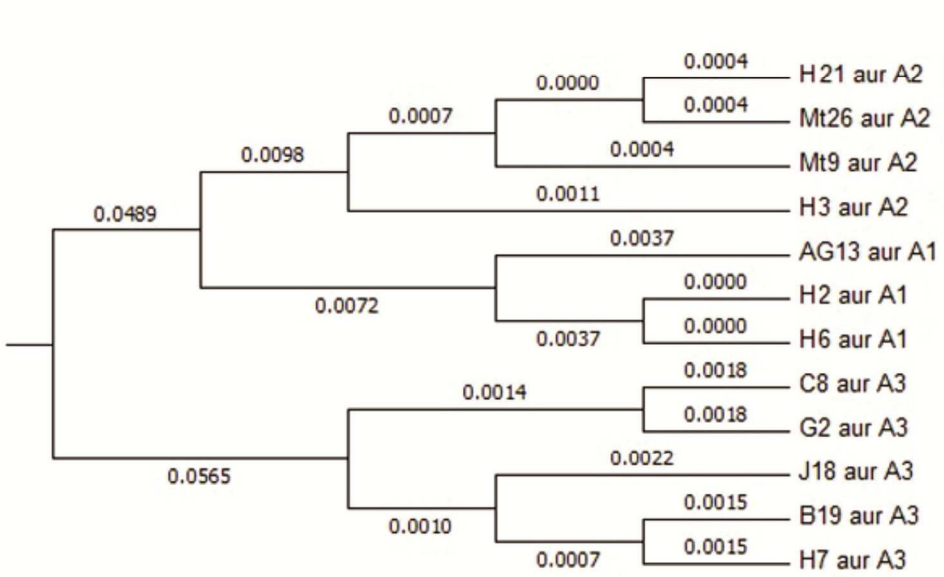
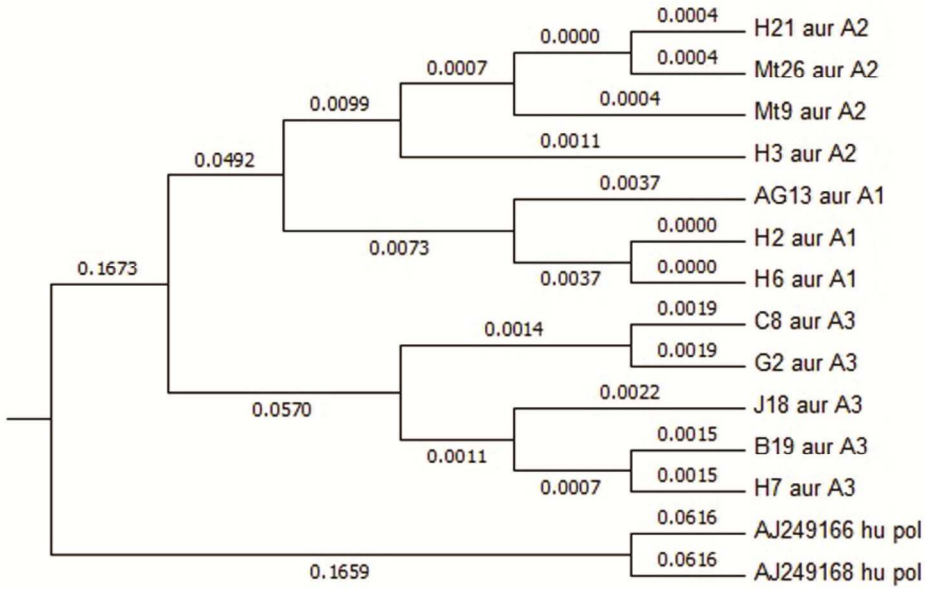
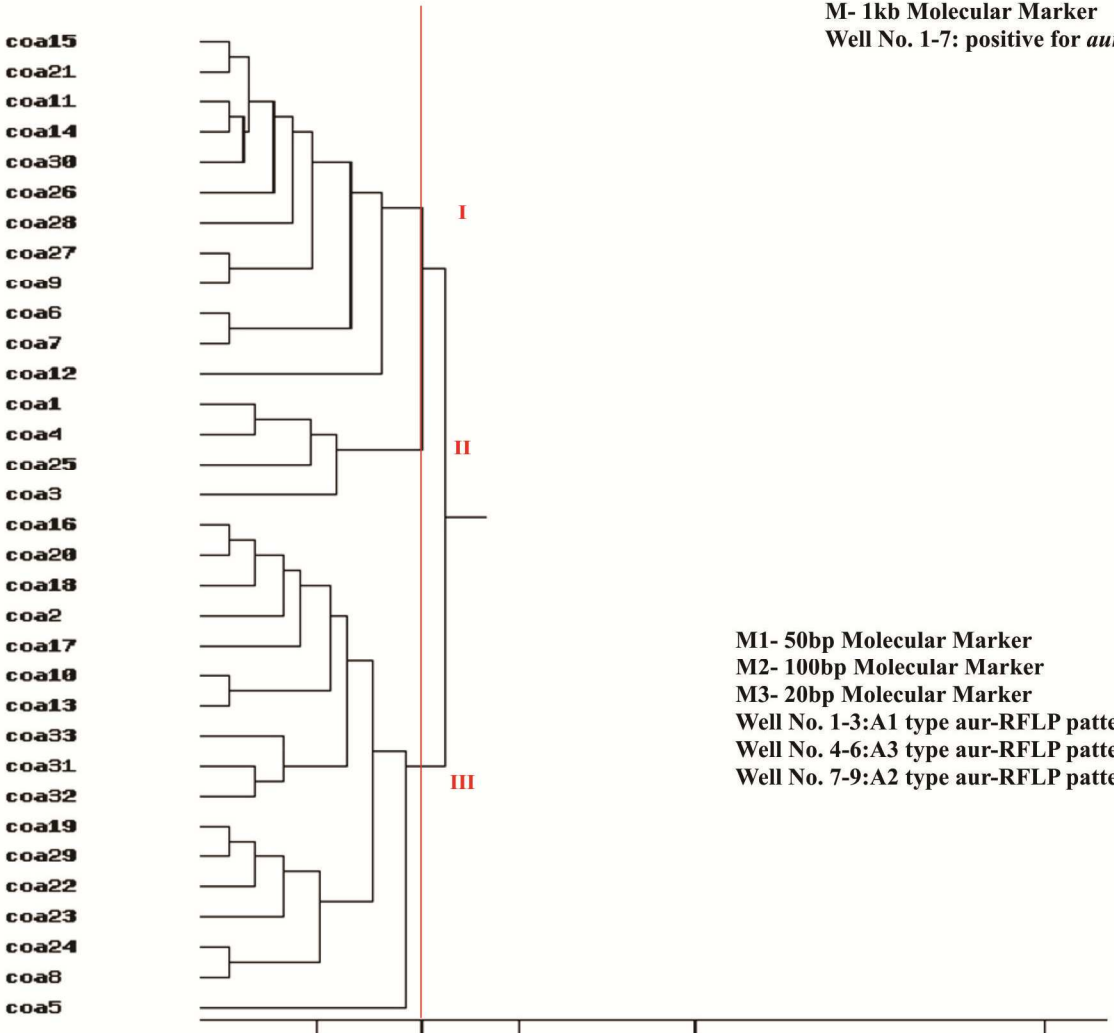


Fig. 34: Phylogenetic cluster analysis of various aur-RFLP patterns of *S. aureus* isolates

Comparison method: Dice
Clustering method: UPGMA







M- 1kb Molecular Marker
Well No. 1-7: positive for *aur* gene

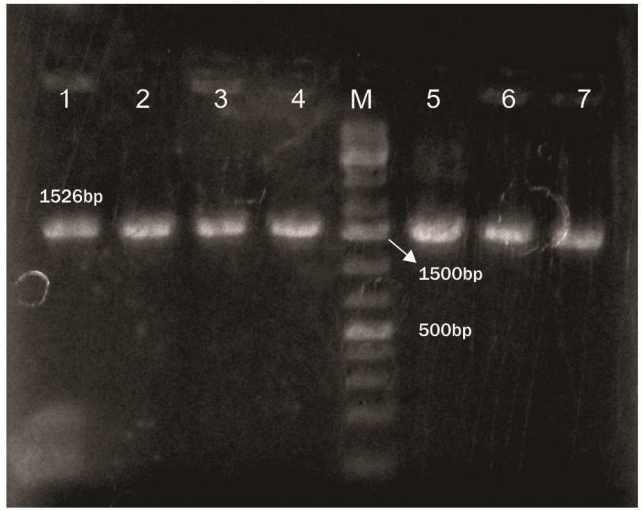
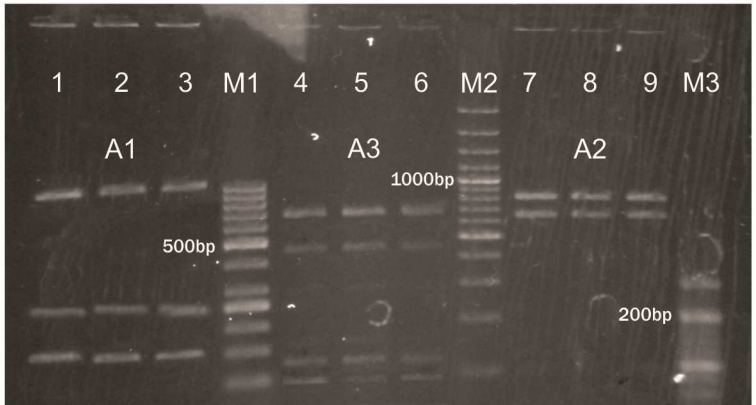


Fig. 32: Detection of *aur* gene among *S. aureus* isolates

M1- 50bp Molecular Marker
M2- 100bp Molecular Marker
M3- 20bp Molecular Marker
Well No. 1-3:A1 type aur-RFLP pattern
Well No. 4-6:A3 type aur-RFLP pattern
Well No. 7-9:A2 type aur-RFLP pattern



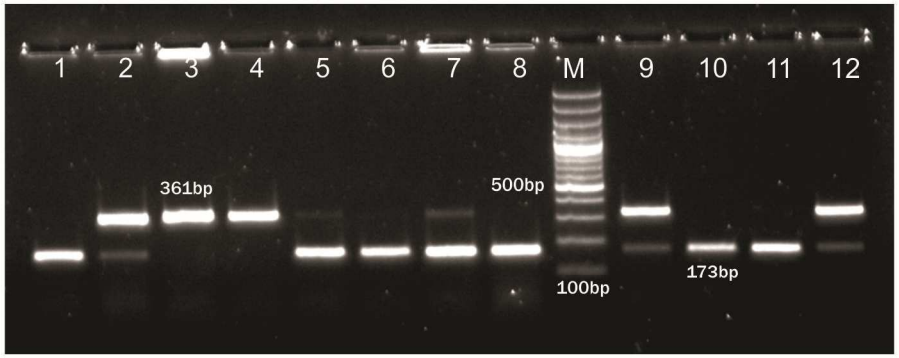


Fig. 28: Detection of *cap5K* and *cap8K* genes among *S. aureus* isolates by Duplex PCR

M- 100bp Molecular Marker
Well No. 1, 6, 8, 10, 11: Positive for *cap8K* gene
Well No. 3 and 4: Positive for *cap5K* gene
Well No. 2, 5, 7, 9, 12: Positive for both (*cap5K* and *cap8K*)genes

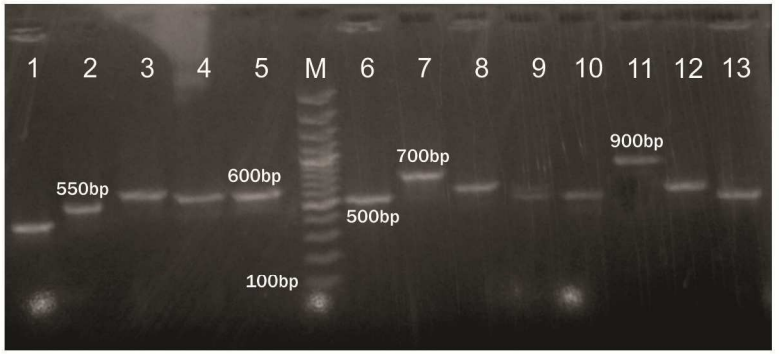
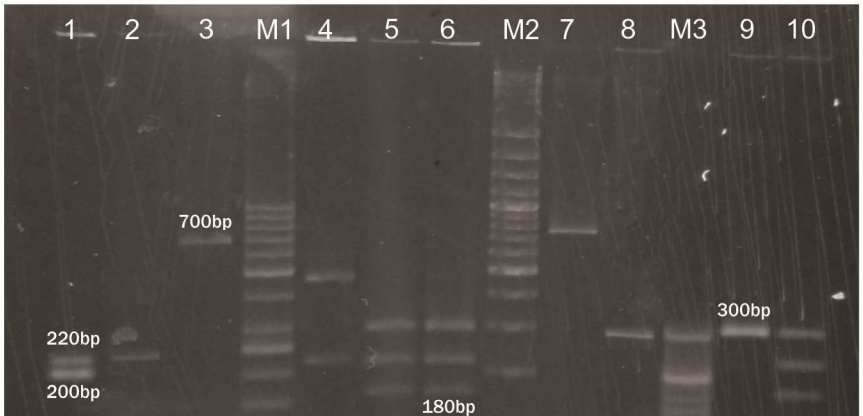


Fig. 29: Detection of *coa* gene among *S. aureus* isolates

M- 100bp Molecular Marker
Well No. 1-13: *coa* positive with variable band sizes



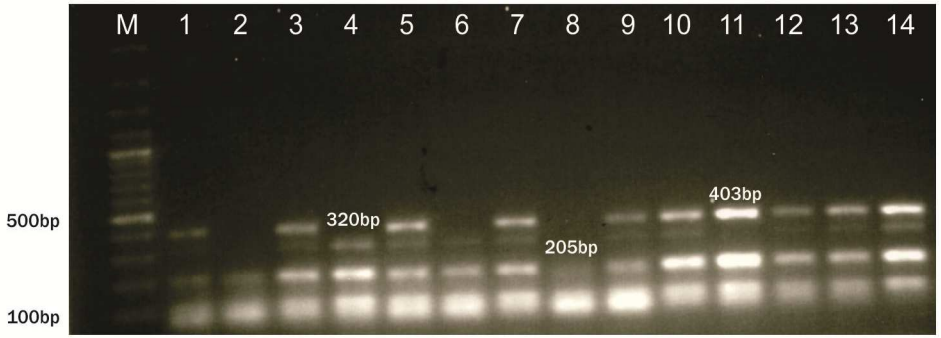


Fig. 24: Detection of *clfB, scn* and *sak* genes among *S. aureus* isolates by Multiplex PCR

M- 100bp Molecular Marker
Well No. 1-14: Variable presence of *clfB* (205 bp), *scn* (320 bp) and *sak* (403 bp) genes in *S. aureus* isolates
Well No. 1, 3, 5, 7, 9, 10, 12, 13, 14: Positive for all three genes
Well No. 2, 8: Positive for *clfB* gene
Well No. 4, 6: Positive for *clfB* and *scn* genes
Well No. 11: Positive for *sak* & *clfB* gene

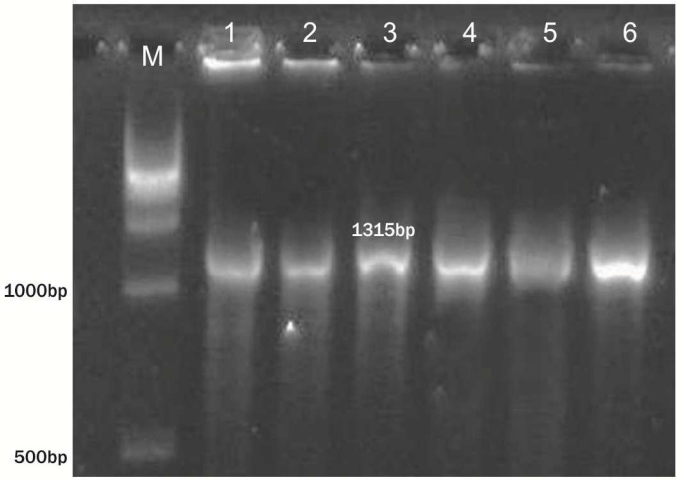
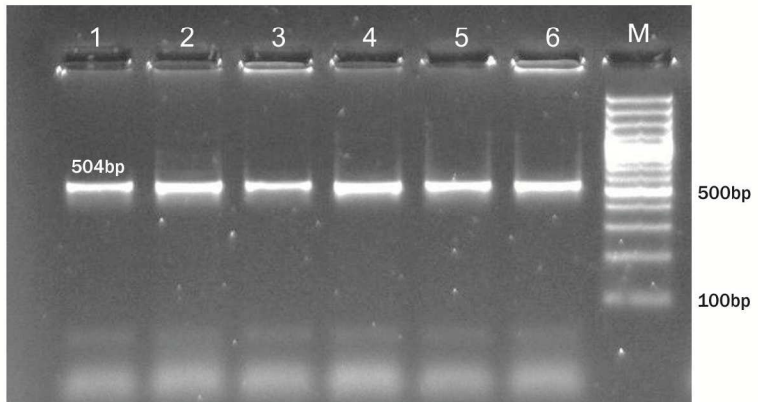
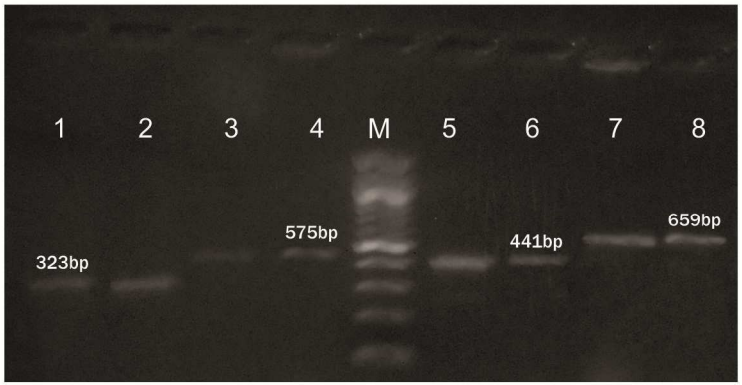
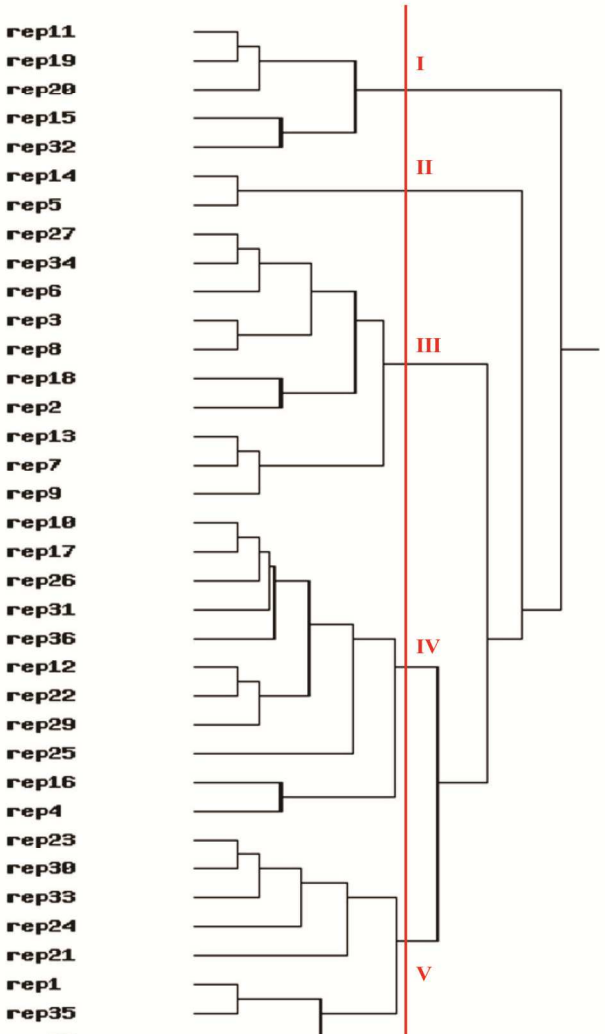


Fig. 25: Detection of *icaA* gene among *S. aureus* isolates

M- 500bp Molecular Marker
Well No. 1-6: Positive for *icaA* gene







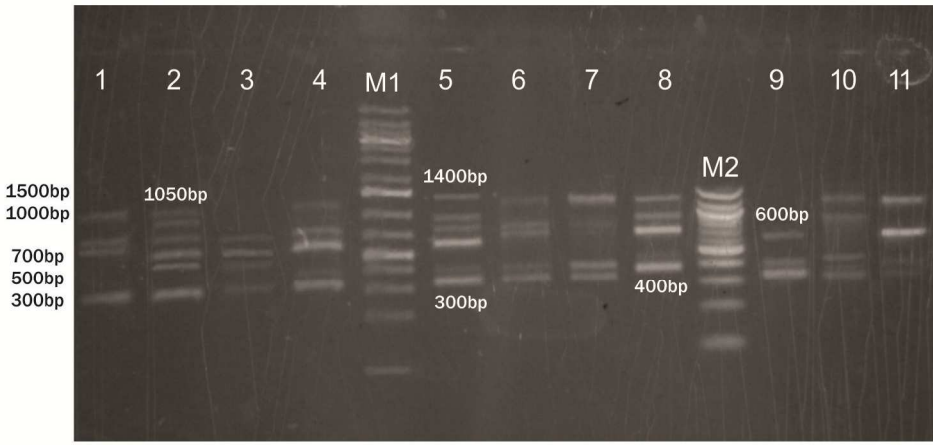
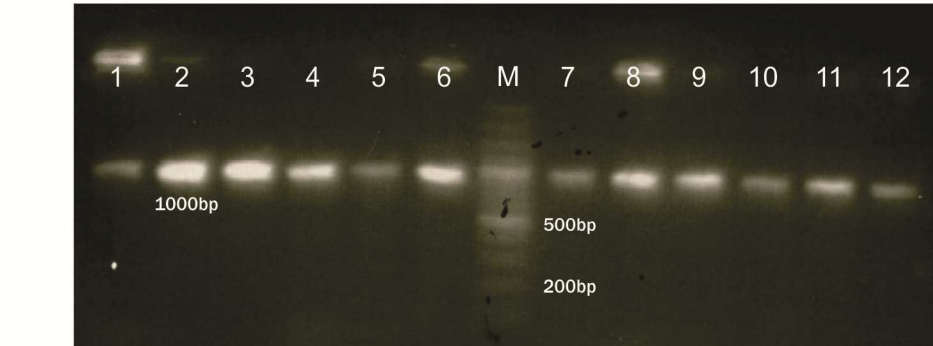


Fig. 22: Various rep-patterns (repetitive element sequence-based PCR of *S. aureus* isolates

M1- 1kb Molecular Marker
M2- 100bp Molecular Marker
Well No. 1-11: rep- patterns in *S. aureus* isolates



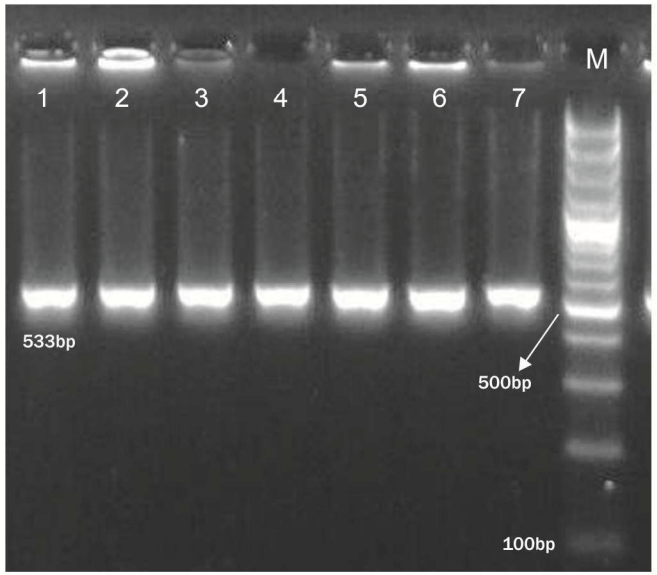


Fig. 15: Detection of *mecA* gene among *S. aureus* isolates

M- 100bp Molecular Marker
Well No.1-7: *mecA* gene positive isolates

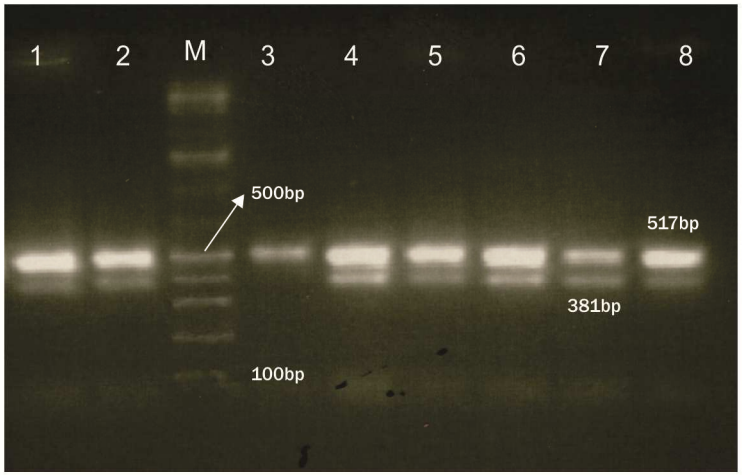
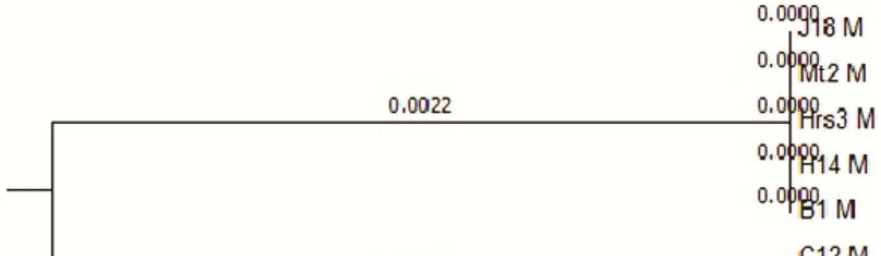
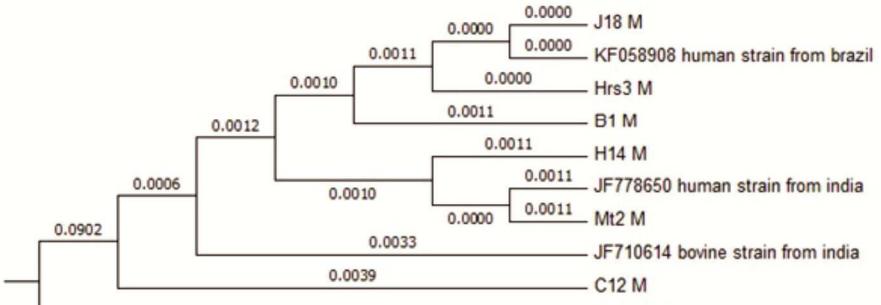


Fig. 16: Detection of *blaZ* and *icaD* genes among *S. aureus* isolates by Duplex PCR

M- 100bp Molecular Marker
Well No.3: *blaZ* gene positive isolate
Well No. 1, 2, 4-8: *blaZ* (517bp) and *icaD* (381bp) genes positive isolates





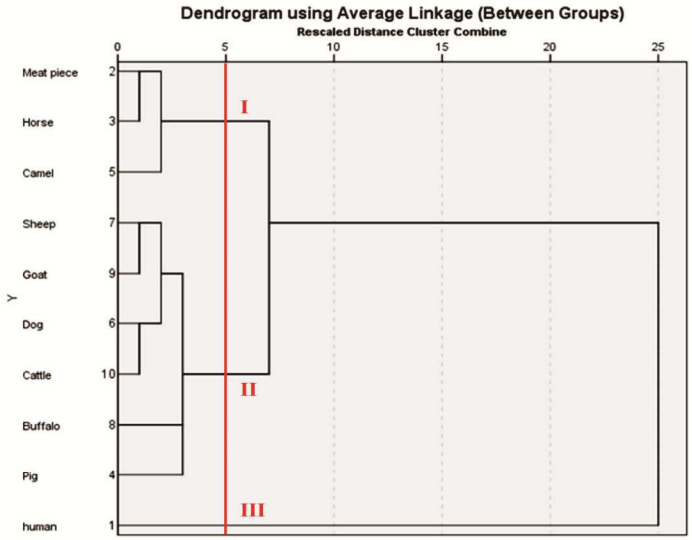


Fig. 12: Hierarchical ascendant cluster analysis of MIC for *S. aureus* isolates using SED and the between-groups linkage method.

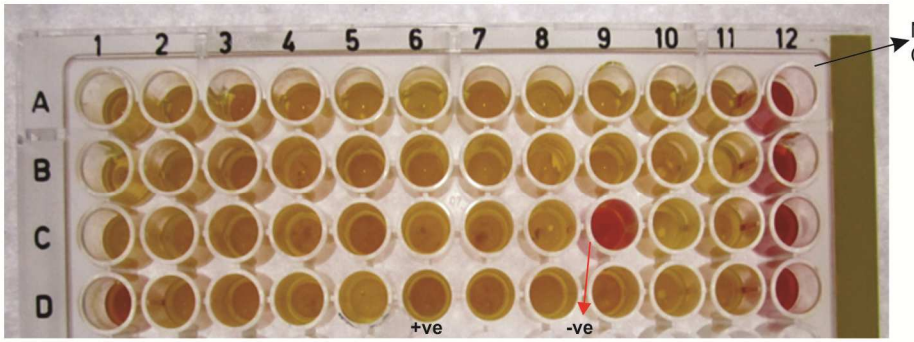
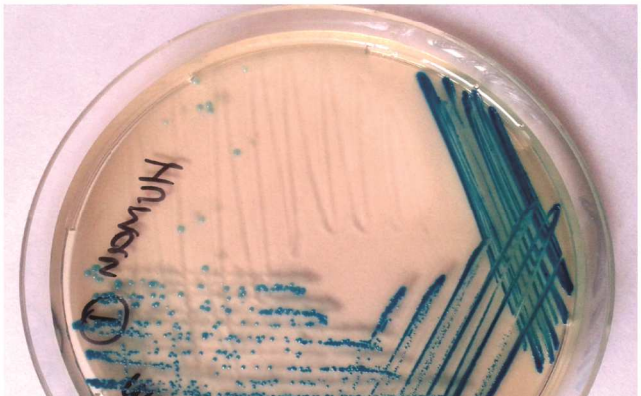


Fig. 14: Detection of β-lactamase production among *S. aureus* isolates



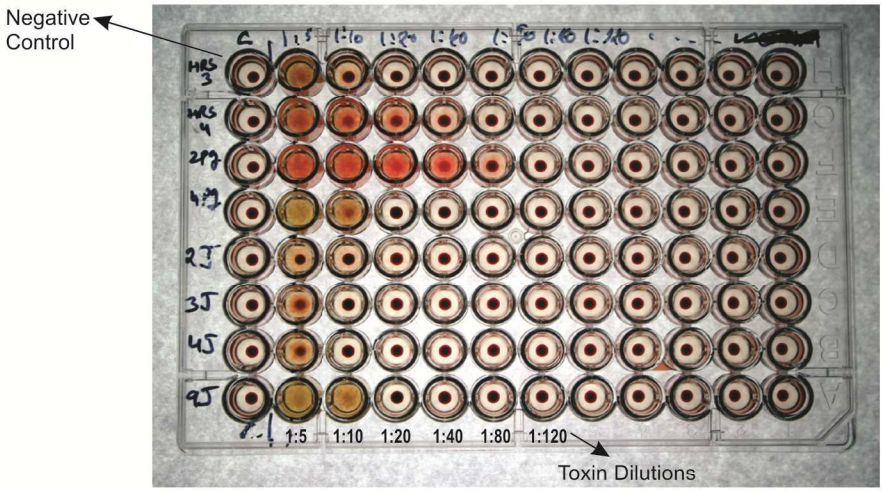


Fig. 8: Titration of delta (δ) hemolysin toxin produced by *S. aureus* isolates

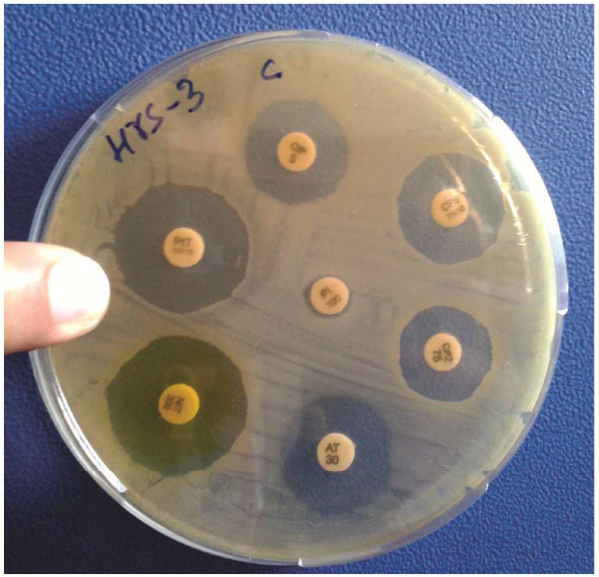
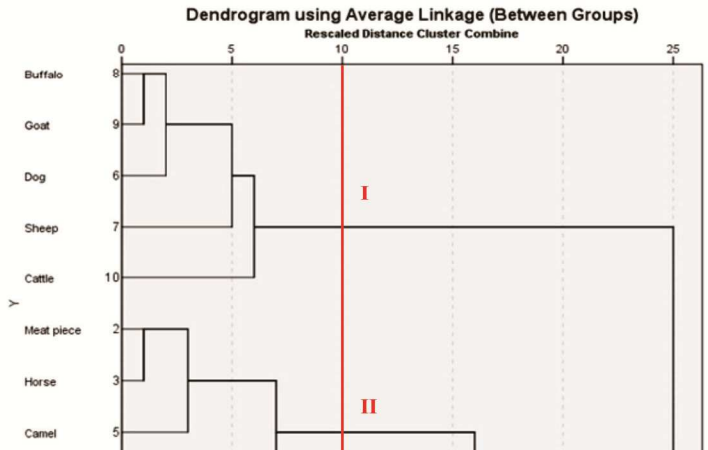
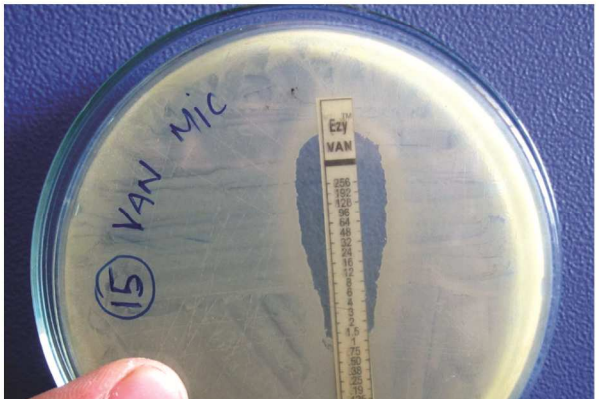


Fig. 9: Antibiotics sensitivity patterns of *S. aureus* isolates





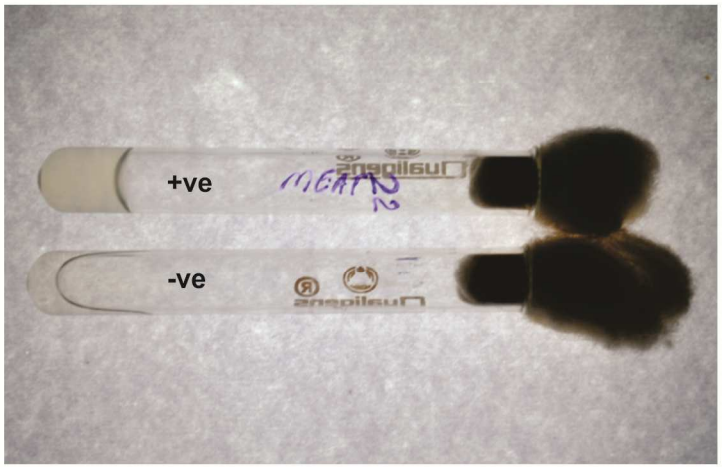


Fig. 4: Coagulation reaction of *S. aureus* isolate

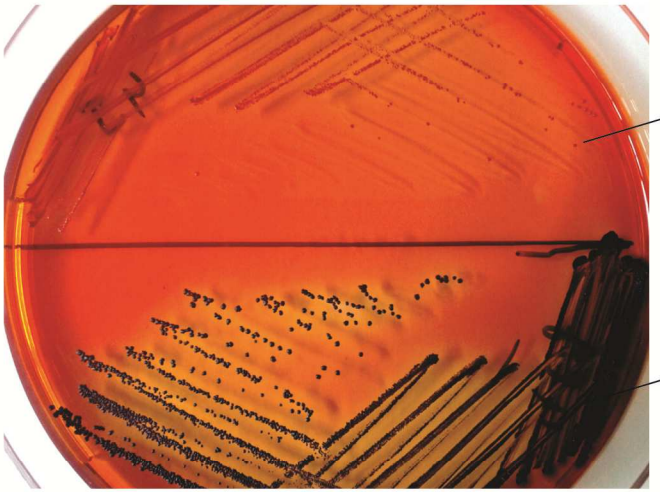
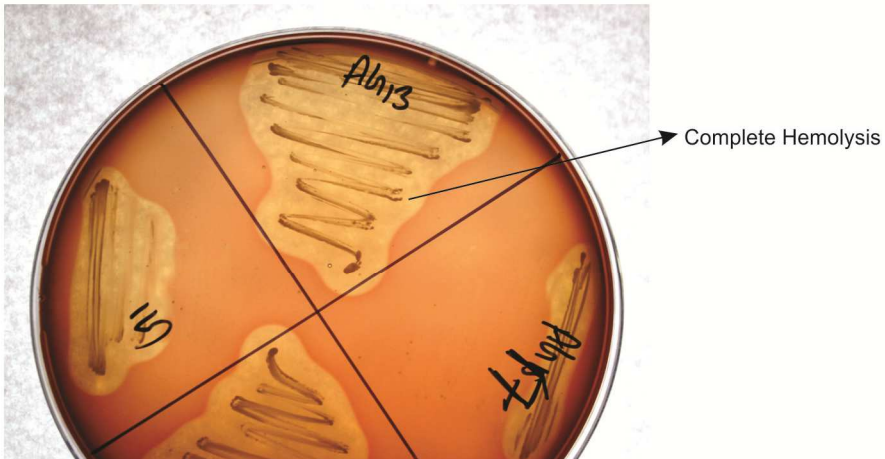
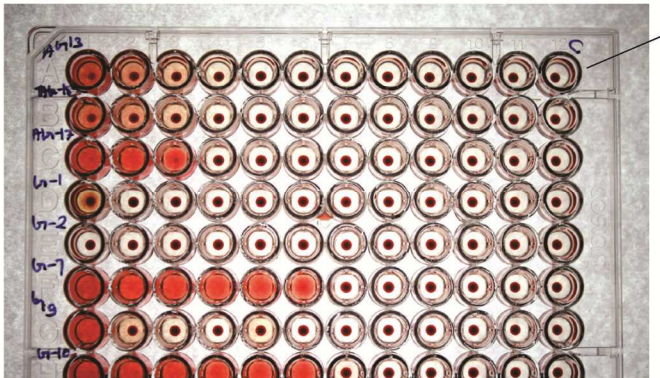


Fig.5 Slime Production in *S. aureus* isolates grow on Congo Red Agar





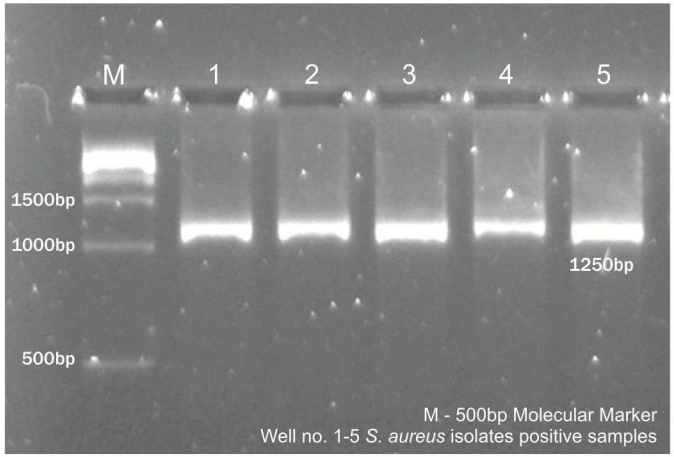


Fig.1: 23r RNA based species specific genotypic confirmation of *S. aureus* isolates

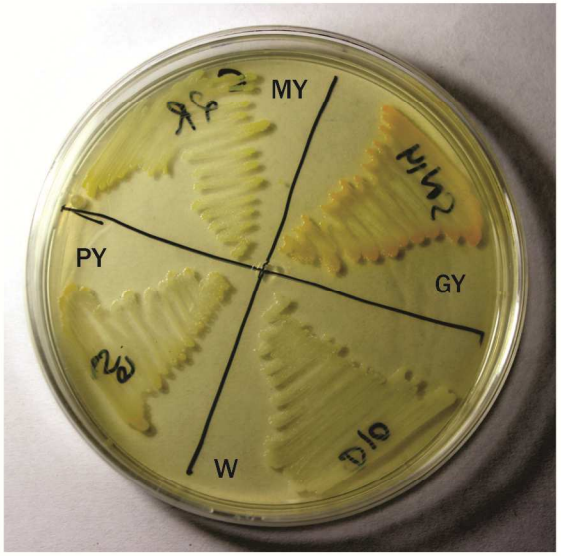


Fig.2: *Staphylococcus aureus* isolates growth on Nutrient Agar