Studies on Phenotypic and Genotypic Evaluation of Biofilm Production and Antimicrobial Resistance in *Staphyloccocus aureus* Isolated from Milk

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BY

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Enrolment No.: V-1210/13

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(2022)

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ABST	:	Antibiotic Susceptibility Test
A/S	:	Ampicillin and Sulbactam
AMR	:	Antimicrobial Resistance
BHI	:	Brain Heart Infusion
BTB	:	BromoThymol Blue
C	:	Chloramphenicol
CAZ	:	Ceftazidime
CEC	:	Cefotaxime/Clavulanic acid
CIP	:	Ciprofloxacin
CLSI	:	Clinical Laboratory and Standards Institute
CMT	:	California Mastitis Test
CNS	:	Coagulase Negative Staphylococci
CTR	:	Ceftriaxzone
CTX	:	Cefotaxime
CX	:	Cefoxitin
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxynucleotide Triphosphates
E	:	Erythromycin
EC	:	Electrical Conductivity
EDTA	:	Ethylenediamine tetraacetic Acid
EMB	:	Eosine Methylene Blue Agar
ESBL	:	Extended spectrum β-lactamase
EtBr	:	Ethidium bromide
GEN	:	Gentamicin
IMP	:	Imipenem
LE	:	Levofloxacin

LZ	:	Linizolid
MDR	:	Multidrug Resistant
MET	:	Methicillin
MHA	:	Mueller Hinton Agar
MLA	:	MacConkey Lactose agar
MRSA	:	Methicillin Resistant *Staphylococcus aureus*
MSA	:	Mannitol Salt Agar
NA	:	Nutrient Agar
NFW	:	Nuclease Free Water
NSS	:	Normal Saline Solution
OF	:	Ofloxacin
OX	:	Oxacillin
PCR	:	Polymerase Chain Reaction
pH	:	Log hydrogen ion concentration
rpm	:	Revolutions per minute
S	:	Streptomycin
SCC	:	Somatic Cell Count
SCM	:	Sub Clinical Mastitis
SOP	:	Standard Operating Procedure
spp.	:	Species
TE	:	Tetracycline
TAE	:	Tris-Acetate-EDTA
Taq	:	Thermus aquaticus
VA	:	Vancomycin

LIST OF TABLES

Table No.	Title
Table 1	Sample collection from different sources
Table 2	Interpretation and scoring of the California Mastitis Test
Table 3	Primers for *nuc* gene of *S. aureus*
Table 4	Composition of PCR reaction mixture of *nuc* gene
Table 5	Amplification conditions for *nuc* gene
Table6	Primers for biofilm forming genes
Table 7	Composition of PCR reaction mixture of *bap* and *icaA* genes
Table 8	Amplification conditions for *bap* and *icaA* genes
Table 9	Detail of Antibiotic discs used in antibiotyping
Table 10	Detail of E-strips used in E-test (Epsilometer test)
Table 11	Primer for antibiotic resistance genes
Table 12	Composition of PCR reaction mixture of *mecA* and *vanA* genes
Table 13	Amplification conditions for *mecA* and *vanA* genes
Table 14	Scoring of Milk samples by California Mastitis Test (CMT)
Table 15	Prevalence of *S. aureus* in milk from different sources
Table 16	Overall Prevalence of *S. aureus* in milk from different sources
Table 17	Molecular detection of *nuc* gene
Table 18	Screening of biofilm producing *S. aureus* by Congo Red Agar (CRA) Assay
Table 19	Screening of biofilm forming *S.aureus* by Tube Method (Biofilm production capability and formation intensity)
Table 20	Screening of biofilm forming *S.aureus* by Tissue Culture Plate Assay (Biofilm production capability and formation intensity)
Table 21	Comparision of biofilm assays (TCP,TM,CRA)
Table 22	Source wise prevalence of Biofilm forming *S. aureus* isolates

Table 23	Percent positivity of *bap* and *ica*A gene
Table 24	Antibiogram of biofilm forming *S. aureus* isolated from Raw, Pooled raw, Mastitic and Pasteurized milk
Table 25	Antibiogram of biofilm forming *S. aureus* isolated from Raw milk
Table 26	Antibiogram of biofilm forming *S. aureus* isolated from Pooled raw mik
Table 27	Antibiogram of biofilm forming *S. aureus* isolated from mastitic milk
Table 28	Antibiogram of biofilm forming *S. aureus* isolated from Pasteurized milk
Table 29	Resistant *S. aureus* isolates
Table 30	Resistance pattern of *S. aureus* isolates
Table 31	Resistance pattern of MDR strain
Table 32	Minimum Inhibitory Concentration(MIC) by E-test
Table 33	Percent positivity of antibiotic resistance gene (*mecA and vanA*)

LIST OF FIGURES

Figure No.	Title
Figure 1	Gel formation in mastitic positive sample
Figure 2	Pre enrichment of samples in Buffered Peptone Water
Figure 3	Jet black colored colonies of *Staphylococcus spp.* on Baired-Parker Agar
Figure 4	Yellow colored colonies of *Staphylococcus spp.* on Nutrient Agar
Figure 5a &b	Yellow colored colonies with yellow zone of *S. aureus* on Mannitol Salt Agar
Figure 6a &b	IMViC Test
Figure 7	Catalase Test
Figure 8	Coagulse Test
Figure 9	Gram positive cocci with purple stain
Figure 10	Prevalence of *S. aureus* in different types of milk
Figure 11	Species specific PCR amplicon (*nuc*) of *S. aureus* resolved after electrophoresis in 1.5 % agarose gel
Figure 12	Colonies on Congo Red Agar
Figure 13	Biofilm formation by Tube Method
Figure 14	Biofilm formation by Tissue Culture Plate Assay
Figure 15	Comparison of TCP, TM and CRA
Figure 16	Agarose gel showing absence of *bap* gene (971 bp) of *S. aureus*
Figure 17	Biofilm forming *icaA* gene (188 bp) resolved after electrophoresis in 1.5 % agarose
Figure 18	Molecular Phylogenetic analysis by Maximum Likelihood method
Figure 19	Pattern of resistance against antimicrobials tested for *S. aureus* isolates

Figure 20a -d	Antibiotic Sensitivity Test
Figure 21a-b	Minimum Inhibitory Concentration by E-Test
Figure 22	Antibiotic resistance *mecA* gene (310bp) resolved after electrophoresis in 1.5% agarose
Figure 23	Agarose gel showing absence of *vanA* gene (1030 bp) of *S. aureus*

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Contents

S. No.	*Particulars*	*Page No.*
1	*Introduction*	***1-4***
2	*Review of Literature*	***5-18***
3	*Materials and Methods*	***19-37***
4	*Results*	***38-63***
5	*Discussion*	***64-69***
6	*Summary and Conclusions*	***70-73***
8	*Bibliography*	***i-xvii***
	CV	

Studies on Phenotypic and Genotypic Evaluation of Biofilm Production and Antimicrobial Resistance in *Staphyloccocus aureus* Isolated from Milk

ABSTRACT

The present research work was conducted to investigate the phenotypic and genotypic evaluation of biofilm production and antimicrobial resistance in *Staphyloccocus aureus* isolated from milk. The *S. aureus* were detected by phenotypic and genotypic methods and biofilm formation capability was evaluated by three different assays viz. Tissue Culture Plate Assay, Tube Method and Congo Red Agar Assay. Antimicrobial resistance was observed by Antibiotic sensitivity test and Minimum inhibitory concentration was evaluated by E test. Biofilm forming (*bap* and *ica*A) and resistance (*mec* and *van*A) genes were screened by simplex PCR. In this investigation**,** a total of 378 samples comprising of 252 raw milk, 40 pooled raw milk, 56 mastitic milk and 30 pasteurised milk were collected over a period of one year from dairy farms, gaushalas, local milk shops, vendors and grocery shops from Mathura district of U.P. Samples were screened for the isolation of *S. aureus* and total of 121 *Staphylococcus spp and* 106 *S. aureus* were obtained that confirmed biochemically and with prevalence of *S. aureus* in raw, pooled raw, mastitis and pasteurized milk was 18.65%, 57.5%, 51.78% and 23.33% with overall prevalence of 28.04%. In the milk of local milk shops, vendors and grocery shops (pasteurized milk) prevalence of *S. aureus* 70.0%, 45.0% and 23.33%, respectively and it was found that *S. aureus* was more prevalent in milk collected from local shops as comparison to vendors and pasteurised milk. All the phenotypically confirmed *S. aureus* isolates were *nuc* gene bearers with prevalence of *nuc* gene in raw milk, pooled raw milk, mastitic raw milk and pasteurized milk were18.65%, 57.50%, 51.78% and 23.33%, respectively. Confirmed *S. aureus* isolates (106) were analyzed for biofilm formation by three different methods CRA, TM and TCP. In CRA assay 17.92% isolates were biofilm former and 82.07% non-biofilm former. In TM, overall, 39.62%, 31.13% and 29.25% *S. aureus* were strong, moderate and weak biofilm producer. In TCP assay, overall, 46.23%, 31.13% and 22.64% *S. aureus* were found to be strong, moderate and weak biofilm producer. Among all three assays highest number of biofilm forming *S. aureus* were revealed by TCP (77.36%), in comparison to TM (70.75%) and CRA assay (17.92%) and also TCP assay was proved to be the golden standard method for the screening of biofilm forming isolates. Prevalence of biofilm former *S. aureus* was highest in mastitic milk followed by pooled raw milk, raw milk, and pasteurized milk which was 96.55%, 82.60%, 70.21% and 28.57%. Isolates were screened for biofilm genes *bap* and *ica*A but none of the isolate possessed *bap* gene (0%) and the percent positivity of *icaA* gene was 81.13%. All the 49 strong biofilm forming *S. aureus* were subjected to antimicrobial sensitivity testing against 18 antibiotics with the range of resistance from 10.20- 69.38%. Isolates were found sensitive towards the antibiotics Cefoxitin, Ceftriaxone, Vancomycin 95.91%, 95.91% and 85.71%, respectively. It was also observed that 20 isolates were resistant to more than 3 classes of antibiotics and 40.81% (20/49) biofilm forming *S. aureus* isolates were multi drug resistant (MDR). The percent positivity of *mec*A and *van* A gene was 22.64% and zero % in *S. aureus*. This study revealed that biofilm forming *S. aureus* were obtained from raw milk of animals and may be a sustainable source of contamination of dairy products with this pathogen. So, there is need of paying more attention to the cleaning and sanitizing processes of food contact surfaces to ensure the public health.

Introduction

CHAPTER- 1

INTRODUCTION

India ranks first in milk production and consumption in the world with milch animal population of 125.34 million (20th Livestock Census 2020, DADF). Dairy is the single largest agricultural commodity, contributing 5% of the national economy and employing more than 8 crore farmers directly. The milk production in India has increased from 55.6 million tonnes in 1991-1992 to 187.7 million tonnes in 2018 - 2019, reporting 237.58 % growth (Subbanna et al., 2021). Milk production during 2019-20 and 2020-21 is 198.44 million tonnes and 209.96 million tonnes respectively showing an annual growth of 5.81% and all India per capita availability of milk is 427 gms per day in 2020-21 (BAHS, 2021-22).

Agriculture and animal husbandry are the backbones of Indian economy and rural based economic sector being inherently suffered with several setbacks including infectious diseases (Kumar et al., 2017). According to Ministry of food processing industry, Uttar Pradesh is the highest milk producing state in India with 30,519 metric tonnes contributing around 18% to the total milk production, followed by Rajasthan, Andhra Pradesh, Gujarat and Punjab contributing 11%, 10%, 8% and 7%, respectively (BAHS, 2021-22).

S. aureus is a common bacterium that has long been recognized as a challenge in both human and veterinary medicine (Bissong and Ateba, 2020). *S. aureus* is an important zoonotic pathogen, which can cause serious infection in humans and animals (Pizauro et al., 2019). It multiplies in skin, mucous membranes and skin glands and causes varied infections such as inflammation of bones, meninges, rashes and septicemia (Hennekinne et al., 2012). *S. aureus* is also associated with food poisoning in humans, Staphylococcal Scaled Skin Syndrome (SSSS), post-operative wound infections, inflammation of lungs (pneumonia) and nosocomial bacteremia in humans, clinical and subclinical mastitis in bovine (Torres et al., 2019). The present employed control measures in developing countries look to be compromised due to ignorance in handling the situation regarding prevention and control of the spread of particularly endemic and emerging *Staphylococcus aureus* strains (Wang et al., 2018).

Mastitis is one of the major problematic diseases of dairy animals throughout the world which is associated with different etiological agents but *S. aureus* is one of

the major causes which are responsible for this dairy scourge (Raza et al., 2013). Several vital factors like the use of antimicrobials, supervision of the herd and individual characteristics of the host produce continuous selective pressure on *S. aureus* strain are likely to be better adapted to survive and become more pathogenic and virulent. Extensive and unsystematic use of antibiotics in human and veterinary medicine is the key reason for emerging of resistant strains of *S. aureus* (Sheela et al., 2015).

This bacterium produces wide range of virulence factors like Toxic Shock Syndrome Toxin-1 (TSST-1), Staphylococcus Enterotoxin (SE) and Panton-Valentine leukocidin (PVL) are regarded as the major cause of *S. aureus* associated food poisoning (Hoque et al., 2018). It is reported that more than 90% of *S. aureus* associated food poisoning outbreaks were attributed to the classical SEs (Tarekgne et al., 2016). The TSST-1 toxin could result in toxic shock syndrome by reducing the host immune response, while PVL could destruct host leukocyte and cause tissue necrosis (Hoque et al., 2018). Staphylococcal enterotoxins (SEs) are protein exotoxins identified in 1959 and differ in respect of their amino-acid homology, nucleotide, genes location, molecular weight and iso electric point value. The group of enterotoxins also contains pyrogenic toxins, toxic shock syndrome toxin (TSST-1), exfoliative toxin A (ETA), exfoliative toxin B (ETB) and Streptococcal Scarlet fever toxin (Kadariya et al., 2014). SEs are resistant to heat and proteolytic enzymes like trypsine, pepsin, chymotrypsine, papain and renin, but this resistance depends on the temperature and pH. These *Staphylococcal* enterotoxin-encoding genes are located on the chromosomal DNA, on pathogenicity islands in phages, transposones and plasmids. In humans, Staphylococcal enterotoxins are accountable for food poisoning and these enterotoxins can be isolated from milk samples infected with mastitis (Hermans et al., 2004).

The other important virulence factor of *S. aureus* is its ability to form biofilm *in vivo* which greatly influence its pathogenicity (Andrade et al., 2021). The biofilm producing bacteria exhibit high resistance to disinfectants, antibiotics and host immune system clearance (Hait et al., 2021). The significance of biofilm is well recognized in medical, environmental and industrial contexts. Several severe infections are reported to be a result of biofilm formation which leads to chronic diseases in most cases and lead to global public health concern. As a result they

reduce the effectiveness of treatments and increase morbidity, mortality and health care cost (Sargeant et al., 1998; Akindolire et al., 2015).

The pathogenic *S. aureus* can be identified by molecular techniques by targeting the virulent genes. The biofilm forming ability of the isolates can be characterized by phenotypic methods Congo Red Agar Assay, Tube Method and Tissue Culture Plate Assay methods and genotypic characterization can be done by screening of biofilm forming genes (Bissong and Ateba, 2020).

Maintenance of quality milk production by dairy industry in developing countries like India is a major problem and it is due to lack of efficient hygienic techniques for milking, storing of milk and maintaining herd health conditions (Kutar et al., 2015). This is the reason that in many parts a payment based system is in progress based on the quality of the milk. The consumption of contaminated milk may present with serious health hazards to humans. The public health significance of *S. aureus* is amplified by the fact that in humans, this bacterium is associated with both nosocomial and community-acquired infection. *S. aureus* is one of the significant causes of udder infection in dairy animals (Xu et al., 2015). Colonization impacts negatively on milk production and may additionally represent an infection to people who work in close contact with cows or consume raw milk (Liu et al., 2017). Thus colonization of dairy herds and subsequent contamination of raw milk by *S. aureus*, especially those expressing a multi-drug resistance (MDR), and biofilm producing ability, remains an important issue for both the dairy producer and public health (Sharma et al., 2017).

Most of the dairy farms located in Mathura region area are small-scale farms owned by independent farmers who usually supply fresh milk to processing plants, as well as to the local communities for direct consumption. In such cases, if proper hygienic practices are not observed during the milking process, milk intended for human consumption may become contaminated and eventually pose serious health hazards to the consumers. This highlights the importance of the screening of milk and other animal products, for the presence of pathogenic biofilm producing *S. aureus*. Furthermore, the persistence of biofilm-producing isolates in the dairy environment enhances the dispersal of virulence factors though the transfer of genetic material to other bacteria (Salina et al., 2020).

Thus, the aim of this study was to correlate biofilm-forming potentials, antimicrobial resistance, and virulent genes in *S. aureus* isolated from raw and pasteurized milk. Hence, present investigation was conducted with the following objectives:

Objectives:

1. To assess the prevalence of *S. aureus* in cattle raw milk and pasteurized milk.
2. To study the pathogenicity of *S. aureus* isolates by molecular detection of virulent genes.
3. To study the phenotypic and genotypic evaluation of biofilm producing *S. aureus* and its antimicrobial resistance pattern.

Review of Literature

CHAPTER- 2

REVIEW OF LITERATURE

2.1. Historical perspective

Staphylococci were first discovered by the Scottish surgeon, Sir Alexender Ogston (1880) and since then it has been found associated with a myriad of human and animal diseases (Melo et al., 2013). He named the round microorganism in infected tissue as “Staphylococcus” (Greek staphyle means bunch of grapes; kokkos means berry). Nocard identified staphylococci from mastitis in sheep in 1887 and then in 1890, Guillebeau stated that these organisms were responsible for mastitis in cattle.

2.2. Classification of *Staphylococcus*

Staphylococcus comprises of different species which have been classified and differentiated on the basis of a variety of phenotypic characteristics such as morphology, and biochemical reactions. Pigment was the initial criterion used to classify staphylococcal species, and in 1885, Rosenbach recognized members of the genus *Staphylococcus* based on the colour of colonies. Staphylococci forming orange-yellow colonies were named *S. aureus (or S. pyogenes aureus)* by Rosenbach, while staphylococci forming white colonies were named *S. albus* (or *S. pyogenes albus*). Another characteristic feature which was described for differentiation between *Staphylococci* was the coagulase test which involves the investigation of the ability of *S. aureus* to clot blood plasma (Kloos, 1980) which paved way for the separation of Staphylococci into two main groups Coagulase positive *S. aureus* (CPS) and Coagulase negative *S. aureus* (CNS). Based on different studies carried by different researchers, at present the genus *Staphylococcus* comprises of 37 species and 17 subspecies (Baird-Parker, 1963; Devriese et al., 1985; Kloos and Schleifer, 1975; Schleifer and Kloos, 1975 and Bannerman and Peacock, 2006). Lipid markers are being increasingly used for both the classification and identification of Gram-positive bacteria especially valuable data have been derived from the analysis of fatty acids (Collins et al., 1982), isoprenoid quinones (Collins & Jones, 1981) and polar lipids (Collins et al., 1982).

2.3. Morphology and Identification

S. aureus is a gram-positive, catalase-positive, usually oxidase-negative, facultative anaerobic coccus, which belongs to the family of Micrococcaceae and the group of Staphylococci. Different phenotypic methods are been proposed to identify *S. aureus* isolates from humans and animals from other species of *Staphylococcus*. These methods include anaerobic fermentation of mannitol, production of coagulase, production of heat stable thermonuclease and production of acetoin from glucose (Roberson et al., 1994; Devriese, 1985).

2.4. Reservoirs and sources of *Staphylococcus aureus*

Staphylococci are normal commensals colonizing about one-third of the population (Sargeant et al., 1998). In recent years, a new strain of *S. aureus*, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), has been recognized as a novel pathogen that has become a rapidly emerging cause of human infections (Price et al., 2012). *S. aureus* is generally considered to be a contagious udder pathogen, which mainly spreads within and between cows at milking and udder is the main source of infection (Ferguson, 2007). Several studies investigating potential reservoirs and fomites of *S. aureus* in dairy farms have been performed and most of these studies investigated extra-mammary sites associated (Piccinini et al., 2009). The probability of udder infection increases if the host has direct contact with the reservoirs of pathogens or indirect contact via fomites (Hait et al., 2021). Reservoirs and fomites of pathogens such as *S. aureus* can be traced by phenotyping and genotyping of the bacteria. Various types of foods serve as an optimum growth medium for *S. aureus* and involved in food-borne Disease (SFD) like meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, especially cream-filled pastries, cakes, and sandwich fillings (Argudin et al., 2010).

2.5. Transmission of *S.aureus*

S. aureus is transmitted from an infected to an uninfected mammary gland during the milking process. Shared equipment, udder cloths, and even milker’s hands can transmit *S. aureus* between cows, if good hygienic practices are not followed. Environmental factors, such as bedding, housing and feedstuffs can also be contaminated and play a role in spreading *S. aureus* infections (Papadopoulos et al., 2018). Also, Spencer et al. (1953) were able to maintain *S. aureus* in vitro on sterile

straw for at least 49 days, which raises the possibility that bedding may be a source of the organisms (Roberson et al., 1994). To cause mastitis, *S. aureus* must enter the mammary gland through the teat sphincter and contaminated wash cloths or milking equipment may bring the bacteria to the teat canal, teat ducts and grow in milk producing tissue and cause mastitis (Sergelidis and Angelidis, 2015).

2.6. Virulence factor of *Staphylococcus* spp.

S. aureus expresses many potential virulence factors. (1) Surface proteins that promote colonization of host tissues. (2) Factors that probably inhibit phagocytosis (capsule, immunoglobulin binding protein A). (3) Toxins that damage host tissues and cause disease symptoms. Coagulase-negative staphylococci are normally less virulent and express fewer virulence factors. The success of *S. aureus* as a pathogen is attributable, in part, to the diverse range of virulence factors produced (Gordon and Lowy, 2008). The virulence factors facilitate the invasion and colonization of host tissue, evasion of the host's immune defence mechanisms, aid in acquisition of nutrients and dissemination of the bacteria within the host tissue (Ferry et al., 2005). Among the vast array of virulence factors produced are numerous enzymes and cytotoxins, such as coagulase, collagenase, exfoliative toxins, hemolysins, hyaluronidase, leukocidins, lipases, nucleases and staphylokinase (Weiss et al., 2009). The genome of *S. aureus* is circular and 2800 kbp size chromosome, prophages, one or more plasmids, transposones, insertion sequences and other incompletely characterized accessory genetic elements. The conserved portion of the *S. aureus* core genome includes the house keeping genes necessary for cell growth and division. The define DNA fragments in *S. aureus* chromosome which can replicate on their own are insertion sequences, bacteriophages, pathogenicity islands and staphylococcal cassette chromosomes. The transfer of these elements in and out of *S.aureus* is responsible for the pathogenicity of the strain, since many of these elements encode virulence or antimicrobial resistance genes (Fox et al., 2005). Several Staphylococcal enterotoxins (SEs) have been identified, SEA, a highly heat-stable SE, is the most common cause of SFD worldwide. *S. aureus* produces wide arrays of toxins. Staphylococcal enterotoxins (SEs) are a family of nine major serological types of heat stable enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ) that belong to the large family of pyrogenic toxin super antigens (Kadariya et al., 2014).

The production of biofilm is considered a major virulence factor that, besides protecting from host defence mechanisms, also shields bacteria against antimicrobial agents (Andrade et al., 2021). Biofilm formation involves two steps: the adhesion of bacteria on the surface of the mammary gland mediated by a capsular antigen called capsular polysaccharide/adhesin (PS/A), followed by accumulation, maturation, and separation phases associated with the production of polysaccharide intercellular adhesion (PIA), essential to the spread of the staphylococci (Simojoki et al*.,* 2012). Cell proliferation and biofilm formation are known virulence factors mediated by the presence of the *ica* locus, comprising four genes, *icaA*, *icaD*, *icaB*, and *icaC*, organized in an operon (*icaADBC*) with the regulatory gene *icaR*. This operon is responsible for expression of PIA, present in the cellwall. The surface protein BAP (biofilm-associated protein) is responsible for promoting the primary fixation in inert surfaces. The expression of *bap* leads to biofilm formation even without the presence of the *ica A D B C* operon. In dairy herds, there is an evidence of the importance of *bap* in mastitis caused by *S. aureus* (Cucarella et al., 2004).

2.7. Antimicrobial resistance

Due to its seriousness and prevalence, nosocomial infection caused by MRSA has been listed as one of the three most difficult infectious diseases in the world by the World Health Organization (Becker and Wardenburg, 2015). Therefore, monitoring the antibiotic resistance of *S. aureus* in raw milk is very important for predicting the speed and type of antibiotic resistance development and for the decision-making of animal antibiotic treatment from the perspective of food safety (Liu et al., 2017). Biofilm formation can enhance the virulence of bacteria, including *S. aureus*, and is considered one of the important virulence factors of *Staphylococcus* (Lee et al., 2014; Bissong and Ateba, 2020). The production of a biofilm not only enables bacteria to tolerate poor environments, but also reduces the penetration rate of antibiotics, promotes the horizontal spread of determinants of antibiotic resistance, and ultimately complicates the treatment of infections caused by these bacteria (Mathur et al., 2006).

In 1997, the first strain of *S. aureus* with reduced susceptibility to vancomycin and teicoplanin was reported from Japan (Hiramatsu et al., 1997). Shortly after, two additional cases were reported from United States. However, first clinical isolate of vancomycin resistant *S. aureus* (VRSA) was reported from United States in 2002

(CDC, 2002). More recently some workers have reported vancomycin resistant staphylococcal stains from Brazil (Palazzo et al., 2005) and Jordan (Bataineh et al., 2006). This emergence of VRSA/VISA may be due to building of selective pressure of vancomycin. Vancomycin, a glycopeptide is currently the main antimicrobial agent available to treat life-threatening infections with MRSA. Until recently vancomycin resistance among gram-positive bacteria had been thought to be uncommon but the confirmed reports of vancomycin resistance in Enterococcus spp; *S. aureus* and CNS have been reported from various part of world.

The true mechanism of vancomycin resistance in *S. aureus* is not known. It was initially feared that *S. aureus* would acquire the *van* gene that code for vancomycin resistance in Enterococcus spp; this phenomenon was successfully accomplished in the laboratory (Noble et al., 1992). Further, Showsh et al. (2001) have demonstrated the presence of sex pheromone in *S. aureus* that promotes plasmid transfer in Enterococcus spp. Release of these pheromones by *S. aureus* with proximity to vancomycin-resistant enterococci causes the transfer of plasmids encoding van gene to the *S. aureus*.

2.8. *Staphylococcus* Outbreaks

S. aureus food-borne disease (SFD) is one of the most common Food borne Diseases and is of major concern in public health programs worldwide (Kou et al., 2021). It is one of the most common causes of reported FBD in the United States (Murray et al., 2005). The first documented event of SFD due to the consumption of contaminated cheese was investigated by Vaughan and Sternberg in Michigan, USA, in 1884 (Hennekinne et al., 2012). Annually, an estimated 76 million illnesses, 3,25,000 hospitalizations, and 5,000 deaths are caused by food-borne diseases in the United States (Mead et al., 1999). Annually, an estimated 76 million illnesses, 3,25,000 hospitalizations, and 5,000 deaths are caused by food-borne diseases in the United States (Scallan et al., 2011). However, SEC and SEE are also implicated with SFD. Several Staphylococcal enterotoxins (SEs) have been identified, SEA, a highly heat-stable SE, is the most common cause of SFD worldwide. Outbreak investigations have found that improper food handling practices in the retail industry account for the majority of SFD outbreaks (Kadariya et al., 2014). Over 13,000 cases of SFD occurred in Japan in 2000 as a result of contamination of milk at a dairy-food

production plant (Asao et al., 2003). Six SFD outbreaks in France in 2009 were caused by SEE present in soft cheese made from unpasteurized milk.

2.9. Work done

2.9.1. Work done around the world

Murray (2005) studied at involving 7126 cases indicated that case fatality rate of SFD is 0.03% all deaths were in elderly patients and detected attack rate of SFD can be up to 85%.

Capurro et al. (2010) studied mastitis within 5 dairy herds of Sweden caused by *S. aureus*. Overall 82 (11%) of quarter mastitis sample were positive for *S.aureus* and 27% of the lactating cows were *S.aureus* in one or more Quarter mastitis sample.

Haran et al. (2012) detected the herd prevalence of *S.aureus* including MRSA in bulk tank milk from Minnesota farms. Herd prevalence of methicillin-susceptible *S. aureus* was 84%, while MRSA was 4%. All isolates of *S. aureus* carried a 98 to100% sequence similarity of 16S rRNA genes.

Xu et al. (2015) revealed prevalence of *Staphylococci* is (104/209, 49.8%) and prevalence of *S.aureus* (28/104) is 26.9% at China. In *S.aureus* isolates, the most prevalent virulence genes were *coa*, *lg* and *eno* (100%) followed by *hla* (96.4%), *hlb* (92.9%), *fib* (92.9%), *clfA* (89.3%), *clfb* (85.7%) and *nuc* (85.7%).

Hoque et al. (2018) revealed that overall prevalence of *S.aureus* from herd, cow and quarter level 72.7, 74.0 and 62% respectively from California. *S. aureus* strains were resistance to atleast one microbials and 49% strains for to 2 or more antimicrobials. Entrotoxin gene profile were detected and mecA was found in 20% isolates indicating the emergence & spread of methicillin resistant *S. aureus*.

Shahmoradi et al. (2019) in vitro biofilm formation assays determined 3 (6%) strains of *S. aureus* to be strong biofilm forming, 15 (30%) of the isolates were determined to be moderate biofilm forming and, 32 (64%) were determined to be weak biofilm forming isolated from clinical samples of human being. All strains were determined to be penicillin, amoxicillin and clavulanic acid resistant.

Mphahlele et al. (2020) in this study *S. aureus* was isolated from cow milk as 1.7% (50/2862) in South Africa. All (100%) isolates were resistant to at least one antimicrobial, while 62% (31/50) were resistant to three or more antimicrobials

(62%; 31/50). Most *S. aureus* isolates were resistant to erythromycin (62%; 31/50) and ampicillin (62%; 31/50). Almost half of *S. aureus* isolates were resistant to oxacillin (46%; 23/50).

Bissong and Ateba (2020) studied at south west province of South Africa and reported a total of 77 *S. aureus* were isolated from the milk samples, out of which 7 (9.1%) and 70 (90.9%) isolates were from pasteurized and unpasteurized milk. Seventy (90.9%) isolates were biofilm producers while the ica biofilm-forming genes were detected among 75.3% of the isolates, with *icaA* being the most prevalent (63.6%). *S.aureus* show multidrug resistance to atleast 3 antibiotics. 5 resistance genes, namely *blaZ* (37.7%, *vanC* (37.7%), *tetK* (31.2%), *tet L* (27.3%), *msr A/B* (20.8%) were detected.

Andrade et al. (2021) found *S. aureus* prevalence as (131/508, 25.5%) from goat milk and (60/138, 43.47%) from sheep milk and 75% were biofilm producer. The *nuc* gene was detected in (35/137, 25.54%) *S. aureus* isolates.

Hait et al. (2021) phenotypically characterized the biofilm forming isolates by the microtiter plate method and Congo red agar (CRA). The microtiter plate results indicated moderate to high biofilm formation for 96% of the isolates, with 4% exhibiting.

Salina et al. (2020) studied on detection of *ica A*, *ica D*, *bap* genes and biofilm production in *S. aureus* and non aureus *Staphylococci* isolated from subclinical and clinical bovine mastitis. Prevalence of *S.aureus* was 13.35% (100/749) from subclinical mastitis. The distribution of the frequencies of *icaA*, *icaD*, and *bap* genes in *S. aureus* isolates were 82%, 83%, and 58%, respectively.

2.9.2. Work done in India

Mathur et al. (2006) studied evaluation of three different methods for detection of biofilm formation in *Staphylococci* in India*.* A total of 152 clinical isolates of Staphylococcal spp. was screened by tissue culture plate, tube method and congo red agar.

Kumar et al. (2009) studied distribution of antibiotic resistance gene of *S. aureus* isolates in India. Phenotypic antibiotic resistance percent in *S. aureus* isolates was revealed tetracycline (36.7), gentamycin (30.5), streptomycin (26.6),

kanamycin (25.8) and penicillin G (22.7). All the isolates were susceptibe to vancomycin, among isolates 10.2% were observed as methicillin resistant.

Chavahan et al. (2012) studied biofilm forming adhesion genes (*icaA*) in *S. aureus* bovine mastitis from seven states of India. Biofilm marker *icaA* gene was revealed in 58.6 (88/150) % *S. aureus* strains by PCR.

Bhattacharya et al. (2016) studied vancomycin resistant pattern of *S. aureus* isolated from 352 milk sample of clinical & subclinical mastitic bovine & caprine from different districts of West Bengal. *S. aureus* was 61.36% (216/352) prevalent and all the isolates were resistant to cefoxitin and oxacillin and possessed *mec A* gene.

Kumar et al. (2017) studied drug resistance against *S. aureus* and processed 368 bovine raw milk sample from 13 districts of Rajasthan India .Out of them 73 are *S. aureus* positive strains, prevalence is 19.84% while 14.4% shows coagulase activity.

2.9.3. Work done in DUVASU, Mathura

Sharma et al. (2015) studied about methicilllin resistant *Staphylococcus aureus* (MRSA) among bovines milk prevalence in DUVASU, Mathura. The incidence of *S.aureus* was higher (50%) in clinical mastitis comparision to that of subclinical mastitis (17.50%). They found 100% resistance against penicillins followed by vancomycin (88.89%), nalidix acid (77.78%).

Kutar et al. (2015) to analyze the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) from cattle and buffaloes with clinical or subclinical mastitis in Uttar Pradesh, India. Out of total 125 mastitic milk samples, incidence was higher (56%) in clinical mastitis than in subclinical (20%). A very high percent of isolates were resistant to cotrimoxazole (63.46%), followed by streptomycin (57.69%), gentamicin (55.76%), cephalexine (42.30%), amoxycillin (38.46%) and erythromycin (36.53%). The presence of *mecA* gene considered as a molecular marker of methicillin resistance and was detected only in 5 (9.61%) of 52 *S. aureus* isolates by PCR.

2.10. Detection of *Staphylococcus aureus*

2.10.1. Isolation of *Staphylococcus aureus by* Conventional methods

Generally Mannitol Salt Agar (MSA) is used in the clinical laboratory to differentiate pathogenic *Staphylococcus spp.* from the non-pathogenic *Micrococcus*

spp. The growing bacteria are able to ferment mannitol and produce an acid pH giving rise to yellow colour colonies on the surface of MSA medium (Kateete et al., 2010). The most important biochemical test is coagulase, which differentiate pathogenic *S. aureus* from non-pathogenic strains. All pathogenic *S. aureus* strains are coagulase positive. The other important conventional biochemical tests which are generally used to characterize *S. aureus* are catalase positive and oxidase negative (Quinn et al., 2000). Growth on blood agar medium decides whether the bacterial isolate is haemolytic or nonhaemolytic. The haemolytic bacteria are generally pathogenic. Haemolysis of *S. aureus* was tested on 5 % sheep blood agar medium.

2.10.2. Molecular Techniques

For decades, the *nuc* gene has been considered the golden standard for *Staphylococcus aureus* identification and is still used presently (Kateete et al., 2010, Torres et al., 2019, McClure et al., 2017). However, the *nuc* gene has been detected in staphylococci of animal origin other than *S. aureus* (Gudding, 1983). The staphylococcal nuclease is a thermostable nuclease encoded by the *nuc* gene (Schleifer et al., 2009), which hydrolyzes DNA and RNA in host cells, causing tissue destruction and spreading of staphylococci (Hu, Y et al., 2013), also promoting the escape of microorganisms when retained by neutrophil extracellular traps (NETs), allowing the bacteria to evade this host defence mechanism (Berends et al., 2010).. Thermonuclease (*nuc*) gene species specific published primer sequence of Brakstad et al. (1992) was employed for the amplification of *nuc* gene for molecular identification of *S. aureus*.

2.10.3. Biofilm production

Biofilm are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002) .Within a biofilm, bacteria communicate with each other by production of chemotactic particles or phenoheromones, a phenomenon called quorum sensing (Hait et al., 202l). Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesions and presence of surfactants are some factor which influence biofilm formation (Thomas et al., 2007). Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial

agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold (Stewart and Costerton, 2001). There are a number of mechanisms by which numbers of microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Breyers & Ratner, 2004). Biofilm formation comprises a sequence of steps. As biofilm formation mechanisms will only be discussed briefly, the reader is directed to several excellent comprehensive reviews on this area (Verstraeten et al., 2008). At present, processes governing biofilm formation that have been identified include 1. pre-conditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface, 2. Transport of planktonic cells from the bulk liquid to the surface, 3. Adsorption of cells at the surface; 4. Desorption of reversibly adsorbed cells, 5. Irreversible adsorption of bacterial cells at a surface, 6. Production of cell–cellsignalling molecules, 7. Transport of substrates to and within the biofilm, 8. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cell growth, replication, and EPS production, 9. Biofilm removal by detachment or sloughing (Breyers & Ratner, 2004). The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Pereira, & Vieira, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and EPS production are important for biofilm formation and development (Allison, 2003).

The production of biofilm is considered a major virulence factor that, besides protecting from host defence mechanisms, also shields bacteria against antimicrobial agents (Berends et al., 2010). Staphylococcal PIA is encoded by the ica operon (Otto, 2018), and biofilm-associated protein (Bap) is a surface protein connected to the cell wall encoded by the *bap* gene (Cucarella et al., 2001). Marques et al. (2016) studied that the isolates of *S. aureus* were characterized by pheno-genotypic and MALDI TOF-MS assays and tested for genes such as *icaA*, *icaD*, *bap*, *agr RNAIII*, *agrI*, *agrII*, *agrIII*, and *agrIV*, which are related to slime production and its regulation. All the studied isolates were biofilm producers and mostly presented *icaA* and *icaD*.

The presence of biofilm-forming strains of *S. aureus* in food processing environments and in food are equally important as biofilms are involved in cross-contamination events. The two-step process in biofilm development first involves the bacteria adherence to a surface followed by the bacteria multiplying to form a multilayered biofilm associated with the production of polysaccharide intercellular adhesion (PIA) (Naseer et al., 2021). PIA is produced and secreted by the proteins encoded in the intercellular adhesion (icaABCD) operon (Miao et al., 2019). *S. aureus* adherence is facilitated by protein adhesions of the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which is most frequently anchored to the cell wall peptidoglycan (Foster and Höök, 1998). *S. aureus* genes that encode MSCRAMMs include bone sialoprotein binding protein (*bbp*), collagen binding protein (*cna*), elastin binding protein (*ebpS)*, encoding laminin binding protein (*eno*), encoding fibrinogen binding protein (*fib*), fibronectin binding proteins A (*fnbA*), fibronectin binding proteins A (*fnbB*), clumping factors A (*clfA*), and clumping factors B (*clfB*) genes (Tristan et al., 2003). The icaABCD operon is responsible for the slime production.

Biofilm analysis was determined by using different methods, the microtiter plate method and observations on Congo red agar (CRA) by various research worker (Mathur et al., 2006; Melo et al., 2013). Phenotypic biofilm examination using CRA identified biofilm producers as black colonies with dry crystalline consistency and non-biofilm-producing colonies developed smooth colonies that ranged from pink to red in color. Weak and moderate biofilm formers produced darkened brownish or black smooth colonies (Hait et al., 2021). A microtiter plate assay was performed to test biofilm formation in vitro following a described method (Melo et al., 2013). Biofilm detection using the microtiter plate method used a sterile flat-bottom 96-well plate. Based on the resulting absorbance values, the isolates were classified into three categories: positive result (high-level biofilm former) OD > 0.240, moderate result (intermediate level biofilm former) OD 0.240–0.120, and negative result (non-biofilm former) OD < 0.120.

2.11. Drug resistance profile of *S. aureus*

In recent time, antibacterial resistance in bacteria from animal origin and its effect on human health drawn much attention worldwide (Aarestrup, 1995). Antibiotic resistant pathogens constitute an important and growing threat to various living

organisms. Among varied antimicrobial drugs, β-lactam antibiotics are currently used in veterinary medicine and thus provide opportunity for selection pressure in development of β-lactam resistance (Li et al., 2007).

The indiscriminate use of antibiotics can lead to development of resistant strains and result in the increase in the cost of treatment. The production of β–lactamases encoded by the structural *blaZ* gene and by the production of an altered form of penicillin binding protein 2A (PBP-2A) which is encoded by the mecA gene is responsible for β-lactam resistance in Staphylococci (Fuda et al., 2005). Recently, another form of MRSA i.e, *mecC* MRSA recognized which encodes a divergent mec gene which can colonize and cause disease in humans and a varied range of other host species. Although reports of *mecC* MRSA are currently rare, they present a potential diagnostic problem where there is reliance on *mecA* or *PBP2a/2* detection for MRSA diagnosis, and their emergence raises a several questions for future research (Paterson et al., 2012). Extensive and inadvertent use of antimicrobials both in human and in veterinary medicine is the key reason for emergence of resistant strains of *S. aureus* (Rabello et al., 2005). MRSA affects both human and animal populations (Weese, 2010). The rise MRSA isolates in livestock has attained particular attention during recent years. There is a potential risk to the cattle, farm workers and the veterinarians who are exposed to MRSA in bovine mastitis cases (Juhász-Kaszanyitzky et al., 2007). The concern of the appearance and transfer of antimicrobial resistant bacteria or genetic determinants from animals to humans via food chain is increasing (Piddock, 1996). India is lacking comprehensive information on the prevalence of antimicrobial resistant in bovine mastitis pathogens in milk.

2.11.1. Antibiotic Susceptibility Test (ABST)

The study of resistance pattern of any bacterial isolate involves both qualitative and quantitative determination. The qualitative determination of resistance in each *S. aureus* isolate gives the antibiogram and also the incidence of resistance for each isolate to different antibiotics and generally carried by the Kirby-Bauer disc diffusion method.

Kumar et al. (2009) studied phenotypic antibiotic resistance percent in *S.aureus* isolates was revealed tetracycline (36.7), gentamycin (30.5), streptomycin (26.6), kanamycin (25.8) and penicillin G (22.7). All the isolates were susceptibe to vancomycin, among isolates 10.2% were observed as methicillin resistant.

Rambabu (2013) reported 56.66 %, 53.33 %, 43.33 %, 36.66 % and 33.33 % of *S. aureus* isolates from bubaline mastitis were resistant to antibiotics vancomycin, amoxycillin +sulbactam, oxacillin, methicillin, ceftriaxone + sulbactam, respectively. The ABST results are suitable for selection of antibiotics for treatment.

Kutar et al., 2015 reported a very high percent of isolates were resistant to cotrimoxazole (63.46%), followed by streptomycin (57.69%), gentamicin (55.76%), cephalexine (42.30%), amoxycillin (38.46%) and erythromycin (36.53%).

2.11.2. MIC by E-test

E-test was a new approach for quantitative antibacterial susceptibility developed in the late 1980s by Bolmstrom and Eriksson (1988). The method is a simple and routine used to determine in vitro the MICs on agar medium. The antimicrobial gradient method incorporates the principle of disc diffusion and agar dilution tests. The E-test is based on the diffusion of an antibiotic agent from a plastic strip onto an agar media to provides quantitatively rapid and reproducible MICs results for tested isolates (Baquero et al., 1992). Some important advantages of the E-test are rapidity, simply and reliability and does not require a special equipment. The E-test method is applicable to a variety of microorganism and drug, that it offers a quantitative test of MICs result which may be interpreted easily compared to standardized methods.

2.12. Public Health Significance

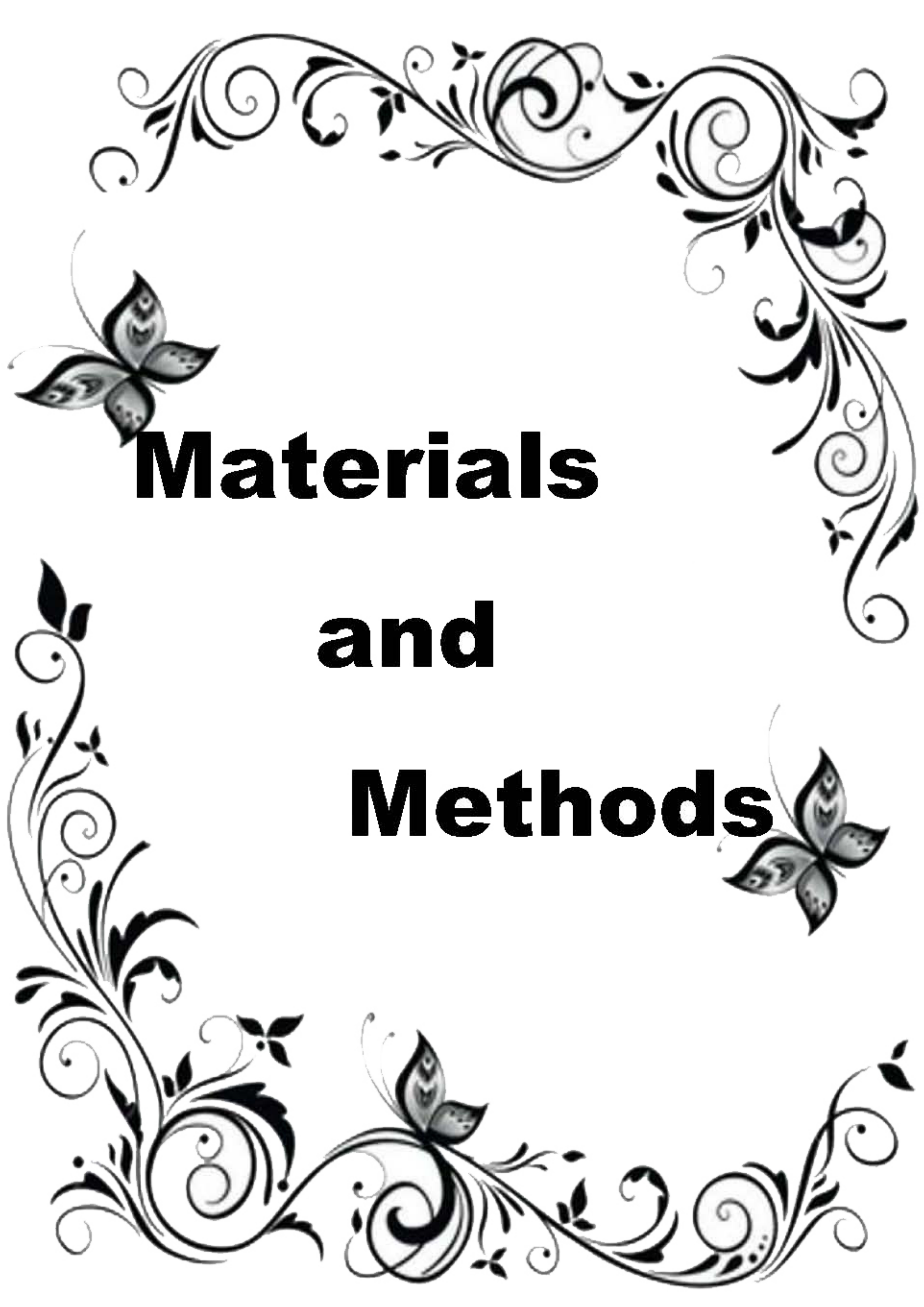
S. aureus is a versatile virulent opportunistic pathogen for humans and animals and is responsible for various infections, such as wound infections and toxin-mediated syndromes (food-poisoning, scalded skin bacteremia (Chambers and DeLeo, 2009).

S. aureus is one of the main pathogens causing mastitis in dairy animals worldwide syndrome and toxic shock syndrome) as well as systemic and life-threatening diseases such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis.

It is an important opportunistic pathogen of raw milk, and the enterotoxin causes significant food poisoning. Monitoring of biofilm forming and the antibiotic resistant of *S. aureus* in milk is helpful for a risk assessment of *S. aureus*. The production of biofilm is considered a major virulence factor that, besides protecting

from host defence mechanisms, also shields bacteria against antimicrobial agents. Furthermore, the persistence of biofilm-producing isolates in the dairy environment enhances the dispersal of virulence factors though the transfer of genetic material to other bacteria (Kadaria et al., 2014)

The presence of MDR *S. aureus*, and especially MRSA, in animals and dairy products represents a potential threat for the spread of this pathogen in the community. The results indicated that human, animal and environmental sources could be involved in the contamination of dairy products along their production chain. The ability of *S. aureus* to develop or acquire strategies which provide resistance to different antimicrobials is an additional approach in the impressive arsenal of this pathogen. *S. aureus* is capable of acquiring antibiotic resistance determinants and therefore *S. aureus* isolates often exhibit resistance to multiple classes of antimicrobial agents (Rybak and LaPlante, 2005). Methicillin resistant *S. aureus* (MRSA) is practically resistant to all available b-lactam antimicrobial drugs. MRSA represents a serious public health issue due to its ability to colonize and infect humans and animals (Petinaki and Spiliopoulou, 2012). MRSA was first recognized as a hospital-associated pathogen (HA-MRSA) (Jevons et al., 1963). However, since 1990, community associated MRSA (CA-MRSA) began to cause infections outside the health-care environment (Rybak and LaPlante, 2005). In recent years, the incidence of MRSA infections has increased in livestock and a third epidemiological type was recognized, the livestock associated MRSA (LA-MRSA) (Graveland et al., 2011). While humans are considered as the main reservoir for new pathogenic strains affecting livestock (Cuny et al., 2013), animals can act as a source of *S. aureus* zoonotic infections, especially the clones that possess no host specificity. It has also been demonstrated that handling and consumption of MRSA contaminated food of animal origin could provide a potential vehicle for transmission to humans (EFSA, 2009; Feingold et al., 2012; Wendlandt et al., 2013).



Materials and Methods

CHAPTER- 3

MATERIAL AND METHODS

Place of study:

The study was performed at Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura, Uttar Pradesh, India.

3.1. Materials

3.1.1. Bacterial strains

The positive culture of *Staphylococcus aureus* ATCC 25923 is procured from the Department of Veterinary Microbiology, DUVASU, Mathura and used as positive control in entire research.

3.1.2. Chemicals and reagents

Nutrient Agar, Baired Parker Agar, Mannitol Salt Agar, Congo Red Agar, Muller Hinton Agar, Buffered Peptone Water, Brain Heart Infusion Broth, Di sodium hydrogen orthophosphate, Potassium di hydrogen orthophosphate, Potassium chloride, Proteinase K, Sodium chloride, Sodium acetate, Sucrose, Hydrogen peroxide, Crystal Violet, Ethidium Bromide, Agarose, Rabbit plasma, Antibiotic discs, E-strips were procured from Himedia, India. CMT reagent was procured from Weizur, India. Nuclease free water, oligonucleotides primers, Master Mix, 100 bP gene ruler, DNA gel loading dye (6X), TAE Buffer (Tris-acetate EDTA) were obtained from Thermo scientific, USA.

3.1.3. Kits

GeneJET Genomic DNA Purification kit was procured from Thermo Scientific, USA and Gram staining was obtained from Himedia, India.

3.1.4. Equipments

Bacteriological incubator (REMI, India), Hot air oven (Tanco), Autoclave (Waiometra), Refrigerated centrifuge (Eppendorf, Germany), Vortex (Scientific Industries), Water bath (Grant), Thermal cycler (BIO RAD, UK), Gel documentation

system (Bio RAD, UK), Horizontal electrophoresis (GeNei, India), ELISA reader (Thermo scientific), Nanodrop (Eppendorf, Germany) were used in the study.

3.1.5. Glassware and plastic ware

All the glassware was procured from M/S J-Sil, India Private Ltd. Micro centrifuge tubes, micropipette tips were purchased from M/S Xygen/Axiva, India.

3.2. Methods

3.2.1. Site of sample collection

A total of 378 milk samples were collected from healthy and mastitic cows (Dairy Farms and Gaushalas), local milk shops, vendors and pasteurized milk (Grocery Shops) in and around Mathura region of Uttar Pradesh during November, 2021 to February, 2022. Detail of sample collection was given in Table 1.

Table 1: Sample collection from different sources

S. No.	Source	Place of Collection	No. of samples / site	Total samples
1.	**Raw milk (From individual animal of Dairy Farms & Gaushalas)**	Livestock Dairy Farm	36	252
		Karnaval Dairy	36	
		Bhagwati Gaushala	36	
		Hasanand Gaushala	36	
		Bhaktivedanta Swami Gaushala Iscon Temple	36	
		Govind Dham Gaushala	36	
		Gayatri Tapobhumi Gaushala	36	
2.	**Pooled raw milk (Vendors)**	Aurangabad	2x10 (2 Samples from each vendor)	20
		Acharya Nagar		
		Motikunj		
		Motienclave		
		Natwar Nagar		
3.	**Pooled raw milk (Local milk shops)**	Aurangabad	2x10 (2 Sample from each shops)	20
		Acharya Nagar		
		Motikunj		
		Motienclave		
		Natwar Nagar		
	Sub Total Pooled raw milk			**40**

4.	**Mastitic raw milk (From individual animal of Dairy Farms & Gaushalas)**	Clinics TVCC & Livestock Dairy Farm	8	56
		Karnaval Dairy	8	
		Bhagwati Gaushala	8	
		Hasanand Gaushala	8	
		Bhaktivedanta Swami Gaushala, Iscon Temple	8	
		Govind Dham Gaushala	8	
		Gayatri Tapobhumi Gaushala	8	
5.	**Pasteurized Milk (Grocery Shops)**	Aurangabad	10x3 (3 Sample from each shop)	30
		Acharya Nagar		
		Motikunj		
		Motienclave		
		Natwar Nagar		
	Total samples			**378**

3.2.2. Collection of milk samples

Raw milk samples (252) were collected directly from udder of healthy cows while mastitic milk samples (56) were taken from affected quarter only. For collection of milk sample firstly teat was cleaned with 70% ethyl alcohol and initially few streams of milk was discarded from the teat and approximately 10 ml of milk sample was collected aseptically in sterile container. Pooled raw milk samples (40 ml) were collected aseptically from local milk shops and vendors into sterile vials. Pasteurized milk samples (30) were collected randomly from grocery shops of Parag and Param brand. All the collected samples were labeled kept on ice, brought to the laboratory and processed within 24 hours.

3.2.3. Screening of samples for detection of clinical mastitis

California Mastitis Test (CMT) was used for screening of clinical mastitis sample method as per Schalm et al. (1971) with slight modifications. The CMT is quick and precise test for diagnosis of mastitis. The CMT was conducted to diagnose the presence of clinical mastitis as per Shitandi and Kihumbu (2004). 10 ml of milk from affected quarters of the udder was placed in each of four shallow cups of paddle and an equal amount (10 ml) of the CMT reagent was added. A gentle circular motion was applied in a horizontal plane and the reactivity of the samples was observed.

Positive samples showed gel formation within a few seconds. The CMT reagent reacts with the white blood cells and the mixture thickens or gels in proportion to the amount of infection present. The result was scored based on the gel formation and categorized as negative if there was no gel formation and positive if there was gel formation ranging from +1 to +3 (Table 2). The mastitic milk samples that showed reactivity in CMT were considered as positive for clinical mastitis.

Table 2: Interpretation and scoring of the California Mastitis Test

CMT Score	Average somatic cell count (cells/ml)	Description of visible reaction
N (Negative)	1,00,000	Mixture remains liquid, with no evidence of thickening
T (Trace)	3,00,000	The slight thickening that forms is seen best by tipping the paddle back and forth and observing the mixture as it flows over the bottom of the cup. Trace reactions tend to disappear with continued rotation of the paddle. Readings were recorded within 10 seconds.
1 (Weak positive)	9,00,000	A distinct thickening of the liquid forms, but there is no tendency toward a gel formation. With some milk, the thickening may disappear after prolonged rotation of the paddle (20 seconds or more). Readings were recorded within 10 seconds.
2 (Distinct positive)	2,700,000	Mixture thickens immediately, and a gel formation is suggested. As the mixture is swirled, it tends to move in toward the centre, exposing the bottom of the outer edge of the cup. When the motion is stopped, the

		mixture level out and covers the bottom of the cup. Readings were recorded within 10 seconds.
3 (Strong positive)	8,100,000	A gel is formed, which causes the surface of the mixture to become elevated like a partially fried egg. There is usually a central peak that remains projecting above the main mass, even after the rotation of the paddle is stopped.

3.3. Sample processing for isolation and identification of *S. aureus*

Milk samples were processed for isolation of *Staphylococcus aureus* as per standard bacteriological procedure given by Kou et al. (2021) with slight modifications.

3.3.1. Enrichment of samples

The milk samples (1ml) were inoculated in the Buffered Peptone Water (BPW) (9 ml) and incubated overnight at 37°C for 24 hrs.

3.3.2. Plating on selective media for isolation of *Staphylococcus* spp./ *S. aureus*

The loopful culture growth from BPW was streaked on Baird Parker Agar and incubated at 37°C for 24 hrs. *Staphylococcus* spp. produced peculiar jet black colored colonies over this agar and further single jet black color colony was streaked on Mannitol salt agar (MSA) and incubated at 37°C for 24hrs.. On MSA, coagulase positive staphylococci produced golden yellow colonies surrounded by yellow zone while coagulase negative staphylococci (CNS) produce red colored colonies. Further single colony from MSA was picked and streaked on Nutrient agar slant and incubated at 37°C for 24 hours. The slants were stored at 4°C till further proceeding.

3.3.3. Gram's staining

On Gram's staining Staphylococci appeared as Gram- positive cocci with purple stain and having grape like structure.

3.3.4. Biochemical characterization

All the *S. aureus* isolates were biochemically confirmed as per the method described by Barrow and Feltham (1993). A single colony from each isolate was picked up from Nutrient agar and inoculated in 5 ml Brain heart infusion broth (BHI) and incubated at 37°C for 4-6 hours until inoculum turbidity is 0.5 McFarland. The tests were performed for Indole, Methyl red, Voges-proskauer, Citrate utilization, catalase, coagulase and oxidase test was also performed along with IMViC.

i. Catalase test

The test was performed by adding 3% hydrogen peroxide to a colony present on agar plate onto the grease free glass slide. Catalase-positive cultures produce oxygen and bubbling effervescence.

ii. Coagulase test

The test was performed using Coagulase test (slide) kit of Himedia containing discs of rabbit plasma. One drop of distilled water was placed on a glass microscope slide and a heavy suspension of the organism being tested was prepared. One coagulase disc was added and rubbed about in the suspension, using the tip of a wire loop. At once a second drop of distilled water was added and mixed again. Coagulase-negative organisms remain evenly suspended. Macroscopic clumping occurred within 30 seconds in a positive test.

iii. Oxidase test

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 as per method of Faller and Schleifer (1981).

iv. Indole production

Tube of tryptone broth was inoculated with the culture and incubated 24 ± 2 h at 35°C ± 0.5°C. Indole production was tested by adding 0.2-0.3 mL of Kovacs' reagent and appearance of distinct red color in upper layer indicated positive reaction.

v. Methyl Red Test (MR)

Glucose phosphate (MR-VP) tubes were inoculated and incubated for up to 4 days at 35°C ± 0 .5°C. Five drops of methyl red solution was added to each tube. In positive tests distinct red color was observed and in negative tests yellow color developed.

vi. Voges-Proskauer (VP) test

Glucose phosphate(MR-VP) broth was inoculated and incubated 24 ± 2 h at 35°C± 0.5°C. 1 mL was transferred to serological tube and 0.6 mL α-naphthol solution was added along with 0.2 mL 40% KOH, and shaken. Let stand it undisturbed. The test was positive if eosin pink or red color developed.

vii. Citrate test

Tube of Simmons citrate agar was inoculated and incubated for 18-24 h at 35°C ± 0.5°C and development of blue colour indicated positive reaction.

3.4. Molecular detection of *S. aureus* isolates

3.4.1. Isolation of bacterial DNA

Gram-Positive Bacterial DNA purification protocol

Gram-positive bacterial lysis buffer was prepared by adding: 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, add lysozyme to 20 mg/mL immediately before use.

i. Approximate 2×10^9 bacterial cells were harvested in a 1.5 or 2 mL microcentrifuge tube by centrifugation for 10 min at 5000 × g and supernatant was discarded.
ii. The pellet was resuspended in 180 µL of Gram-positive bacteria lysis buffer and incubated for 30 min at 37 °C.
iii. 200 µL of lysis solution and 20 µL of Proteinase K were added and mixed thoroughly by vortexing or pipetting to obtain a uniform suspension.
iv. The samples were incubated at 56°C for 30 min in water bath until the cells were completely lysed.
v. Then 20 µl of RNase A solution was added and mixed by vortexing and incubated the mixture for 10 min at room temperature.

vi. Further 400 µL of 50% ethanol was mixed by pipetting or vortexing.

vii. Prepared lysate was transferred to a Gene JET Genomic DNA purification column inserted in a collection tube. The columns were centrifuged for 1 min at 6000×g. collection tube containing the flow-through solution was discarded. Further, GeneJET Genomic DNA purification column were placed into a new 2 mL collection tube.

viii. 500 µL of Wash Buffer I (with ethanol added) was added and centrifuged for 1 min at 8000 × g and then flow-through was discarded and purification column placed back into the collection tube.

ix. 500 µL of Wash Buffer II (with ethanol added) was added to the Gene JET Genomic DNA purification column and centrifuged for 3 min at maximum speed (≥12000 × g). Collection tube containing the flow-through solution was discarded and transfered to the Gene JET Genomic DNA purification column to a sterile 1.5 ml micro centrifuge tube.

x. 200 µL of Elution Buffer was added to the center of the Gene JET Genomic DNA purification column membrane to eluted genomic DNA.

xi. It was further incubated for 2 min at room temperature and centrifuged for 1 min at 8000×g.

xii. Discarded the purification column and used the purified DNA immediately in downstream applications or store at -20 °C.

3.4.2. Quantification of bacterial DNA

DNA quantification was carried out in nanodrop by taking 1 µL of elution buffer used for DNA extraction. The nanodrop was calibrated at 260 nm as well as at 280 nm wavelength, then 1 µL of test sample was taken and concentration was measured at A260/A280 ratio the values measured and this ratio of around 1.9 (1.85-1.95) indicated best quality of DNA.

3.4.3. Molecular detection of housekeeping *nuc* gene of *S .aureus*

Biochemically confirmed, Gram and catalase positive, staphylococci isolates were further confirmed for *S. aureus* by using species-specific primer targeting *nuc* gene of *S. aureus* using simplex-PCR. Oligonucleotide primers were custom synthesized from Eurofins Genomics, India Private Limited as details were given in table 3.

Table 3: Primers for *nuc* gene of *S. aureus*

S. No.	Gene	Primer Sequence 5'-3'	Amplicon Size (bp)	Reference
1.	***nuc*** **F**	5'GCGATTGATGGTGGATACGGTT3'	**267**	Brakstad et al. (1992)
	nuc **R**	3'AGCCAAGCCTTGACGAACTAAAGC5'		

3.4.4. Amplification of bacterial DNA by simplex PCR

The chromosomal DNA of studied organisms was obtained by using protocol given in kit (Thermo scientific). For genotypic identification of *S. aureus.* The *nuc* gene was targated as per method given by Brakstad et al. (1992). Compositon of reaction mixture and cyclic conditions for *nuc* gene were mentioned in table 4 & 5 respectively.

3.4.5. Agarose gel electrophoresis

The resulting PCR products were analyzed in agarose gel electrophoresis as described by Sambrook and Russel (2001). The 1.5 % agarose gel was prepared in 1X TAE buffer having ethidium bromide (0.5µg/mL). The mixture was poured onto a gel-casting tray fitted with comb and allowed to solidify. The gel was immersed in an electrophoresis tank containing 1X TAE buffer. Further 1 µL of 6× DNA loading dye (Thermo Scientific) was mixed with 5 µL of DNA sample and loaded into the wells. The GeneRuler of 100 bp DNA Ladder, r (Thermo Scientific) were run along with samples. The amplified products were electrophoresed for 60-90 min at 80 V. The gel was then visualized under Gel documentation system (Bio-Rad).

Table 4: Composition of PCR reaction mixture of *nuc* gene

S. No.	PCR Reaction Contents (25µl)	*nuc* gene
1.	**Master Mix**	12.5µL
2.	**Forward Primer**	2.0µL
3.	**Reverse Primer**	2.0µL
4.	**DNA template**	1.0 µL
5.	**Nuclease –free water**	7.5 µL
Total		**25 µL**

Table 5: Amplification conditions for *nuc* gene

S. No.	PCR Conditions	*nuc* gene	
1.	**Initial denaturation**	Time	Temp.
		5 min	94 °C
2.	**Denaturation**	1 min	94 °C
3.	**Annealing**	30 sec	55 °C
4.	**Extension**	90 sec	72°C
5.	**Final Extension**	4 min	72°C
	No. of Cycles	**36**	

3.5. Biofilm production

3.5.1. Phenotypic biofilm production

Phenotypically and genotypically confirmed isolates of *S. aureus* were subjected to observed the biofilm forming capacity *in vitro* by the following CRA, TP and TCP methods.

3.5.1.1. Congo Red Agar (CRA) method

Congo red agar (CRA) method that is a qualitative assay for detection of biofilm producer microorganism. The CRA assay was performed as per Panda et al. (2016). The CRA medium was constituted by mixing 0.8 g of Congo red and 36 g of sucrose to 37 g/L of Brain heart infusion (BHI) agar and incubated for 24 hours at 37°C. The morphology of colonies that undergone different colors was differentiated as biofilm producers or non-biofilm producers. Black colored colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink color were considered non-biofilm producers.

3.5.1.2. Tube Method (TM)

Tube method (TM) also a qualitative assay for detection of biofilm producer was performed as per method described by Christensen et al.(1985). To perform Tube method BHI broth with 2% sucrose (10 ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 h at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried tubes were then stained with crystal violet (0.1%) for half an hour. The excess stain was removed and tubes were then dried and observed for biofilm formation. A visible film lined in the wall and bottom of the tube were considered positive for biofilm formation. Tubes were examined based on presence of visible film either on wall or bottom or at both the sites, and the amount of biofilm formation was scored as weak, moderate (Biofilm either on bottom or wall of tube) or strong (Biofilm at bottom and wall of test tube). The experiment was performed in duplicate.

3.5.1.3. Tissue Culture Plate Assay (TCP)

Tissue culture plate method was performed as method given by Mathur et al. (2006). According to this method isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18–24 h at 37°C

in a stationary condition. The broth with visible turbidity was diluted to 1 in 100 with fresh medium. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and only broth served as a control to check sterility and nonspecific binding of the medium. These plates were incubated for 24 hours at 37°C. After incubation, the content of the well was gently removed and then were washed 4 times with 0.2 ml of phosphate buffer saline (sessile" organisms in platewere fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a Enzyme-Linked Immunosorbent Assay auto reader at wavelength of 570 nm and were graded as per Christensen et al. (1985). These OD values were considered as an index of bacteria adhering to the surface and forming biofilms. The OD value less than 0.120 considered as none adherence and weak biofilm former while mean OD values 0.120-0.24 showed moderate adherence and moderate biofilm former while OD_> 0.240 showed strong adherence and categorized at strong biofilm former.

3.5.2. Genotypic characterization of Biofilm Forming *S.aureus*

3.5.2.1. Molecular Detection of Biofilm genes (*bap* & *icaA)*

Phenotypically confirmed biofilm forming *S. aureus* were subjected to simplex PCR to target biofilm forming *bap* & *icaA* gene. Oligonucleotide primers detail of *bap* & *icaA* gene were mentioned in table 6, Composition of PCR reaction mixture and amplification conditions were mentioned in table 7 and 8, respectively. Monoplex PCR for *bap* and *icaA* genes were carried out as per methods given by Cucarella et al.(2004) and Notcovich et al.(2018) with slight modifications. Agarose gel electrophoresis was carried out as per the procedure given in section 3.4.5. of material and methods.

Table 6: Primers for biofilm forming genes

S. No.	Gene Name	Primer sequence(5'-3')	Amplicon size	References
1.	***bap*** **F**	CCCTATATCGAAGGTGTAGAATTGCAC	971	Cucarella et al. (2004)
	bap **R**	GCTGTTGAAGTTAATACTGTACCTGC		
2.	***icaA*** **F**	ACACTTGCTGGCGCAGTCAA	188	Notcovich et al. (2018)
	icaA **R**	TCTGGAACCAACATCCAACA		

Table 7: Composition of PCR reaction mixture of *bap* and *ica*A genes

S. No.	PCR Reaction Contents (25µl)	*bap* gene	*icaA* gene
1.	Master Mix	12.5 µL	12.5 µL
2.	Forward Primer	2.0 µL	2.0 µL
3.	Reverse Primer	2.0 µL	2.0 µL
4.	DNA template	1.0 µL	1.0 µL
5.	Nuclease –free water	7.5 µL	7.5 µL
Total		**25 µL**	**25 µL**

Table 8: Amplification conditions for *bap* and *icaA* genes

S. No.	PCR conditions	Thermal conditions *bap*		Thermal conditions *icaA*	
		Time	Temp.	Time	Temp.
1.	Initial denaturation	2 min	94°C	2min	94°C
2.	Denaturation	1 min	94°C	1 min	94°C
3.	Annealing	20 sec	42°C	1 min	52°C
4.	Extension	1 min	72°C	2 min	72°C
5.	Final Extension	10 min	72°C	4 min	94°C
	No.of Cycles	**39**		**25**	

3.6. Gene sequencing of PCR products

A total of 03 PCR products were prepared from *S. aureus* strains isolated from different milk i.e., raw, mastitic and pasteurized. Approximately 100 µl of PCR product was prepared for each gene. The PCR products were sent to Eurofins, genomic India Pvt. Ltd Bangalore, Karnataka for sequencing. The sequencing reads were obtained by both forward and reverse primer used for the amplification of virulent *nuc* genes.

3.6.1 Sequence analysis

The sequences obtained were subjected to BLAST analysis (www.ncbi.nlm. nih.gov/BLAST) to ascertain that the obtained sequence was of *S. aureus*. Afterwards the nucleotide as well as deduced amino acid sequences of *S. aureus* were aligned with those of other available sequences in GenBank database using the MegAlign Programme of Lasergene Software (DNASTAR). Further to analyse the evolutionary significance the phylograms were also constructed using Maximum Likelihood method by using MEGA ver. 7.0 software. The evolutionary history was inferred by using the Maximum Likelihood method (Tamura and Nei, 1993) and evolutionary analyses were conducted in MEGA7 (Kumar et al., 2017).

3.7. Antimicrobial resistance profile

3.7.1. Antibiotic Sensitivity Test (ABST)

Antimicrobial susceptibility of the bacterial isolates was determined by disc diffusion (Bauer-Kirby) method as recommended by Clinical and Laboratory Standards Institute (CLSI, 2018) and interpreted as susceptible, intermediate and resistant. A total of 18 antibiotics comprising often different antimicrobial classes commonly used in the dairy farms and human clinical cases were selective in this study (Table 15). *S. aureus* ATCC 25923 were used as the control strains. Single colony of *S. aureus* from were grown in the nutrient broth and incubate overnight at 37ºC. Turbidity was compared with 0.5 McFarland (Hi-Media) turbidity standards equivalent to the cell density of 1.5 X 10^8 CFU/ml. The 100 µl of culture broth of each isolate was inoculated on MHA plate and spread evenly by using sterilized cotton swabs, the plates were kept upright for an hour so that the culture gets absorbed onto the plates evenly. Antibiotic discs were placed on MHA to each other end. The plates were incubated at 37ºC for 24 hours and the zone of inhibition surrounding the antibiotic discs was measured with the scale.

Table 9: Detail of Antibiotic discs used in antibiotyping

S. No.	Antibiotic groups	Name of Antibiotics	Abbreviation	Concentration (µg)
1	β – lactam	i. Oxacillin	OX	1
		ii. Methicillin	MET	5
		iii. Ampicillin + Sulbactam	A/S	10
2	Cephlosporin	iv. Cefoxitin	CX	30
		v Ceftriaxone	CTR	30
		vi. Ceftazidime	CAZ	30
		vii. Cefotaxime	CTX	30
3	Macrolide	viii. Erythromycin	E	15
4	Carbapenem	ix. Imipenem	IPM	10
5	Glycopeptide	x. Vancomycin	VA	30
6	Aminoglycosides	xi. Gentamicin	GE	10
		xii. Streptomycin	S	10

7	**Amphenicol**	**xiii. Chloramphenicol**	C	30
8	**Quinolone**	**xiv. Oflxacin**	OF	5
		xv. Ciprofloxacin	CIP	50
		xvi. Levofloxacin	LE	5
9	**Oxazolidinones**	**xvii Linizolid**	LZ	30
10	**Tetracycine**	**xviii. Tetracycline**	TE	10

3.7.2. Multidrug Resistance Profile (MDR)

Isolates showing resistance to three or more classes of antimicrobials were considered as multidrug resistant (MDR). MDR *S. aureus* were determined as per the method given by Magiorakaos et al. (2012) with slight modifications.

3.7.3. Minimum Inhibitory Concentration by E-Test (Epsilometer Test)

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC of few drugs was determined as per the method given by Sharma et al. (2017). Found effective against *S. aureus* isolates resistant against multiple drugs. Detail of E-strips used in the study was mentioned in table 10.

3.7.3.1. Preparation of inoculum

For each isolate, 10 µl inoculums was plated onto sterilized Muller Hinton Agar (MHA) plate and incubated at 37 °C for 16-18 hrs. Further, 4-5 morphologically similar colonies were selected from the fresh agar plate and transferred into a sterile Tryptone soya broth (TSB) and incubated at 30-37°C for 2-8 hours until light to moderate turbidity develops. The turbidity of suspension was adjusted with that of standard 0.5 McFarland.

Procedure:

i. Mueller Hinton Agar plate was prepared to perform Epsilometer test.
ii. Sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculums and rotated the soaked swab firmly against the upper inside wall of the tube to express excess fluid.

iii. Entire agar surface of the plate was streaked with the swab three times, the plate was turned at 60° angle between each streaking.

iv. Removed Ezy MIC ™ strip container from refrigeration and keep it at room temperature for 15 minutes before opening.

v. Removed one applicator from the self-sealing bag and stored at room temperature.

vi. Applicator was holded in the middle and gently pressed its broader sticky side on the centre of Ezy MIC ™ strip.

vii. Applicator was lifted along with attached Ezy MIC ™ strip and placed the strip at a desired position on agar plate swabbed with test culture and gently turn the applicator clockwise with fingers.

viii. 60 secs the Ezy MIC ™ strip be adsorbed and firmly adhered to the agar surface.

ix. Plates are incubated at 37°C for 24 hours.

x. MIC was recorded where the ellipse intersect the MIC scale on the E-strip.

Table 10: Detail of E-strips used in E-test (Epsilometer test)

S. No.	Group of E-strip	Name of E-strip	Abbreviation	Concentration (µg/ml)
1	β – lactam	i. Oxacillin	OXA	0.016-256
		ii. Ampicillin/Sulbactam	AMS	0.016-256
2	Cephalosporin	iii. Ceftriaxone	CTR	0.016-256
3	Macrolide	iv. Erythromycin	ERY	0.016-256
4	Carbapenem	v. Imipenem	IPM	0.002-32
5	Glycopeptide	vi. Vancomycin	VAN	0.016-256
6	Aminoglycosides	vii. Gentamicin	GEN	0.016-256
		viii. Streptomycin	STR	0.016-256
7	Amphenicol	ix. Chloramphenicol	CHL	0.016-256
8	Quinolone	x. Ofloxacin	OFX	0.002-32
		xi. Ciprofloxacin	CPH	0.016-256
		xii. Levofloxacin	LEV	0.002-32
9	Oxazolidinones	xiii. Linezolid	LNZ	0.016-256
10	Tetracycline	xiv. Tetracycine	TET	0.016-256

3.7.4. Genotypic identification of methicillin and vancomycin resistance gene (*mec A* and *vanA* gene)

S.aureus isolates were screened for methicillin and vancomycin resistance genes (*mecA* and *vanA*) as per the protocol of Tiwari and Sen. (2006) with slight modifications and list of primers mentioned in table 11. The composition of PCR reaction mixture and amplification conditions for *mecA* and *vanA* gene were depicted in table 12, 13 respectively. Agarose gel electrophoresis was carried out as per method described in the section 3.4.5 of material & methods.

Table 11: Primers for antibiotic resistance genes

S. No.	Gene Name	Primer sequence(5'-3')	Amplicon size	References
1.	***mecA* F**	GTAGAAATGACTGAACGTCCGATAA	310	Tiwari and Sen. (2006)
	***mecA* R**	CCAATTCCACATTGTTTCGGTCTAA		
2.	***vanA* F**	CATGAATAGAATAAAAGTTGCAATA	1030	Tiwari and Sen. (2006)
	***vanA* R**	CCCCTTTAACGCTAATACGACGATCAA		

Table 12: Composition of PCR reaction mixture of *mecA* and *van*A genes

S.N.	PCR Reaction Contents (25µl)	*mecA* gene	*vanA* gene
1.	**Master Mix**	12.5 µL	12.5 µL
2.	**Forward Primer**	2.0 µL	2.0 µL
3.	**Reverse Primer**	2.0 µL	2.0 µL
4.	**DNA template**	2.0 µL	2.0 µL
5.	**Nuclease –free water**	6.5 µL	6.5 µL
Total		**25 µL**	**25 µL**

Table 13: Amplification conditions for *mecA* and *vanA* genes

S. No.	PCR conditions	Thermal conditions *mecA*		Thermal conditions *vanA*	
		Time	Temp.	Time	Temp.
1.	**Initial denaturation**	4 min	94°C	10 min	94°C
2.	**Denaturation**	45 sec	94°C	30 sec	94°C
3.	**Annealing**	45 sec	56°C	45 sec	50°C
4.	**Extension**	30 sec	72°C	30 sec	72°C
5.	**Final Extension**	2 min	72°C	10 min	72°C
	No.of Cycles	**30**		**30**	

Results

CHAPTER- 4

RESULTS

4.1. Screening of samples for mastitis

A total of 56 samples collected from cows with symptom of mastitis were screened mastitis by CMT (California Mastitis Test). All the tested samples produced gel formation with CMT reagent (Fig.1) and thus 100% samples were found positive for mastitis (Table 14).

Table 14: Scoring of milk samples by California Mastitis Test (CMT)

S.No.	Sample ID	CMT Score	Outcome
1	MMC1	2	Distinct positive
2	MMC2	3	Strong positive
3	MMC3	3	Strong positive
4	MMC4	2	Distinct positive
5	MMC5	2	Distinct positive
6	MMC6	3	Strong positive
7	MMC7	2	Distinct positive
8	MMC8	3	Strong positive
9	MMK9	2	Distinct positive
10	MMK10	2	Distinct positive
11	MMK11	3	Strong positive
12	MMK12	2	Distinct positive
13	MMK13	3	Strong positive
14	MMK14	2	Distinct positive
15	MMK15	2	Distinct positive
16	MMK16	3	Strong positive
17	MMB17	3	Strong positive
18	MMB18	2	Distinct positive
19	MMB19	3	Strong positive
20	MMB20	2	Distinct positive
21	MMB21	3	Strong positive
22	MMB22	3	Strong positive
23	MMB23	3	Strong positive

24	**MMB24**	3	Strong positive
25	**MMH25**	3	Strong positive
26	**MMH26**	2	Distinct positive
27	**MMH27**	3	Strong positive
28	**MMH28**	3	Strong positive
29	**MMH29**	2	Distinct positive
30	**MMH30**	3	Strong positive
31	**MMH31**	2	Distinct positive
32	**MMH32**	3	Strong positive
33	**MMI33**	3	Strong positive
34	**MMI34**	3	Strong positive
35	**MMI35**	2	Distinct positive
36	**MMI36**	2	Distinct positive
37	**MMI37**	3	Strong positive
38	**MMI38**	2	Distinct positive
39	**MMI39**	3	Strong positive
40	**MMI40**	3	Strong positive
41	**MMG41**	2	Distinct positive
42	**MMG42**	2	Distinct positive
43	**MMG43**	3	Strong positive
44	**MMG44**	2	Distinct positive
45	**MMG45**	3	Strong positive
46	**MMG46**	2	Distinct positive
47	**MMG47**	3	Strong positive
48	**MMG48**	3	Strong positive
49	**MMT49**	2	Distinct positive
50	**MMT50**	3	Strong positive
51	**MMT51**	2	Distinct positive
52	**MMT52**	3	Strong positive
53	**MMT53**	2	Distinct positive
54	**MMT54**	3	Strong positive
55	**MMT55**	3	Strong positive
56	**MMT56**	2	Distinct positive

Fig. 1: Gel formation in mastitic positive sample

Fig. 2: Pre enrichment of samples in Buffered Peptone Water

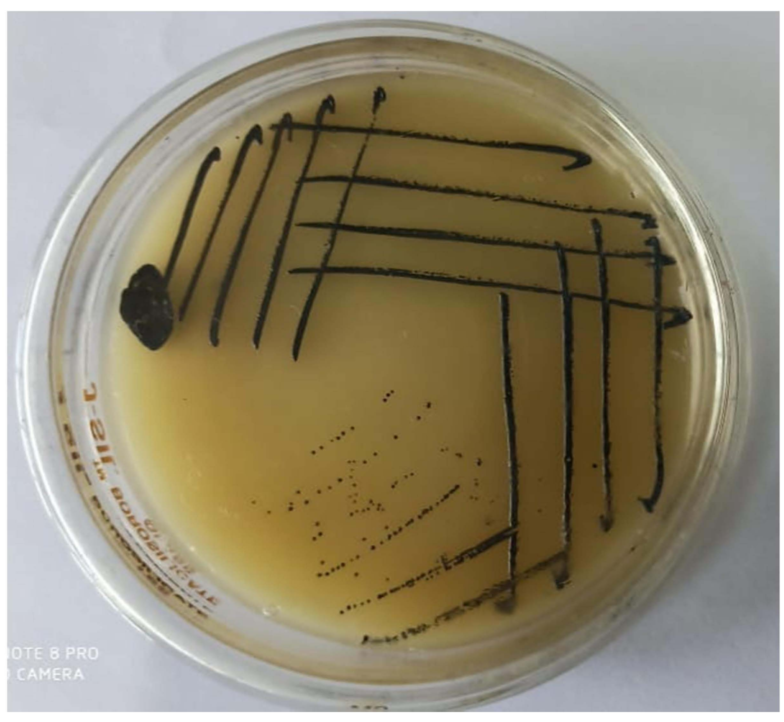


Fig. 3: Jet black colored colonies of *Staphylococcus spp.* on Baired-Parker Agar

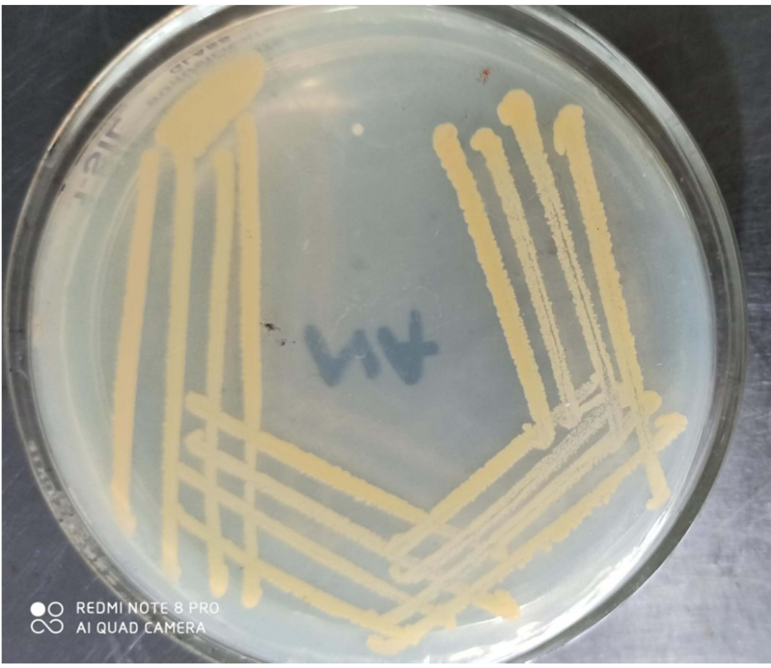
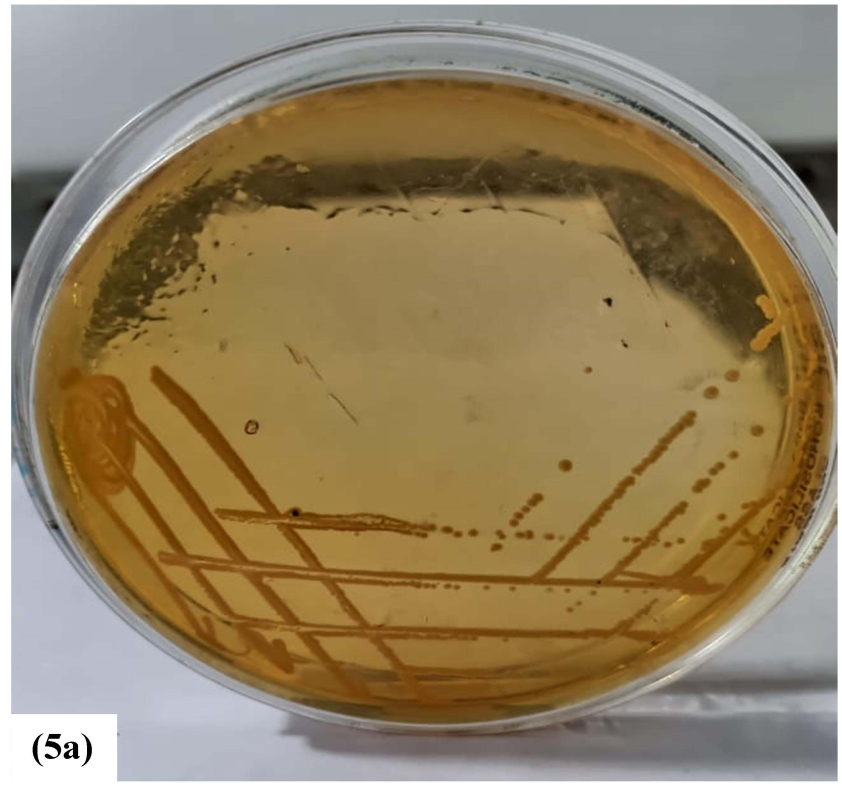


Fig. 4: Yellow colored colonies of *Staphylococcus spp.* on Nutrient Agar



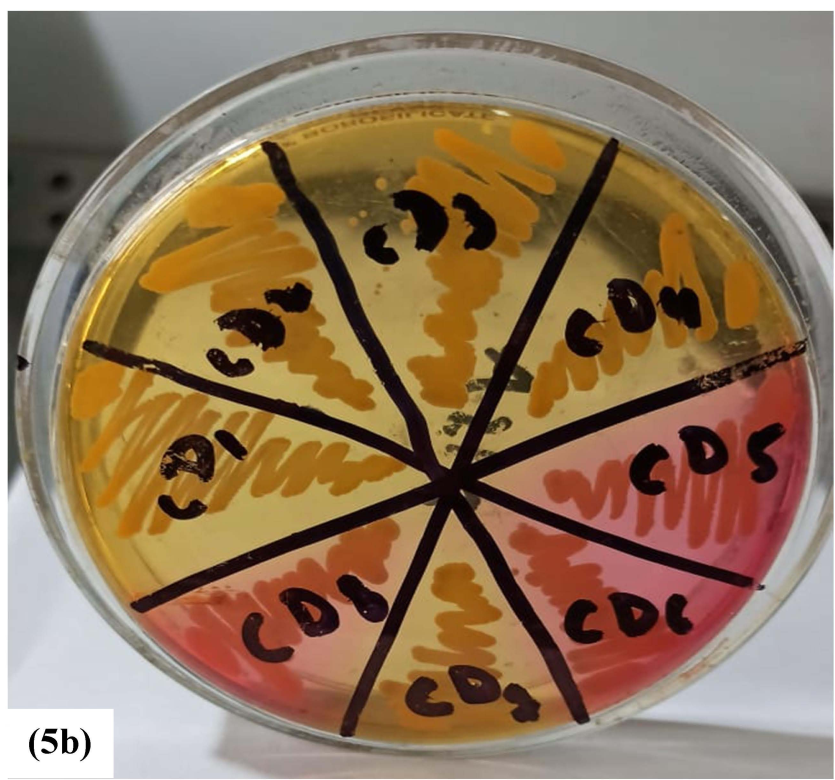


Fig. 5(a) & (b): Yellow colored colonies with yellow zone of *S. aureus* on Mannitol Salt Agar

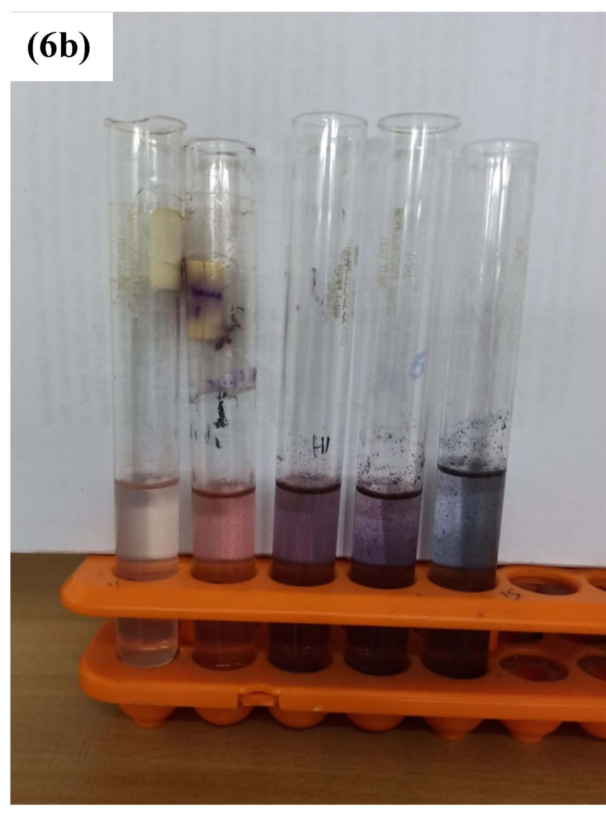


Fig. 6a & b: IMViC Test

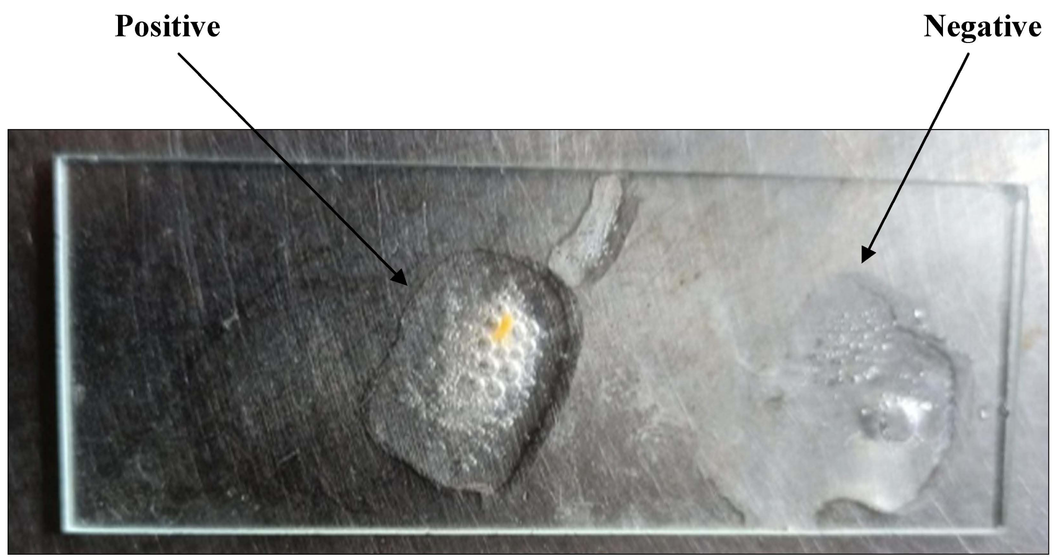


Fig.7: Catalase Test

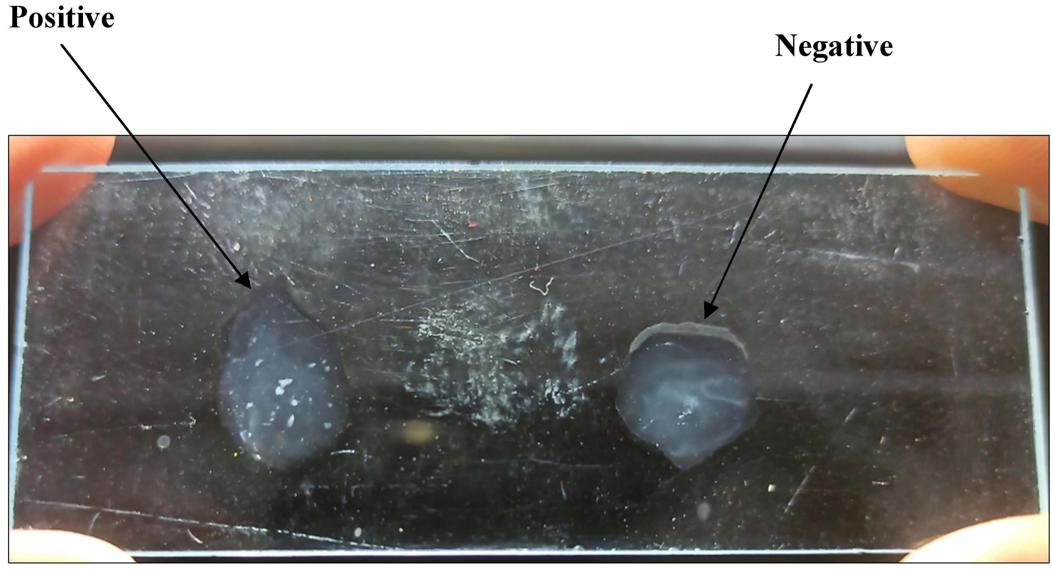


Fig. 8: Coagulase Test

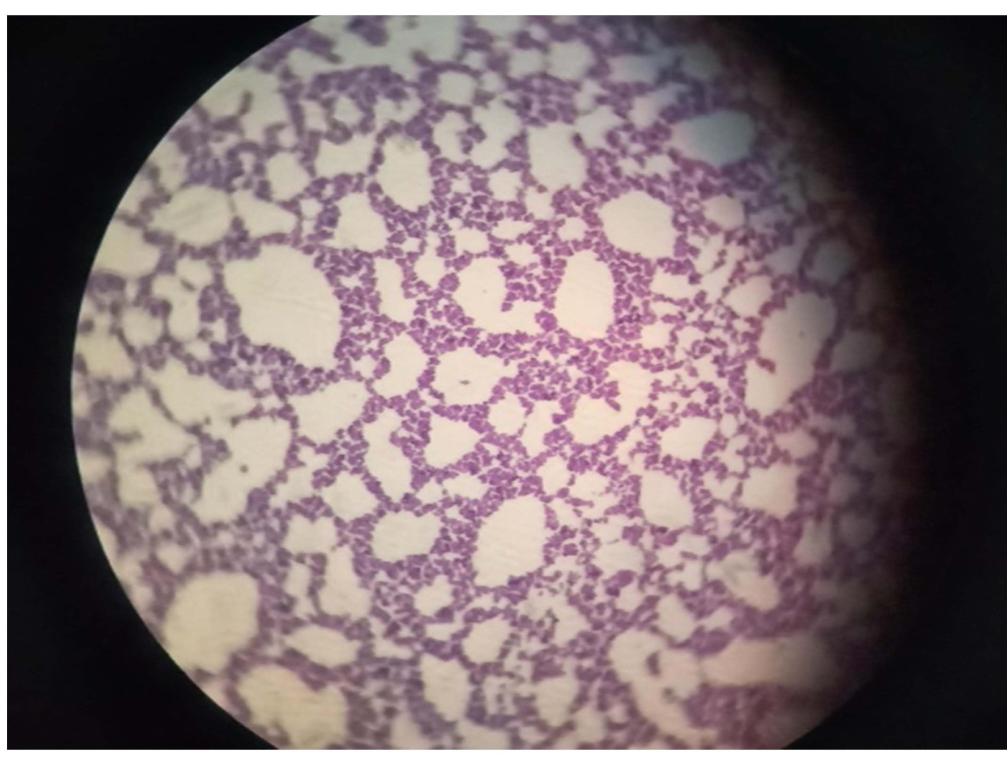


Fig. 9: Gram positive cocci with purple stain

4.2. Isolation and identification of *S. aureus*

For the isolation of *S. aureus,* a total of 378 milk samples were processed for enrichment (Fig. 2) and selective plating on selective media. Out of 378 samples, 121 samples produced the jet black colored colonies on Baired Parker agar (Fig. 3) and yellow colored colonies on Nutrient agar (Fig. 4) and isolates were categorized as *Staphylococcus.* Further for isolation of *S. aureus* colonies from Baired Parker agar were picked and streaked over Mannitol salt agar and 106 *Staphylococcus* isolates produced yellow colored colonies surrounded by yellow zone and isolates were identified as *S. aureus* (Fig. 5a & 5b)

4.2.1. Biochemical characterization of *S. aureus*

The presumptive isolates of *S. aureus* were biochemically confirmed by IMViC (Fig.6a & 6b), catalase (Fig.7) and coagulase test (Fig.8). All presumptive *S. aureus* were Gram positive cocci in grapes like bunches (Fig. 9) and were catalase positive and oxidase negative.

4.3. Prevalence of *S. aureus*

Prevalence of *S. aureus* in raw milk, pooled raw milk, mastitic milk and pasteurized milk were 18.65%, 57.5%, 51.78% and 23.33% with overall prevalence of 28.04%. Highest prevalence of *S. aureus* in raw milk was at Gayatri Tapobhumi Gaushala with 25% while in mastitic milk 62.5% from Hasanand Gaushala. Outcome showed that *S. aureus* was more prevalent in milk of local shops than vendors and prevalence of *S. aureus* in pooled raw milk were highest in vendors of Aurangabad region with 75% while from local shops was highest in Natwar Nagar with 100% prevalence. Prevalence values of *S. aureus* at different places were mentioned in Table (15 & 16) (Fig. 10).

Table 15: Prevalence of *S. aureus* in milk from different sources

S.No.	Sources	Source of Collection	Place of Collection	Samples	*Staphylococcus spp.*	*S. aureus*	Prevalence of *S. aureus* (%)
1.	**Raw Milk (From individual animal)**	Dairy Farms and Gaushalas	Livestock Dairy Farm	36	5	4	11.11
			Karnaval Dairy	36	9	8	22.22
			Bhagwati Gaushala(Birla)	36	6	6	16.67
			Hasanand Gaushala	36	9	8	22.22
			Bhaktivedanta Swami Gaushala Iscon Temple	36	6	5	13.89
			Govind Dham Gaushala	36	8	7	19.44
			Gayatri Tapobhumi Gaushala	36	10	9	25.0
	Subtotal raw milk			**252**	**53**	**47**	**18.65**
2.	**Pooled raw milk**	Vendors	Acharya Nagar	4	2	1	25.0
			Aurangabad	4	3	3	75.0
			Moti enclave	4	1	1	25.0
			Moti kunj	4	2	2	50.0
			Natwar Nagar	4	3	2	50.0
				20	**11**	**9**	**45.0**
3.	**Pooled raw milk**	Local shops	Acharya Nagar	4	2	2	50.0
			Aurangabad	4	4	3	75.0
			Moti enclave	4	3	2	50.0
			Moti kunj	4	3	3	75.0

			Natwar Nagar	4	4	4	100.0
				20	16	14	70.0
	Subtotal pooled raw milk			**40**	**27**	**23**	**57.5**
	Total raw milk			**292**	80	70	**24.00**
4	**Mastitic raw milk (From individual animal)**	Dairy farm and Gaushalas	Clinics TVCC & Livestock Dairy Farm	8	5	4	50.0
			Karnaval Dairy	8	5	4	50.0
			Bhagwati Gaushala	8	4	4	50.0
			Hasanand Gaushala	8	6	5	62.50
			Bhaktivedanta Swami Gaushala Iscon Temple	8	4	4	50.0
			Govind Dham Gaushala	8	5	4	50.0
			Gayatri Tapobhumi Gaushala	8	4	4	50.0
	Subtotal mastitic milk			**56**	**33**	**29**	**51.78**
5.	**Pasteurized milk**	Grocery shops	Acharya Nagar	6	2	2	33.33
			Aurangabad	6	3	2	33.33
			Moti enclave	6	1	1	16.67
			Moti kunj	6	1	1	16.67
			Natwar Nagar	6	1	1	16.67
	Subtotal pasteurized milk			**30**	**8**	**7**	**23.33**
	Total samples			**378**	**121**	**106**	**28.04**

Table 16: Overall Prevalence of *S. aureus* in milk from different sources

S. No.	Source	No. of samples	Prevalence of *Staphylococcus spp*. (%)	Prevalence of *S. aureus* (%)
1.	Raw milk (Dairy farms & Gaushalas)	252	21.03 (53/252)	18.65 (47/252)
2.	Pooled raw milk (Vendors)	20	55.0 (11/20)	45.0 (09/20)
3.	Pooled raw Milk (Local Shops)	20	80.0 (16/20)	70.0 (14/20)
	Subtotal pooled raw milk	40	67.5 (27/40)	57.5 (23/40)
	Total Raw milk	292	27.39 (80/292)	24.00 (70/292)
4.	Mastitic milk (Dairy farms & Gaushalas)	56	58.9 (33/56)	51.78 (29/56)
5.	Pasteurized milk (Grocery shops)	30	26.6 (08/30)	23.33 (07/30)
	Total	**378**	**32.01 (121/378)**	**28.04 (106/378)**

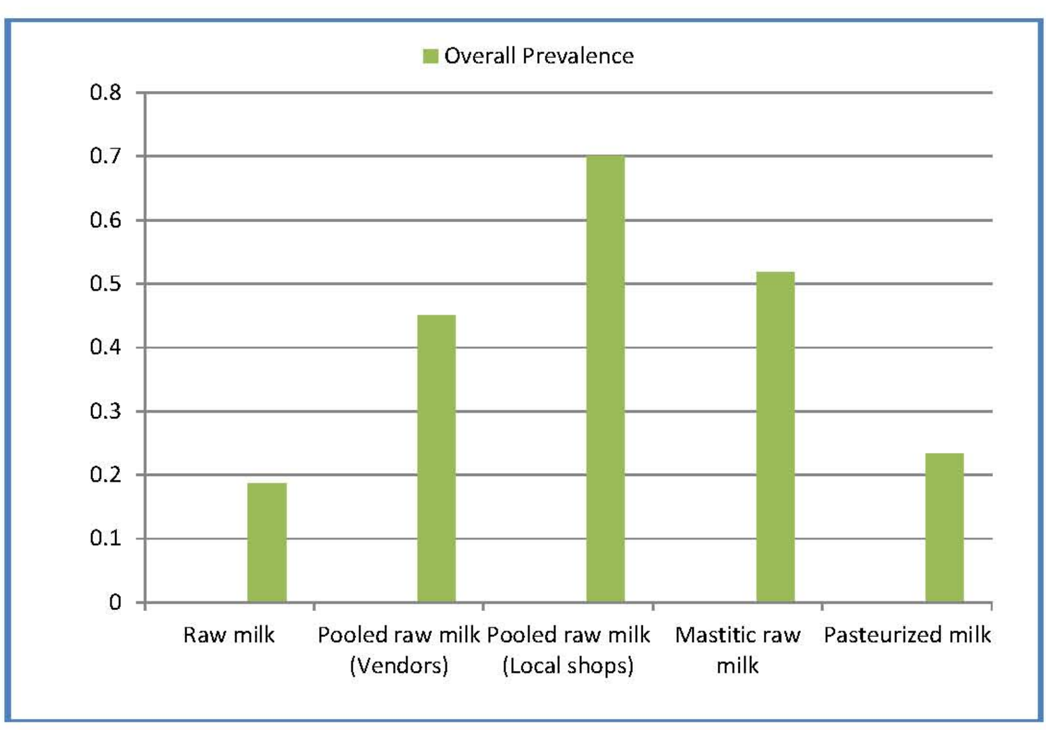


Fig.10. Prevalence of *S. aureus* in different types of milk

4.4. Molecular characterization of *S. aureus*

4.4.1. & 2. Isolation and quantification of DNA

Bacterial DNA was isolated by Gene JET Genomic DNA Purification Kit and purity of DNA was tested by Nanodrop. The absorbance ratio of the isolated DNA at 260/280 came within range of around 1.80 -1.90 that indicates the good quality of DNA.

4.4.3. Genotypic identification of *S. aureus* by *nuc* gene

All the biochemically confirmed isolates of *S. aureus* were subjected to simplex PCR for the species specific *nuc* gene. All the phenotypically confirmed isolates were *nuc* gene bearers. Prevalence of *nuc* gene in raw milk, pooled raw milk, mastitic raw milk and pasteurized milk was 18.65%, 57.50%, 51.78% and 23.33% respectively (Table. 17 & Fig. 11).

Table 17: Molecular detection of *nuc* gene

S. No.	Source	Total Samples	Screened *S. aureus*	*S. aureus* +ve for *nuc* gene	Prevalenc of *nuc* gene (%)
1.	**Raw milk (Dairy farms & Gaushalas)**	252	47	47	18.65 (47/252)
2.	**Pooled raw milk (Vendors)**	20	09	09	45.0 (09/20)
3.	**Pooled raw milk (Local Shops)**	20	14	14	70.0 (14/20)
	Sub total pooled raw milk	40	23	23	57.5 (23/40)
	Total raw milk	292	70	70	24.00 (70/292)
4.	**Mastitic raw milk (Dairy farms & Gaushalas)**	56	29	29	51.78 (29/56)
5.	**Pasteurized milk (Grocery Shops)**	30	07	07	23.33 (07/30)
	Total	**378**	**106**	**106**	**28.04**

4.5. Biofilm Production

4.5.1. Phenotypic characterization

Phenotypically and genotypically confirmed 106 isolates of *S. aureus* were analyzed for biofilm formation by three different described methods CRA, TM and TCP. On the basis of these assays the isolates were categorized strong, moderate and weak biofilm formers.

4.5.1.1. Congo Red Agar (CRA) Assay

In this assay, out of 106 *S. aureus* isolates, 19 produced (17.92%) the black colored colonies and 87 (82.07%) produced red colored colonies on Congo red agar. Thus, in this screening assay 10.63% biofilm forming *S. aureus* were revealed from raw milk, 17.39% from pooled raw millk, 27.59% from mastitic milk and 28.57% from pasteurized milk with overall prevalence of 17.92% from all milk sources by CRA assay (Table. 18 & Fig. 12).

4.5.1.2. Tube Method (TM)

In tube method, biofilm forming isolates were screened on the basis of slime produced by isolates either on bottom or surfaces of test tubes. In this assay 27.65% *S. aureus* were strong and moderate biofilm former each obtained from raw milk while in pooled raw milk strong and moderate former were 52.17% and 30.43%, respectively and 17.39% were non biofilm former. Tube method screened 48.28 and 42.86% were strong biofilm producer in mastitic and pasteurized milk, isolates produced biofilm on top as well as bottom of test tube while 37.93% and 28.57% were moderate biofilm producer. Overall, by tube method 39.62%, 31.13% and 29.25%. *S. aureus* were strong, moderate and weak biofilm producer. (Table. 19 & Fig. 13).

4.5.1.3. Tissue Culture Plate Assay

In TCP assay, biofilm formation was evaluated on the basis of OD values measured on 570 and same number of *S. aureus* (106) were also screened through TCP, in raw and pooled raw milk 38.29% and 47.82% were strong biofilm former with more than 0.240 OD values, 31.91% and 34.78% were moderate biofilm producer while 29.78% and 17.39% isolates showed OD values less than 0.120 and categorized as weak biofilm producer. In comparision to all types of milk highest number of strong biofilm former was obtained in mastitic milk 65.51% and lowest from pasteurized milk 14.29%. Overall, 46.23%, 31.13% and 22.64% *S. aureus* were found to be strong, moderate and weak biofilm producer through TCP assay. (Table. 20 & Fig. 14).

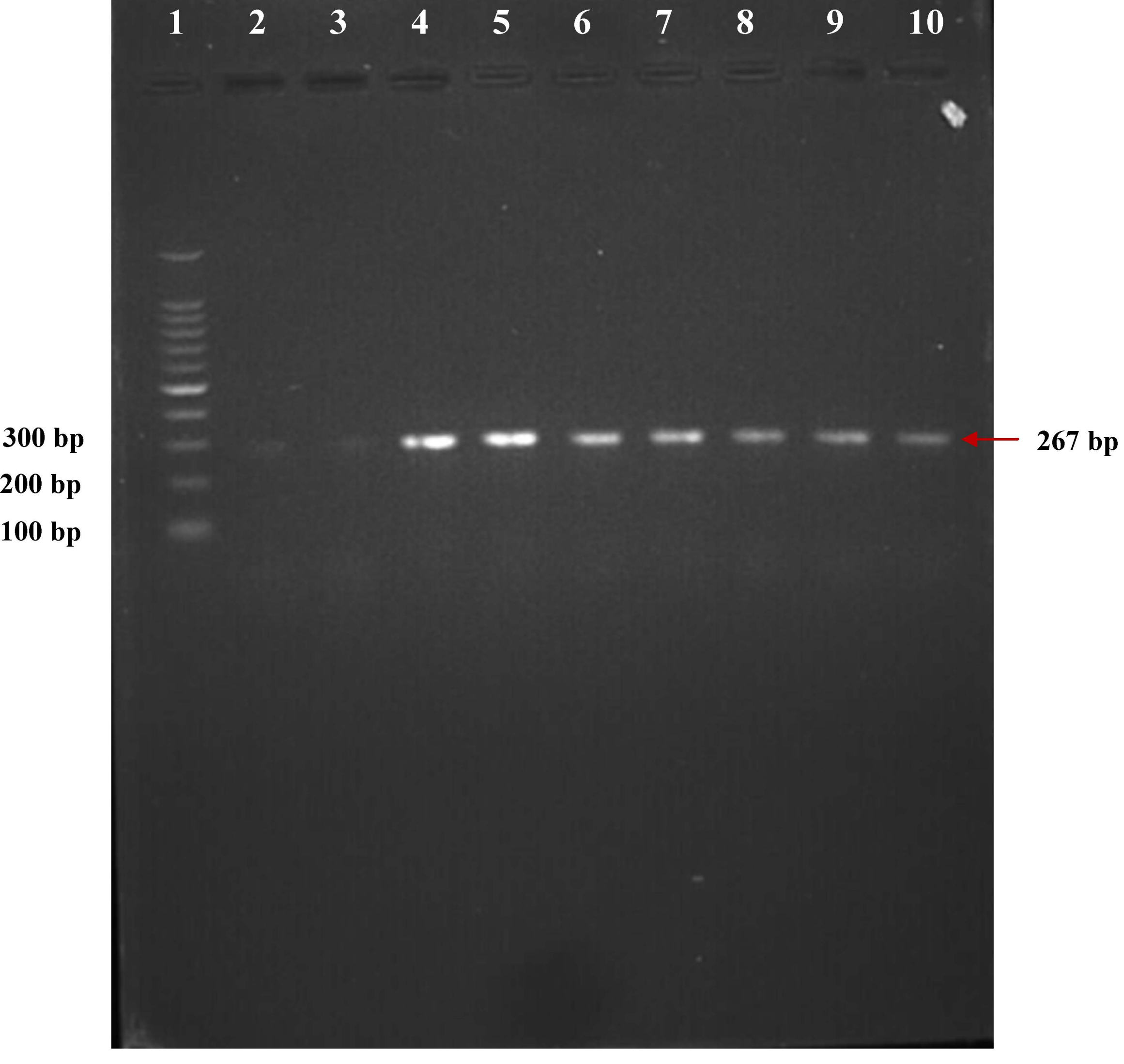


Fig. 11: Species specific PCR amplicon (*nuc*) of *S. aureus* resolved after electrophoresis in 1.5 % agarose gel

Lane 1 : 100bp DNA ladder

Lane 2 to 9 : *nuc* gene of *S. aureus* of 267 bp

Lane 10 : Positive Control

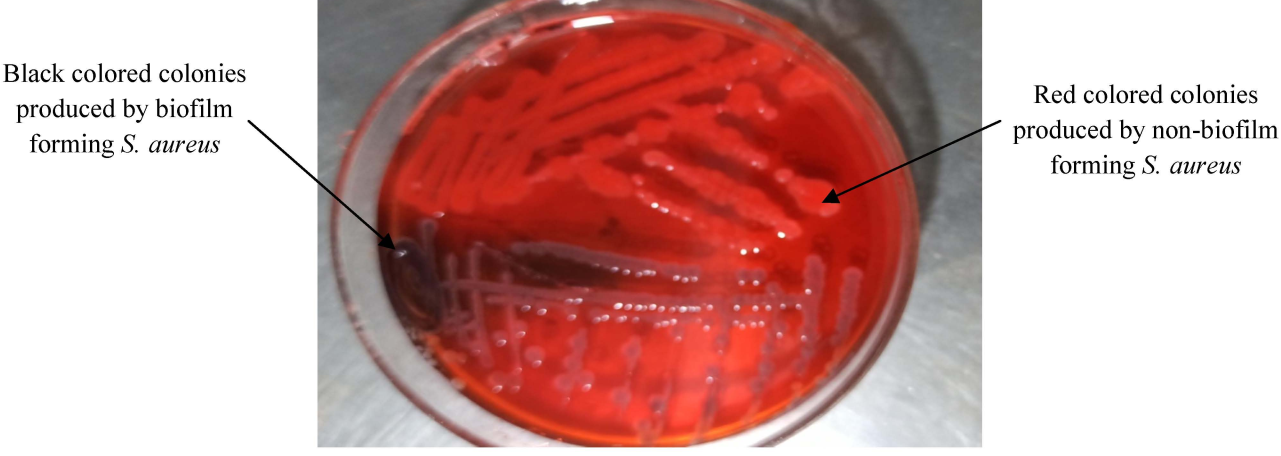


Fig. 12: Colonies on Congo Red Agar

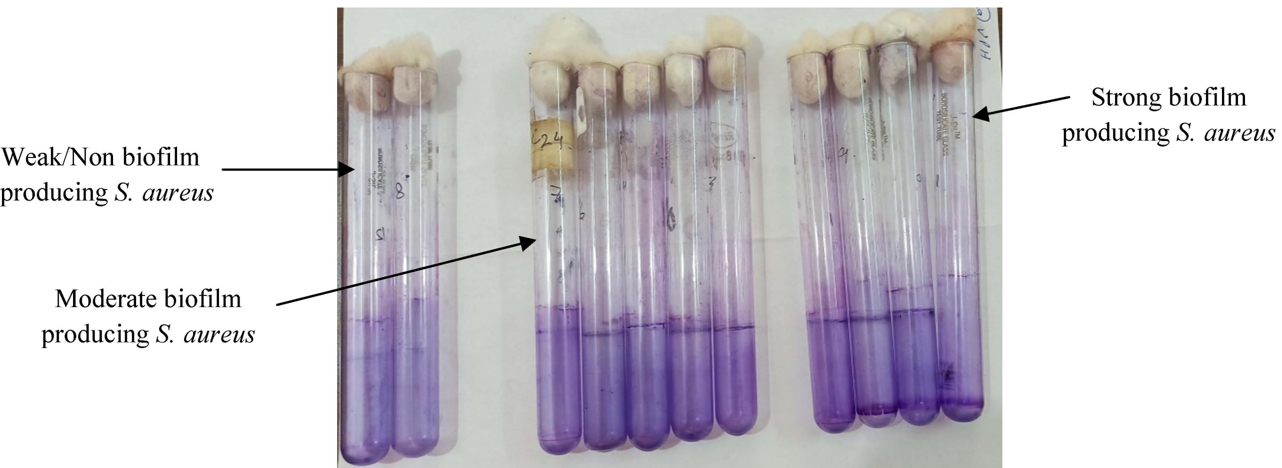


Fig. 13: Biofilm formation by Tube Method

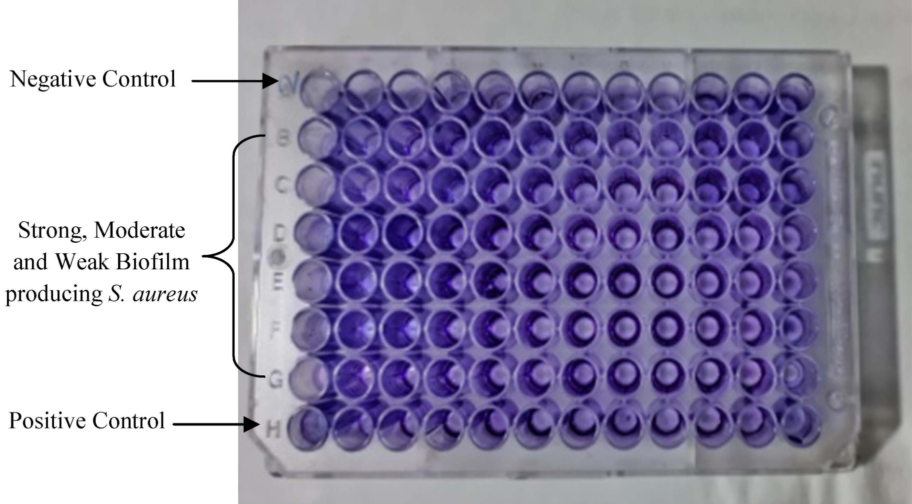


Fig. 14: Biofilm formation by Tissue Culture Plate Assay

Table 18: Screening of biofilm producing *S. aureus* by Congo Red Agar (CRA) Assay

S.No.	Colour of colony on CRA agar	Biofilm formation category	Raw Milk (%)	Pooled Raw Milk (%)	Sub Total Raw milk (%)	Mastitic Milk (%)	Pasteurized Milk (%)	Total (%)
Screened *S. aureus* isolates			**47**	**23**	**70**	**29**	**07**	**106**
1	Black colony with dry crystalline consistency (+)	Positive	10.63 (5/47)	17.39 (4/23)	12.86 (9/70)	27.59 (8/29)	28.57 (02/ 07)	17.92 (19 /106)
2	Red colony (-)	Negative	89.36 (42/47)	82.60 (19/23)	87.14 (61/70)	72.41 (21/29)	71.43 (05/07)	82.08 (87/106)

Table 19: Screening of biofilm forming *S. aureus* by Tube method (Biofilm production capability and formation intensity)

S. No.	Stained line at bottom or brim of test tube	Biofilm formation category	Raw Milk (%)	Pooled Raw Milk (%)	Total raw milk (%)	Mastitic Milk (%)	Pasteurized Milk (%)	Total (%)
Screened *S. aureus* isolates			**47**	**23**	**70**	**29**	**07**	**106**
1	No visible film line	None/Weak	44.68 (21/47)	17.39 (4/23)	35.71 (25/70)	13.79 (04/29)	28.57 (02/07)	29.25 (31/106)
2	Visible film line at top or bottom/	Moderate	27.65 (13/47)	30.43 (7/23)	28.57 (20/70)	37.93 (11/29)	28.57 (02/07)	31.13 (33/106)
3	Visible film line at top and bottom	Strong	27.65 (13/47)	52.17 (12/23)	35.71 (25/70)	48.28 (14/29)	42.86 (03/07)	39.62 (42/106)

Table 20: Screening of biofilm forming *S. aureus* by Tissue Culture Plate Assay (Biofilm production capability and formation intensity)

S. No.	Mean OD values	Adherence	Biofilm formation Category	Raw Milk (%)	Pooled Raw milk (%)	Total raw milk (%)	Mastitic Milk (%)	Pasteurized milk (%)	Total (%)
Screened *S. aureus* isolates				**47**	**23**	**70**	**29**	**07**	**106**
1	<0.120	Weak/ none	Weak/None	29.78 (14/47)	17.39 (4/23)	25.71 (18/70)	3.45 (01/29)	71.43 (05/07)	22.64 (24/106)
2	0.120-0.240	Moderate	Moderate	31.91 (15/47)	34.78 (8/23)	32.86 (23/70)	31.03 (09/29)	14.29 (01/07)	31.13 (33/106)
3	>0.240	Strong	Strong	38.29 (18/47)	47.82 (11/23)	32.86 (23/70)	65.51 (19/29)	14.29 (01/07)	46.23 (49/106)

4.5.1.4. Comparison of Biofilm Assays (TCP, TM and CRA)

Among three assays that were used for the screening of biofim formation highest number of biofilm forming *S. aureus* were revealed by the TCP (77.36%) in comparison to TM (70.75%) and CRA assay (17.92%). Among three test CRA proved to be least fruitful as only 17.92% isolates were biofilm producer by this test. In the other two assays TM and TCP strong biofilm producer were 39.62 and 46.23% respectively while moderate were 31.13% in both the assay. Thus, TCP assay was proved to be the golden standard method for the screening of biofilm forming isolates. (Table. 21 & Fig. 15).

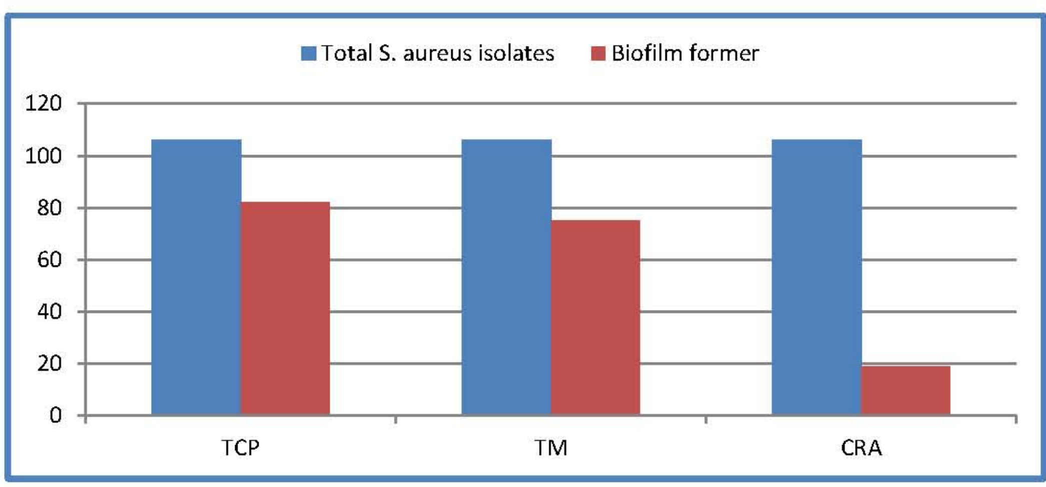


Fig.15: Comparison of TCP, TM and CRA

4.5.1.5 Overall Prevalence of Biofilm producing *S. aureus*

In the present study, Gaushalas, dairy farms, local shops, vendors and grocery shops of Mathura district were screened for biofilm forming *S. aureus*. Prevalence of biofilm forming *S. aureus was* highest in mastitic milk followed by pooled raw milk, raw milk and pasteurized milk was 96.55% ,82.60%, 70.21%and 28.57% (Table. 22).

Table 21: Comparison of Biofilm Assays (TCP, TM & CRA)

***S. aureus* isolates**	**Biofilm formation**	**Tissue Culture Plate Assay (TCP) (%)**	**Tube Method (TM) (%)**	**Congo Red Agar Assay (CRA) (%)**
n=106	High	46.23 (49/106)	39.62 (42/106)	17.92 (19/106)
	Moderate	31.13 (33/106)	31.13 (33/106)	-
	Weak/none	22.64 (24/106)	29.25 (31/106)	82.07 (87/106)
	Total Biofilm former (Strong + Moderate)	77.36 (82/106)	70.75 (75/106)	17.92 (19/106)

Table: 22 Source wise prevalence of Biofilm forming *S. aureus* isolates

S. No.	Source	Total samples	*S. aureus* with (*nuc* gene)	Prevalence of *S. aureus* (%)	Biofilm forming *S. aureus* by TCP (% positive)			Total biofilm former (% positive) TCP	Biofilm forming *S. aureus* by TM (% positive)			Total biofilm former (% positive) TM	Biofilm forming *S. aureus* by CRA	
					S	M	W/N	S+M	S	M	W/N	S+M	P	N
1	Raw milk	252	47	18.65 (47/252)	38.29 (18/47)	31.91 (15/47)	29.78 (4/47)	70.21 (33/47)	27.65 (13/47)	27.65 (13/47)	44.68 (21/47)	55.31 (26/47)	10.63 (5/47)	89.36 (42/47)
2	Pooled raw milk	40	23	57.50% (23/40)	47.82 (11/23)	34.78 (8/23)	17.39 (4/23)	82.60 (19/23)	52.17 (12/23)	30.43 (7/23)	17.39 (4/23)	82.60 (19/23)	17.39 (04/23)	82.60 (19/23)
	Subtotal raw milk	292	70	23.97% (70/292)	41.43 (29/70)	32.86 (23/70)	25.71 (18/70)	74.29 (52/70)	35.71 (25/70)	28.57 (20/70)	35.71 (25/70)	64.29 (45/70)	12.86 (9/70)	87.14 (61/70)
3	Mastitic milk	56	29	51.79 (29/56)	65.51 (19/29)	31.03 (9/29)	3.45 (1/29)	96.55 (28/29)	48.28 (14/29)	37.93 (11/29)	13.79 (4/29)	86.20 (25/70)	27.59 (08/29)	72.41 (21/29)
4	Pasteurized milk	30	07	23.33% (7/30)	14.29 (1/7)	14.29 (1/7)	71.43 (5/7)	28.57 (2/7)	42.86 (3/7)	28.57 (2/7)	28.57 (2/7)	71.42 (5/7)	28.57 (02/07)	71.42 (05/07)
	Total	378	106	28.04 (106/378)	46.22 (49/106)	31.11 (33/106)	22.64 (24/106)	77.36 (82/106)	39.62 (42/106)	31.13 (33/106)	29.25 (31/106)	70.75 (75/106)	17.92 (19/106)	82.08 (87/106)

Figures in parenthesis indicate the percentage

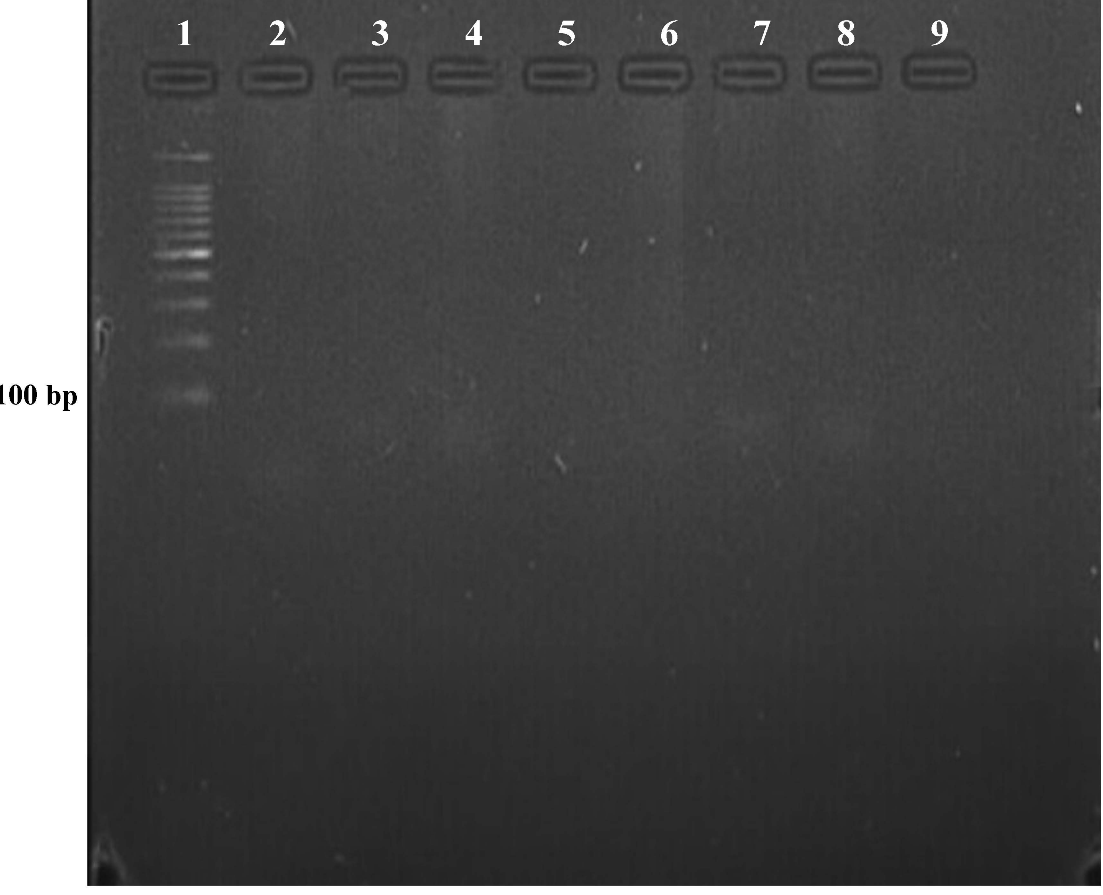


Fig. 16: Agarose gel showing absence of *bap* gene (971 bp) of *S. aureus*

Lane 1- 100 bp DNA ladder

Lane 2 to 9- No PCR amplicon

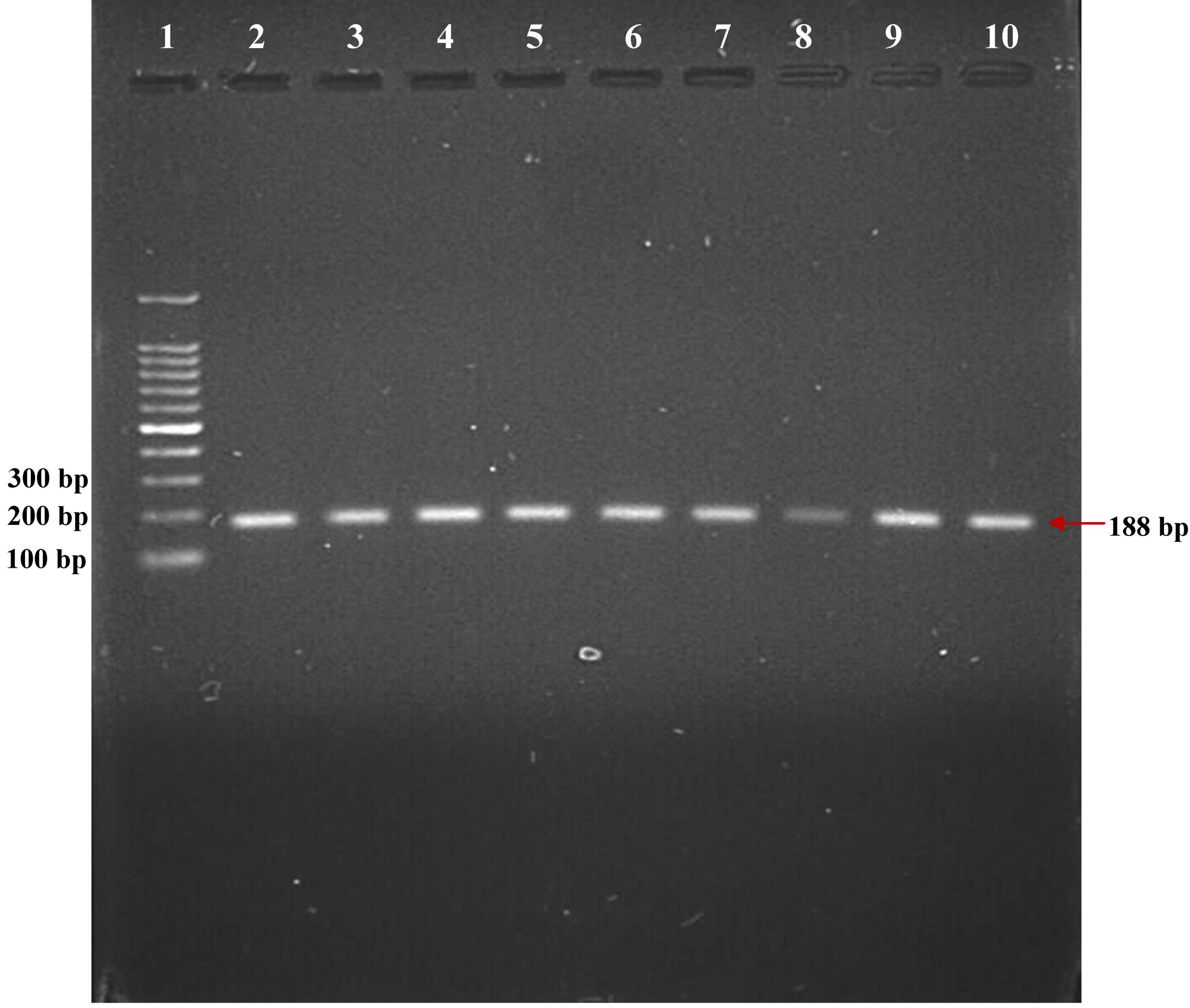


Fig. 17: Biofilm forming *icaA* gene (188 bp) resolved after electrophoresis in 1.5 % agarose

Lane 1- 100 bp DNA ladder

Lane 2 to 9- Biofilm forming *icaA* gene (188 bp) of *S. aureus*

Lane 10-Positive Control

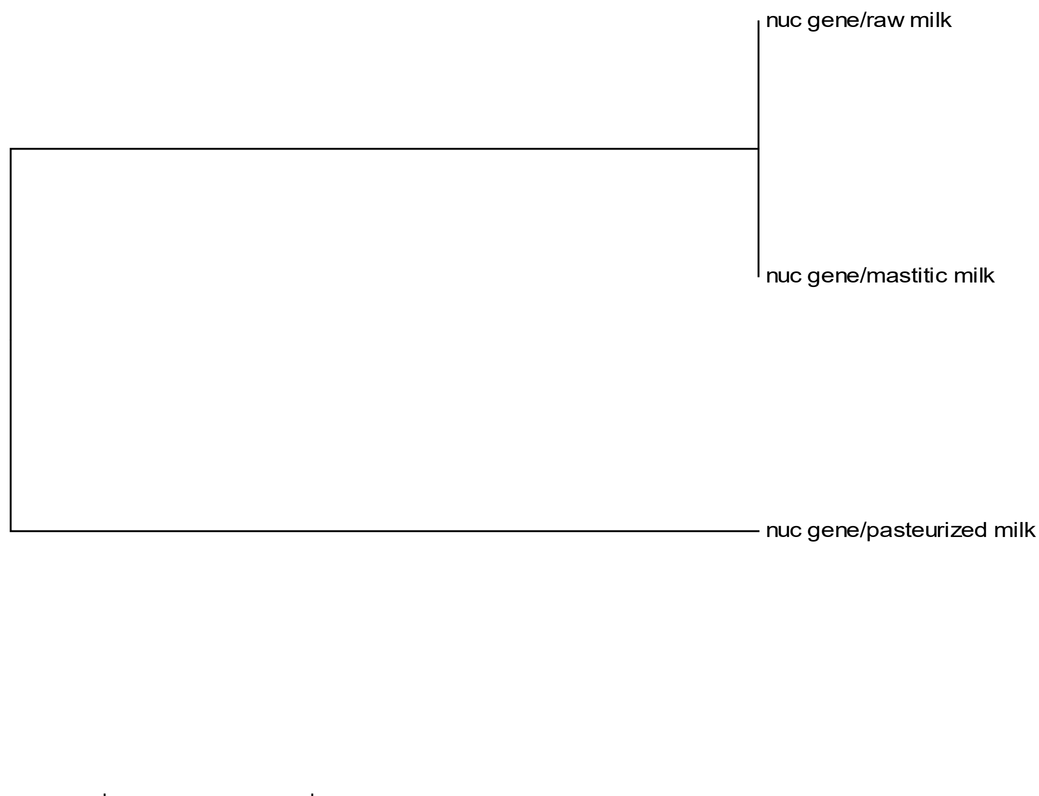


Figure. 18 Molecular Phylogenetic analysis by Maximum Likelihood method.
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-375.24) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 3 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 280 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

4.5.2. Genotypic characterization of biofilm forming *S. aureus*

4.5.2.1. Molecular detection of biofilm forming genes (*bap* and *ica*A)

After the phenotypic characterization biofilm forming isolates were screened for biofilm genes *bap* and *ica*A by simplex PCR. Outcome showed that none of the isolates possessed *bap* gene and prevalence was zero % in *S. aureus* isolates (Fig.16). The another gene *icaA* of ica locus was prevalent in *S. aureus* isolates and the percent positivity of this gene in raw milk, pooled raw milk, mastitic milk and pasteurized milk was 74.46%, 91.30%, 96.55% and 28.57% with overall percent positivity of 81.13%. (Table. 23 & Fig.17).

Table 23: Percent positivity of *bap* & *icaA* gene

S. No.	Source	Total Sample	*S. aureus*	Percent positivity of *bap* gene (%)	Percent positivity of *icaA* gene (%)
1	**Raw Milk**	252	47	0.0 (0/47)	74.46 (35/47)
2	**Pooled Raw Milk**	40	23	0.0 (0/23)	91.30 (21/23)
	Total Raw mlk	292	70	0.0 (0/70)	80.00 (56/70)
3	**Mastitic Raw Milk**	56	29	0.0 (0/29)	96.55 (28/29)
4	**Pasteurized Milk**	30	07	0.0 (0/07)	28.57 (2/07)
	Total	**378**	**106**	**0.0 (0/106)**	**81.13 (86/106)**

4.6. Phylogenetic Analysis

The size of PCR products (n=3) of *nuc* genes were confirmed by sequencing Eurofins, genomic India Pvt. Ltd Bangalore, Karnataka. Details of sequenced sample Sequencing reads were obtained by both forward and reverse primer used for amplification of *nuc* gene (267 bp). The nucleotide sequences of *nuc* gene are in processing of submission in the GenBank database. The deduced amino acid sequence of *nuc* gene was compared that were belonged to raw, mastitic and pasteurized milk (Fig. 18). In the phylogram *nuc* gene of raw and mastitic milk were originated from single clade while the *nuc* gene of pasteurized milk was showing a separate branch.

4.7. Antimicrobial resistance profile of *S. aureus*

4.7.1. Antibiotic sensitivity Test (ABST)

All the 49 strong biofilm forming *S. aureus* were subjected to antimicrobial sensitivity testing against various groups of antimicrobials for antibiotic sensitivity

test. Zone of inhibition was recorded and interpretated the resistance, intermediate and sensitivity of isolates. The resistance for *S. aureus* isolates for following antibiotics viz. Oxacillin, Methicilin, Ampicillin/Sulbactam, Ceftazidime, Cefotaxime, Erythromycin, Imipenem, Gentamicin, Streptomycin, Chloramphenicol, Ofloxacin, Ciprofloxacin, Levofloxacin, Linizolid and Tetracycline was measured as 69.38%, 53.06%, 32.65%, 10.20%, 14.28%, 32.65%, 10.20%, 10.20%, 20.51%, 10.20%, 59.18%, 24.49%, 24.45%, 28.57% and 18.37%, respectively with the range from (10.20- 69.38). Isolates were found sensitive towards the antibiotics Cefoxitin, Ceftriaxone, Vancomycin 95.91%, 95.91% and 85.71%, respectively (Table 24 & Fig. 19). In different milk sources raw milk, pooled raw milk, mastitic milk and pasteurized milk resistance, intermedate and sensitivity pattern were mentioned in table 25, 26, 27, 28 & Fig. 20a, 20b, 20c, 20d, respectively.

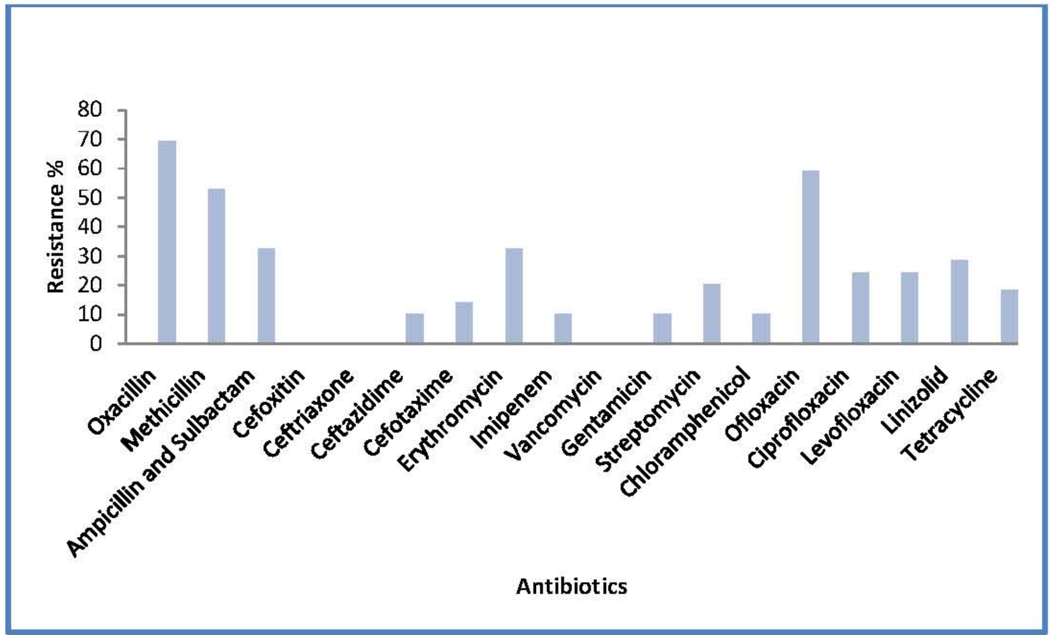
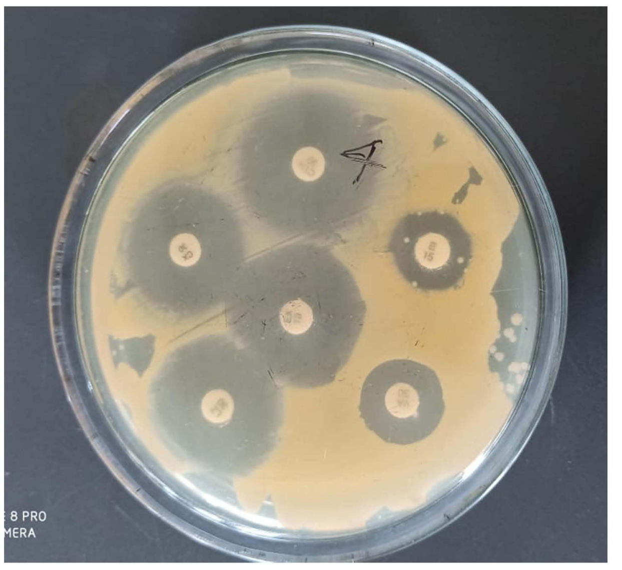


Fig. 19: Pattern of resistance against antimicrobial tested for *S. aureus* isolates

4.7.2. Multi drug resistant (MDR) *S. aureus*

The antibiotics resistance and its pattern for *S. aureus* isolates were written in table 29 and table 30, respectively. To found the MDR strains, it was obsereved that 20(40.81%) isolates were resistant to three or more than 3 class of antibiotics (Penicillin, Quinolones and Macrolide) and were categorized as multi drug resistant. Thus, 19 resistance patterns were observed in MDR strains ranging from 3 to 12 antibiotics (Table 31).



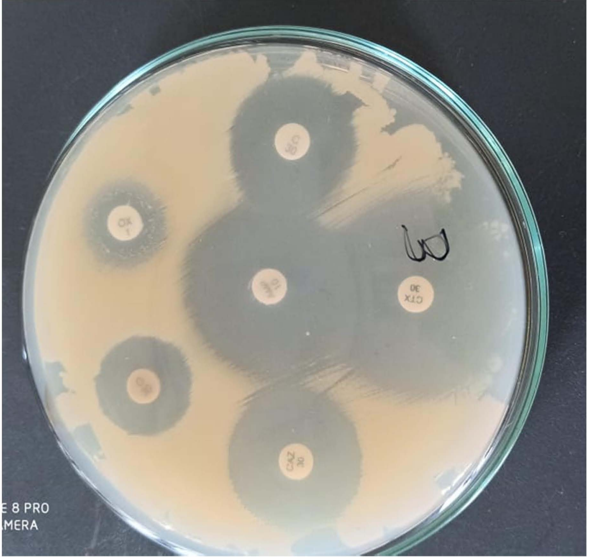
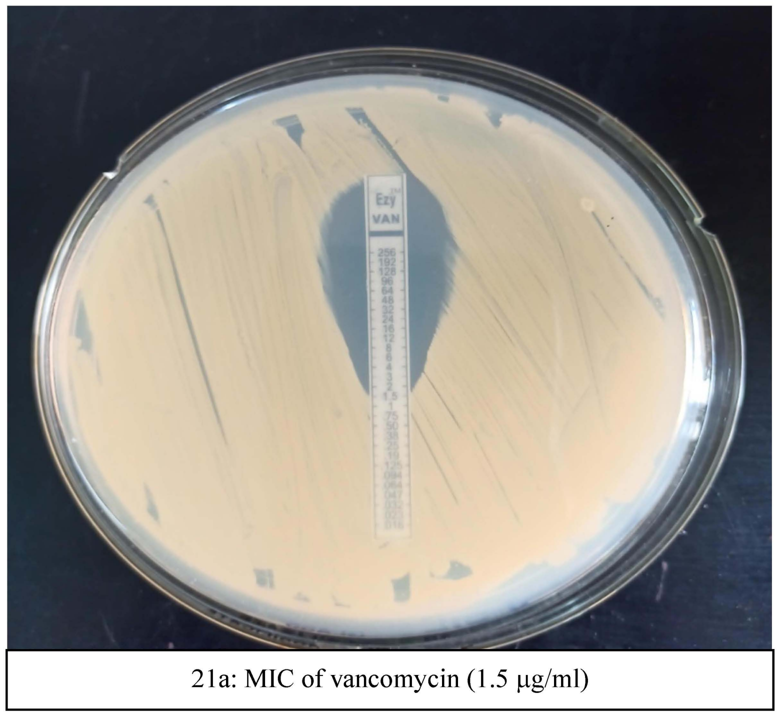


Fig. 20a-d: Antibiotic Sensitivity Test (Zone of inhibition produced by Biofilm forming *S. aureus)*



21a: MIC of vancomycin (1.5 µg/ml)

21b: MIC of Linizolid (1.0 µg/ml)

Fig. 21a & b: Minimum Inhibitory Concentration by E-Test

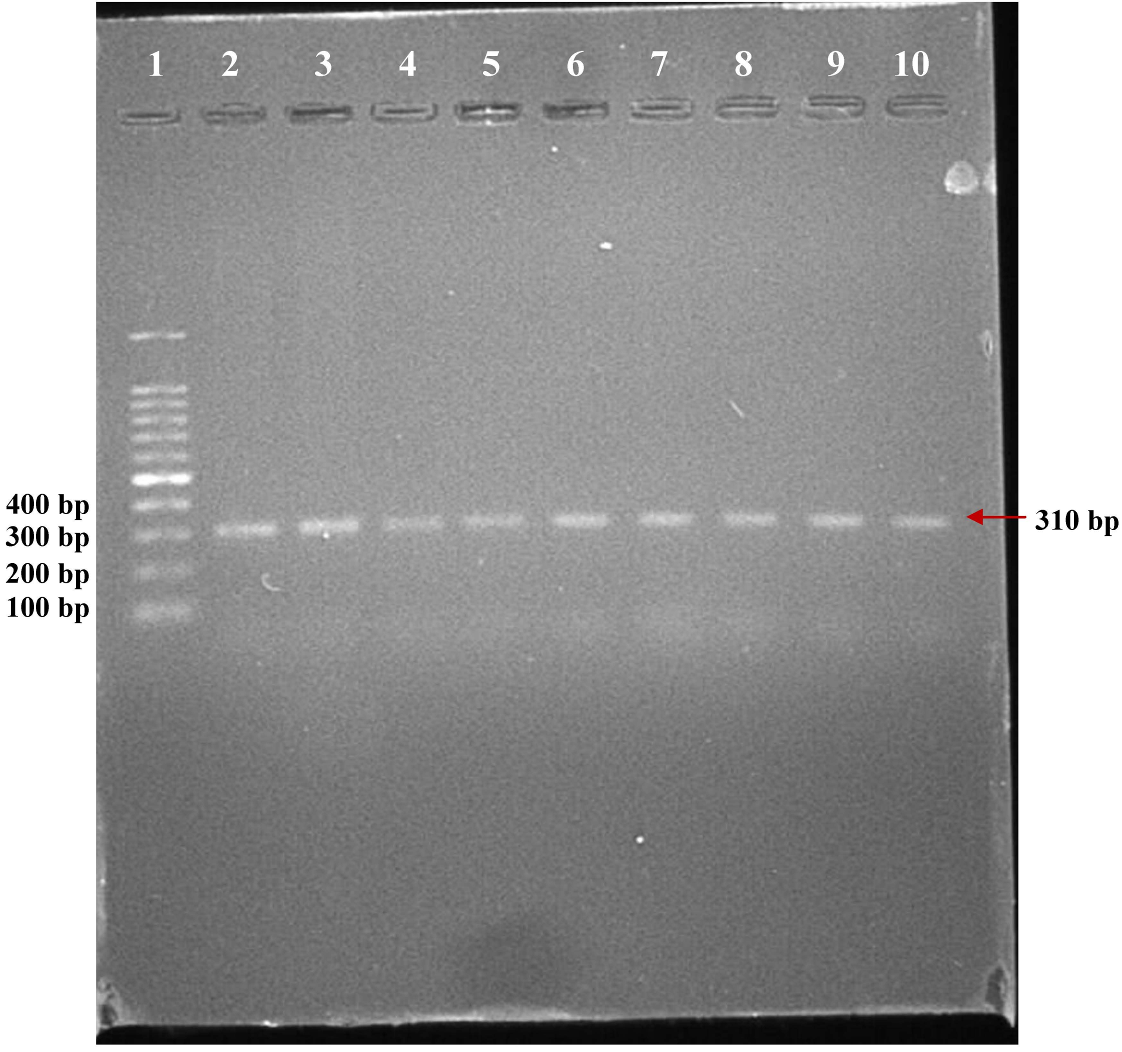


Fig. 22: Antibiotic resistance *mecA* gene (310bp) resolved after electrophoresis in 1.5% agarose

Lane 1- 100bp DNA ladder

Lane 2 to 9- *mecA* gene of *S. aureus*

Lane 10- Positive Control

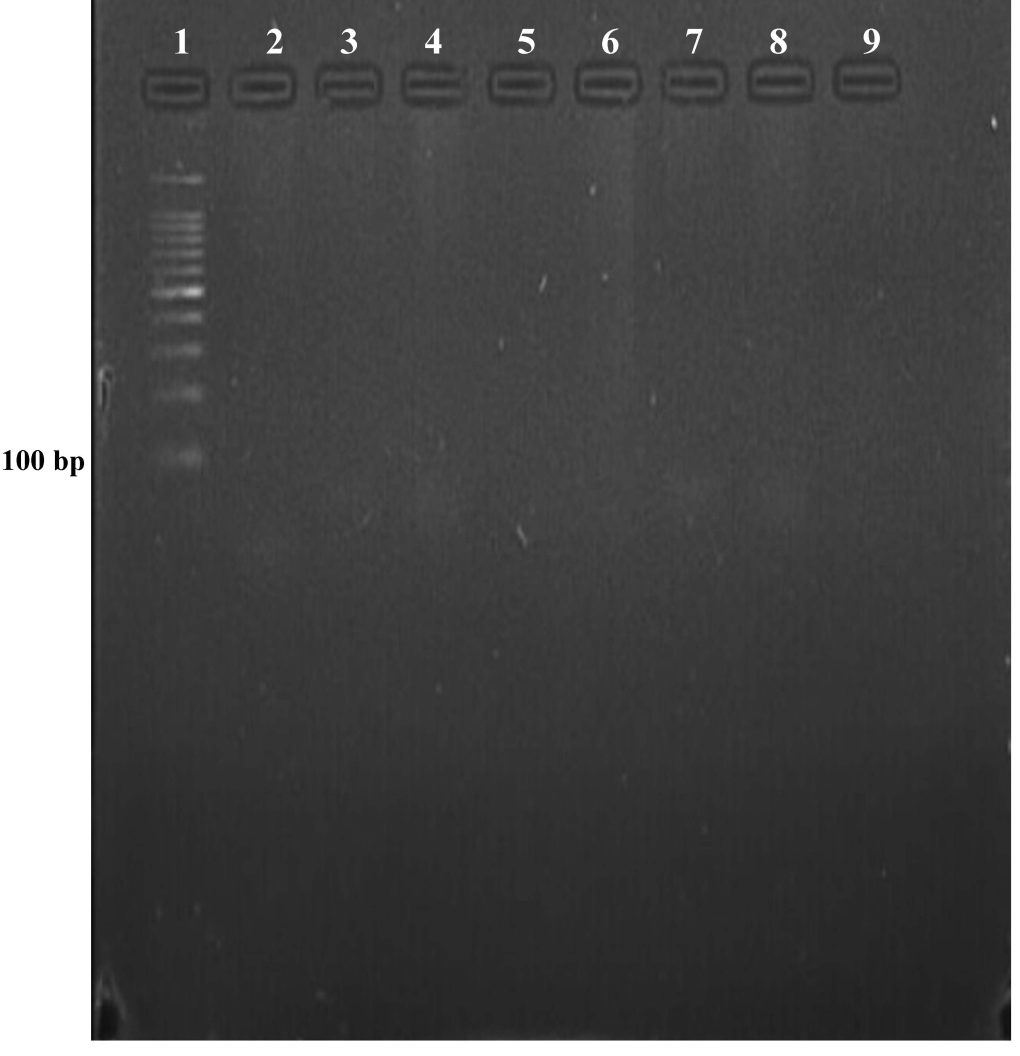


Fig. 23: Agarose gel showing absence of *vanA* gene (1030 bp) of *S. aureus*

Lane 1- 100 bp DNA ladder

Lane 2 to9- No PCR amplicon

4.7.3. Minimum Inhibitory Concentration by E Test

Seven *S. aureus* strains from different milk sample were subjected to E-test to determine the MIC for a group of antibiotics. The following E-strips Oxacillin, Ampicillin / Sulbactam, Ceftriaxone, Erythromycin, Imipenem, Vancomycin, Gentamicin, Streptomycin, Chloramphenicol, Ofloxacin, Ciprofloxacin, Levofloxacin, Linezolid, Tetracycline range of MIC obtained for seven strains were from 0.064 - 0.94, 0.19-0.50, 0.25-0.64, 0.25-1.0, 0.032-0.47, 0.75-2.0, 0.25-1.0, 1.5-4.0, 3.0-32.0, 0.125-0.38, 0125-0.5 0.023-0.64, 0.50-1.0, 0.19-0.75 respectively. A low MIC range of 0.023-0.64 was obtained against Levofloxacin and very high MIC value of 3.0-32.0 recovers with Chloramphenicol. (Table. 32 & Fig.20a, 20b)

4.7.4 Prevalence of Antibiotic Resistance Gene (*mecA* & *vanA*)

All *S. aureus* isolates were further subjected to simplex PCR for *mecA* and *van*A gene. The positivity of *mecA* gene in raw milk, pooled raw milk, mastitic milk and pasteurized milk were 10.63%, 17.39%, 48.28% and 14.28% respectively (Table.33 & Fig. 21).

The vanA gene was zero % prevalent as none of isolates of *S. aureus* carried this gene (Table.33 & Fig.22).

Table 24: Antibiogram of biofilm forming *S. aureus* isolated from Raw, Pooled raw, Mastitic and Pasteurized milk

S. No.	Antibiotic Used	Abbreviation	Concentration(µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	**Oxacillin**	OX	1	34 (69.38)	6 (12.24)	9 (18.37)
2	**Methicillin**	MET	5	26 (53.06)	8 (16.32)	15 (30.61)
3	**Ampicillin and Sulbactam**	A/S	10	16 (32.65)	3 (6.12)	30 (61.22)
4	**Cefoxitin**	CX	30	0 (0.0)	2 (4.08)	47(95.91)
5	**Ceftriaxone**	CTR	30	0 (0.0)	2(12.24)	47 (95.91)
6	**Ceftazidime**	CAZ	30	5 (10.20)	5 (10.20)	39 (79.59)
7	**Cefotaxime**	CTX	30	7 (14.28)	8 (16.33)	34 (69.39)
8	**Erythromycin**	E	15	16 (32.65)	25 (51.02)	8 (16.33)
9	**Imipenem**	IPM	10	5 (10.20)	5 (10.20)	39 (79.59)
10	**Vancomycin**	VA	30	0 (0.0)	7 (14.28)	42 (85.71)
11	**Gentamicin**	GEN	10	5 (10.20)	5 (10.20)	39 (79.59)
12	**Streptomycin**	S	10	8 (20.51)	2 (4.08)	39(79.59)
13	**Chloramphenicol**	C	30	5 (10.20)	16 (32.65)	28 (57.14)
14	**Ofloxacin**	OF	5	29 (59.18)	12 (24.49)	8 (16.33)
15	**Ciprofloxacin**	CIP	50	12 (24.49)	8 (16.33)	29 (59.18)
16	**Levofloxacin**	LE	5	11 (24.45)	23 (46.94)	15 (30.61)
17	**Linizolid**	LZ	30	14 (28.57)	9 (18.37)	26 (53.06)
18	**Tetracycline**	TE	10	09 (18.37)	30 (61.22)	10 (20.41)

Figures in parenthesis indicate the percentage

Table 25: Antibiogram of biofilm forming *S. aureus* isolated from Raw milk

S. No.	Antibiotic Used	Abbreviation	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	**Oxacillin**	OX	1	12 (66.66)	01 (5.55)	05 (27.77)
2	**Methicillin**	MET	5	07 (38.88)	03 (16.66)	08 (44.44)
3	**Ampicillin and Sulbactam**	A/S	10	07 (38.88)	01 (5.55)	10 (55.55)
4	**Cefoxitin**	CX	30	0 (0.0)	0 (0.0)	18 (100.0)
5	**Ceftriaxone**	CTR	30	0 (0.0)	1(5.55)	17 (94.44)
6	**Ceftazidime**	CAZ	30	02 (11.11)	1(5.55)	15 (83.33)
7	**Cefotaxime**	CTX	30	02 (11.11)	02 (11.11)	14 (77.77)
8	**Erythromycin**	E	15	05 (27.77)	11(61.11)	02(11.11)
9	**Imipenem**	IPM	10	1 (5.55)	0 2 (11.11)	15 (83.33)
10	**Vancomycin**	VA	30	0 (0.0)	2 (11.11)	16 (88.88)
11	**Gentamicin**	GE	10	1 (5.55)	1 (5.55)	16 (88.88)
12	**Streptomycin**	S	10	2 (11.11)	1 (5.55)	15 (83.33)
13	**Chloramphenicol**	C	30	02 (11.11)	05 (27.27)	11 (61.11)
14	**Ofloxacin**	OF	5	09(50.0)	05 (27.77)	04 (22.22)
15	**Ciprofloxacin**	CIP	50	01(55.55)	03 (16.66)	14 (77.77)
16	**Levofloxacin**	LE	5	04 (22.22)	08 (44.44)	06 (33.33)
17	**Linizolid**	LZ	30	04 (22.22)	02 (11.11)	12(66.66)
18	**Tetracycline**	TE	10	2 (11.11)	10 (55.55)	6 (33.33)

Figures in parenthesis indicate the percentage

Table 26: Antibiogram of biofilm forming *S. aureus* isolated from Pooled raw milk

S. No.	Antibiotic Used	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	**Oxacillin (OX)**	1	07 (63.63)	02 (18.18)	02(18.18)
2	**Methicillin (MET)**	5	08 (72.72)	03 (27.27)	0(0.0)
3	**Ampicillin and Sulbactam (A/S)**	10	02(18.18)	01 (9.09)	08(72.72)
4	**Cefoxitin (CX)**	30	0 (0.0)	01 (9.09)	10 (90.90)
5	**Ceftriaxone (CTR)**	30	0 (0.0)	0(0.0)	11(100.0)
6	**Ceftazidime (CAZ)**	30	2 (18.18)	2 (18.18)	07 (63.63)
7	**Cefotaxime (CTX)**	30	02 (18.18)	03 (27.27)	06 (54.54)
8	**Erythromycin (E)**	15	06 (54.54)	04 (36.36)	01 (9.09)
9	**Imipenem (IPM)**	10	01 (9.09)	2 (18.18)	8 (72.72)
10	**Vancomycin (VA)**	30	0 (0.0)	02 (18.18)	09 (81.81)
11	**Gentamicin (GE)**	10	2 (18.18)	01 (9.09)	08 (72.72)
12	**Streptomycin (S)**	10	02 (18.18)	0(0.0)	09 (81.8)
13	**Chloramphenicol (C)**	30	01(9.09)	05 (45.45)	05 (45.45)
14	**Ofloxacin (OF)**	5	09 (81.81)	01 (9.09)	01 (9.09)
15	**Ciprofloxacin (CIP)**	50	01 (9.09)	02 (18.18)	08 (72.72)
16	**Levofloxacin (LE)**	5	02 (18.18)	06 (54.54)	03 (27.27)
17	**Linizolid (LZ)**	30	02 (18.18)	02 (18.18)	07 (63.63)
18	**Tetracycline (TE)**	10	03 (27.27)	06 (54.54)	02 (18.18)

Figures in parenthesis indicate the percentage

Table 27: Antibiogram of biofilm forming *S. aureus* isolated from mastitic milk

S. No.	Antibiotic Used	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	**Oxacillin (OX)**	1	14 (73.68)	3 (15.79)	2 (10.53)
2	**Methicillin (MET)**	5	10 (52.63)	02 (10.52)	07 (36.84)
3	**Ampicillin and Sulbactam (A/S)**	10	07 (36.84)	1 (5.26)	11 (57.89)
4	**Cefoxitin (CX)**	30	0 (0.0)	1 (5.26)	18 (94.73)
5	**Ceftriaxone (CTR)**	30	01 (5.26)	01(5.26)	18(94.73)
6	**Ceftazidime (CAZ)**	30	1(5.26)	2 (10.52)	16 (84.21)
7	**Cefotaxime (CTX)**	30	03 (15.78)	03 (15.78)	13 (68.42)
8	**Erythromycin (E)**	15	05 (26.31)	9 (47.36)	5(26.31)
9	**Imipenem (IPM)**	10	03 (15.78)	01 (05.26)	15 (78.94)
10	**Vancomycin (VA)**	30	0 (0.0)	3 (15.78)	16 (84.21)
11	**Gentamicin (GEN)**	10	2 (10.52)	03 (15.78)	14 (73.68)
12	**Streptomycin (S)**	10	04 (21.05)	0 (0.0)	15 (78.94)
13	**Chloramphenicol (C)**	30	2 (10.53)	6 (31.57)	11 (57.89)
14	**Ofloxacin (OF)**	5	10 (52.63)	06 (31.57)	03 (15.78)
15	**Ciprofloxacin (CIP)**	50	9 (47.36)	3 (15.79)	7 (36.84)
16	**Levofloxacin (LE)**	5	4 (21.05)	9 (15.79)	6 (31.57)
17	**Linizolid (LZ)**	30	8 (42.10)	5 (26.32)	6 (31.58)
18	**Tetracycline (TE)**	10	3 (15.79)	14(73.68)	2 (10.53)

Figures in parenthesis indicate the percentage

Table 28: Antibiogram of biofilm forming *S. aureus* isolated from Pasteurized milk.

S. No.	Antibiotic Used	Abbreviation	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	**Oxacillin (OX)**	OX	1	1 (100.0)	0 (0.0)	0 (0.0)
2	**Methicillin (MET)**	MET	5	1 (100.0)	0 (0.0)	0 (0.0)
3	**Ampicillin and Sulbactam (A/S)**	A/S	10	0 (0.0)	0 (0.0)	1 (100.0)
4	**Cefoxitin (CX)**	CX	30	0 (0.0)	0 (0.0)	1 (100.0)
5	**Ceftriaxone (CTR)**	CTR	30	0 (0.0)	0 (0.0)	1 (100.0)
6	**Ceftazidime (CAZ)**	CAZ	30	0 (0.0)	0 (0.0)	1 (100.0)
7	**Cefotaxime (CTX)**	CTX	30	0 (0.0)	0 (0.0)	1 (100.0)
8	**Erythromycin (E)**	E	15	0 (0.0)	1 (100.0)	0 (0.0)
9	**Imipenem (IPM)**	IPM	10	0 (0.0)	0 (0.0)	1 (100.0)
10	**Vancomycin (VA)**	VA	30	0 (0.0)	0 (0.0)	1 (100.0)
11	**Gentamicin (GEN)**	GEN	10	0 (0.0)	0(0.0)	1 (100.0)
12	**Streptomycin(S)**	S	10	0 (0.0)	1 (100.0)	0 (0.0)
13	**Chloramphenicol (C)**	C	30	0 (0.0)	0 (0.0)	1 (100.0)
14	**Ofloxacin (OF)**	OF	5	1 (100.0)	0 (0.0)	0 (0.0)
15	**Ciprofloxacin (CIP)**	CIP	50	1 (100.0)	0 (0.0)	0 (0.0)
16	**Levofloxacin (LE)**	LE	5	1 (100.0)	0 (0.0)	0 (0.0)
17	**Linizolid (LZ)**	LZ	30	0 (0.0)	0 (0.0)	1 (100.0)
18	**Tetracycline (TE)**	TE	10	1 (100.0)	0 (0.0)	0 (0.0)

Figures in parenthesis indicate the percentage

Table 29: Antibiotic Resistance pattern of *S. aureus* isolates

S. No.	Strain	OX	MET	A/S	CX	CTR	CAZ	CTX	E	IPM	VA	GE	S	C	OF	CIP	LE	LZ	TE
1	RM1	R	R	R	-	-	R	R	R	R	-	-	R	-	R	-	-	-	R
2	RM2	R	R	R	-	-	R	R	R	-	-	-	R	R	R	R	-	R	R
3	RM3	R	R	R	-	-	-	-	R	-	-	-	-	-	R	-	R	-	-
4	RM4	R	R	R	-	-	-	-	R	-	-	R	-	-	R	-	-	-	-
5	RM5	R	R	R	-	-	-	-	R	-	-	-	-	-	R	-	-	R	-
6	RM6	R	R	R	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-
7	RM7	R	R	R	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-
8	RM8	R	-	-	-	-	-	-	-	-	-	-	-	-	R	-	R	-	-
9	RM9	R	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-
10	RM10	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	RM11	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R	-
12	RM12	R	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-
13	RM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	RM14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
15	RM15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	RM16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-
17	RM17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	RM18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	RM19	R	R	R	-	-	R	R	R	R	-	R	R	-	R	R	-	-	-
20	RM20	R	R	R	-	-	R	R	R	-	-	R	R	-	R	-	-	-	R
21	RM21	R	R	-	-	-	-	-	R	-	-	-	-	-	R	-	-	R	-
22	RM22	R	R	-	-	-	-	-	R	-	-	-	-	-	R	-	R	-	-
23	RM23	R	R	-	-	-	-	-	R	-	-	-	-	-	R	-	-	-	-
24	RM24	R	R	-	-	-	-	-	R	-	-	-	-	-	R	-	-	-	-
25	RM25	R	R	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-

26	RM26	-	R	-	-	-	-	-	-	-	-	-	-	-	R	-	-	R	R
27	RM27	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-
28	RM28	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	R	-	-
29	RM29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	MM1	R	R	R	-	-	R	R	R	R	R	R	R	-	R	R	-	-	R
31	MM2	R	R	R	-	-	-	R	R	R	R	R	-	-	R	R	-	R	-
32	MM3	R	R	R	-	-	-	R	R	R	-	R	-	R	R	R	R	-	-
33	MM4	R	R	R	-	-	-	-	R	-	-	R	-	-	R	R	-	R	-
34	MM5	R	R	R	-	-	-	-	R	-	-	-	-	-	R	R	-	-	-
35	MM6	R	R	R	-	-	-	-	-	-	-	-	-	-	R	R	-	-	-
36	MM7	R	R	R	-	-	-	-	-	-	-	-	-	-	R	R	-	R	-
37	MM8	R	R	-	-	-	-	-	-	-	-	-	-	-	R	R	-	-	-
38	MM9	R	R	-	-	-	-	-	-	-	-	-	-	-	R	R	-	R	R
39	MM10	R	R	-	-	-	-	-	-	-	-	-	R	-	R	-	R	-	-
40	MM11	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-
41	MM12	R	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-
42	MM13	R	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	R	-
43	MM14	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
44	MM15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R
45	MM16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	MM17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	MM18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R	-
48	MM19	-	-	-	-	-	-	-	-			-	-	-	-	-	-	-	-
49	PM1	R	R	-	-	-	-	-	-	-	-	-	-	-	R	R	R	-	R

R-Resistance

Table 30: Antibiotic Resistance pattern of *S. aureus* isolates

S. No.	Resistance pattern of MDR strains	Resistant for groups of antibiotics	No of antibiotics
1	OX-MET-A/S- CAZ-CTX-E- IPM- S- OF-TE	7	10
2	OX-MET-A/S- CAZ-CTX -E- S-C-OF-CIP-LZ-TE	8	12
3	OX-MET-A/S-E- OF-LE	3	6
4	OX-MET-A/S-E-GE-OF	4	6
5	OX-MET- A/S-E-OF-LZ	4	6
6	OX-MET-A/S-OF	2	4
7	OX-MET-A/S-OF	2	4
8	OX-OF-LE	2	3
9	OX-OF	2	2
10	OX	1	1
11	OX-LE-LZ	3	3
12	OX-C	2	2
13	-	0	0
14	LE	1	1
15	-	0	0
16	LZ	1	1
17	-	0	0
18	-	0	0
19	OX-MET-A/S-CAZ-CTX-E-IPM-GE-S-OF-CIP-TE	7	12
20	OX-MET-A/S-CAZ-CTX-E- GE-S-OF-TE	6	10
21	OX-MET-E-C-LZ	4	5
22	OX-MET-E-C-LE	4	5
23	OX-MET-E	2	3
24	OX-MET-E	2	3
25	OX-MET-C	2	3
26	MET-E-OF-LZ-TE	5	5
27	OF	1	1
28	OF-LE	1	2
29	-	0	0
30	OX-MET-A/S-CAZ-CTX-E-IPM-GE-S-OF–CIP-TE	7	12
31	OX MET-A/S-CTX-E-IPM-GE-S-OF-CIP-LZ	7	11
32	OX MET-A/S-CTX-E-IPM-S-C-OF-CIP-LE	7	11
33	OX MET-A/S-E-S--OF-CIP-LZ	5	8
34	OX MET-A/S-E-OF-CIP	3	6
35	OX MET-A/S-OF—CIP	2	5
36	OX MET -A/S-OF-CIP-LZ	3	6
37	OX MET-OF-CIP	2	4
38	OX MET-OF-CIP-LZ-TE	4	6
39	OX MET-OF-LE	3	4
40	OX-LZ	2	2
41	OX-C	2	2
42	OX-LZ	2	2
43	OX MET-LE	2	3
44	LZ-TE	2	2
45	-	0	0
46	-	0	0
47	LE-LZ	2	2
48	-	0	0
49	OX-MET-OF-CIP-LE	3	5

Table: 31 Antibiotic Resistance pattern of MDR strain

S. No.	Resistance pattern of MDR strain	No. of MDR Strain	No. of Antibiotics
1	OX-MET-A/S- CAZ-CTX -E-S-C-OF-CIP-LZ-TE	01	12
2	OX-MET-A/S-CAZ-CTX-E-IPM-GE-S-OF-CIP-TE	02	12
3	OX- MET-A/S-CTX-E-IPM-GE-S-OF-CIP-LZ	01	11
4	OX -MET-A/S-CTX-E-IPM-S-C-OF-CIP-LE	01	11
5	OX-MET-A/S- CAZ-CTX-E- IPM- S- OF-TE	01	10
6	OX-MET-A/S-CAZ-CTX-E- GE-S-OF-TE	01	10
7	OX MET-A/S-E-S-OF-CIP-LZ	01	8
8	MET-E-OF-LZ-TE	01	5
9	OX MET-OF-CIP-LZ-TE	01	6
10	OX-MET-A/S-E-GE-OF	01	6
11	OX-MET- A/S-E-OF-LZ	01	6
12	OX-MET-E-C-LZ	01	5
13	OX-MET-E-C-LE	01	5
14	OX-MET-A/S-E- OF-LE	01	6
15	OX MET-A/S-E-OF-CIP	01	6
16	OX MET -A/S-OF-CIP-LZ	01	6
17	OX-MET-OF-CIP-LE	01	5
18	OX MET-OF-LE	01	4
19	OX-LE-LZ	01	3

Table 32: Minimum Inhibitory Concentration (MIC) of *S. aureus* E-test

S. No.	Name of E-strip	Abbreviation	Concentration (µg/ml)	Strain 1	S 2	S 3	S 4	S 5	S 6	S 7
1	**Oxacillin**	OXA	0.016-256	0.64	-	0.94	0.064	0.125	0.064	0.094
2	**Ampicillin/Sulbactam**	AMS	0.016-256	0.5	0.24	0.38	0.5	0.38	0.19	0.25
3	**Ceftriaxone**	CTR	0.016-256	0.64	-	0.50	0.38	0.64	0.25	0.38
4	**Erythromycin**	ERY	0.016-256	0.75	-	0.50	0.75	0.38	0.25	1.0
5	**Imipenem**	IPM	0.002-32	0.47	-	0.032	.094	0.032	0.094	0.032
6	**Vancomycin**	VAN	0.016-256	1.5	1.5	1.0	2.0	1.0	2.0	0.75
7	**Gentamicin**	GEN	0.016-256	0.75	1.0	0.50	0.38	0.25	1.0	0.50
8	**Streptomycin**	STR	0.016-256	3.2	-	4.0	3.2	4.0	1.5	1.5
9	**Chloramphenicol**	CHL	0.016-256	4.0	32.0	3.0	8.0	12.0	24.0	16.0
10	**Ofloxacin**	OFX	0.002-32	0.19	0.125	0.38	0.125	0.125	0.19	0.19
11	**Ciprofloxacin**	CPH	0.016-256	0.19	0.25	0.38	0.50	0.125	0.25	0.25
12	**Levofloxacin**	LEV	0.002-32	0.64	0.50	0.047	0.032	0.023	0.047	0.50
13	**Linezolid**	LNZ	0.016-256	1.0	1.0	0.75	1.0	1.0	0.75	0.50
14	**Tetracycine**	TET	0.016-256	0.5	-	0.38	0.75	0.50	0.25	0.19

Table 33: Percent positivity of antibiotic resistance gene (*mecA* and *vanA*)

S. No.	Source	Total Samples	*S. aureus* isolates	Percent positivity of *mecA*	Percent positivity of *vanA*
1.	**Raw Milk**	252	47	10.63 (5/47)	0.0 (0/47)
2.	**Pooled Raw Milk**	40	23	17.39 (4/23)	0.0 (0/23)
	Total raw milk	292	70	12.85 (9/70)	0.0 (0/70)
3.	**Mastitic Milk**	56	29	48.28 (14/29)	0.0 (0/29)
4.	**Pasteurized Milk**	30	7	14.28 (1/7)	0.0 (0/7)
	Total	**378**	**106**	**22.64 (24/106)**	**0 (0/106)**

Discussion

CHAPTER-5

DISCUSSION

Staphylococcus aureus is an important zoonotic pathogen, which can cause serious infection in humans and animals. *S. aureus* is a primary pathogen of global public health concern and is ranked third in the world among reported foodborne pathogens (Umaru et al., 2016). About 40.0% of mastitis cases in some countries are caused by *S. aureus* (Kateete et al., 2013; Basanisi et al., 2017). However, *S. aureus* may be discharged in the milk of dairy animals after infection may cause threat to consumer safety (Li et al., 2017). Therefore, bovine mastitis is a global challenge, as it not only damages the health of animals, but also reduces milk production and increases the cost of medical care, which eventually leads to huge economic losses in the dairy industry (Botaro et al., 2015). Due to contamination of raw milk by pathogenic microorganisms, particularly *S. aureus* having the capability to produce toxin biofilm and multidrug resistance. These virulence characteristics of *S. aureus* in raw milk has attracted widespread attention especially biofilm formation that can enhance the virulence of bacteria (Lee et al., 2014; Bissong and Ateba, 2020). The production of a biofilm not only enables bacteria to tolerate poor environmental conditions but also reduces the penetration rate of antibiotics and promotes the horizontal spread of determinants of antibiotic resistance and ultimately complicates the treatment of infections caused by *Staphylococcus* (Wang et al., 2018).

In this study, the *S. aureus* was isolated from 378 milk samples collected from different regions of Mathura. In raw milk *S. aureus* was 18.65% prevalent results were consistent with the study of Li et al. (2017) who reported 22.0% in milk of healthy cows from China. Thaker et al. (2013) and Nhatsave et al. (2021) determined 6% and 41% prevalence of *S. aureus* in raw milk from Gujarat in India and Mozambique, respectively, prevalence values were lower and higher to current study. Prevalence of *S. aureus* in pooled raw milk was 57.5% (23/40) collected from vendors and retail milk sellers the outcomes were higher to 34.0% prevalence of *S. aureus* in bulk tank milk from bovine in Greece (Papadolous et al., 2018).

In raw milk taken from mastitis cows prevalence of *S. aurues* was 51.78% and this prevalence value is similar to 47.20% reported in China (Wang et al., 2018).

From the clinical and subclinical mastitic milk isolation rate of *S. aureus* was 61.36% and 75.40% from India and Newzealand, respectively, the values are quite higher to current study (Bhattachcharya et al., 2016; Notovich et al., 2018).

In pasteurized milk isolation rate of *S. aureus* was 23.33 in current study, these findings were similar with work of Bissong and Ateba (2020) who reported 29.2% of *S. aureus* in South Africa. Similarly, Akindolire et al. (2015) revealed 13.0% *S. aureus* in pasteurized milk from South Africa that is lower to this study. In present study, overall prevalence of *S. aureus* in milk was reported as 28.04 % from various sources of milk (raw, pooled raw, mastitic and pasteurized milk) and almost similar values of 32.5% were reported form raw, bulk milk and pasteurized milk at South Africa (Akindolire et al., 2015).

Overall our data indicate that *S. aureus* is common and frequently detected in pooled raw milk and raw milk of dairy cows with mastitis in Brij region of Mathura. *S. aureus* contaminates many sources of raw milk which are usually related to mastitis or human carrier. Failure to follow good animal husbandry and food processing method will lead to contamination in raw and pasteurized milk. Difference in prevalence is largely due to differences in the type of livestock breeding system, animal species, milking method and the surrounding environment conditions. So, the collection, production, transportation and sale of raw milk at local shops and by vendors should be standardized. In the gaushalas and dairy farm it is necessary to carry out corresponding professional training for the operators to minimize *S. aureus* contamination of milk.

In the current study all the phenotypically characterized *S. aurues* were *nuc* gene bearer and this gene was 28.04% prevalent in *S. aureus* isolated from various milk sources. In the various studies researchers revealed 74.0%, 74.2% and 85.24% *nuc* genes from various milk sources in South Africa and India.(Akindolire et al., 2015; Bissong and Ateba, 2020; Pati and Mukherjee, 2016).

Biofilm production is recognized as an important virulence factor for bacteria of the genus *Staphylococcus spp* (Cucarella et al., 2002; Vasudevan et al., 2003; Fox et al., 2005). Hence, formation of biofilms by *S. aureus* is a major concern for the dairy industry and is frequently associated with a lack of monitoring of operational standards established for processing milk (Zadoks et al., 2002). In the screening of

biofilm forming *S. aureus* by TCP assay overall 28.04% isolates showed the ability to produce biofilms out of which 46.22%, 31.11% and 22.64% of *S. aureus* were strong, moderate and weak biofilm former. Results are dissimilar with finding of Lee et al. (2014) their work determine 9.7% strong 6.5% moderate and 29.0% weak with overall 45.2% biofilm former isolated from mastitis, subclinical mastitis and bulk tank milk. Kou et al. (2021) reported all the isolates 100% had the ability to form a biofilm out of which 66.1% (41/62) were strong biofilm forming strains and Szweda et al. (2012) revealed 57.6% (76/32) biofilm forming *S. aureus* through TCP assay both the values are higher to the current study.

In mastitic milk high prevalence of biofilm forming *S. aureus* 96.55%**,** 86.20%, 27.59% was evaluated by TCP, TM and CRA assay. In accordance with this study 93.4% and 27.17% biofilm forming *S. aurues* were revealed from TCP and CRA assay in mastitis milk from Newzealand as per Notcovich et al. (2018) and from Brazil 98.9% and 85.0% by the two assays in mastitis milk (Melo et al., 2012). Results of TCP assay are quite similar to our study while there is wide variation in findings of CRA. The discrepancies in the categorization of biofilm phenotypes could results from differences in the interpretation of results. Therefore standardizing the method and interpretation of biofilm forming is crucial. At the same time, *S. aureus* from different abilities to form biofilm and it is important to fully explain these various sources (Bissong and Ateba, 2020). It has been previously demonstrated that the phenotypic expression of biofilm production, ability is influenced by number of factor including composition of medium also (Atshan and Shamsuddin, 2011; Zmantar et al., 2010).

In our study none of the *S. aurues* strain harbored the *bap* gene with 0% prevalence, these results are in agreement with previous reports, where the bap gene was absent in the *S. aureus* isolates belonged to milk samples (Vautor et al., 2008; Szweda et al., 2012; Notcovich et al., 2018). In contrast to the current results, in other studies, the *bap* gene was 27.0%, 5.0% and 15.6% reported from the various type of milk in the work of Sharma et al. (2017), Marques et al. (2017) and Bissong and Ateba (2020), respectively. Moreover our results confirmed the hypothesis proposed by Vautor and coworkers that the *bap* gene had not spread yet among the *S. aureus* isolates of animal and human origin and its prevelance is very low (Vautor et al., 2008). The other biofilm forming *ica* gene cluster acts directly on the production of an

extracellular adhesion polysaccharide, which is associated with pathogenic *S. aureus* infections (Melchior et al., 2011). The *ica*A was also targated and in raw and mastitic milk percent positivity of this gene was 74.46% and 96.55%, respectively. In accordance with this study high prevalence of this gene 95.7%, 85.0% and 100% were reported in mastitic milk isolates from Brazil and Newzeland , respectively (Melo et al., 2013; Marques et al., 2017 and Notcovich et al., 2018). In raw milk 100% *ica A* gene bearer isolates were reported in the study of Sharma et al. (2017) outcome are not in agreement with our study. In pasteurized milk *icaA* gene was 28.57% prevalent in this study while the high isolation rate 63.9% was obtained from South Africa (Bissong and Ateba, 2020).

Biofilm formation is a resistance mechanism present in bacterial strains and the ability to form a biofilm is highly influenced by the environment in which bacterial colonies develop and the different environments had influenced the expression of the *ica* genes for biofilm synthesis. The expression levels of these genes may have varied between different studies. The presence of the ica locus in bacteria isolated from chronic staphylococcal infections in other species suggests that the ica locus is highly conserved within *Staphylococcus* spp. (Singh et al., 2013).

The misuse and over use of antibiotics in livestock is known to have contributed tremendously to the emergence of resistance strain. Antibiotic uses in food producing animal tend to be increasing worldwide. Consequently, the screening of animal product for antimicrobial resistance pathogen is pertinent. In this study, 18 antibiotic belonging to the 10 classes of antimicrobial were screened against *S. aureus* isolated from raw and pasteurized milk and resistance was observed against all the antibiotics except Cefoxitin, Ciprofloxacin and Vacomycin. However the higher resistance was observed against Oxacilin (69.38%), Ofloxacin (59.18%) and Methicillin (53.06%). In this study Cefoxitin, Ciprofloxacin and Vancomycine showed low resistance of 0%. These results are in line with report from previous antibiotic studies on various type of milk, Oxacillin was 60-100% and more than 70.0% resistant in study Akindolire et al. (2015) and Naseer et al. (2020) while 44.47% Ofloxacin resistant isolates were revaled in study of Sharma et al. (2015). In accordance to this study Cefoxitin was 100.0% and 80% sensitive in the study of Marques et al. (2017) and Naseer et al. (2020). In contrast to this study, Vancomycin resistant *S. aureus* was 100.0%, 83.1%, 70.0% revealed in work of Akindolire et al.

(2015); Naseer et al. (2020) and Bissong and Ateba (2020) respectively. In this study methicillin resistant gene *mec* was screened in isolates and 12.85% isolates were *mecA* gene bearer isolated from raw and pooled raw milk while 48.28% from mastitic milk, 14.28% from pasteurized milk with overall prevalence of 22.64%. In accordance with this study 21.0% *S. aureus* were found with *mec* gene from China kou et al. (2021) while higher and lower prevalence values 100% and 0.0% were obtained in raw milk from India (Sharma et al., 2017; Mekonnen et al., 2018). In our study 48.28% *S. aureus* isolates of mastitic milk were possessed *mec*A gene and in the bovine mastitis milk 100%, 20% and 0% *mec* gene bearer *S. aureus* were revealed in the work of Bhattacharya et al. (2016); Hoque et al. (2019) and Schimdt et al. (2017) from India, Bangladesh and South Africa, respectively. Methicillin-resistant *S. aureus* (MRSA) represents those *S. aureus* strains that have acquired the *mec*A gene encoding penicillin-binding protein 2a, which mediates resistance to methicillin and all other b-lactam antibiotics, so it represents a global health problem (Arsic et al., 2012). Due to its seriousness and prevalence, nosocomial infection caused by MRSA has been listed as one of the three most difficult infectious diseases in the world by the World Health Organization (Becker and Wardenburg, 2015).

In current study none of the isolate was found positive for *van*A gene from various sources of milk. In accordance to our study *van*A gene was 0% prevalent in various studies (Akpaka et al., 2017; Bissong and Ateba, 2020). A prevalence value of 17.7% were obtained with *van A* gene, that was higher to this study (Kou et al., 2021). Resistance gene *vanA* are equally detected in our isolates as phenotypically all the strains were sensitive to Vancomycin and genotypically negative for *van*A gene. Our study is consistent with Zehra et al. (2017) which reported a significant relationship between phenotypic and genotypic resistance in *S. aureus* strain. The phenotypic resistance without the gene may be caused by other attribute such as point mutation, biofilm formation and antibiotic tolerance.

Antibiotic treatment is an important measure to control bovine mastitis and human infection. *S. aureus* has significant resistance to antibiotics and the ability to evade the human immune system (Liu et al., 2017). More and more studies have reported that *S. aureus* has developed drug resistance and has evolved from single-drug resistant to multi-drug resistant (MDR), making it increasingly difficult to solve the problem of antibiotic resistance. In the present study 40.81% *S. aureus* were multi

drug resistant, in the various studies across the world MDR *S. aureus* were reported 62%, 32.5% and 90.0% as per Mphahlele et al. (2020); Bissong and Ateba (2020); Sharma et al. (2017). Outcome of MDR are variable similar, lower and higher to current study. Therefore, monitoring the antibiotic resistance of *S. aureus* in raw milk is very important for predicting the speed and type of antibiotic resistance development and for the decision-making of animal antibiotic treatment from the perspective of food safety (Liu et al., 2017).

Summary and Conclusions

CHAPTER- 6

SUMMARY AND CONCLUSIONS

The present study entitled "Studies on Phenotypic and Genotypic Evaluation of Biofilm Production and Antimicrobial Resistance in *Staphyloccocus aureus* Isolated from Milk" was conducted in the department of Veterinary Public Health, College of Veterinary Science & Animal Husbandry, DUVASU, Mathura. This longitudinal study was undertaken to provide the information on the prevalence of biofilm forming *S. aureus* in raw cow milk, pooled milk from local shops and vendors and pasteurised milk around Mathura region of Uttar Pradesh, India. The *S. aureus* were isolated and identified by conventional methods characterized by molecular technique and investigated to biofilm production by CRA, TM and TCP methods and further subjected to antmicrobial resistance studies.

In this investigation, a total of 378 samples comprising of 252 raw milk, 40 pooled raw milk, 56 mastitic milk and 30 pasteurised milk were collected over a period of one year from dairy farms, Gaushalas, local milk shops, vendors and grocery shops from Mathura districts of U.P. These samples were screened for the isolation of *S. aureus***.** A total of 121 *Staphylococcus spp and* 106 *S. aureus* were obtained, confirmed biochemically and further subjected to simplex PCR for the screening of housekeeping *nuc* gene in *S. aureus* isolates**.** A total of 106 strains were nuc gene bearer and further subjected to biofilm production.

The results showed that out of 106 *S. aureus* isolates, prevalence of *S. aureus* in raw milk, pooled raw milk, mastitis milk and pasteurized milk were 18.65%, 57.5%, 51.78% and 23.33% with overall prevalence of 28.04%. In the milk collected from local milk shops, vendors and grocery shops (pasteurized milk) prevalence of *S. aureus* 70.0%, 45.0% and 23.33%, respectively. Outcome showed that *S. aureus* was more prevalent in milk collected from local shops as comparison to vendors and pasteurised milk. All the phenotypically confirmed *S. aureus* isolates were *nuc* gene bearers with prevalence of *nuc* gene in raw milk, pooled raw milk, mastitic raw milk and pasteurized milk were18.65%, 57.50%, 51.78% and 23.33%, respectively.

Phenotypically and genotypically confirmed 106, *S. aureus* isolates were analyzed for biofilm formation by three different methods CRA, TM and TCP. On

the basis of these assays the isolates were categorized strong, moderate and weak biofilm formers. In CRA assay out of 106 *S. aureus* isolates, 19 produced the black colored colonies and 87 produced red colored colonies on Congo Red Agar, thus in this screening assay 17.92% isolates were biofilm forming *S. aureus* from all milk sources, out of which 10.63% were revealed from raw milk, 17.39% from pooled raw millk, 27.59% from mastitic milk and 28.57% from pasteurized milk. In tube method, biofilm forming isolates were screened on the basis of slime produced by isolates either on bottom or surfaces of test tubes. In this assay 27.65% *S. aureus* were strong and moderate biofilm former each obtained from raw milk while in pooled raw milk strong and moderate former were 52.17% and 30.43%, respectively and 17.39% were non biofilm former. Tube method screened 48.28 and 42.86% were strong biofim producer in mastitic and pasteurized milk, isolates produed biofilm on top as well as bottom of test tube while 37.93% and 28.57% were moderate biofilm producer. Overall, by tube method 39.62%, 31.13% and 29.25%. *S. aureus* were strong, moderate and weak biofilm producer. In TCP assay, biofilm formation was evaluated on the basis of OD values measured on 570 and same number of *S. aureus* (106) were also screened through TCP, in raw and pooled raw milk 38.29% and 47.82% were strong biofilm former with more than 0.240 OD values, 31.91% and 34.78% were moderate biofilm producer while 29.78% and 17.39% isolates showed OD values less than 0.120 and categorized as weak biofilm producer. In comparison to all types of milk highest number of strong biofilm former was obtained in mastitic milk 65.51% and lowest from pasteurized milk 14.29%. Overall, 46.23%, 31.13% and 22.64% *S. aureus* were found to be strong, moderate and weak biofilm producer through TCP assay. Among all three assays highest number of biofilm forming *S. aureus* were revealed by TCP in comparison to TM and CRA assay with 77.36%, 70.75% and 17.92% biofilm producer. Thus TCP assay was proved to be the golden standard method for the screening of biofilm forming isolates. Thus, Prevalence of biofilm former *S. aureus* was highest in mastitic milk followed by pooled raw milk, raw milk, and pasteurized milk was 96.55%, 82.60%, 70.21% and 28.57 collected from different gaushalas, dairy farms , local shops, vendors and grocery shops of Mathura districts.

After the phenotypic characterization biofilm forming isolates were screened for biofilm genes *bap* and *ica*A and outcome showed none of the isolate possessed *bap* gene and prevalence was zero % in *S. aureus* isolates. The another gene *icaA* of ica locus was prevalent in *S. aureus* isolates and the percent positivity of this gene in raw milk, pooled raw milk, mastitic milk and pasteurized milk was 74.46%, 91.30%, 96.55% and 28.57% respectively, with overall percent positivity of 81.13%.

All the 49 strong biofilm forming *S. aureus* were subjected to antimicrobial sensitivity testing against various groups of antimicrobials for antibiotic sensitivity test and zone of inhibition was recorded and interpretated the resistance, intermediate and sensitivity of isolates. The resistance for *S. aureus* isolates for following antibiotics viz. Oxacillin, Methicilin, Ampicillin / Sulbactam, Ceftazidime, Cefotaxime, Erythromycin, Imipenem, Gentamicin, Streptomycin, Chloramphenicol, Ofloxacin, Ciprofloxacin, Levofloxacin, Linizolid and Tetracycline were measured as 69.38%, 53.06%, 32.65%, 10.20%, 14.28%, 32.65%, 10.20%, 10.20%, 20.51%, 10.20%, 59.18%, 24.49%, 24.45%, 28.57% and 18.37%, respectively with the range from 10.20- 69.38. Isolates were found sensitive towards the antibiotics Cefoxitin, Ceftriaxone, Vancomycin 95.91%, 95.91% and 85.71%, respectively. It was also observed that 20 isolates were resistant to more than 3 classes of antibiotics like Penicillin, Quinolones and Macrolides and 40.81% (20/49) biofilm forming *S. aureus* were found to be multi drug resistant (MDR).

Seven *S. aureus* strains from different milk sample were subjected to E- test to determine the MIC for a group of antibiotics. For the following E- strips, Oxacillin, Ampicillin / Sulbactam, Ceftriaxone, Erythromycin, Imipenem, Vancomycin, Gentamicin, Streptomycin, Chloramphenicol, Ofloxacin, Ciprofloxacin, Levofloxacin, Linezolid, Tetracycline range of MIC obtained were from 0.064 - 0.94, 0.19-0.50, 0.25-0.64, 0.25-1.0, 0.032-0.47, 0.75-2.0, 0.25-1.0, 1.5-4.0, 3.0-32.0, 0.125-0.38, 0125-0.5 0.023-0.64, 0.50-1.0, 0.19-0.75, respectively. A low MIC range of 0.023-0.64 was obtained against Levofloxacin and very high MIC value of 3.0-32.0 recover with Chloramphenicol.

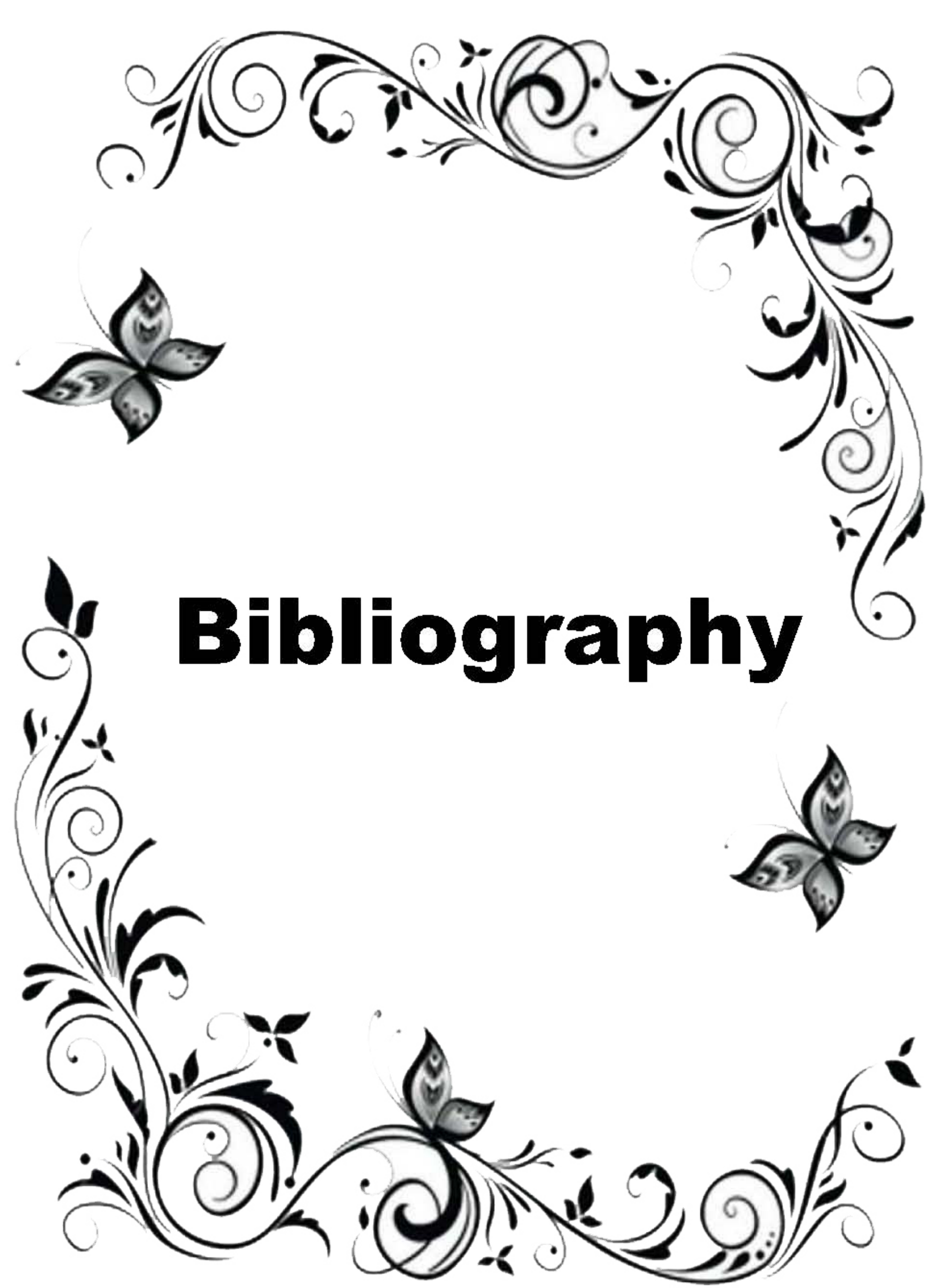
All *S. aureus* isolates were further subjected to simplex PCR for the antibiotic resistance genes *mecA* and *van*A. The percent positivity of *mec*A gene was highest in mastitic milk, pooled raw milk, pasteurized milk and in raw milk were 48.28%,

17.39%, 14.28% and 10.63%, respectively .The *van* A gene was zero % prevalent as none of isolates of *S. aureus* carried this gene.

Results of the present study indicated that the biofilm forming *S. aureus* were isolated from the different types of milk (raw milk, pooled raw milk, mastitic raw milk and pasteurized milk). So, milk may be source of infection of virulent biofilm forming multidrug resistant *S. aureus* for humans and showing the zoonotic importance of the *S. aureus*.

Conclusions

1. Prevalence of *S. aureus* in raw, mastitic and pasteurized milk was 51.78%, 24.0% and 23.33% respectively, with overall prevalence of 28.04% and the phenotypically confirmed *S. aureus* isolates were 100% positive for virulent *nuc* gene. As these *S. aureus* were obtained in cow raw milk and other types of milk, so there is possibility that milk get contaminated during milking by milk handler, during distribution by vendors and post pasteurization contamination in pasteurization plant. There may be public health hazard by consuming raw milk or milk products. Henceforth, proper boiling of milk is very essential.
2. Prevalence of biofilm former *S. aureus* was highest in mastitic milk followed by pooled raw milk, raw milk, and pasteurized milk and the biofilm forming *icaA* gene was prevalent in *S. aureus* isolates with overall percent positivity of 81.13%. Biofilm forming attribute of isolates make it more virulent by increasing adhesiveness of *S. aureus* on surfaces and that surface continuously act as source of infection in raw milk.
3. Among the biofilm forming screening method TCP was proved to be golden standard method as compared to TM and CRA assay. It will be helpful in the screening of other biofilm forming food borne pathogens.
4. High resistance of strong biofilm former *S. aureus* were detected against oxacillin and methicillin as well as one resistance gene and the isolates were multidrug resistant. The presence of potential biofilm producing and antibiotic resistant *S. aureus* in milk intended for human consumption may present with severe health challenges. Consequently, there is a need to enhance control measures, especially in the dairy sector, to curb the spread of pathogenic *S. aureus* and to limit the use of antibiotics.



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Basic Animal Husbandry Statistics Annual Report 2021-22, pp. 4

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Appendix - I

REAGENTS USED FOR BACTERIOLOGICAL MEDIA

1. Nutrient agar medium

Peptone	10.0 g
Beef extract	5.0 g
Sodium chloride	5.0 g

pH 7.2 ± 0.2 @ 25°C

2. Mannitol salt agar

Ingredients	gms/litre
Proteose Peptone	10,000
HM peptone B	1,000
Sodium chloride	75,000
D- Mantol	10,000
Phenol red	0.025
Agar	15,000

PH 7.4 +- 0.2 @ 25°C

3. BHI Broth (Brain Heart Infusion)

Ingredients	gms / litre
HM infusion powder	12.500
BHI powder	5.000
Protease peptone	10.000
Dextrose (Glucose)	2.000
Sodium chloride	5.000
Disodium phosphate	2.500

pH 7.2 ± 0.2 @ 25°C

4. Buffer peptone water:

Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

pH 7.2 ± 0.2 @ 25°C

5. Phosphate Buffer Saline

Sodium chloride	8 gm
Sodium hydrogen phosphate	1.16gm
Potassium Di hydrogen phosphate	0.2 gm
Potassim chloride	0.2 gm
Distilled Water	1000 ml

pH 7.2

6. **Glucose Phosphate Peptone Water (GPPW)**

Peptone	0.5 g
Di- potassium hydrogen phosphate	100 ml

pH 7.2 ± 0.2 @ 25°C

7. **Composition of Congo Red Agar Media**

Ingredients	gm/lit
BHI Broth	37 gm
Agar	10 gm
Sucrose	5 gm
Congo Red Dye	0.8 gm

DW up to 1000 ml

Mixed all ingredients in 1 L Distilled Water and autoclave at 1210 C for 15 min.

8. Composition of Mueller Hinton Agar method

Beef Extract	2 g/L
Starch	1.5 g/L
Casein Hydrolysate	17.5 g/L
Agar	17 g/L
DW	Upto 1L

Final pH 7.3 ± 0.3 (at 15°C)

9. Composition of Baired Parker Agar

Ingredients	gms/litre
Tryptone	10,000
HM Peptone B	5,000
Yeast extract	1,000
Glycine	12,000

Sodium Pyruvate	10,000
Lithium chloride	5,000
Agar	20,000

Final pH 7.0+- 0.2 (at 25°C)

Appendix II

REAGENTS USED FOR BACTERIOLOGICAL STAINING

1. Crystal Violet

Ammonium oxalate 0.8 g

Dissolve into 80.0 ml distilled water

Crystal violet 2.0 g

Dissolve into 20.0 ml of 95% ethyl alcohol.

Mix the two solutions together and allow them to stand overnight at room temperature (25°C). Filter through coarse filter paper before use. Store at room temperature (25°C).

2. Gram's iodine

Iodine (crystalline) 1.0 g

Potassium iodide 2.0 g

Grind both well with mortar Distilled water 300.0 ml Store at room temperature (25°C).

Covered bottle with foil- (to protect solution from light).

3. Decolorizer

Ethyl alcohol (95%) 50 ml

Acetone 50 ml

4. Preparation of Safranin

Safranin-O 2.5 g

Dissolve into 100.0 ml 95% ethyl alcohol

Safranin (from step 1) 10.0 ml

Distilled water. 90.0 ml

Store at room temperature (25°C).

Appendix III

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. **Tris-acetate- EDTA (TAE) buffer (10X)**

   Tris base 48.4 g

   Glacial acetic acid 11.42 ml

   0.5 M EDTA (pH 8.0) 7.44 g

   Distilled water was added to make the final volume 1000 ml

   A working solution of 1× was used.

2. **Ethidium bromide stock solution (10mg/ml)**

   Ethidium bromide 100 mg

   Distilled water 10 ml

   The solution was mixed and stored at 4ºC. A concentration of 0.5 µg/ml was used in preparing agarose gel.

3. **DNA ladder marker (Working solution)**

   DNA ladder marker 1 part

   6X loading dye 1 part Nuclease free water 4 part

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