STUDY ON OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *Staphylococcus* FROM CLINICAL AND SUBCLINICAL MASTITIC MILK SAMPLES AND THEIR ANTIMICROBIAL SUSCEPTIBILITY IN BIHAR

THESIS

BY

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(BVC/ M/VPH/002/2017-18)

submitted to

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विश्वविद्यालय

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DEPARTMENT OF VETERINARY PUBLIC HEALTH & EPIDEMIOLOGY
Bihar Veterinary College, Patna-800014
(Bihar Animal Sciences University Patna, Bihar)

CERTIFICATE-I

This is to certify that the thesis entitled **"Study on occurrence and molecular characterization of *Staphylococcus* from clinical and subclinical mastitic milk samples and their antimicrobial susceptibility in Bihar."** submitted in partial fulfillment of requirement for the degree of **Master of Veterinary Science (Veterinary Public Health)** of the faculty of post-Graduate Studies, **Bihar Animal Sciences University Patna, Bihar** is the bonafide research carried out by **Dr. Kislay Kumar, Registration No- BVC/M/VPH/002/2017-18,** under my supervision and guidance. No part of the thesis has been submitted for any other Degree or Diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

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Dedicated to….

My Parents,
Wife & Son

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Date _______________ **(KISLAY KUMAR)**
Place _______________

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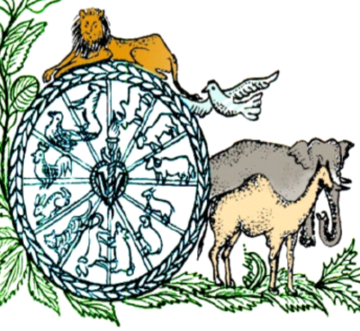
Abbreviations

% : Percentage
µg : Micro gram
µl : Micro liter
µ : Micron
bp : Base pair
Cfu : Colony forming units
conc. : Concentration
DNA : Deoxy-ribonucleic acid
dNTPs : Deoxy-nucleotide triphosphates
DW : Distilled water
et al : et alibi
g : Gram
h : Hour
i.e. : That is
M : Molar
mA : Milli ampere
mg : Milligram
ml : Milliliter
mM : Millimole
MOI : Multiplicity of infection
MSA : Mannitol Salt Agar
Mw : Molecular weight
NA : Nutrient Agar
NaCl : Sodium chloride
ºC : Degree centigrade
NSS : Normal Saline Solution
p mol : Pico mole
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
pH : - Log hydrogen ion concentration
rpm : Revolutions per minute
TAE : Tris Acetate EDTA
Taq : *Thermus aquaticus*
Tris : Tris-hydroxy methyl aminoethane
UV : Ultraviolet
V : Volts
Viz. : Namely
w/v : Weight by volume

ABSTRACT



ABSTRACT

Staphylococcus is one of the most prevalent and clinically significant pathogens that cause the mastitis and public health concern. The present study was performed to Study on occurrence and molecular characterization of *Staphylococcus* from clinical and subclinical mastitic milk samples and their antimicrobial susceptibility in Bihar.

The samples were collected from four different districts of Bihar. By conventional method and PCR 86.50% samples were found positive for *Staphylococcus*, of which 80.00% were from Gaya district, 88.00% from Patna district, 96.00% from Vaishali district and 82.00% from Sitamarhi district. Coagulase test showed that 60.69% isolates from different mastitic milk samples were confirmed as coagulase positive *Staphylococci* (CoPS) while, other 39.31% isolates as coagulase negative *Staphylococci (CoNS)*. The district wise distribution of coagulase positive *Staphylococci* showed 54.55%, 70.73%, 56.25% and 62.50% isolates from Gaya, Patna, Vaishali and Sitamarhi district, respectively. The results obtained from species specific *16S rRNA* gene PCR showed that 56.64% (98/173) isolates as *S. aureus*. The *Staphylococcus* isolates from Gaya, Patna, Vaishali, Sitamarhi showed the distribution of *S. aureus* species specific *16S rRNA* gene in 57.50%, 52.27%, 50.00%, 68.29% isolates respectively. MRSA were also detected by PCR targeting *mecA* gene in 38.73% of *Staphylococcus* isolates. The district wise distribution of *mecA* gene among *Staphylococcus* isolates is 25.00%, 43.18%, 43.75% and 41.46% in Gaya, Patna, Vaishali and Sitamarhi district respectively.

The antibiogram study of isolates reveals the high resistance of *Staphylococcus* to Oxacillin (62.42%), Cefoxitin (53.76%), Rifampicin (47.4%) and Erythromycin (38.15) while moderate resistance observed against Gentamycin (31.79%) & Clindamycin (29.48%) and low resistance against Ciprofloxacin (13.29%), Teicoplanin (7.32%), Tetracycline (1.73%).

Antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Gaya district showed maximum resistance of 72.5% to Cefoxitin followed by a resistance of 35 to 55.00% to Oxacillin Erythromycin, Gentamicin, and Rifampicin, while 20 to 28% resistant to Ciprofloxacin, Clindamycin and Tetracycline.

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Patna district showed maximum resistant 84.09% to Rifampicin followed by 75.00% to Oxacillin, 70.45% to Cefoxitin, 45.45% to Clindamycin, 36.36% to Erythromycin, 18.18% to Gentamicin, 6.81% to Ciprofloxacin and 2.27% to Tetracycline.

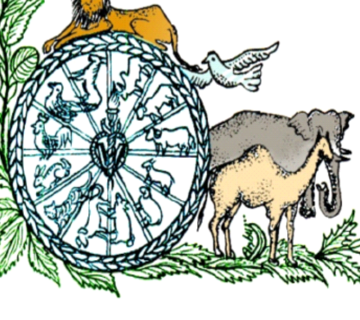
The antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Vaishali district showed maximum resistance of 62.50% to Oxacillin followed by 43.75% to Erythromycin, 41.66% to Clindamycin, 39.58% to Rifampicin, 33.33% to Cefoxitin and Gentamicin, 14.58% to Ciprofloxacin and 06.25% to Tetracycline.

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Sitamarhi district showed maximum resistance of 58.53% to Oxacillin followed by 43.90% to Cefoxitin, 31.70% to Rifampicin, 26.82% to Gentamicin, 19.51% to Erythromycin, 17.07% to Tetracycline, 07.31% to Teicoplanin, 04.87% to Ciprofloxacin and 02.43 to Clindamycin.

In all 173 Staphylococci isolates characterized in the present study, the occurrence of oxacillin sensitive but *mecA* positive *Staphylococcus* (OS-MRS) isolates 10.40% were also made. We also found occurrence of multidrug resistant (MDR) *Staphylococcus* i.e. isolates resistant to more than two antibiotic groups as 53.76% (93/173) in the region.

Thus from the present study it can be concluded that *Staphylococcus* is widely prevalent in clinical and subclinical mastitic animals. The current study also showed a high-level multidrug resistance among *Staphylococcus*. From the study, it could be stated that *mec*A gene harboring MRS were highly resistant to other antibiotic also in addition to oxacillin. The resistance patterns founded in this study posse an alarming condition for future use of antibiotics to treat animals.

Introduction



1 INTRODUCTION

India has vast livestock resources which play an important role in the economy of India. Livestock sector contributes 4.11% to the country GDP and 25.6% of total Agriculture GDP of India. About 20.5 million people depend upon livestock sector for their livelihood and it contributes 16% to the income of small farm households as against an average of 14% for all rural households. Animal husbandry provides livelihood to two-third of rural community in the country and provides employment to about 8.8% of the population in India (Anon, 2018). India continue to be the largest producer of milk in world with production of 155.5 million tonnes and 165.4 million tonnes during 2015-16 and 2016-17, respectively which shows an annual growth of 6.37% with per capita availability of 355 grams milk per day in 2016-17 (Anon, 2018).

Mastitis is an inflammation of the mammary gland affecting predominantly dairy cattle. It is the most important threats of livestock sector affecting the world's dairy industry including India. It has been reported that the worldwide monetary losses due to mastitis is approximately US $35 billion per lactating season (Wellenberg *et al*., 2002) with an average cost of management of clinical mastitis case as US $179 (Bar *et al*., 2008). In India 50% of milking animals are reported to be affected with mastitis (Dua, 2001) leading to an estimated annual loss of INR.7165 crores (Bansal and Gupta, 2010). The economic loss in India is reported as massive with higher magnitude than in developed countries (Dhanda and Sethi, 1962; Dua, 2001; Sarma *et al*., 1987; Hillerton *et al*., 1992). In India, per lactation monetary losses was reported as INR.1390 of which 37% accounts for veterinary expenses and 49% is owing to loss of value from milk (Sinha *et al*., 2014). Approximately 70 percent of this cost is associated with a reduction in milk production. A large portion of milk yield reduction and increase in cost of production results from irreversible damage to the mammary tissue (Oliver and Calvinho, 1995).

The clinical cases of mastitis are categorized by the occurrence of one or more clinical signs such as abnormal milk, udder inflammation and other signs including raised temperature, tiredness, anorexia while sub clinical mastitis are categorized by reduced milk production with poor quality. In modern dairy herds, subclinical mastitis is the major form of mastitis affecting more than 20 to 50% of cows (Wilson *et al.,* 1997; Pitkala *et al*., 2004). Sub-clinical mastitis remains to be an incomprehensible and latent form that poses more serious economic concern to the dairy sector, as its incidence is much higher in dairy herd (Shaheen *et al*., 2016).

Mastitis is caused by a wide number of pathogens and can be categorized into contagious/spreadable and environmental mastitis. Pathogens causing contagious mastitis include *Staphylococcus* spp., *Mycoplasma* spp., *Corynebacterium* bovis and *Streptococcus agalactiae.* However, environmental mastitis can be defined largely as those intra-mammary infections caused by pathogens residing in the animal's environment such as *Klebsiella* spp., *E. coli, Streptococcus uberis* and *Streptococcus dysgalactiae* (Ababe *et al.*, 2016).

The genus *Staphylococcus* belongs to family Staphylococcaceae (Firmicutes) which is a Gram-positive coccus of 0.5-1.5 µm diameter, grape-like appearance although pairs and short chains may be seen, non-motile, facultative anaerobe and non-spore former. Staphylococci may be divided into coagulase positive and negative based on their ability to coagulate rabbit/human plasma. Significant pathogenic species of coagulase-positive *Staphylococcus* (CoPS) and coagulase negative *Staphylococcus* (CoNS) are *S. aureus* and *S. epidermidis,* respectively, which are commonly isolated from the environment and infected animals and humans (Chu *et al*., 2012**;** Wirtu *et al.,* 2018). Coagulase negative staphylococci has been reported very infrequent as the cause of subclinical or clinical mastitis. Mastitis in cattle may be caused by *S. capitis, S. caseolyticus S. chromogenes, S. haemolyticus, S. cohnii, S. epidermidis, S. simulans, S. warneri, S. hyicus,* and, *S. hominis*, however in cattle and sheep by *S. xylosus* and in goats by *S. caprae;* **(**Wirtu *et al.*, 2018)**.** Moreover, they can damage mammary tissue to rise the somatic cell counts and reduction in milk quality and production **(**Taponen and Pyorala 2009).

Among the genus *Staphylococcus, S. aureus* is one of the most broadly studied species. It has capacity to colonize and infect a variety of host species, including wildlife, farm and companion animals and humans. In case of animals, it causes chronic and deep infections in the mammary glands (Hamid *et al*., 2017**)**. *Staphylococcus* can cause severe blood infections and necrotizing fasciitis in humans, wound infection and mastitis in cattle, horses, pigs, and goats, exudative epidermitis in pigs, pyoderma in horses, dogs and cat. *S. aureus* is the most prevalent and contagious pathogen present in 80% of subclinical bovine mastitis and 30-40% of all mastitic cases resulting into an estimated annual loss of 35 billion US dollar, worldwide **(**Reshi *et al.,* 2015; Wells *et al*.,1998). However, in Asia, recent reports indicated the changing trends of mastitis etiology from *Staphylococcus aureus* to coagulase negative Staphylococci as a major mastitis causing pathogen (Sharma *et al*., 2012).

There are large number of antimicrobials used for prevention and control of diseases, enhancing growth and increasing feed efficiency in food producing animals (CDC, 2005). There are various factors involved in the development of antibiotic resistance some of which includes indiscriminate use of antibiotics, unethical prescription practices, lack of education among farmers/patients, limited diagnostic facilities, unauthorized sale of antimicrobials, poor functioning of drug regulatory mechanisms and use of antimicrobials as growth promotor in feed industry. However irrational antibiotic usage has been considered as the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Philips *et al.,* 2004; Levy, 1982; Ayukekbong *et al*. 2017).

Now a days, *Staphylococcus* and more commonly *S. aureus* are widely reported as resistant to several β-lactam antibiotics including methicillin known as methicillin-resistant *S. aureus* (MRSA). Methicillin resistance is caused by the attainment of a *mecA* gene resulting in the production of an alternative penicillin-binding protein 2a (PBP2a), which has lower affinity for β-lactam antibiotics **(**Hamid *et al.,* 2017; Rich and Roberts 2004).

Methicillin resistant *Staphylococcus aureus* (MRSA) have been widely studied and well characterized in comparison to methicillin resistant *Staphylococcus* (MRS) in India and abroad (Mahato *et al*., 2017; Monecke *et al*., 2012; Salisbury *et al*., 1997; Fessler *et al*., 2010; Rich, 2005; Turkyilmaz *et al*., 2010; Zhou *et al*., 2014). Infections produced by methicillin resistant staphylococci (MRS) are more damaging due to limited options of drug treatment and long duration. Recent reports from different parts of the world revealed MRS as an emerging cause of infection and major threat of the dairy industry and public health due to its zoonotic potential (Luthje and Schwarz, 2006; Rahman, 2015., Walther and Perreten, 2007; Moodley and Guardabassi, 2009; Tomasz *et al*., 1991). Emergence of antibiotic resistant strains making the surveillance of antibiotic susceptibility very crucial because amplified drug acquaintance and practice of antibiotics in animal diseases also poses a hazard to human health whose effect and impact is still not well characterized. (Taponen and Pyorala, 2009; Fessler *et al*., 2010; Preethirani *et al*., 2015; Srednik *et al*., 2015). Molecular characterization of the isolates will help and give insight for the differentiation of genotypic and phenotypic basis for the emergence of multidrug resistant organism and their characterization.

Keeping in view of the above importance of members of Genus *Staphylococcus* along with *S. aureus* in mastitis, the present study was performed with the following objectives.

1. To study the occurrence of clinical and subclinical mastitis in Agro Climatic Zone - I & II of Bihar.
2. To isolate and characterize staphylococci from clinical and subclinical mastitic milk samples.
3. To determine antibiotic resistance pattern of these isolates.

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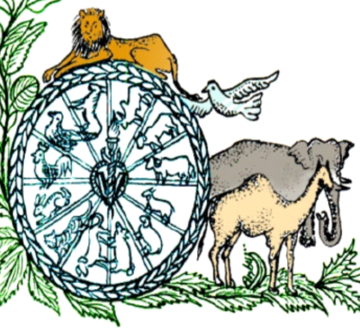
Review of Literature



2 REVIEW OF LITERATURE

Milk is one of the most important highly nutritious food for all age group of humans. It is universally accepted as a complete food due to presence of nearly all essential components. However, the quality as well as quantity of milk has been greatly affected by the mastitis which is a one of the most expensive disease of dairy animals (Awale *et. al.,* 2012). Globally, mastitis contributes about 38% in the total direct costs of the common production diseases (Kossaibati and Esslemont, 1997). In India, the economic losses, due to mastitis is increased to about 115 folds in last five decades (Dua, 2001).

Mastitis is considered as complex multi-factorial disease, the factors related with the animal, environment and pathogen determine its occurrence (Ondiek *et. al.,* 2013). Mastitis in animals may be clinical and characterized by production of abnormal milk with or without secondary symptoms such as swollen quarters, elevated body temperature and/or other systemic signs. (Malinowski and Gajewski 2010) It is usually detected by the presence of abnormal milk such as flakes, clots, or a watery appearance with swollen, red, hot, and hard udder (Abrahmsen *et. al*., 2014). There is immediate drop in milk production that will not regain upto a level of previous production at least up to 60 days following the clinical onset of mastitis. (Hundera *et al.,* 2005) The non-observable form of mastitis i.e., no visible abnormalities found either in milk or the udder, is known as subclinical mastitis (Kathiriya *et. al.,* 2014). It is reported that more than 50% of animals in a herd can have subclinical mastitis at any given time (Tomita and Hart, 2001). It is characterized by a sudden rise in milk somatic cell count observed in normal milk from normal udders indicating the presence of subclinical mastitis. Animals which have subclinical mastitis are usually not producing milk to their full potential and can serve as a potential source of infection to healthy udders (Tomita and Hart, 2001).

The mastitis in animals may be caused by the organisms which do not normally live on the surface of skin or in the udder, but enter the teat canal when animals comes into contact with a contaminated environment (Hogan and Smith, 1987). This type of mastitis is called as environmental mastitis and *Str. uberis, Str. dysagalactiae*, coliforms, etc. are some organism which play a major role (Radostits *et al.,* 2007).

Mastitis may be a contagious infection caused by various infectious agents and among infectious agents, bacterial pathogens are major threat to mammary gland. These microorganisms are often contagious, widely distributed in the environment of dairy animals and thus increase prevalence rate of intramammary infections. (Sharif and Muhammed,2009). The major contagious pathogens involved in mastitis are *Streptococcus agalactiae*, *Staphylococcus aureus*, *Corynebacterium bovis* and *Mycoplasma* species. Among these, *S. aureus*, is currently the most frequently isolated contagious pathogen in subclinical and chronic bovine mastitis worldwide (Zecconi, 2006).

Staphylococcus aureus, a Gram-positive coccus, and have been the most predominant contagious pathogen of bovine mastitis with a characteristic pathogenicity and poses serious problems to the dairy industry as well as drawing public concerns. According to Natzke *et al*., 1972; Quinn, *et al.,* 2002. around 19 to 40% of cows were infected with this organism and that infected cows produce less milk as compared to non-infected cows. *S. aureus* predominantly cause subclinical mastitis resulting in chronic infection that can pursue for the life of the animal (Riollet *et al.*, 2000; Tomita and Hart, 2001).

Status of Mastitis due to *Staphylococcus* in abroad:

Shoukry and Shabana (1997) studied in Ismailia Governorate and examined 173 quarter milk samples from 297 dairy cows and 178 buffaloes for sub-clinical mastitis and reported an incidence of 22.20 per cent in cows and 14.60 per cent in buffaloes. They reported *S. aureus* as the most prevalent microorganism in dairy cows, while dairy buffaloes dominated by *E. coli*.

Workineh *et al*., (2002) in Ethiopia reported clinical and subclinical mastitis as 21.5% and 38.2%, respectively and most of the pathogen isolated was *Staphylococcus* which was as reported 57 percent.

Aasmae *et al*., (2003) conducted a study to investigate the antibacterial resistance of the pathogens causing clinical mastitis in dairy cows from Estonia. The bacteria most frequently isolated were *S. aureus* (169 out of 543) and *S. agalactiae* (81 out of 543).

Mdegela *et al*., (2004) in a study on mastitis in Kibaha and Morogoro districts of Tanzania, reported that out of the 919-quarter milk samples cultured, 8.2 % were positive for aerobic bacteria with predominant isolates being *S. epidermidis* (2.8 %), *S. aureus* (1.7 %), *S. agalactiae* (1.2 %) and *S. intermedius* (1.1 %).

Oliveria *et al*., (2004) examined 196 buffalo milk samples from southern Pernambuco State, Brazil and found *Staphylococcus spp*. in 38.77% samples whereas CoNS was isolated in 42% samples.

Khan and Muhammad (2005) studied quarter-wise comparative prevalence of mastitis in buffaloes and crossbred cows in Pakistan. They reported *S. aureus* with highest frequency (45%), followed by *Streptococcus agalactiae* (23%), *E. coli* (18%) and *Bacillus spp*. (14%) in buffaloes. In case of crossbred cows, *S. aureus*, *Streptococcus agalactiae*, *E. coli* and *Bacillus spp*. were isolated from 48, 30, 13 and 8% milk samples respectively.

Dhakal *et al*., (2006) in their study in Chitwan, Nepal reported subclinical mastitis in buffalo and the major cause reported was CoNS for subclinical mastitis whereas for clinical mastitis the major causative bacteria reported was CoNS and coliform.

Piepers *et al*., (2007) studied the prevalence and distribution of mastitis pathogens in sub-clinically infected dairy cows in Flanders, Belgium. They collecetd 1,78,668 quarter milk samples from 1087 cross-sectional dairy herd screenings performed in three consecutive years. In the study they reported that more than 50 per cent of all intra mammary infection were caused by non-aureus Staphylococci whereas *S. aureus* was isolated from 18 per cent of the culture-positive quarters. It was concluded in the study that *non-aureus* Staphylococci were the predominant cause of intra mammary infection, warranting more research regarding the epidemiology and pathogenicity of the species.

Morales *et al*., (2008) studied the prevalence of pathogens associated with bovine mastitis in bulk tank milk in Mexico. They found the prevalence of *Mycoplasma* as 55 per cent; *S. aureus* 30 per cent; *S. uberis* 37.5 per cent and CoNS 38.3 per cent.

Vanderhaeghen *et al*., (2010) studied in Belgian cows, about the Methicillin-resistant *S. aureus* (MRSA) ST398 associated with clinical and subclinical mastitis. They reported the occurrence of *mecA* gene in 9.3 per cent of the *S. aureus* isolates. Further in their study on characterization of MRSA strains it was shown that all strain was resistant to Tetracycline.

Botrel *et al*. (2010) studied the distribution and antimicrobial resistance on 1631 bacterial isolates from clinical and subclinical cases in dairy cows in Rhone-Alpes, France. The proportion of CoNS in clinical and subclinical mastitis were (15.8 %) and (13.7 %), respectively.

Bal *et al*., (2010) studied the prevalence and antimicrobial susceptibilities of *Staphylococci* from subclinical mastitis cases in Turkey and identified 100 CoNS. The most frequently isolated species were *S. haemolyticus* (27 %) and *S. simulans* (24 %).

Rahman *et al*., (2010) studied the prevalence of sub clinical mastitis (SCM) in dairy cows at Bangladesh. They reported that 51.3% cow had been suffering from SCM with different bacterial infection, of which 17.1% cows had mono-bacterial infection and 10.8% cows had mixed bacterial infections including *S. aureus* (3.13%).

Mekibib *et al*., (2010) carried out bacterial isolation from 153 bovine milk samples suffering from mastitis in Ethiopia and reported incidence of *S. aureus* (47.1%) followed by CoNS (30.1%).

Junaidu *et al*., (2011) reported prevalence of mastitis in lactating cows in some selected commercial dairy farms in Sokoto Metropolis. Out of 100 samples analyzed, fifty-two samples were positive for bacterial isolation and out of the bacterial isolates *Staphylococcus species* were reported in majority of the samples as *S. aureus* (22.8%), *Staphylococcus epidermidis* (10.9%), *Streptococcus spp*. (14.1%), *Corynebacterium spp.* (15.2%), *Bacillus spp*. (7.6%), *E coli* (9.78%) and *Klebsiella spp*. (4.35%)

Benhamed *et al*., (2011) studied in Aran West Algeria and recorded prevalence of *S. aureus* (30.76%), *Streptococcus spp*. (30.76%) and *E. coli* (23.07%) in mastitic milk of cattle.

Ayano *et al*., (2013) in their study examined 546 milking cows in Holeta district of Ethiopia, out of which 224 (41.02%) cases were subclinical mastitis and major pathogens isolated from subclinical mastitis were *Staphylococcus aureus* (13.8%), *Streptococcus uberis* (12.1%), *Staphylococcus epidermidis* (11.7%), *Escherichia coli* (11.6%), *Streptococcus dysagalactiae* (10.6%), *Pseudomonas aeruginosa* (9.7%), *E. coli* O157:H7 (6.9%), *Micrococcus* species (6.5%) and *Streptococcus agalactiae* (6.4%) and others (10.7%).

Vazquez *et al*., (2013) collected 1087 samples from mastitic cow in Maxico and reported a group of pathogens including the CoNS, *S. aureus, S. agalactiae* and Coliform bacteria in 464 (15.6%), 175 (5.9%), 200 (6.8%), and 123 (4.1%) samples respectively.

Cervinkova *et al*., (2013) studied 669 individual cow milk samples from asymptomatic cows for the presence of microorganisms with the potential to cause mastitis at Czech Republic. They reported that 53.5% of samples were coagulase-negative staphylococci while other included *streptococci* and *enterococci*.

Mehmeti *et al*., (2016) studied on 152 milk samples from cows with clinical mastitis from different farms in Kosovo and reported the presence of *Staphylococci* in 58.55% of Staphylococci, of which 65.17% were coagulase negative and 34.83% coagulase positive. They have isolated *S. aureus* from 27 samples, *S. epidermidis* from 25, and *S. chromogenes* from 15, while other species of *Staphylococci* were isolated from 22 samples.

Abebe *et al*., (2016) studied the prevalence of mastitis and identified the cow-and herd-level potential risk factors of mastitis. They reported 74.7%. prevalence of mastitis at herd-level in Ethiopia. The corresponding cow-level prevalence reported was 62.6%, of which 59.2% and 3.4% were sub-clinical and clinical mastitis cases, respectively. *S. aureus* was isolated from 51.2% of the milk samples from animals suffering from mastitis.

Saglam *et al*., (2017) studied in Turkey, studied on CMT positive milk samples, a total of 49.7% *Staphylococcus spp*. was isolated as a distribution of 63.24% coagulase-positive Staphylococci and 36.75% coagulase-negative Staphylococci.

Derib *et al*., (2017) performed a cross sectional study at Wolaita Sodo town, Southern Ethiopia and isolated MRSA. They reported the occurrence of mastitis in 40.9% of animals out of which, 4.66% and 36.18% were clinical and subclinical respectively. They have reported 28 (71.8 %) *S. aureus* with multidrug resistance and methicillin resistance.

Wirtu *et al*., (2018) reported isolation of *S. aureus* from milk samples of mastitic cattle in selected dairy farms in Bishoftu Town, Ethiopia and reported 28.26% sub-clinical mastitis at cow herd level and 16.40% at quarter level. They reported isolation of *S. aureus* from 7.03% milk samples.

Status of *Staphylococcus* resistance to antibiotic in abroad:

Luthje and Schwarz (2006) in their studies screened cases of subclinical mastitis in the cattle of Germany and isolated 298 CoNS. They reported that CoNS have a low level of resistance to all antimicrobial agents tested (0–7.4%) except Ampicillin (18.1%).

Dhakal *et al*., (2006) in their study in Chitwan, Nepal reported the resistance pattern of the isolates revealing Enrofloxacin and Gentamicin as the most sensitive drug.

Turutoglu *et al*., (2006) carried out the antibiotic susceptibility test on 103 *S. aureus* and 136 CoNS isolates in Turkey. They reported that only 35 isolates were susceptible to all antibiotics tested, while the remaining 204 isolates were resistant to at least to one of the antibiotics. Among Staphylococci, 18 isolates of *S. aureus* and 31 isolates of CoNS were found phenotypically resistant to Methicillin along with Penicillin G, Ampicillin, Amoxycillin and Cloxacillin.

Bal *et al*., (2010) studied the prevalence and antimicrobial susceptibility of Staphylococci from subclinical mastitis cases in Turkey and revealed the highest resistance to Penicillin G (58 %), Ampicillin (48%), Neomycin (20%), and Oleandomycin (14%).

Vanderhaeghen *et al*., (2010) studied the methicillin-resistant *S. aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. The presence of *mecA* gene was investigated in 118 *S. aureus* strains originating from mastitis milk samples. which was detected in 11 (9.3 %) isolates.

Botrel *et al*., (2010) studied the distribution and antimicrobial resistance on 1631 bacterial isolates from clinical and subclinical cases in dairy cows in Rhone-Alpes, France. In their study they observed antibiotic resistance in very low proportion of isolates, except for Penicillin G in Staphylococci, as well as for Macrolides and Tetracycline in Streptococci.

Persson *et al*., (2011) conducted nationwide survey on the microbial etiology of cases of subclinical mastitis in dairy cows in Sweden. They reported that 45.16% *S. aureus* isolates as multidrug resistant. All isolates were susceptible toward vancomycin and Linezolid. Whereas high resistance was observed against Oxacillin and Cefoxitin.

Mehmeti *et al*., (2016) studied on 152 milk samples from cows with clinical mastitis from different farms in Kosovo and reported *Staphylococci* in 89 samples, they also observed that most of the isolates were resistant to two or more antibiotics. The highest resistance was found penicillin and ampicillin (> 65%), followed by Tetracycline, Oxacillin, Streptomycin, Chloramphenicol (> 23%), and less than 3% to Erythromycin.

Derib *et al*., (2017) in a cross-sectional study at Wolaita Sodo town, Southern Ethiopia, isolated MRSA and their resistance to different antimicrobials were studied. They have reported 28 (71.8 %) *S. aureus* with multidrug resistance and Methicillin resistance.

Work done at India related to *Staphylococcus* and Mastitis

Mallikarjunaswamy and Murthy (1997) studied the in vitro antibiotic sensitivity of 214 bacterial isolates from cases of bovine subclinical mastitis from 162 dairy cows in Bangalore. The major bacteria identified were *S. epidermidis* (80), *S. aureus* (66), *S. intermedius* (2), *Str. agalactiae* (34).

Paul *et al*., (2000) reported the incidence of sub-clinical mastitis in cow 25.78% than in buffaloes 10.6% in and around Chennai. The predominant bacteria in mastitic milk were *Staphylococcus spp*. followed by *Streptococcus spp.* and *E. coli.*

Datta and Rangenkar (2001) tested 173 milk samples from 45 cows for mastitis in India. They reported the predominance of *Staphylococcus species* (60.9%) in mastitis cases, followed by *Streptococcus species* (23.9%), *Pseudomonas species* (6.5%) and *Bacillus species* (2.2%). Majority of the isolates were moderately sensitive to gentamicin and oxytetracycline and resistant to ampicillin and streptomycin.

Sharma and Prasad (2003) studied in Himachal Pradesh and reported *Staphylococcus* species in 54.05 % of mastitic animals followed by *Streptococcus* species (14.41 %), *E. coli* (11.71 %), *Bacillus species* (9.01 %) and *Corynebacterium species* (7.21 %).

Balakrishnan *et al*., (2004) in Namakal, India isolated 40 bacterial isolates from 65 milk samples of which 35 percent were *S. aureus,* 17.5 percent were *S. agalactiae*, and 2.5 percent were *S. dysgalactaie.*

Harini and Sumathi (2011) studied in Kanakapura taluk, Ramanagara district of Karnataka state to find out the incidence of subclinical mastitis (SCM) and to assess the antibiotic sensitivity pattern of the causative organisms in lactating cows. The predominant bacterial isolates recovered were *Staphylococcus aureus* (58%) and *Escherichia coli* (23.5%) followed by *Staphylococcus epidermidis* (8%), *Streptococcus sp.* (5.5%), *Klebsiella sp.* (3%) and *Bacillus sp.* (2%).

Ranjan *et al*., (2011) studied on bovine mastitis in different climatic conditions in Jharkhand, India. They reported 27.37% milk positive for *Staphylococcus aureus* and was the most prevalent organism followed by coagulase negative *Staphylococcus spp*. (12.63%).

Pankaj *et al*., (2012) studied in Hissar, India and reported the prevalence of sub clinical mastitis in crossbred and indigenous cows characterized etiological agent/s involved along with their antimicrobial sensitivity pattern. They reported quarter wise and animal wise prevalence of mastitis as 64.21 % & 39.83 % respectively and revealed 38.66 % coagulase positive Staphylococci and 29.33 % coagulase negative Staphylococci.

Mubarack *et al*., (2012) studied on prevalence of bovine mastitis in dairy cows in and around Coimbatore district, Tamilnadu, India. They reported the prevalence of mastitis at cow level as 66.0%, out of which 26.06 % and 75.30 % were clinical and subclinical, respectively.

Haque *et al*., (2013) studied in Ranchi, India and reported *S. aureus* as the most prevalent causative agents for bovine mastitis (37.25%) followed by mixed infection of another *Staphylococcus spp.* (16.67%), *E. coli* (11.76%), *Streptococcus* spp. (7.84%), *Pseudomonas* spp. (6.86%) and *Klebsiella* spp. (1.96%).

Sharma *et al.,* (2015) in Mathura, India screened bovine mastitic milk samples and reported that the overall incidence of *Staphylococcus aureus* in clinical and sub clinical mastitis was 33.75%. The incidence of *Staphylococcus aureus* was lower (17.50%) in subclinical mastitis in comparison to that of clinical mastitis (50.00%).

Preethirani *et al*., (2015) in a study in south India screened 190 milk sample and isolated the coagulase-negative Staphylococci (64.8%), *Staphylococcus aureus* (7.3%).

Mausam *et al*., (2016) reported prevalence of *S. aureus* in 56.67% bovine milk samples of Bihar.

Krishnamoorthy *et al*., (2017) studied the prevalence of subclinical and clinical mastitis and major bacterial pathogens viz., *Staphylococcus species*, *Streptococcus species* and *Escherichia coli* in dairy animals by using meta-analysis in India. They reported the prevalence of SCM and CM 41% and 27% respectively in India. Prevalence estimate of *Staphylococcus sp., Streptococcus sp.* and *Escherichia coli* were 45%, 13% and 14%, respectively.

Devi and Dutta *et al*., (2018) reported the incidence of bovine sub clinical mastitis (SCM) in dry pregnant cows of organized and unorganized farms in an around Khanapara, Guwahati, Assam. They reported 66.67% incidence of SCM in organized farm however 93.33% incidence level was reported from unorganized farms.

Verma *et al*., (2018) studied bovine mastitic case in and around Meerut (U.P) region. They reported Staphylococci as the major prevalent pathogens (42.55%) in mastitis.

Jyothi *et al*., (2018) studied to estimate the prevalence of bacterial pathogens in and around Hyderabad region of Telangana state, India. They reported *Staphylococcus* spp. as the most predominant bacteria followed by *E. coli,* Streptococci spp. and *Pseudomonas* spp.

Thakur *et al.,* (2018) studied to isolate and identify the major pathogens from clinical and subclinical mastitis milk samples and their antibiotic sensitivity evaluation at Bihar Agricultural University, Sabour and adjoining villages. They reported occurrence of clinical and subclinical mastitis as 15% & 85% respectively in the Bhagalpur, Bihar. Upon microbiological testing and 16S ribosomal DNA sequencing the major pathogen associated with clinical and subclinical mastitis were identified as coagulase negative *Staphylococcus spp.* (*S. saprophyticus, S. haemolyticus, S. agnetis*) 54%, followed by coagulase positive *Staphylococcus aureus* (26%) and *Streptococcus* and *Bacillus spp*.

Status of *Staphylococcus* resistance to antibiotic in India:

Mallikarjunaswamy and Murthy (1997) reported *Staphylococcus isolates* from mastitic milk sample from dairy farms in and around Bangalore sensitive to Chloramphenicol, but resistant to Streptomycin, Neomycin and Penicillins. The *S. aureus* isolates were highly sensitive to Ciprofloxacin (100 %), Ceftizoxime (100 %), Gentamicin (98.4 %), Cloxacillin (98.48 %), Ciprofloxacin (93.93 %), Tetracycline (93.9 %) and Co-trimoxazole (92.42 %) and moderately sensitive to Neomycin (86.36 %). Most of the isolates showed resistance to Penicillin (68.18 %) and Streptomycin (57.57 %).

Datta and Rangenkar (2001) reported that majority of the isolates obtained from 173 mastitic milk samples, are moderately sensitive to Gentamicin and Oxytetracycline and resistant to Ampicillin and Streptomycin.

Bulla *et al.,* (2005) studied in vitro sensitivity of 52 isolates from buffaloes sub-clinical intra-mammary infection towards 20 antimicrobials and reported variation in their sensitivity pattern in India. All the *Staphylococci* were sensitive to chloramphenicol, cloxacillin, ampicillin, oleandomycin, enrofloxacin, Ampiclox and Novobiocin. More than 80 per cent of these isolates showed sensitivity towards

Nitrofurantoin, Kanamycin, Neomycin, Polymixin-B, Erythromycin, Amoxicillin, Amoxicillin+Clavulanic acid and Spiramycin. All the strains of Streptococci were found sensitive to Penicillin-G, Chloramphenicol, Cloxacillin, Ampicillin, Enrofloxacin, Oleandomycin, Ampiclox and Erythromycin.

Kumar *et al.,* (2010) studied mastitic milk sample in India and reported that the isolates were also highly resistant to antibiotics, i.e. 29.9% to Gentamicin and 26.2% Ciprofloxacin, and 22.22% resistant to Clindamycin, Gentamicin, and Tetracycline.

Basappa *et al*., (2011) investigated about the prevalence and antimicrobial susceptibility of CoNS isolated from bovine mastitis in and around Dharwad region. The study revealed that the highest numbers of CoNS were susceptible to Ceftriaxone 83.88% followed by Cefotaxime 79.41%, Methicillin 76.47%, Ciprofloxacin 73.52%, Erythromycin 70.05%, Amikacin 66.11%, Gentamycin 42.94%, Amoxicillin 36.76%, Ampicillin 29.41%, and the lowest susceptibility was shown in Penicillin 23.23%. The results also indicated that the increase in prevalence and antibiotic resistance pattern of the CoNS isolated from bovine mastitis exhibited the highest degree of susceptibility to ceftriaxone of all the tested antimicrobial agents.

Harini and Sumathi (2011) studied in and around Kanakapura taluk, Ramanagara district of Karnataka state to find out the incidence of subclinical mastitis (SCM) and to assess the antibiotic sensitivity pattern of the causative organisms in lactating cows. They revealed higher sensitivity for Ciprofloxacin (89%), Ofloxacin (85%), Enrofloxacin (82%), Gentamicin (80%) and Chloramphenicol (75%), and resistant to Tetracycline in antibiogram studies of bacterial isolates.

Preethirani *et al*., (2015) in a study in South India, revealed that CoNS were highly resistant to Methicillin, Amoxycillin/Sulbactam and Penicillin-G (72% to 85.6%), and displayed intermediate resistance to Ceftriaxone/Sulbactam, Cefoxitin and Cefotaxime (48.8% to 52.8%) while they were least resistant to Co-trimoxazole, Chloramphenicol and Gentamicin (3.2% to 4.0%).

Kumar *et al.,* (2010) studied and reported a high prevalence of MRSA in the tested isolates (13.1%) in mastitic milk sample.

Sharma *et al*., (2015) reported the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) among bovine milk samples in Mathura, India. They revealed the drug sensitivity pattern of isolates Ciprofloxacin, Clindamycin, Gentamicin, Levofloxacin, Norfloxacin, Tetracycline (22.22% each).

Mausam *et al*., (2016) studied and reported *mecA* positive *S. aureus* (MRSA) in 29.33% of bovine milk samples in Bihar. They also reported the antibiotic sensitivity pattern that reveals that 86.36 and 95.45 percent MRSA isolates from mastitic milk sample were resistant to penicillin and ampicillin respectively, whereas 80.49 and 87.80 percent non-MRSA isolates were resistant to penicillin and ampicillin respectively.

Mahato *et al*., (2017) studied bovine mastitis cases in India, and reported high resistance CoNS was observed toward Oxacillin (85.5%) and Cefoxitin (83.9%) and moderate resistance were seen against Rifampicin (37.1%), Clindamycin (32.3%), Erythromycin (25.8%), and Tetracycline (20.9%). Resistance against Ciprofloxacin (11.3%) and Gentamycin (9.7%) were low, while all strains were susceptible to Vancomycin, Teicoplanin and Linezolid.

Hamid *et al.,* (2017) studied in Jammu and Kashmir, they reported that the 22.5% samples positive for *S. aureus* out of that 16.6% *S. aureus* isolates were multidrug resistant and 16.6% isolates were confirmed as MRSA while rest were MSSA.

Mahato *et al.,* (2017) studied on milk samples from mastitic cows for the identification of variable traits among the Methicillin Resistant and Sensitive Coagulase Negative Staphylococci in India. They reported methicillin resistant gene, *mecA,* in 95.16% isolates and out of which 11.9% of *mecA* positive isolates were Oxacillin susceptible and referred as Oxacillin susceptible *mecA* positive staphylococci (OS-MRS).

Verma *et al*., (2018) studied bovine mastitic case in and around Meerut (U.P) region. They revealed that Gentamicin (65.96%) was the most effective antibiotic followed by Ciprofloxacin (41.49%) against *Staphylococcus.*

Jyothi *et al.,* (2018) studied to assess the antibiotic sensitivity pattern of the organisms from milk samples in and around Hyderabad region of Telangana state, India. They reported maximum sensitivity to Ceftiofur with (60%) for *Staphylococcus sp.* and *Pseudomonas species*, 58% for *Streptococcus* sp. and 90% for *E. coli*.

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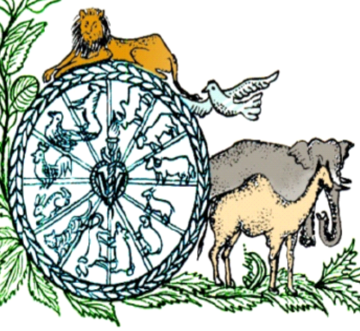
Materials and Methods



3

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Design of study

The present work was taken to study on the occurrence and molecular characterization of *Staphylococcus* from clinical and subclinical mastitic milk samples and their antimicrobial susceptibility pattern in Bihar. The materials and methods employed to achieve the aims and objectives of the present study are described as below.

3.1.2. Sample collection area and location of study

The present work was carried out at the Department of Veterinary Public Health and Epidemiology, Bihar Veterinary College, Bihar Animal Sciences University Patna, Bihar. The study was carried out from cattle clinical and subclinical mastitic milk samples, collected from two agroclimatic zone I (Patna & Gaya) and II (Sitamarhi & Vaishali) of Bihar by standard sampling method. The duration of study was from August 2018 to February 2019 i.e for a period of six months.

3.1.3 Experimental Sample

A total of 200 milk samples from 200 clinical and subclinical mastitic animals (one sample consisted of milk from the four quarter of udder and were pooled together from each animal) were collected aseptically as per the guidelines of National Mastitis Council and processed for isolation and further characterization of *Staphylococcus.* Sample were categorized as clinical and subclinical mastitis by California Mastitis Test. Among 200 milk samples, 137 samples were of subclinical mastitis animals obtained from apparently healthy animals and rest 63 samples were collected from clinical cases of mastitis (Table-1).

3.1.4. Media, Buffers & Reagents

The details of the preparations of media, buffers and other reagents used in the present study have been given in the appendix.

3.1.5. Chemicals used for molecular studies

All the chemicals used in the study were of molecular biology grade except the requirements for bacterial isolation and biochemical characterization. Chemicals were procured from Hi-media (India) and other reputed firms. Chemicals used in the study included *Taq* DNA polymerase,10X PCR amplification buffer, dNTP mixture (thermo scientific, China. Qiagen, Germany. Hi-media, India. Sigma, USA), 6X gel loading dye, 100 bp DNA ladder plus ruler (Hi Media), 0.5 M ethylene diamine tetra acetic acid (0.5 M EDTA), ethidium bromide (Hi-media, India), tris base (Hi-media, India), agarose M (GenNei, India) and nuclease free water (Hi-Media, India).

3.1.6. Plasticwares and glasswares

Plasticwares used in this study were procured from Tarsons (India), Moxcare (India) Hi Media (India), and Genaxy (Hong Kong) whereas glasswares were procured from Tarsons (India), Borosil (India) and Touff (India). Glasswares were thoroughly washed and sterilized wherever necessary following the recommended procedures.

3.1.7. Equipments

Some important equipments used in the study were variable volume pipette set (Thermoscientific, China), pH meter (Labman), thermal cycler (Techne, UK), horizontal gel electrophoresis apparatus (Thermoscientific, China), gel documentation system (Biorad), deep freeze (-20ºC) (Blue Star, India), spinwin micro centrifuge (Diahan, Korea), electronic balance (Denver, USA), autoclave and hot air oven (Instrumentation India, India), incubator (REMI, India), water distillation apparatus (Borosil, India), laminar air flow bench (Ikon instruments, India), microscope (Olympus, India), microwave oven (LG, India) and micropipette (Eppendorf, Germany), water bath (YSI, India), biosafety cabinate level 2 (matrix) and vertex (Tarsons, India) .

3.1.8. Oligonucleotide Primers

Primers used in the study were custom synthesized from Xcelris and Eurofins, India. The details of the primers are given in Table 2.

3.2. METHODS

3.2.1. Collection of samples:

The animal showing clinical signs like swelling and pain of udder, yellow or blood tinged and watery consistency of milk were considered a clinical mastitis cases where as animal suspected for subclinical mastitis were confirmed by performing Califonia Mastitis Test (CMT) as per the method described by Dhakal (2006).

Before proceeding to the collection of milk samples from the animals, teats and udder of the animals were washed thoroughly and left to dry. Few streams of fore milk were discarded and milk from apparently healthy animals were initially screened by CMT as per the methods described by Dhakal (2006). In brief, the milk sample and CMT reagent were mixed in equal volume in the cup and mixed properly with horizontal movement of the cup. The milk samples showing slime or gel formation were considered as subclinical mastitic milk. Approximately, 10 ml milk samples from both mastitic and subclinical mastitic animals were collected in 15 ml sterile tube, marked properly and transported to the laboratory within 12 h of collection under cooled condition.

3.2.2. Isolation and identification of *Staphylococcus* species from milk samples

3.2.2.1. Enrichment:

Approximately 1 ml of clinical and subclinical mastitic milk samples were mixed with 9 ml Brain Heart Infusion broth followed by incubation at 37°C for 24 h (Palilu and Budiarso 2017).

3.2.2.2. Selective plating:

A loopful of inoculum from enrichment broth with turbidity was streaked on mannitol salt agar plate followed by incubation at 37°C for 24 h and examined for the growth of *Staphylococcus*. Both mannitol fermenter and non-fermenter colony from mannitol salt agar plate was picked and examined under oil immersion after Gram's staining as per the method described by Agarwal *et al*., (2003).

3.2.3. Preservation of the isolates

A single colony from each sample either mannitol fermenter and non-fermenter showing characteristic of *Staphylococcus* in Gram's staining was stabbed in nutrient agar (0.9%) with 10.0% NaCl. After overnight incubation at 37°C the stabs were sealed with paraffin film and stored at room temperature for further use.

3.2.4. Biochemical characterization of isolates

The presumptive colonies which showed characteristic morphology as cocci in bunch under microscopic examination were further confirmed by oxidase and catalase test.

The catalase test was performed by taking a fresh colony on a clean and dry glass slide and placing a drop of 3% H_2O_2 on the colony. The evolution of oxygen bubbles after addition of 3% H_2O_2 was considered as positive for catalase test.

The oxidase test was performed by smearing of a fresh colony over the Oxidase disc (Hi-media, India) and development of intense deep-purple colour, within 5-10 seconds or up to 60 sec. was considered as positive.

3.2.5. Extraction of DNA template for PCR

The template DNA was prepared from the biochemically confirmed isolates by the method of boiling and snaps chilling (Kaushik *et al*., 2014). The details of the procedure are as follows:

1) 1.5 ml of overnight grown culture in nutrient broth were taken in 2 ml microfuge tube and centrifuged at 5,000 rpm for 10 min to pellet the bacteria.

2) Supernatant was discarded and 1 ml normal saline was added to the bacterial pellet and vortexed.

3) Again, centrifuged at 5,000 rpm for 4 min to obtain the pellet.

4) Supernatant was discarded and 200 µl distilled water was added to the pellet and kept under boiling water for 8 min.

5) After boiling it was immediately transferred to -20^{0}C till preparation of reaction mixture for PCR assay.

6) Before use the tube was taken out of freezer and kept at room temperature for thawing and centrifuged at 4,000 rpm for 3 min.

7) The supernatant was used as DNA template (bacterial lysate).

3.2.6. Molecular confirmation of staphylococci targeting genus specific *tstaG* gene

A PCR assay was standardized for amplification of *tstaG* gene fragment of *Staphylococcus* isolates as per the method described by Morot-Bizot *et al*., 2004 with some modification. The PCR reaction mixture was prepared in 25 µl reaction volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) each of forward and reverse primers, 1µl (1 unit) *Taq* DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease free water.

The PCR amplification reaction was standardized under the following conditions: an initial denaturation phase of 94^{o}C for 5 min, followed by 40 amplification cycles of denaturation at 95^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and elongation at 72^{o}C for 30 sec, with a final elongation phase at 72^{o}C for 10 min.

3.2.7. Tube Coagulase test

All PCR confirmed isolates of *Staphylococcus* were screened by tube coagulase test using human plasma (from pathological laboratory) as per methods described by Agarwal *et al*., 2003.

Tube coagulase test was performed on overnight grown culture in 0.8 ml of nutrient broth followed by addition of 0.2 ml of human plasma of EDTA-blood. The mixture was kept in water bath preheated at 37^{o}C and observation was made after 4 h. If the test remains negative until four hours at 37^{o}C, the mixture was kept at room temperature and observation was made up to 24 h (at 2-4 h interval).

3.2.8. Molecular characterization of isolates by PCR targeting *16S rRNA* gene of *Staphylococcus aureus*

All the *Staphylococcus* confirmed isolates from different mastitic milk samples were screened for the detection of *S. aureus* targeting species specific *16S rRNA* gene.

A PCR assay was standardized for amplification of *16S rRNA* gene fragment of *S. aureus* isolates as per the method described by Karmakar *et al*., (2016) with some modification. The PCR reaction mixture was prepared in 25 µl reaction volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) each of forward and reverse primers, 1µl (1 unit) *Taq* DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease free water.

The PCR amplification reaction was standardized with an initial denaturation phase of 94°C for 5 min, followed by 35 amplification cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min with a final elongation phase at 72°C for 10 min.

3.2.9. Molecular characterization of isolates by amplification of *mecA* gene

All the *Staphylococcus* confirmed isolates from different mastitic milk samples were screened by PCR for the detection of methicillin resistant *mec*A gene.

A PCR assay was standardized for amplification of *mec*A gene fragment of *Staphylococcus* isolates as per the method described by Braoios *et al*., (2009) with some modification. The PCR reaction mixture was prepared in 25 µl reaction volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) each of forward and reverse primers, 1µl (1 unit) *Taq* DNA polymerase, 5 µl of bacterial lysate and 12 µl nuclease free water.

The PCR amplification reaction was standardized with an initial denaturation phase of 94°C for 5 min, followed by 32 amplification cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 1 min with a final elongation phase at 72°C for 10 min.

3.2.10. Agarose gel electrophoresis

The gel casting tray was placed on a levelled surface and open sides were sealed with adhesive tape. The gel comb was placed across the casting tray, so that the teeth of comb remain 1 mm above the base of tray.

Agarose gel (1.5%) was prepared by boiling molecular grade agarose in 1X Tris Acetate-EDTA buffer to dissolve completely. After boiling and cooling to 50^{0}C, 1% ethidium bromide solution was added to give a final concentration of 0.5 µg/ml of agarose gel solution. The molten agarose was poured onto gel casting tray and left undisturbed for about half an hour to solidify the gel.

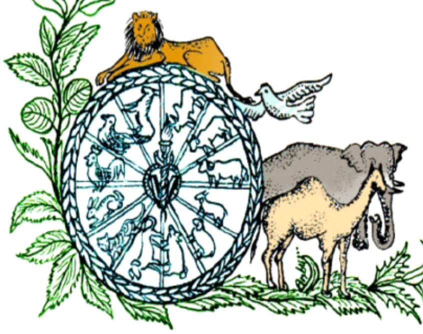
After solidification, the comb was taken out and adhesive tape was removed. The gel with casting tray was then submerged in electrophoresis tank filled with sufficient quantity of 1X TAE electrophoresis buffer (about 1 mm above the surface of the gel) with the wells at cathode end of the tank.

With the help of micropipette 10 µl of PCR product was mixed with 2 µl of loading dye (6X) and loaded into wells. Electrophoresis was performed at 5 V/cm for 1 h and the progress of mobility was monitored by migration of bromophenol dye. To estimate the length of fragments, a 100-bp DNA ladder was run on gel, and the amplicons were observed and documented under UV transilluminator/ gel documentation system.

3.2.11. Antibiotic susceptibility pattern

All the confirmed *Staphylococcus* isolates under study were examined to determine the antibiotic resistance pattern by disc diffusion method (Bauer *et al*., 1966; Mahato *et. al.* 2017) using 9 antibiotic discs viz. Oxacillin (1 µg), Ciprofloxacin (5 µg), Teicoplanin (30 µg), Cefoxitin (30 µg), Erythromycin (15 µg), Rifampicin (5 µg), Clindamycin (2 µg), Gentamicin (10 µg) and Tetracycline (30 µg) (Hi-Media, Mumbai) (Table.3). The test colony was inoculated overnight in nutrient broth at 37^{0}C. About 100 µl of overnight grown culture was spread on Mueller-Hilton agar plates with sterile L-shaped spreader and antibiotic disc were sticked to the plates with forceps. All antibiotic disc containing plates were incubated for 18-24 h at 37^{0}C. The zone of inhibition was measured by the scale (Himedia, India) and results were interpreted according to the guidelines of CLSI.

Results



4 RESULTS

The results of the study for isolation of *Staphylococcus* by conventional method and identification by biochemical and molecular methods from clinical and subclinical mastitic milk samples, and antibiotic susceptibility profiles of the isolates are presented in this section.

4.1Screening of milk samples by California Mastitis Test

The milk samples collected from different animals were screened by CMT for detection of clinical and subclinical mastitis. After the screening by CMT a total of 200 milk sample from subclinical mastitic animal were processed. Out of 200 clinical and subclinical mastitic bovine milk samples processed, 50 samples each were collected from different district of Bihar viz., Gaya, Patna, Vaishali and Sitamarhi. All subclinical milk samples were confirmed on the basis of result obtained from California Mastitis Test, whereas clinical mastitis was confirmed by change in milk colour, consistency and clinical sign. The samples collected from Gaya district comprised of 16 clinical and 34 subclinical mastitic milk samples. Similarly, samples from Patna district comprised of 14 clinical and 36 subclinical, Vaishali district comprised of 16 clinical and 34 subclinical, and Sitamarhi district comprised of 17 clinical and 33 subclinical mastitic milk (Table-1).

4.2. Isolation of *Staphylococus* by conventional method

A total of 200 mastitic milk samples (clinical and subclinical) were processed for isolation of *Staphylococcus* by enrichment in BHI broth with 18-24 h incubation at 37^0C followed by streaking on the Mannitol Salt agar (MS agar). By this conventional enrichment and plating on MS agar 86.50% (173/200) of clinical and subclinical milk samples were found positive for *Staphylococcus* (Table-4, Fig.-1).

The district wise distribution of *Staphylococcus* on the basis of growth on MSA included 80.00% (40/50) from Gaya district, 88.00% (44/50) from Patna district, 96.00% (48/50) from Vaishali district and 82.00% (41/50) from Sitamarhi district (Table-4).

Among the isolates from clinical and subclinical mastitic milk samples of Gaya district, 32% (16/50) and 48% (24/50) belonged to clinical and subclinical, respectively. Similarly, 28% (14/50) and 60% (30/50), 32% (16/50) and 64% (32/50), as well as 34% (17/50) and 48% (24/50) isolates from Patna, Vaishali and Sitamarhi district were isolated from clinical and subclinical milk samples, respectively (Table-4, Fig.-1).

4.3. Biochemical characterization of *Staphylococcus*

A single characteristic colony from each sample were selected for Gram staining, oxidase and catalase test and it was found that all 173 isolates showed characteristic morphology and biochemical reactions of *Staphylococcus.* (Fig.-3).

4.4. PCR detection of *Staphylococcus* genus targeting *tstaG* gene

All biochemically confirmed isolates of *Staphylococci* (N=173) from different clinical and subclinical mastitic milk samples of four district of Bihar were further confirmed by genus specific PCR targeting the amplification of *tstaG* gene. The finding of the PCR study supports the morphological and biochemical results obtained in this study i.e., all 173 isolates were found to harbor *tstaG* gene of 370 bp amplicon size (Table-4, Fig.-4 & 8).

The district wise distribution showed that 40% (16/40), 31.81% (14/44), 33.33% (16/48) & 41.46% (17/41) of staphylococci were from clinical sample of Gaya, Patna, Vaishali and Sitamarhi, respectively. Whereas 60% (24/40), 68.18% (30/44), 66.67% (32/48) & 58.54% (24/41) of staphylococci isolates belonged from subclinical sample of Gaya, Patna, Vaishali and Sitamarhi district respectively (Table-4, Fig.-4 & 8).

4.5. Tube Coagulase test for *Staphylococcus aureus*

All 173 *tstaG* gene harboring isolates of *Staphylococcus* were screened by tube coagulase test using human plasma to determine the distribution of coagulase positive and negative staphylococci. The tube coagulase test showing coagulation reaction with human plasma were considered coagulase positive (Fig-3). A total of 60.69% (105/173) isolates from different mastitic milk samples were confirmed as coagulase positive staphylococci while, other 39.31% (68/173) isolates showed coagulase negative reaction.

The district wise distribution of coagulase positive staphylococci showed that 62.50% (25/40), 54.55% (24/44), 56.25% (27/48) and 70.73% (29/41) isolates belonged to Gaya, Patna, Vaishali and Sitamarhi district, respectively.

Out of 25 coagulase positive isolates of Gaya district, 52.00% (13/25) and 48.00% (12/25) isolates belonged to clinical and subclinical mastitic milk samples, respectively while, among 24 coagulase positive isolates of Patna district encompassed 41.67% (10/24) and 58.33% (14/24), respectively from clinical and subclinical milk samples.

Similarly, among 27 coagulase positive *Staphylococcus* isolates from Vaishali district, 40.74% (11/27) and 59.26% (16/27) were from clinical and subclinical mastitic milk samples, respectively while, 48.28% (14/29) and 51.72% (15/29) isolates were from clinical and subclinical mastitic milk samples, respectively among 29 coagulase positive isolates of Sitamarhi district (Table-4, Fig-3, 9 & 12).

Among all the isolates, 39.31% (68/173) isolates showed coagulase negative reaction. The district wise distribution of coagulase negative staphylococci showed that 37.50% (15/40), 45.45% (20/44), 43.75% (21/48) and 29.26% (12/41) isolates belonged to Gaya, Patna, Vaishali and Sitamarhi district, respectively. Out of 15 coagulase negative isolates of Gaya district, 20.00% (03/15) and 80.00% (12/15) isolates belonged to clinical and subclinical mastitic milk samples, respectively while, among 20 coagulase negative isolates of Patna district encompassed 20.00% (04/20) and 80.00% (16/20), respectively from clinical and subclinical milk samples.

Similarly, among 21 coagulase negative *Staphylococcus* isolates from Vaishali district, 23.81% (05/21) and 76.19% (16/21) were from clinical and subclinical mastitic milk samples, respectively while, 25.00% (03/12) and 75.00% (09/12) isolates were from clinical and subclinical mastitic milk samples, respectively among 12 coagulase negative isolates of Sitamarhi district (Table-4, Fig-12).

4.6. PCR detection of *Staphylococcus aureus* targeting *16S rRNA* gene

All the *Staphylococcus* confirmed isolates (N=173) from different milk samples were screened for the detection of *S. aureus* targeting species specific *16S rRNA* gene PCR. The results obtained from species specific *16S rRNA* gene PCR showed that 56.64% (98/173) isolates produced expected amplicon size of 228bp. (Fig.-5) The occurrence of *S. aureus* among 105 coagulase positive Staphylococci showed that 93.33% (98/105) isolates were coagulase positive *S. aureus.* (Table-4, Fig.-5, 10 & 13).

The *Staphylococcus* isolates from Gaya district (n=40) showed the distribution of *S. aureus* species specific *16S rRNA*gene in 57.50% (23/40) isolates out of which 47.83% (11/23) and 52.17% (12/23) were from clinical and subclinical milk samples, respectively. Similarly, *Staphylococcus* isolates from Patna district (n=44) showed the distribution of *S. aureus* species specific *16S rRNA*gene in 52.27% (23/44) isolates, out of which 43.48% (10/23) and 56.52% (13/23) were from clinical and subclinical mastitic milk samples, respectively (Table-4, Fig.-5, 10 & 13).

The *Staphylococcus* isolates from Vaishali district (n=48) showed the distribution of *S. aureus* species specific *16S rRNA* gene in 50.00% (24/48), out of which 45.83% (11/24) were from clinical and 54.17% (13/24) were from subclinical milk samples. Similarly, *Staphylococcus* isolates from Sitamarhi district (n=41) showed the distribution of *S. aureus* species specific *16S rRNA* gene in 68.29%

(28/41), out of which 46.43% (13/28) were from clinical and 53.57% (15/28) isolates were from subclinical milk samples (Table-4, Fig.-5, 10 & 13).

4.7. Detection of methicillin resistant *Staphylococcus* (MRS) targeting *mecA* gene by PCR

A PCR was standardized for amplification of *mec*A gene of 310 bp amplicon size using the DNA isolated from methicillin resistant *S. aureus* culture maintained in our department (Fig.-6). The *Staphylococcus* isolates (n=173) from different clinical and subclinical mastitic milk samples were screened for the presence of *mecA* gene by PCR. The PCR detected *mecA* gene in 38.73% (67/173) of *Staphylococcus* isolates. The district wise distribution of *mecA* gene among *Staphylococcus* isolates are as follows:

The clinical and subclinical mastitic milk samples of Gaya district showed a distribution of *mecA* gene in 25.00% (10/40) isolates of *Staphylococcus*, out of which 43.75% (07/16) isolates were from clinical and12.50% (03/24) were from subclinical mastitic milk samples (Table-4, Fig.-6, 11 & 14).

Similarly, in Patna, Vaishali and Sitamarhi district *mecA* gene was found in 43.18% (19/44), 43.75% (21/48) and 41.46% (17/41) isolates of clinical and subclinical mastitic milk samples, respectively. The finding of *mec*A gene positive *Staphylococcus* among clinical and subclinical mastitic milk samples showed that 28.57% (04/14) and 50.00% (15/30), 50.00% (08/16) and40.62% (13/32), and 52.94% (09/17) and 33.33% (08/24) isolates, respectively from clinical and subclinical mastitic milk samples from Patna, Vaishali and Sitamarhi district harbored *mec*A gene (Table-4, Fig.-6, 11 & 14).

4.8. Antibiotic susceptibility pattern

Antibiogram study of *Staphylococcus* isolates from clinical and subclinical bovine mastitic milk were performed using 09 antibiotic discs viz. Oxacillin (1 µg), Ciprofloxacin (5 µg), Teicoplanin (30 µg), Cefoxitin (30 µg), Erythromycin (15 µg), Rifampicin (5 µg), Clindamycin (2 µg), Gentamicin (10 µg) and Tetracycline (30 µg) by method described in section 3.2.11. (Fig-7). The antibiogram study of isolates reveals the high resistance of *Staphylococcus* to Oxacillin (62.42%), Cefoxitin (53.76%), Rifampicin (47.4%) and Erythromycin (38.15) while moderate resistance observed against Gentamycin (31.79%) & Clindamycin (29.48%) and low resistance against Ciprofloxacin (13.29%), Teicoplanin (7.32%) and Tetracycline (1.73%) (Fig.-19 & 20, Table-14).

4.8.1. Antibiotic susceptibility pattern of isolates from Gaya district

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Gaya district showed maximum resistance (72.5%) to Cefoxitin followed by a resistance of 55.00% to Oxacillin and Erythromycin, 50.00% to Gentamicin, 35.00% to Rifampicin, 27.50% to Ciprofloxacin, 25.00% to Clindamycin, 20.00% to Tetracycline whereas none of the isolates were found resistant to Teicoplanin (Fig.-15, Table-5 & 6).

The isolates showed a maximum susceptibility of 92.50% to Teicoplanin followed by a sensitivity of 75.00% to Tetracycline, 50.00% to Ciprofloxacin and Gentamicin, 47.50% to Rifampicin, 45.00% to Oxacillin and Clindamycin, 27.50% to Cefoxitin, and 25.00% Erythromycin.

The isolates also showed an intermediate resistance of 30.00% to Clindamycin followed by 22.50% to Ciprofloxacin, 20.00% to Erythromycin, 17.50% to Rifampicin, 7.50% to Teicoplanin and 5.00% to Tetracycline (Fig.-15, Table-5 & 6).

4.8.2. Antibiotic susceptibility pattern of isolates of Patna district

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Patna district showed maximum resistant (84.09%) to Rifampicin followed by 75.00% to Oxacillin, 70.45% to Cefoxitin, 45.45% to Clindamycin, 36.36% to Erythromycin, 18.18% to Gentamicin, 6.81% to Ciprofloxacin and 2.27% to Tetracycline (Fig.-16, Table-7 & 8).

The isolates showed a maximum susceptibility of 95.45% to Tetracycline and Ciprofloxacin followed by a sensitivity of 84.09% to Gentamicin, 77.27% to Teicoplanin, 59.09% to Erythromycin, 40.91% to Clindamycin, 31.82% to Cefoxitin, 27.27% to Oxacillin and 15.91% to Rifampicin. The isolates also showed an intermediate resistance of 25% to Teicoplanin followed by 15.90% to Clindamycin, 6.81% to Erythromycin, 4.54% to Tetracycline and 02.27% to Rifampicin (Fig-16 Table-7 & 8).

4.8.3. Antibiotic susceptibility pattern of isolates from Vaishali district

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Vaishali district showed maximum resistance of 62.50% to Oxacillin followed by 43.75% to Erythromycin, 41.66% to Clindamycin, 39.58% to Rifampicin, 33.33% to Cefoxitin and Gentamicin, 14.58% to Ciprofloxacin and 06.25% to Tetracycline (Fig.-17, Table-9 & 10).

The isolates showed a maximum susceptibility of 91.67% to Tetracycline followed by a sensitivity of 87.50% to Teicoplanin, 77.08% to Ciprofloxacin and 66.67% to Gentamicin and Cefoxitin, 39.58% to Rifampicin, 37.50% to Oxacillin and Erythromycin, and 25.00% to Clindamycin. However, isolates also showed an intermediate resistance of 33.33% to Clindamycin followed by 20.83% to Rifampicin, 18.75% to Erythromycin, 12.50% to Teicoplanin, 08.33% to Ciprofloxacin and 2.08% to Tetracycline. (Fig.-17, Table-9 & 10).

4.8.4. Antibiotic susceptibility pattern of isolates from Sitamarhi district

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Sitamarhi district showed maximum resistance of 58.53% to Oxacillin followed by 43.90% to Cefoxitin, 31.70% to Rifampicin, 26.82% to Gentamicin, 19.51% to Erythromycin, 17.07% to Tetracycline, 07.31% to Teicoplanin, 04.87% to Ciprofloxacin and 02.43 to Clindamycin (Fig.-18 Table-11 & 12).

The isolates showed a maximum susceptibility of 90.24% to Ciprofloxacin followed by a sensitivity of 80.49% to Clindamycin, 75.60% to Tetracycline, 73.17% to Teicoplanin and Gentamicin, 68.29% to Erythromycin, 51.22% to Rifampicin, 56.10% to Cefoxitin and 41.46% to Oxacillin. However, isolates also showed an intermediate resistance of 19.51% to teicoplanin followed by 17.07% to Rifampicin and Clindamycin, 12.19% to Erythromycin, 7.31% to tetracycline and 4.87% to Ciprofloxacin (Fig.-18 Table-11 & 12).

4.8.5. Occurrence of phenotypically Oxacillin sensitive but of *mecA* gene positive Staphylococcus (OS-MRS)

In all 173 staphylococci isolates characterized in the study, the occurrence of oxacillin sensitive but *mecA* positive *Staphylococcus* (OS-MRS) isolates were 10.40%. The district wise distribution of OS-MRS were 07.50%, 11.36%, 12.50%, 09.76% for Gaya, Patna, Vaishali and Sitamarhi respectively (Table-13, Fig-21).

4.8.6. Multidrug resistant (MDR) *Staphylococcus* isolates from mastitis milk sample

On the basis of antibiogram study performed the occurrence of multidrug resistant (MDR) Staphylococcus (Mahato *et al.,* 2017; Anderson *et al.* 2006) i.e. isolates resistant to more than two antibiotic groups was 53.76% (93/173). The district wise distribution of MDR *Staphylococcus* among *Staphylococcus* isolates are as follows:

The mastitis milk samples of Gaya district showed a distribution of MDR *Staphylococcus* in 60.00% (24/40) isolates of *Staphylococcus*. Similarly, in Patna, Vaishali and Sitamarhi district MDR *Staphylococcus* were found in 65.91% (29/44), 50.00% (24/48) and 39.02% (16/41) isolates of mastitic milk samples, respectively (Fig.-22, Table-15).

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Table.1. Detail of samples used under study

S. No.	**Agroclimatic zone**	**District**	**No. of clinical mastitic milk samples**	**No. of subclinical mastitic milk sample**	**Total**
1.	I	Gaya	16	34	50
		Patna	14	36	50
2.	II	Sitamarhi	16	34	50
		Vaishali	17	33	50
Total			**63**	**137**	**200**

Table2. Oligonucleotide primers used in this study

Sl. No.	**Primer Sequences (5 ’- 3 ’)**	**Target gene**	**Product size (bp)**	**Reference**
1.	F: GGCCGTGTTGAACGTGGTCAAATCA	*tstaG*	370	Morot-Bizot *et al*., 2004
	R: TACCATTTCAGTACCTTCTGGTAA			
2.	F: GTAGGTGGCAAGCGTTATCC	*16S rRNA*	228	Karmakar *et al*., 2016
	R: CGCACATCAGCGTCAG			
3.	F: GTA GAA ATG ACT GAA CGT CCG ATAA	*mec*A	310	Braoios *et al*., 2009
	R: CCAATTCC ACATTGT TTCG GTC TAA			

F: Forward primer, R: Reverse primer, bp: Base pair

Table.3. Detail of antibiotic discs used in this study

S. No.	Antibiotic	Symbol
1.	Oxacillin(1µg)	OX
2.	Ciprofloxacin(5µg)	CIP
3.	Teicoplanin(30µg)	TEI
4.	Cefoxitin(30µg)	CX
5.	Erythromycin(15µg)	E
6.	Rifampicin(5µg)	RIF
7.	Clindamycin(2µg)	CD
8.	Gentamicin(10µg)	GEN
9.	Tetracycline(30µg)	TE

Table.4. Occurrence of *Staphylococcus* (CoNS & CoPS), *S. aureus*, MRS in clinical and subclinical mastitis milk samples

Sr No.	District	Samples Type	Total sample Screened	Growth of *Staphylococcus* on mannitol salt agar (%)	*Staphylococcus* Genus Positive (%)	Coagulase positive (%)	Coagulase negative (%)	*16S rRNA* positive (%)	*mecA* positive (%)
1.	Gaya	Clinical	16	32.00 (16/50)	40.00 (16/40)	52.00 (13/25)	20.00 (03/15)	47.83 (11/23)	43.75 (07/16)
		Sub-clinical	34	48.00 (24/50)	60.00 (24/40)	48.00 (12/25)	80.00 (12/15)	52.17 (12/23)	12.50 (03/24)
	Total		50	80.00 (40/50)	100.00 (40/40)	62.50 (25/40)	37.50 (15/40)	57.50 (23/40)	25.00 (10/40)
2.	Patna	Clinical	14	28.00 (14/50)	31.81 (14/44)	41.67 (10/24)	20.00 (04/20)	43.48 (10/23)	28.57 (04/14)
		Sub-clinical	36	60.00 (30/50)	68.18 (30/44)	41.67 (14/24)	80.00 (16/20)	56.52 (13/23)	50.00 (15/30)
	Total		50	88.00 (44/50)	100.00 (44/44)	54.55 (24/44)	45.45 (20/44)	52.27 (23/44)	43.18 (19/44)
3.	Vaishali	Clinical	16	32.00 (16/50)	33.33 (16/48)	40.74 (11/27)	23.81 (05/21)	45.83 (11/24)	50.00 (08/16)
		Sub-clinical	34	64.00 (32/50)	66.67 (32/48)	59.26 (16/27)	76.19 (16/21)	54.17 (13/24)	40.62 (13/32)
	Total		50	96.00 (48/50)	100.00 (48/48)	56.25 (27/48)	43.75 (21/48)	50.00 (24/48)	43.75 (21/48)
4.	Sitamarhi	Clinical	17	34.00 (17/50)	41.46 (17/41)	48.28 (14/29)	25.00 (03/12)	46.43 (13/28)	52.94 (09/17)
		Sub-clinical	33	48.00 (24/50)	58.54 (24/41)	51.72 (15/29)	75.00 (09/12)	53.57 (15/28)	33.33 (8/24)
	Total		50	82.00 (41/50)	100.00 (41/41)	70.73 (29/41)	29.26 (12/41)	68.29 (28/41)	41.46 (17/41)
	Total		**200**	**86.50 (173/200)**	**100.00 (173/173)**	**60.69 (105/173)**	**39.31 (68/173)**	**93.33 (98/105)**	**38.73 (67/173)**

Table.5. ABST profile of *Staphylococcus* isolates from cattle mastitic milk samples of Gaya district

S. No.	Sample no.	Antibiotics								
		OX	**CX**	**CIP**	**E**	**TEI**	**TE**	**RIF**	**CD**	**GEN**
1.	G-1(C)	R	R	S	R	S	S	R	S	R
2.	G-2(C)	R	S	S	I	S	S	I	I	S
3.	G-3(C)	R	R	R	R	S	R	S	S	R
4.	G-4(C)	R	R	I	R	S	S	R	S	R
5.	G-5(C)	S	R	R	S	S	S	I	I	S
6.	G-6(C)	R	R	S	R	S	S	S	S	R
7.	G-7(C)	S	R	I	R	S	S	I	S	R
8.	G-8(C)	R	R	S	R	S	R	R	S	R
9.	G-9(C)	S	R	S	S	S	S	S	S	S
10.	G-10(C)	R	R	R	S	S	R	S	I	S
11.	G-11(SC)	S	S	R	R	S	S	S	S	S
12.	G-13(SC)	S	S	S	R	S	S	I	I	R
13.	G-14(C)	R	R	I	R	S	S	R	R	S
14.	G-15(SC)	R	R	S	R	S	R	S	I	S
15.	G-16(SC)	R	R	R	R	I	S	R	S	R
16.	G-17(SC)	S	R	R	R	S	S	I	S	S
17.	G-18(SC)	S	R	I	I	S	S	S	I	R
18.	G-19(SC)	S	R	S	S	S	S	S	I	S
19.	G-20(SC)	R	R	S	R	S	S	R	R	R
20.	G-21(SC)	R	S	I	R	S	S	R	S	S
21.	G-24(SC)	S	R	S	R	S	S	I	R	R

22.	G-27(C)	S	S	R	R	S	S	S	S	S
23.	G-28(C)	R	S	I	I	S	S	S	I	R
24.	G-29(SC)	R	R	R	R	S	S	S	S	S
25.	G-30(SC)	R	R	R	I	S	S	I	I	S
26.	G-31(SC)	R	R	R	R	S	R	R	I	R
27.	G-32(SC)	S	R	S	R	S	R	S	S	R
28.	G-33(SC)	R	R	S	R	I	R	R	R	R
29.	G-34(SC)	S	R	I	R	S	R	R	S	R
30.	G-36(C)	S	R	S	I	S	S	S	I	R
31.	G-37(SC)	R	R	I	S	S	S	R	R	S
32.	G-38(SC)	S	S	S	S	S	S	S	S	S
33.	G-39(C)	S	S	S	S	S	S	S	S	S
34.	G-40(SC)	R	R	I	S	S	I	S	I	R
35.	G-42(SC)	R	R	S	I	S	S	R	R	R
36.	G-43(SC)	R	R	S	I	S	S	S	R	S
37.	G-44(SC)	S	S	S	S	S	S	S	S	S
38.	G-45(SC)	R	S	S	I	S	I	S	R	S
39.	G-46(SC)	S	R	R	R	I	S	R	R	R
40.	G-47(C)	S	S	S	S	S	S	R	R	S

OX: Oxacillin, CX: Cefoxitin, CIP: Ciprofloxacin, E: Erythromycin, TEI: Teicoplanin, TE: Tetracycline, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin. S-Sitamarhi, P-Patna, V -Vaishali, G-Gaya, S: Sensitive, I: Intermediate, R: Resistant. C- Clinical mastitis; SC-Subclinical mastitis

Table.6. ABST profile of *Staphylococcus* isolates from mastitic milk sample of Gaya district

S. No.	Antibiotic	Number of isolates	Resistant (%)	Intermediate Resistant (%)	Sensitive (%)
1.	**OX**	40	55.00 (22/40)	00.00 (00/40)	45.00 (18/40)
2.	**CX**	40	72.50 (29/40)	00.00 (00/40)	27.50 (11/40)
3.	**CIP**	40	27.50 (11/40)	22.50 (09/40)	50.00 (20/40)
4.	**E**	40	55.00 (22/40)	20.00 (08/40)	25.00 (10/40)
5.	**TEI**	40	00.00 (00/40)	07.50 (03/40)	92.50 (37/40)
6.	**TE**	40	20.00 (08/40)	05.00 (02/40)	75.00 (30/40)
7.	**RIF**	40	35.00 (14/40)	17.50 (07/40)	47.50 (19/40)
8.	**CD**	40	25.00 (10/40)	30.00 (12/40)	45.00 (18/40)
9.	**GEN**	40	50.00 (20/40)	00.00 (00/40)	50.00 (20/40)

Table.7. ABST profile of *Staphylococcus* isolates from cattle mastitic milk samples of Patna district

S. No.	Sample no.	Antibiotics								
		OX	CX	CIP	E	TEI	TE	RIF	CD	GEN
1.	P-2(C)	R	R	S	S	S	S	R	S	S
2.	P-3(SC)	S	R	S	I	I	S	R	S	S
3.	P-4(SC)	R	R	S	S	S	S	R	R	S
4.	P-5(SC)	S	S	S	S	I	S	R	I	S
5.	P-7(SC)	R	R	S	S	S	S	S	R	S
6.	P-8(SC)	R	R	S	S	S	S	R	R	S
7.	P-9(SC)	R	R	S	S	S	S	R	S	S
8.	P-10(SC)	R	R	S	S	S	S	R	S	S
9.	P-11(SC)	R	R	S	R	S	S	R	R	S
10.	P-12(C)	S	R	S	I	I	S	R	S	R
11.	P-13(C)	R	R	S	S	S	S	R	S	S
12.	P-14(C)	R	S	S	S	S	S	R	I	S
13.	P-15(C)	S	S	S	S	S	S	R	S	S
14.	P-16(SC)	R	R	S	R	S	S	R	R	S
15.	P-17(SC)	R	R	S	S	S	S	R	R	S
16.	P-18(C)	R	R	S	I	S	S	R	R	S
17.	P-19(C)	R	R	S	R	I	S	R	R	S
18.	P-20(C)	R	R	S	S	S	S	R	I	S
19.	P-22(SC)	R	R	S	R	S	S	R	R	S
20.	P-24(SC)	R	R	S	S	S	S	R	R	S
21.	P-25(SC)	R	S	S	S	S	R	S	I	S
22.	P-26(SC)	S	S	S	S	S	S	R	S	S

23.	P-27(SC)	R	S	S	S	S	S	R	S	S
24.	P-28(SC)	R	R	R	R	I	S	R	S	R
25.	P-29(C)	R	R	R	R	I	S	R	I	R
26.	P-30(C)	S	S	R	R	I	S	R	R	S
27.	P-31(SC)	S	R	S	R	I	S	R	R	S
28.	P-32(SC)	R	R	S	R	S	S	R	R	S
29.	P-33(SC)	R	R	S	S	S	S	R	R	R
30.	P-34(SC)	R	R	S	R	I	I	R	S	R
31.	P-36(SC)	R	S	S	S	S	S	R	R	R
32.	P-37(SC)	R	S	S	S	S	S	S	R	S
33.	P-38(C)	R	R	S	S	S	S	S	S	S
34.	P-39(SC)	S	S	S	S	S	S	R	S	S
35.	P-40(C)	R	R	S	R	I	S	R	R	S
36.	P-41(SC)	S	S	S	R	S	S	S	S	S
37.	P-42(SC)	R	S	S	S	S	S	R	R	S
38.	P-43(SC)	S	R	S	S	S	S	R	S	S
39.	P-44(C)	S	R	S	R	S	S	S	S	S
40.	P-45(SC)	R	R	S	R	I	S	I	I	R
41.	P-46(SC)	S	S	S	R	S	S	S	S	R
42.	P-47(C)	R	S	S	S	S	S	R	S	S
43.	P-48(SC)	R	R	S	S	S	I	R	R	S
44.	P-49(SC)	R	R	S	S	S	S	R	R	S

OX: Oxacillin, CX: Cefoxitin, CIP: Ciprofloxacin, E: Erythromycin, TEI: Teicoplanin, TE: Tetracycline, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin. S-Sitamarhi, P-Patna, V -Vaishali, G-Gaya. S: Sensitive, I: Intermediate, R: Resistant. C- Clinical mastitis; SC- Subclinical mastitis

Table.8. ABST profile of *Staphylococcus* isolates from mastitic milk sample of Patna district

S. No.	Antibiotic	Number of isolates	Resistant (%)	Intermediate Resistant (%)	Sensitive (%)
1.	**OX**	44	72.73 (32/44)	00.00 (00/44)	27.27 (12/44)
2.	**CX**	44	68.18 (30/44)	00.00 (00/44)	31.82 (14/44)
3.	**CIP**	44	06.82 (03/44)	00.00 (00/44)	93.18 (41/44)
4.	**E**	44	34.09 (15/44)	06.82 (03/44)	59.09 (26/44)
5.	**TEI**	44	00.00 (00/44)	25.00 (11/44)	75.00 (33/44)
6.	**TE**	44	02.27 (01/44)	04.55 (02/44)	93.18 (41/44)
7.	**RIF**	44	81.82 (36/44)	02.27 (01/44)	15.91 (07/44)
8.	**CD**	44	45.45 (20/44)	13.64 (06/44)	40.91 (18/44)
9.	**GEN**	44	18.18 (08/44)	00.00 (00/44)	81.82 (36/44)

Table.9. ABST profile of *Staphylococcus* isolates from cattle mastitis milk samples of Vaishali district

S. No.	**Sample no.**	**Antibiotics**								
		OX	**CX**	**CIP**	**E**	**TEI**	**TE**	**RIF**	**CD**	**GEN**
1.	V-1(C)	R	R	S	R	S	I	R	I	R
2.	V-2(C)	R	S	S	S	S	S	S	I	S
3.	V-3(SC)	R	R	I	R	I	S	R	R	R
4.	V-4(SC)	R	R	I	R	S	S	R	S	R
5.	V-5(SC)	S	R	I	S	S	S	S	S	S
6.	V-6(SC)	R	R	R	R	S	S	S	R	R
7.	V-7(SC)	R	R	R	R	I	S	S	R	R
8.	V-8(C)	S	R	S	I	I	S	I	I	R
9.	V-9(C)	R	R	S	R	S	S	I	I	S
10.	V-10(C)	R	R	S	R	S	S	S	I	S
11.	V-11(C)	S	S	S	S	S	S	R	I	S
12.	V-12(C)	R	R	S	R	S	S	S	I	S
13.	V-13(C)	R	R	S	I	S	S	R	I	S
14.	V-14(C)	R	S	S	S	S	S	S	S	S
15.	V-15(C)	R	S	S	S	S	S	R	S	S
16.	V-16(C)	R	S	S	R	S	S	R	R	R
17.	V-17(C)	S	S	R	R	S	R	S	S	R
18.	V-18(C)	R	S	S	R	S	S	S	S	S
19.	V-19(SC)	R	R	S	I	S	S	I	R	R
20.	V-20(SC)	S	S	S	S	S	S	S	S	S
21.	V-21(C)	R	S	S	I	S	S	I	I	S
22.	V-22(C)	S	S	S	S	S	S	S	S	S
23.	V-23(SC)	S	S	S	R	S	R	R	R	S
24.	V-24(SC)	R	S	S	R	S	S	I	I	R

25.	V-25(SC)	R	S	S	R	S	S	R	R	S
26.	V-26(SC)	R	S	S	S	S	S	S	S	S
27.	V-27(SC)	R	S	S	S	S	S	S	S	S
28.	V-28(SC)	S	S	S	S	S	S	I	I	R
29.	V-29(SC)	R	R	S	R	I	S	R	R	S
30.	V-30(SC)	R	S	S	S	S	S	I	I	S
31.	V-31(SC)	R	S	S	I	S	S	I	I	R
32.	V-33(SC)	S	S	S	I	S	R	R	R	R
33.	V-34(SC)	R	S	S	S	I	S	S	R	S
34.	V-35(SC)	S	S	S	S	S	S	S	R	S
35.	V-36(SC)	S	S	S	R	I	S	I	R	S
36.	V-37(SC)	S	S	R	R	S	S	S	R	R
37.	V-38(SC)	S	R	R	R	S	S	R	S	S
38.	V-39(SC)	S	S	R	R	S	S	R	I	S
39.	V-40(SC)	R	S	R	R	S	S	R	R	S
40.	V-41(SC)	S	S	I	S	S	S	R	R	S
41.	V-42(SC)	R	R	S	S	S	S	R	R	S
42.	V-43(SC)	R	S	S	I	S	S	S	R	S
43.	V-44(SC)	S	S	S	R	S	S	R	S	S
44.	V-45(SC)	R	R	S	S	S	S	R	R	S
45.	V-46(SC)	S	S	S	I	S	S	R	R	R
46.	V-47(SC)	S	S	S	S	S	S	I	R	S
47.	V-48(SC)	R	S	S	I	S	S	S	I	R
48.	V-49(C)	R	S	S	S	S	S	S	I	S

OX: Oxacillin, CX: Cefoxitin, CIP: Ciprofloxacin, E: Erythromycin, TEI: Teicoplanin, TE: Tetracycline, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin. S-Sitamarhi, P-Patna, V -Vaishali, G-Gaya. S: Sensitive, I: Intermediate, R: Resistant. C- Clinical mastitis; SC-Subclinical mastitis

Table.10. ABST profile of *Staphylococcus* isolates from mastitic milk sample of Vaishali district

S. No.	Antibiotic	Number of isolates	Resistant (%)	Intermediate Resistant (%)	Sensitive (%)
1.	**OX**	48	62.50 (30/48)	00.00 (00/48)	37.50 (18/48)
2.	**CX**	48	33.33 (16/48)	00.00 (00/48)	66.67 (32/48)
3.	**CIP**	48	14.58 (07/48)	08.33 (04/48)	77.08 (37/48)
4.	**E**	48	43.75 (21/48)	18.75 (09/48)	37.50 (18/48)
5.	**TEI**	48	00.00 (00/48)	12.50 (06/48)	87.50 (42/48)
6.	**TE**	48	06.25 (03/48)	02.08 (01/48)	91.67 (44/48)
7.	**RIF**	48	39.58 (19/48)	20.83 (10/48)	39.58 (19/48)
8.	**CD**	48	41.67 (20/48)	33.33 (16/48)	25.00 (12/48)
9.	**GEN**	48	33.33 (16/48)	00.00 (00/48)	66.67 (32/48)

Table.11. ABST profile of *staphylococcus* isolates from cattle mastitic milk samples of Sitamarhi district

S. No.	Sample no.	Antibiotics								
		OX	CX	CIP	E	TEI	TE	RIF	CD	GEN
1.	S-1(C)	R	R	S	R	S	R	S	S	R
2.	S-2(C)	R	S	S	S	S	S	S	S	S
3.	S-4(C)	R	S	S	S	S	S	S	I	S
4.	S-5(C)	R	R	S	S	S	I	R	S	S
5.	S-6(SC)	S	S	S	S	S	S	S	S	S
6.	S-8(C)	R	R	S	S	S	S	R	S	S
7.	S-9(SC)	R	R	S	S	S	S	R	S	S
8.	S-10(SC)	R	R	S	S	I	S	I	S	S
9.	S-11(SC)	R	S	S	S	S	R	S	S	S
10.	S-12(SC)	R	S	S	S	S	S	S	S	S
11.	S-13(C)	R	R	S	S	S	S	S	S	R
12.	S-14(SC)	R	S	S	S	S	S	R	I	S
13.	S-16(SC)	S	S	S	S	I	S	R	S	S
14.	S-17(C)	S	S	S	R	I	S	I	I	S
15.	S-18(SC)	S	S	S	S	S	S	I	S	S
16.	S-19(SC)	S	S	S	S	S	S	I	I	S
17.	S-21(SC)	S	S	S	S	S	S	S	S	S
18.	S-22(SC)	R	R	S	R	R	S	R	S	R
19.	S-24(C)	S	S	S	R	S	S	S	S	S
20.	S-25(C)	S	S	S	S	S	S	R	S	S

21.	S-26(SC)	S	R	S	S	I	S	R	S	S
22.	S-27(SC)	R	R	S	I	R	S	R	S	R
23.	S-28(C)	S	S	S	S	S	S	S	S	S
24.	S-29(SC)	S	S	S	R	S	S	S	I	S
25.	S-30(C)	R	R	S	S	S	S	R	S	S
26.	S-31(SC)	R	R	I	I	R	I	R	R	S
27.	S-32(SC)	S	S	R	R	S	S	I	S	S
28.	S-33(C)	R	R	R	R	I	S	R	S	R
29.	S-34(C)	S	S	S	I	S	S	I	S	R
30.	S-36(SC)	R	R	S	S	S	S	S	S	R
31.	S-37(C)	R	S	S	S	S	S	S	S	S
32.	S-38(SC)	R	R	I	R	I	S	S	I	S
33.	S-39(C)	R	S	S	S	S	R	S	S	S
34.	S-40(SC)	S	S	S	S	I	R	S	S	S
35.	S-41(SC)	S	R	S	I	S	R	I	I	R
36.	S-43(SC)	R	S	S	S	S	S	S	S	S
37.	S-44(SC)	R	S	S	S	S	S	S	S	S
38.	S-45(C)	S	R	S	I	S	R	S	S	R
39.	S-47(SC)	S	S	S	S	I	S	S	S	S
40.	S-49(SC)	R	R	S	S	S	I	S	S	R
41.	S-50(C)	R	R	S	S	S	R	R	S	R

OX: Oxacillin, CX: Cefoxitin, CIP: Ciprofloxacin, E: Erythromycin, TEI: Teicoplanin, TE: Tetracycline, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin. S-Sitamarhi, P-Patna, V -Vaishali, G-Gaya. S: Sensitive, I: Intermediate, R: Resistant. C- Clinical mastitis; SC- Subclinical mastitis

Table.12. ABST profile of *Staphylococcus* isolates from mastitic milk sample of Sitamarhi district

S. No.	Antibiotic	Number of isolates	Resistant (%)	Intermediate Resistant (%)	Sensitive (%)
1.	**OX**	41	58.54 (24/41)	00.00 (00/41)	41.46 (17/41)
2.	**CX**	41	43.90 (18/41)	00.00 (00/41)	56.10 (23/41)
3.	**CIP**	41	04.87 (02/41)	04.87 (02/41)	90.24 (37/41)
4.	**E**	41	19.51 (08/41)	12.20 (05/41)	68.29 (28/41)
5.	**TEI**	41	07.32 (03/41)	19.51 (08/41)	73.17 (30/41)
6.	**TE**	41	17.07 (07/41)	07.32 (03/41)	75.61 (31/41)
7.	**RIF**	41	31.71 (13/41)	17.07 (07/41)	51.22 (21/41)
8.	**CD**	41	02.44 (01/41)	17.07 (07/41)	80.49 (33/41)
9.	**GEN**	41	26.83 (11/41)	00.00 (00/41)	73.17 (30/41)

Table.13. Occurrence of phenotypically Oxacillin sensitive *Staphylococcus* but presence of *mecA* gene

S. No.	District	No. of Isolates	Isolates having Oxacillin sensitive but *mecA* gene present	Oxacillin sensitive but *mecA* gene present
1.	Gaya	40	G-9(C), G-13(SC), G-24(SC),	07.50% (03/40)
2.	Patna	44	P-15(C), P-31(SC), P-39(SC), P-43(SC), P-44(C).	11.36% (05/44)
3.	Vaishali	48	V-17(C), V-22(C), V-28(SC), V-41(SC), V-44(SC), V-46(SC).	12.50% (06/48)
4.	Sitamarhi	41	S-19(SC), S-25(C), S-41(SC), S-45(C).	09.76% (04/41)
Total		173	------------	10.40% (18/173)

C- Clinical mastitis; SC- Subclinical mastitis

Table.14. ABST profile of *Staphylococcus* isolates from mastitic milk sample of Bihar

S. No.	Antibiotic	Number of isolates	Resistant (%)	Intermediate Resistant (%)	Sensitive (%)
1.	**OX**	173	62.42 (108/173)	00.00 (00/173)	37.57 (65/173)
2.	**CX**	173	53.76 (93/173)	00.00 (00/41)	46.24 (80/173)
3.	**CIP**	173	13.29 (23/173)	08.67 (15/173)	78.03 (135/173)
4.	**E**	173	38.15 (66/173)	14.45 (25/173)	47.40 (82/173)
5.	**TEI**	173	07.32 (03/173)	19.51 (28/173)	73.17 (142/173)
6.	**TE**	173	01.73 (19/173)	04.62 (08/173)	84.39 (146/173)
7.	**RIF**	173	47.40 (82/173)	14.45 (25/173)	38.15 (66/173)
8.	**CD**	173	29.48 (51/173)	23.70 (41/173)	46.82 (81/173)
9.	**GEN**	173	31.79 (55/173)	00.00 (00/41)	68.21 (118/173)

Table.15. MDR *Staphylococcus* isolates from mastitic milk sample of Bihar

S. No.	District	No. of Isolates	Multidrug resistant Isolates	Multidrug resistant
1.	Gaya	40	G-1(C), G-3(C), G-4(C), G-6(C), G-7(C), G-8(C), G-10(C), G-14(C), G-15(SC), G-16(SC), G-17(SC), G-20(SC), G-21(SC), G-24(SC), G-29(SC), G-31(SC), G-32(SC), G-33(SC), G-34(SC), G-37(SC), G-40(SC), G-42(SC), G-43(SC), G-46(SC).	60.00% (24/40)
2.	Patna	44	P-2(C), P-4(SC), P-7(SC), P-8(SC), P-9(SC), P-10(SC), P-11(SC), P-12(SC), P-13(C), P-16(SC), P-17(SC), P-18(C), P-19(C), P-20(C), P-22(SC), P-24(SC), P-28(SC), P-29(SC), P-30(C), P-31(SC), P-32(SC), P-33(SC), P-34(SC), P-36(SC), P-40(C), P-42(SC), P-45(SC), P-48(SC), P-49(SC).	65.91% (29/44)
3.	Vaishali	48	V-1(C), V-3(SC), V-4(SC), V-6(SC), V-7(SC), V-9(C), V-10(C), V-12(C), V-13(C), V-16(C), V-17(C), V-19(SC), V-23(SC), V-24(SC), V-25(SC), V-29(SC), V-33(SC), V-37(SC), V-38(SC), V-39(SC), V-40(SC), V-42(SC), V-45(SC), V-46(SC).	50.00% (24/48)
4.	Sitamarhi	41	S-1(C), S-5(C), S-8(C), S-9(SC), S-13(C), S-22(SC), S-27(SC), S-30(C), S-31(SC), S-33(C), S-36(SC), S-38(SC), S-41(SC), S-45(C), S-49(SC), S-50(C).	39.02% (16/41)
Total		173	------------	53.76% (93/173)

C- Clinical mastitis; SC- Subclinical mastitis

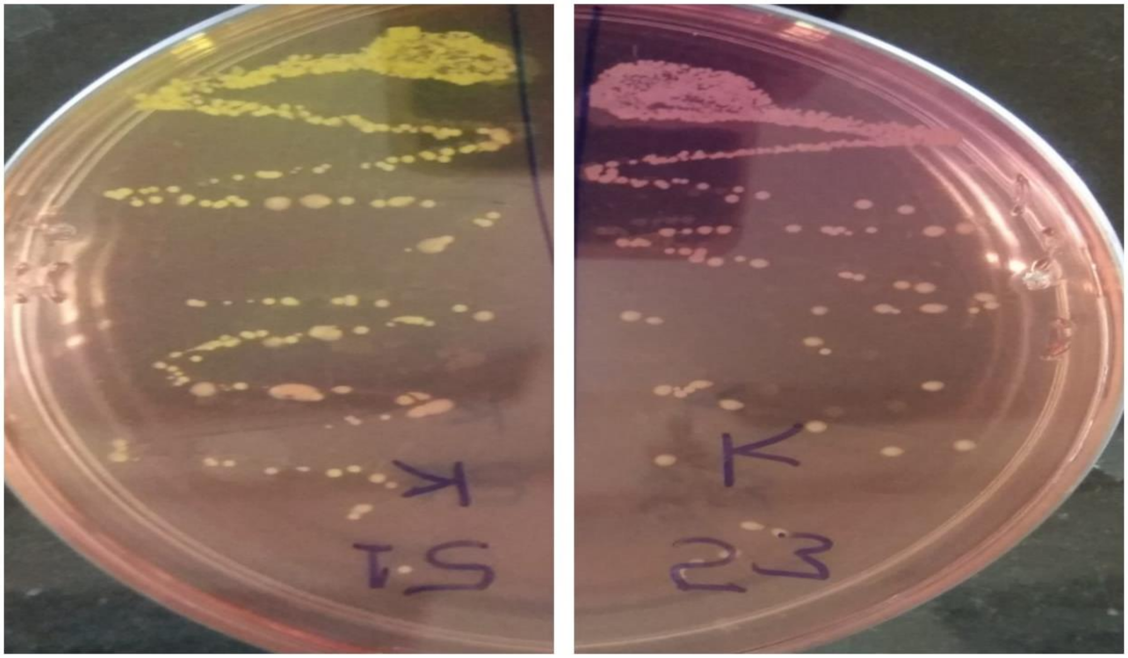


Fig.1. Typical colonies of *Staphylococcus* (*S. aureus* and other than aureus) on Mannitol Salt agar.

Fig. 2. Catalase test of *Staphylococcus aureus* isolate
A: Test Positive B: Test Negative

Fig.3. Tube coagulase test of *Staphylococcus aureus* isolate using human plasma A: Test Negative B: Test Positive

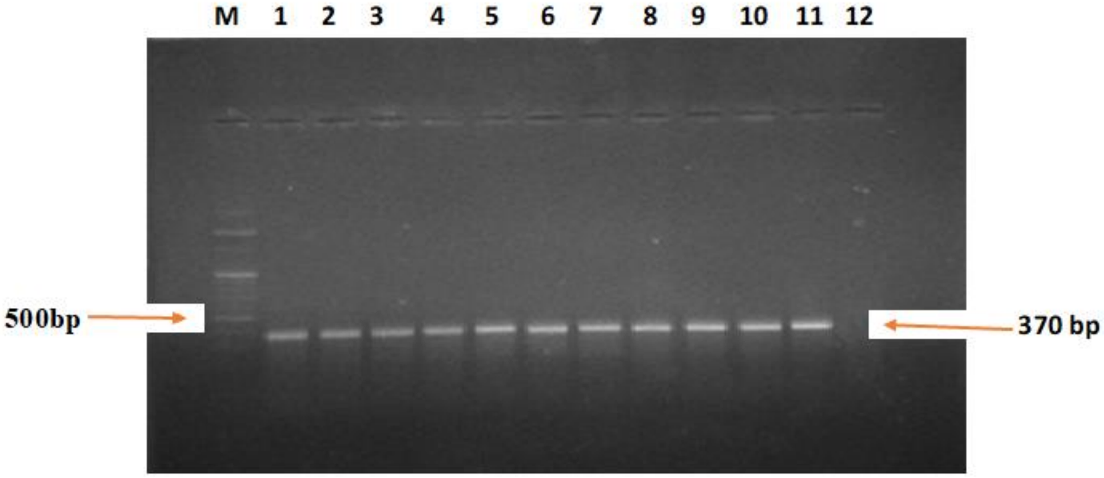


Fig.4. PCR amplification of *tstaG* gene of *Staphylococcus* genus

M: Gene ruler 100 bp DNA ladder

L1: Positive control

L2 – L11: Positive amplicons of *Staphylococcus* genus

L12: Negative control

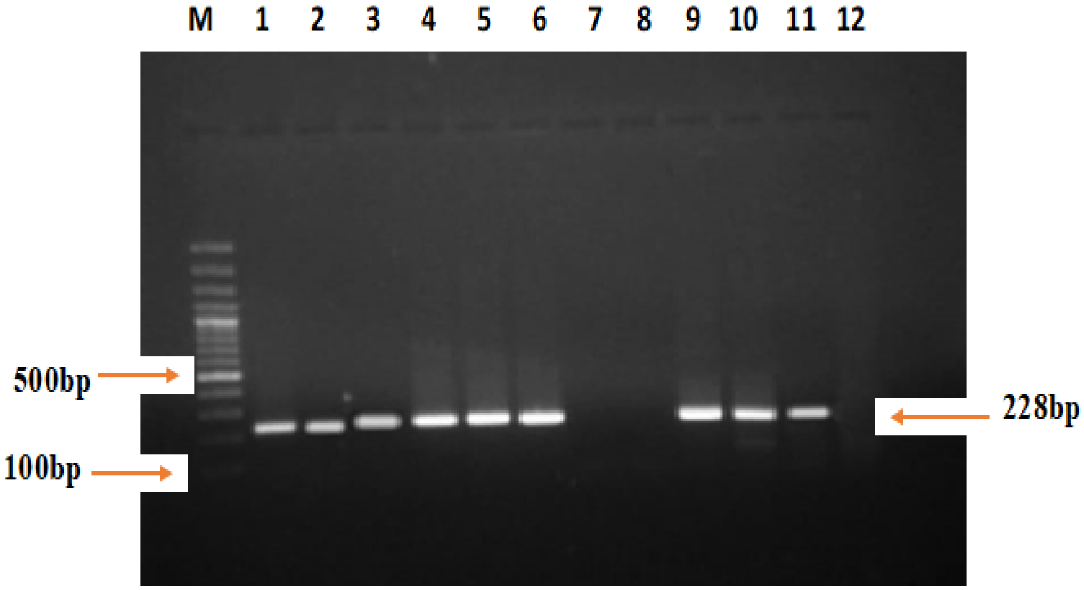


Fig.5. PCR amplification of *16S rRNA* gene of *Staphylococcus aureus*

M: Gene ruler 100 bp DNA ladder

L1: Positive control

L2 – L6: Positive amplicons of *Staphylococcus aureus*

L9 – L11: Positive amplicons of *Staphylococcus aureus*

L7 & L8: Negative amplicons of *Staphylococcus aureus*

L12: Negative control

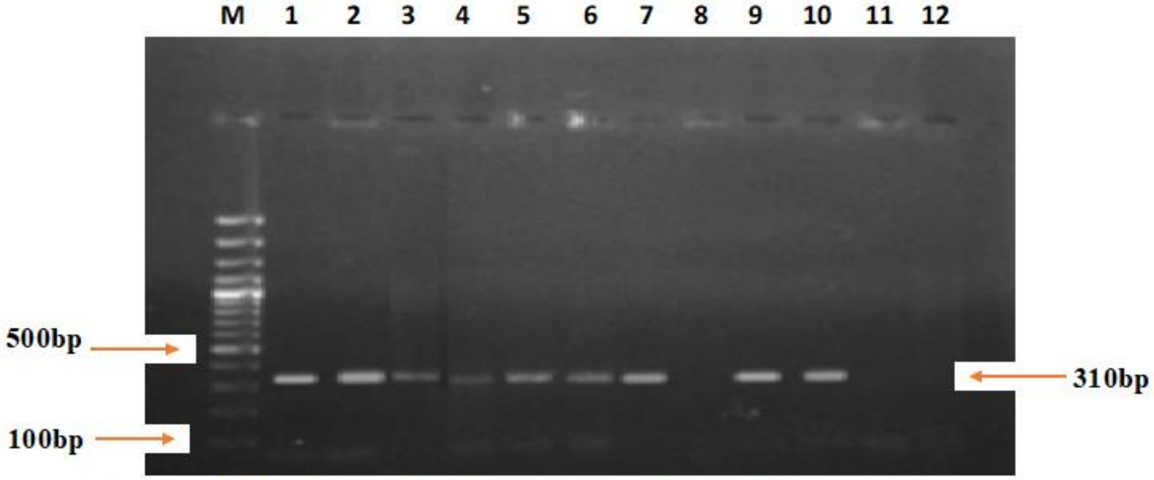


Fig.6. PCR amplification of *mecA* gene of *Staphylococcus*

M: Gene ruler 100 bp DNA ladder

L1: Positive control

L2 – L7: Positive amplicons of *mecA* gene

L9 – L10: Positive amplicons of *mecA* gene

L8 & L11: Negative amplicons of *mecA* gene

L12: Negative control

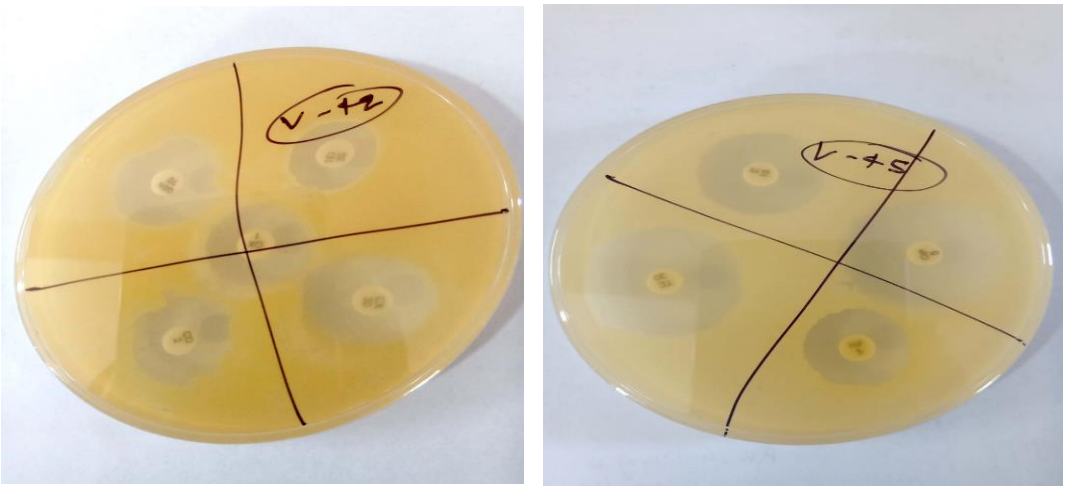


Fig.7. ABST of MRSA isolates on Mueller-Hinton agar

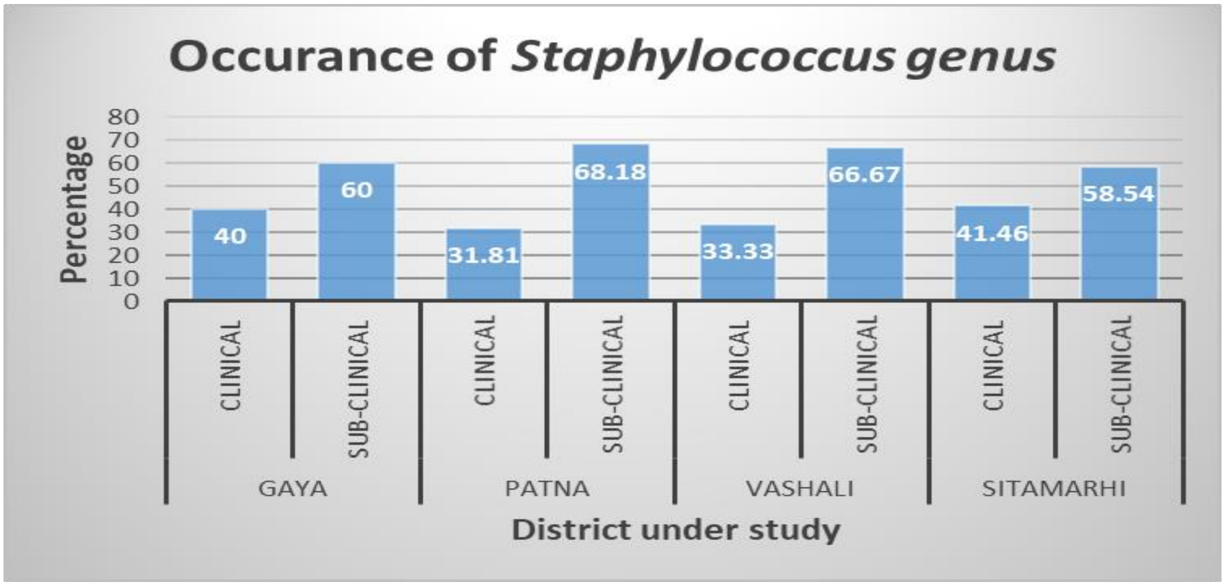


Fig.8. Occurrence of *Staphylococcus* genus in Clinical and subclinical mastitic milk sample

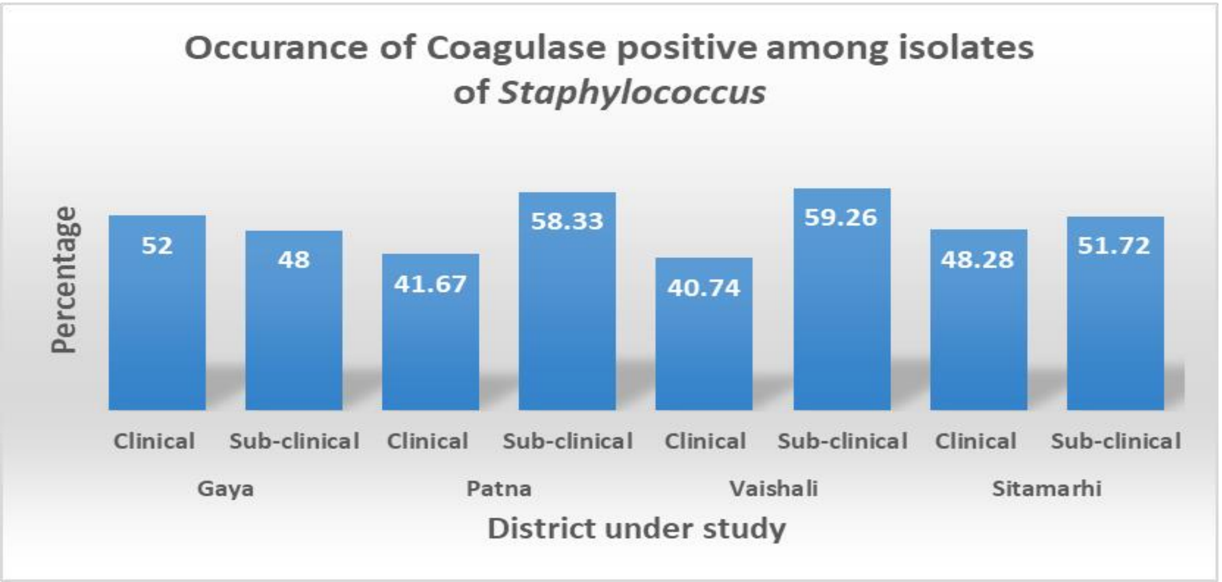


Fig.9. Occurrence of CoPS in Clinical and subclinical mastitic milk sample

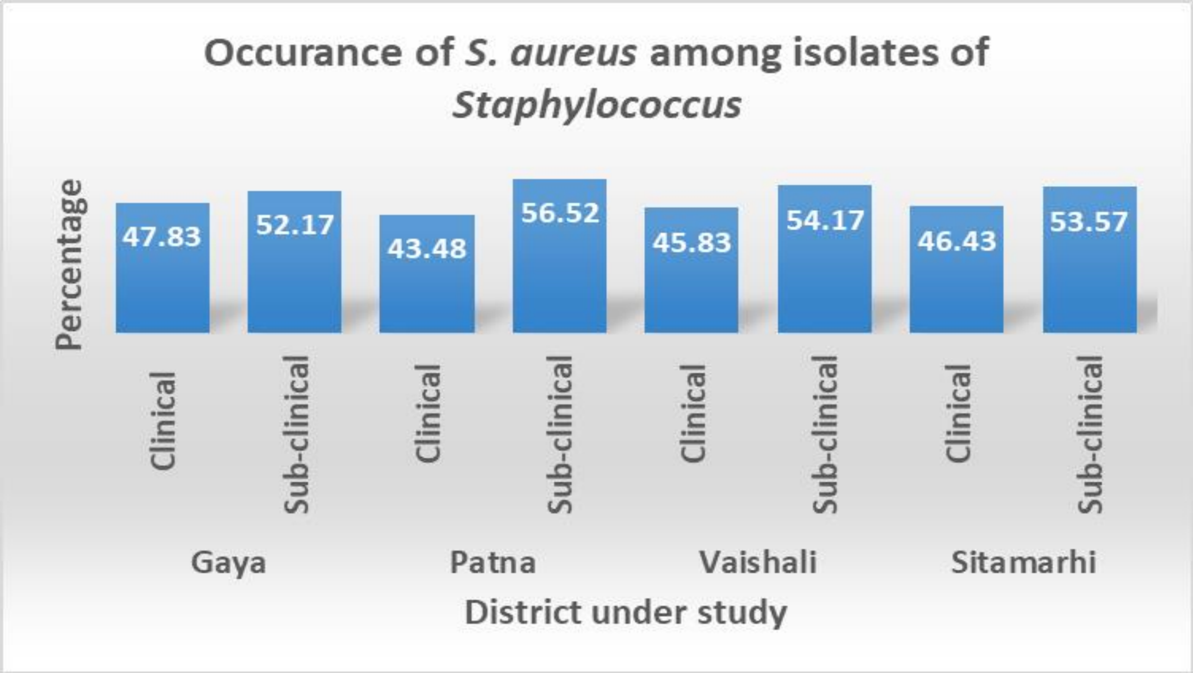


Fig.10. Occurrence of *S. aureus* in Clinical and subclinical mastitic milk sample

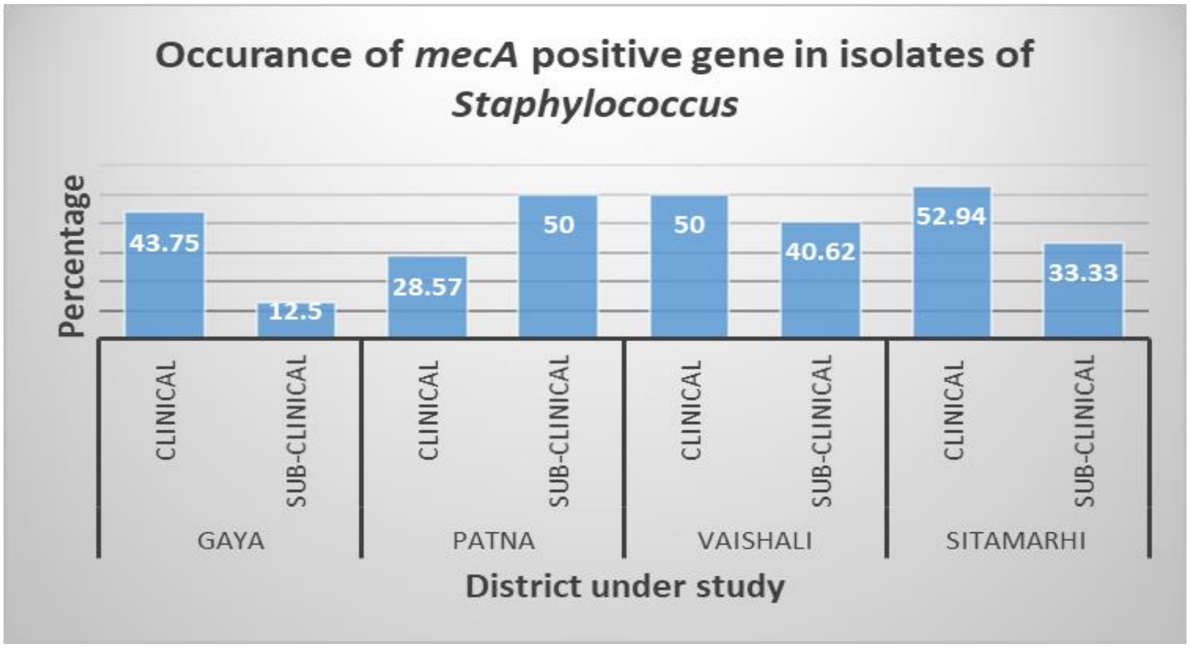


Fig.11. Occurrence of MRS in Clinical and subclinical mastitic milk sample

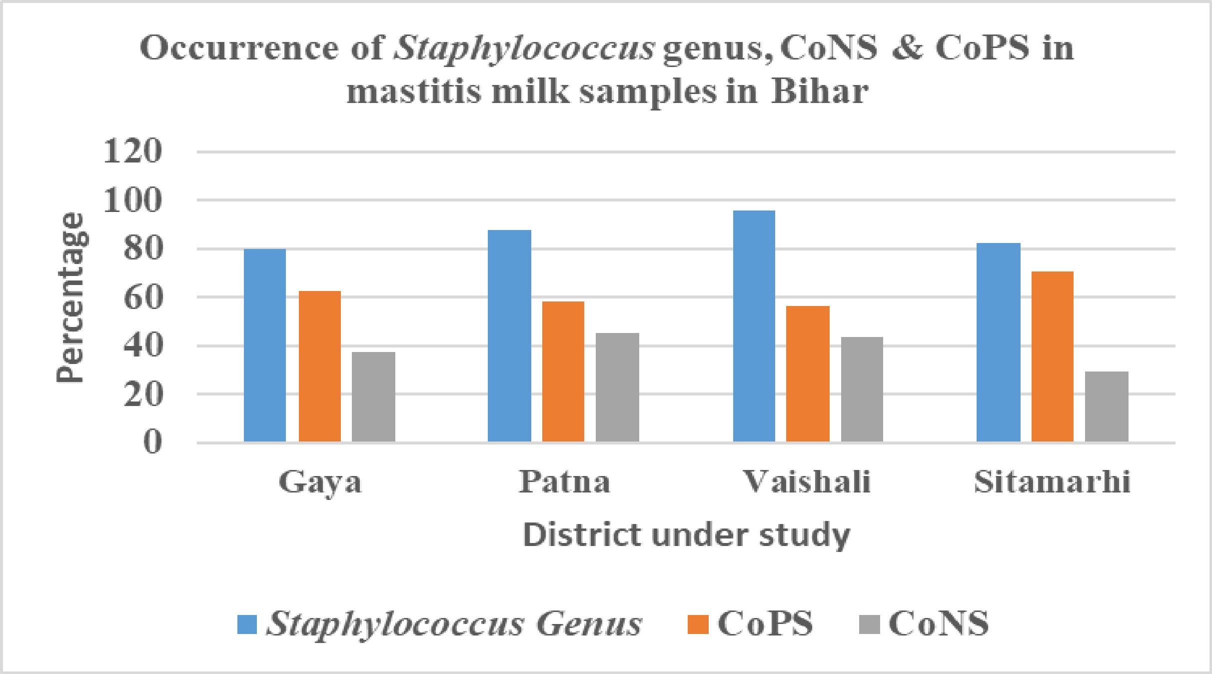


Fig.12. Occurrence of *Staphylococcus,* CoPS and CoNS in mastitic milk samples in Bihar

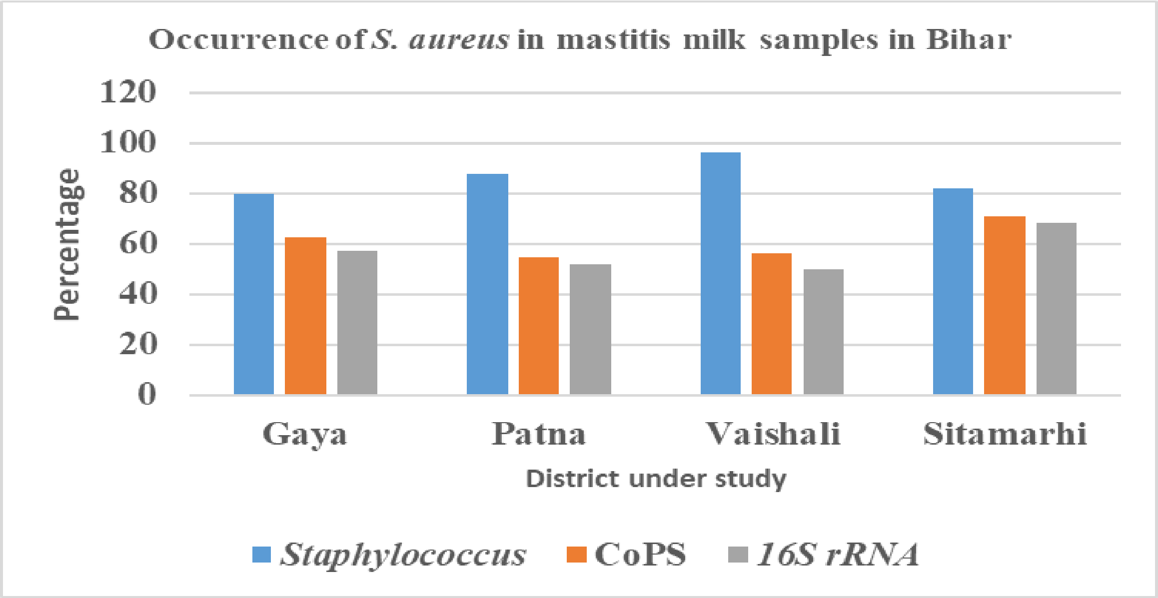


Fig.13. Occurrence of *Staphylococcus aureus* in mastitic milk samples in Bihar

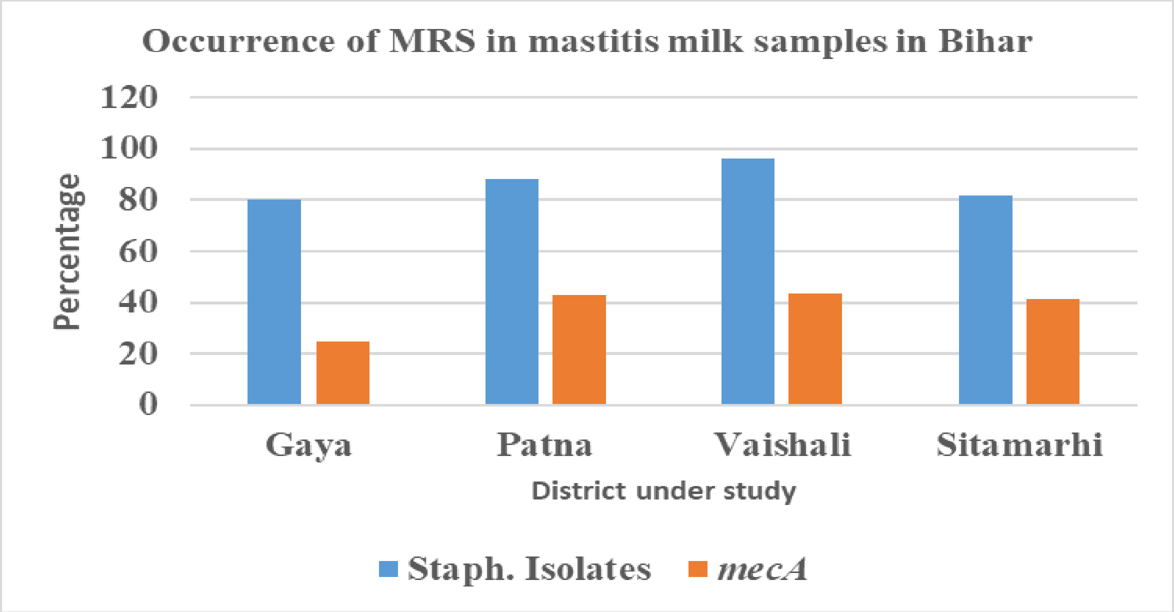
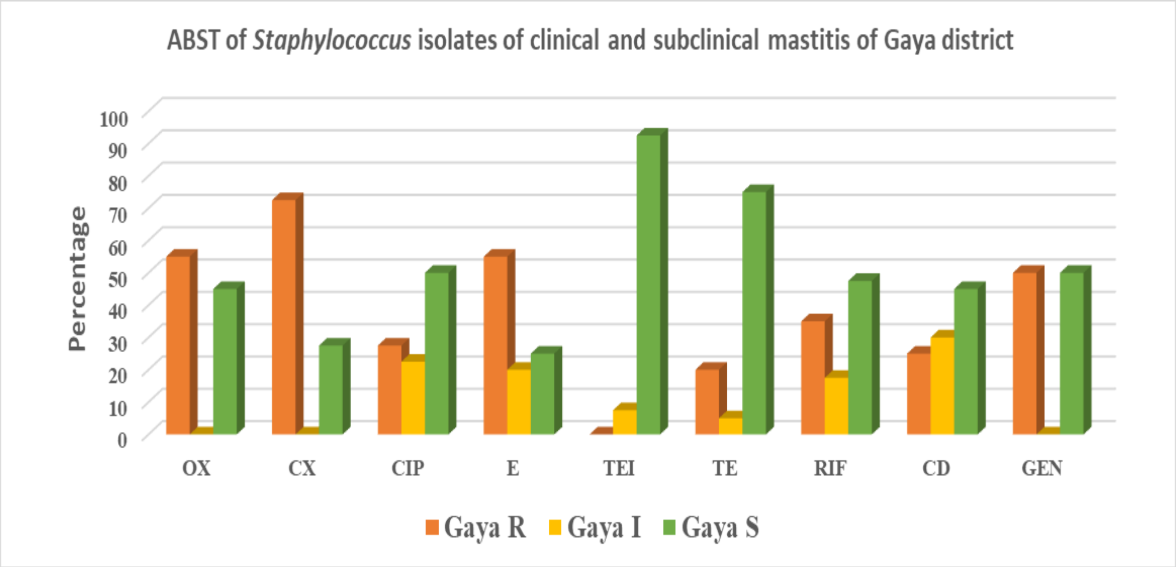
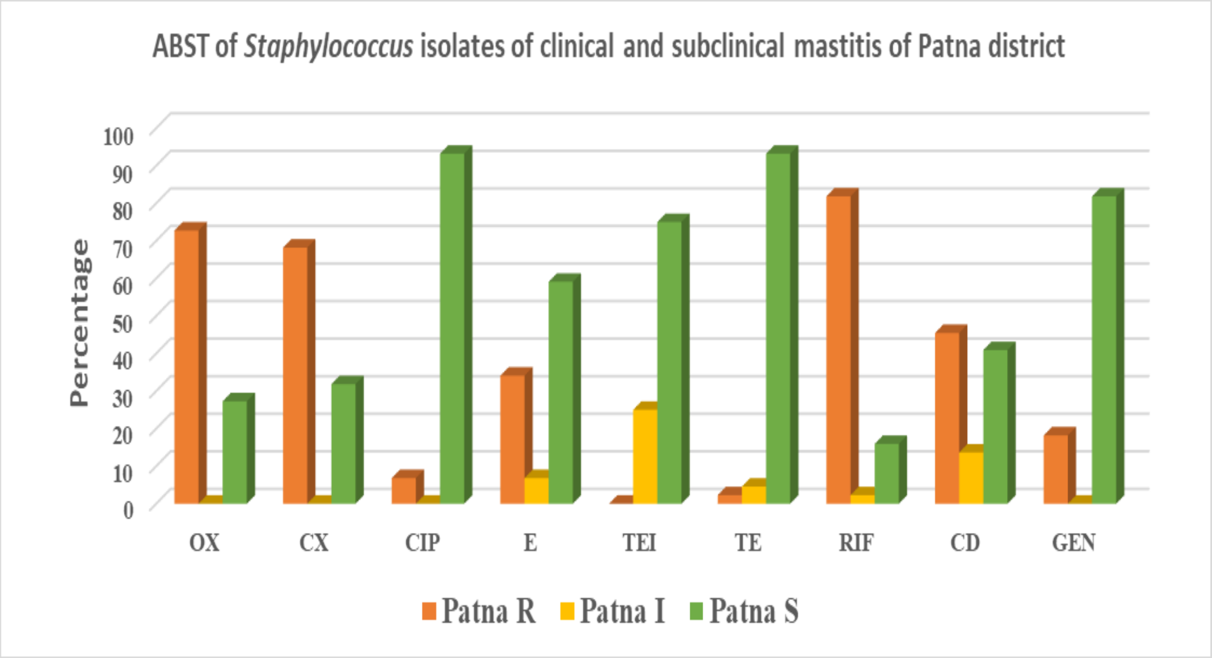


Fig.14. Occurrence of MRS in mastitic milk samples in Bihar



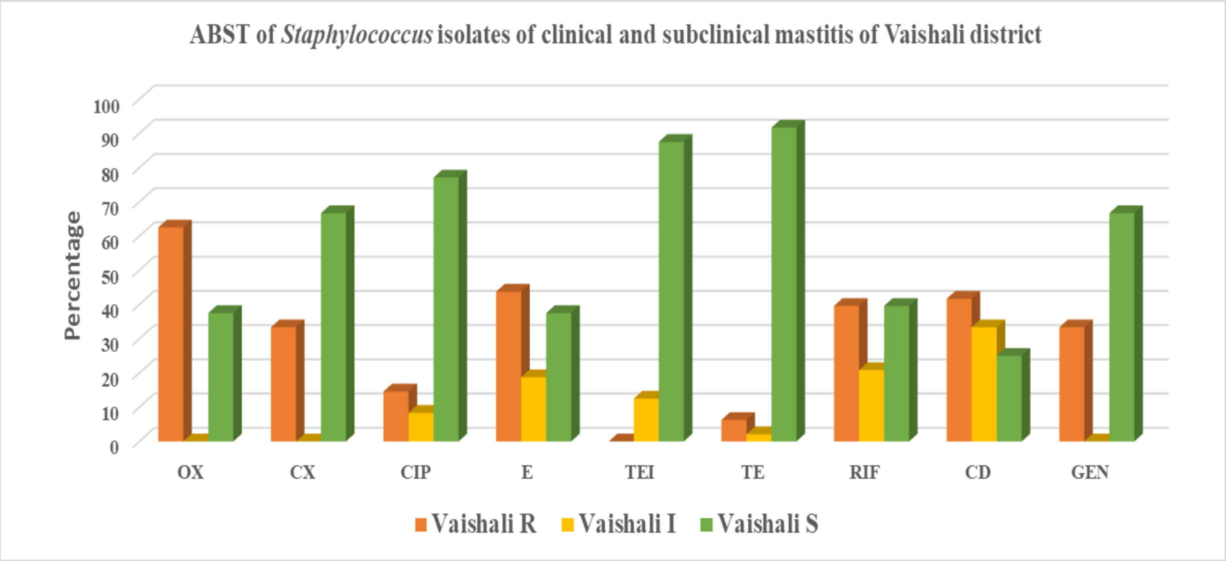
OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.15. Antibiotic sensitivity pattern of *Staphylococcus* isolates of clinical and subclinical mastitic milk sample of Gaya district



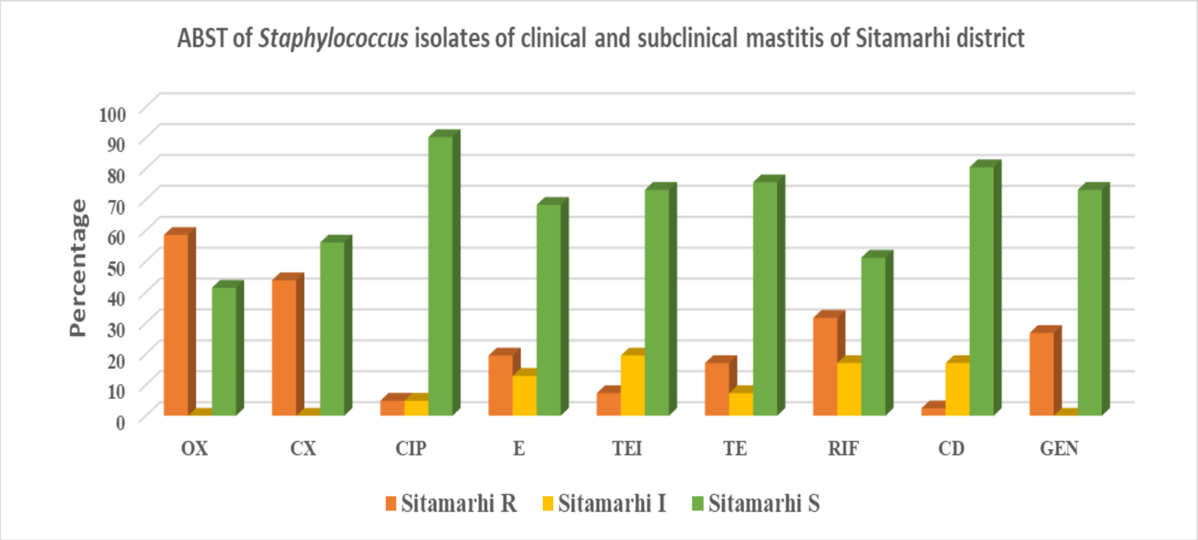
OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.16. Antibiotic sensitivity pattern of *Staphylococcus* isolates of clinical and subclinical mastitic milk sample of Patna district



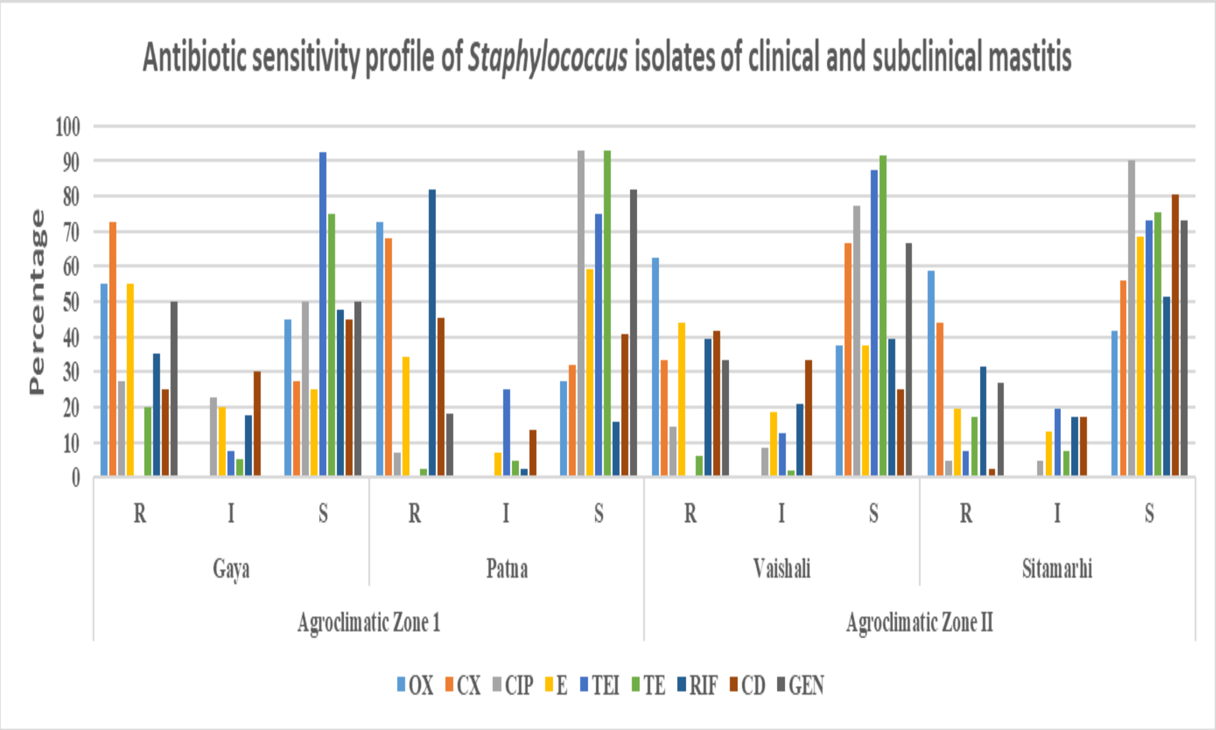
OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.17. Antibiotic sensitivity pattern of *Staphylococcus* isolates of clinical and subclinical mastitic milk sample of Vaishali district



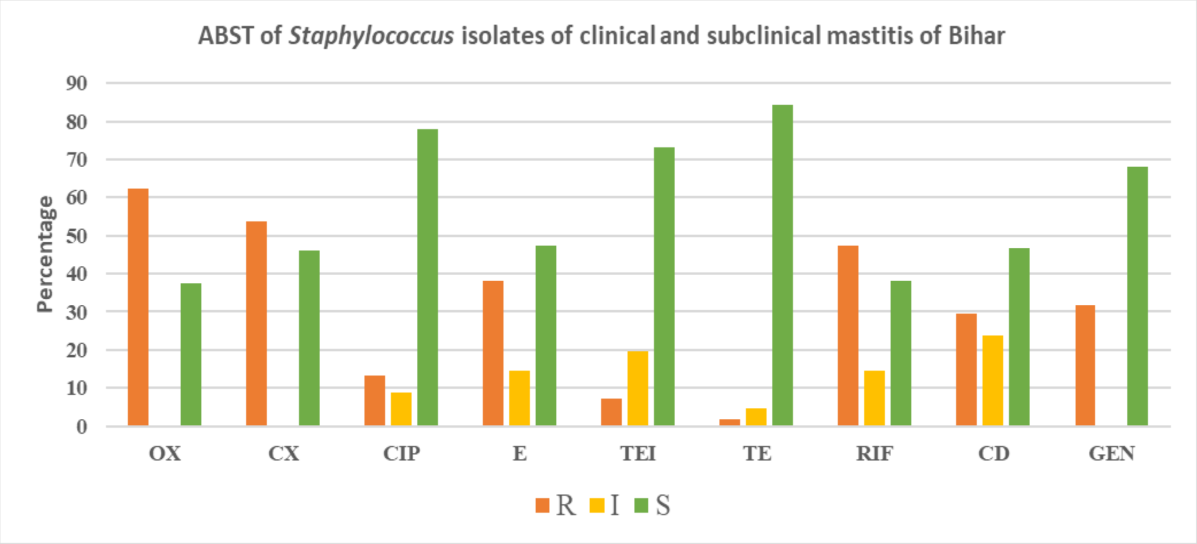
OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.18. Antibiotic sensitivity pattern of *Staphylococcus* isolates of clinical and subclinical mastitic milk sample of Sitamarhi district



OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.19. Antibiotic sensitivity pattern of *Staphylococcus* isolates of clinical and subclinical mastitic milk sample of Bihar



OX: Oxacillin, CIP: Ciprofloxacin, TEI: Teicoplanin, CX: Cefoxitin, E: Erythromycin, RIF: Rifampicin, CD: Clindamycin, GEN: Gentamicin, TE: Tetracycline.

Fig.20. Antibiotic sensitivity pattern of *Staphylococcus* isolates of mastitic milk sample of Bihar

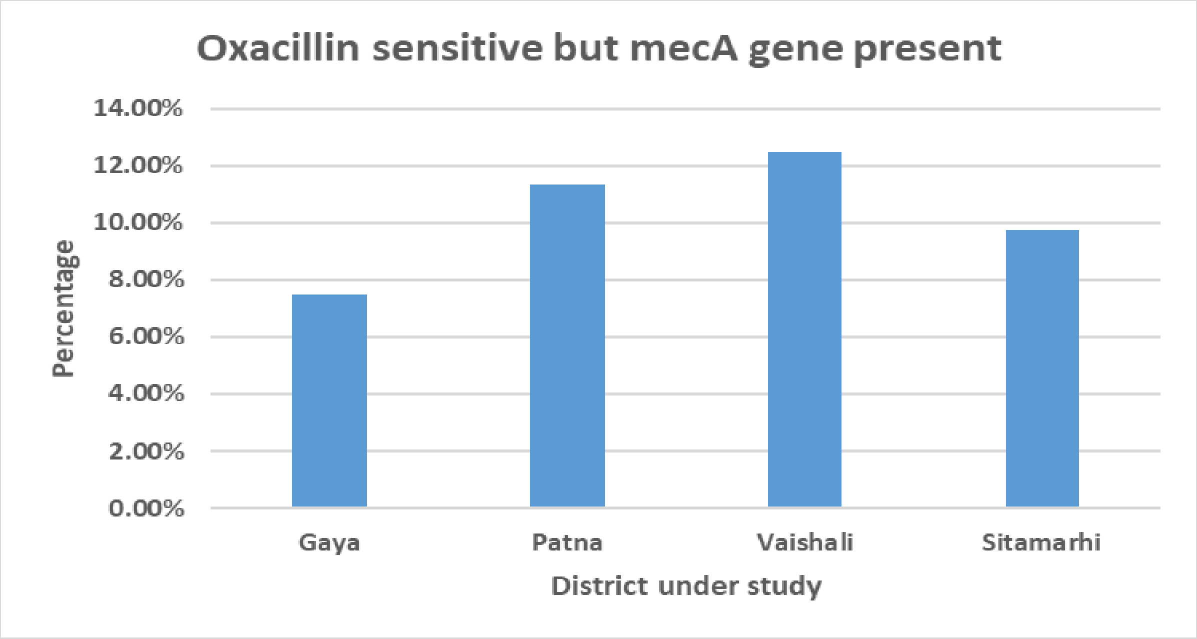


Fig.21. Occurrence of phenotypically Oxacillin sensitive *Staphylococcus* but presence of *mecA* gene

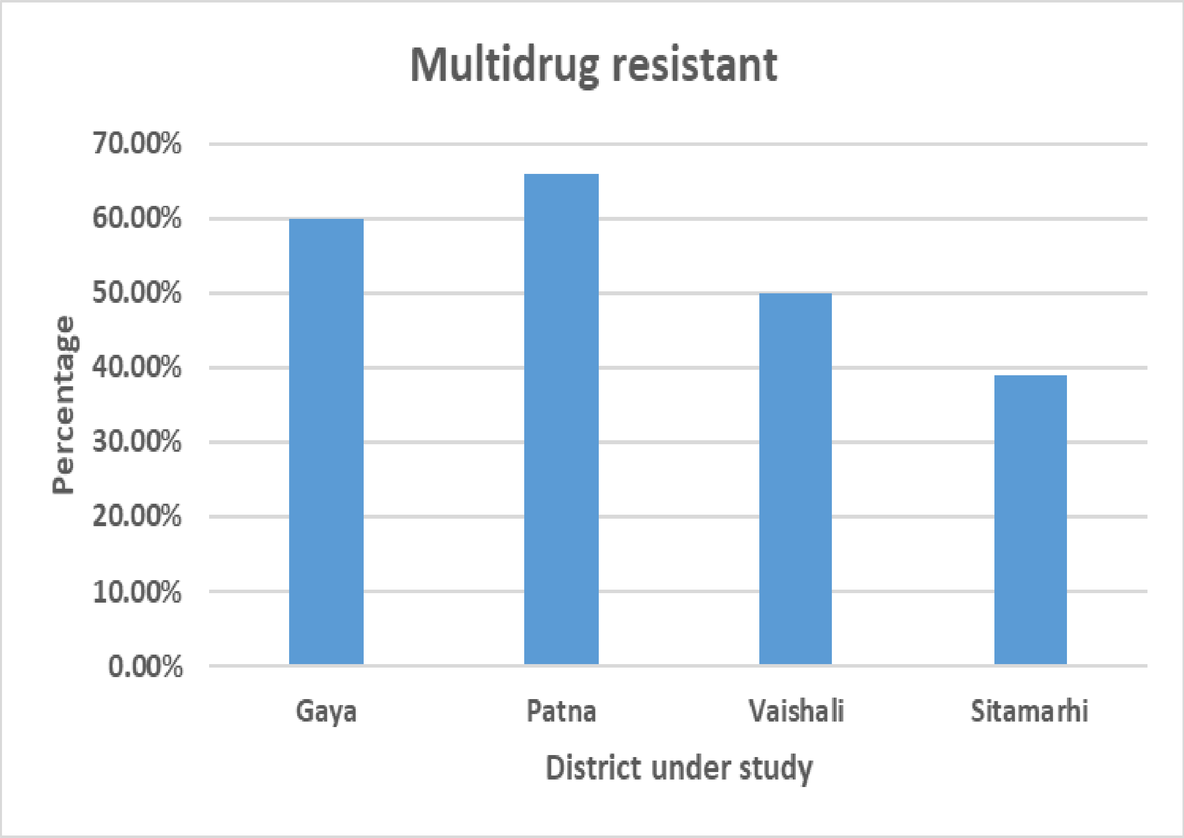
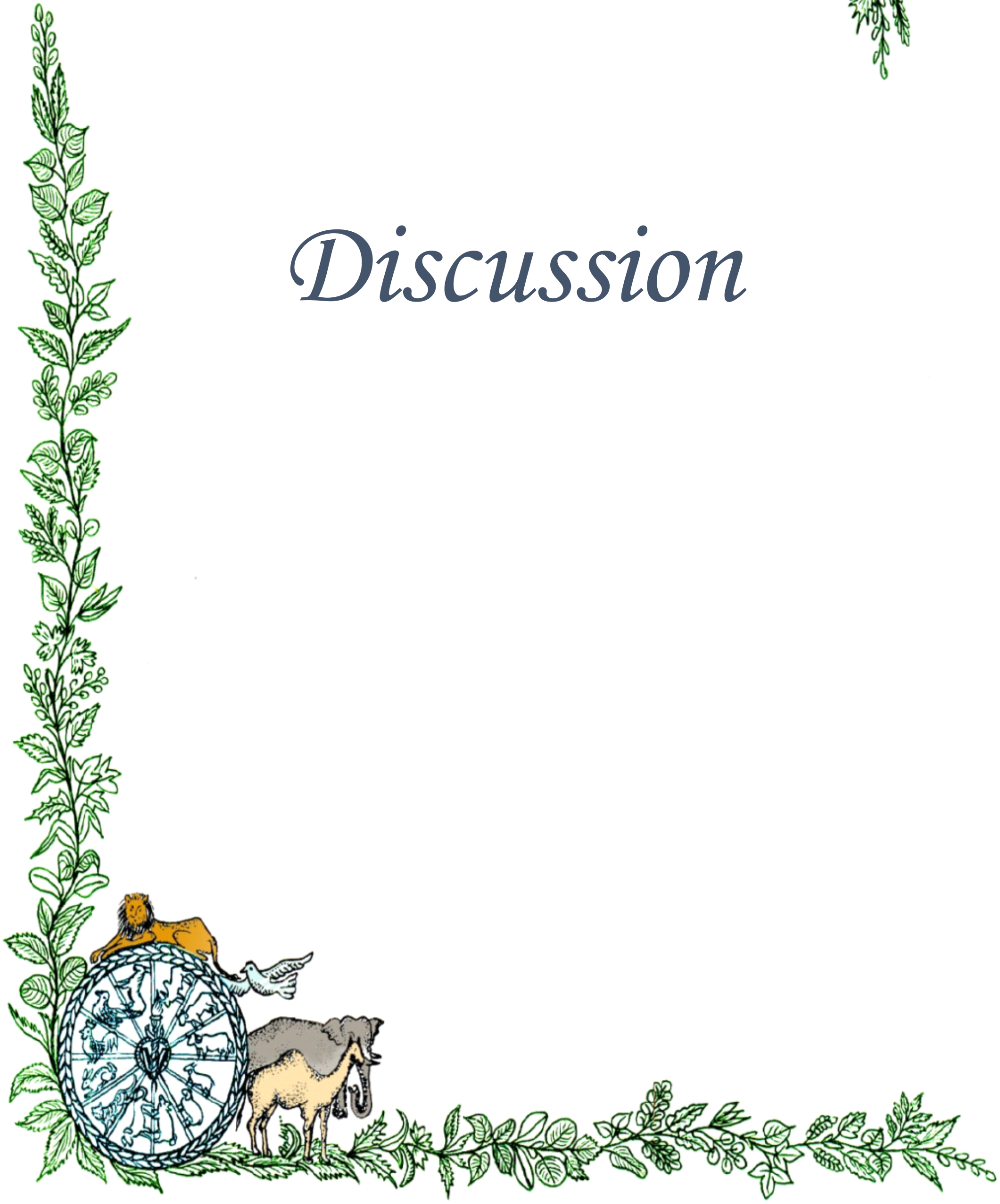


Fig.22. MDR *Staphylococcus* isolates from mastitic milk sample of Bihar

Discussion



5

DISCUSSION

Staphylococcus are Gram-positive facultative anaerobe, catalase-positive cocci of the family Micrococcaceae and considered as the most important microorganism causing mastitis. Among Genus *Staphylococcus*, the species *S. aureus* is responsible for about one-third of cases of clinical and subclinical mastitis (Topuzoglu *et al*., 2015; Li *et al*., 2017) and is considered as economically important disease of the dairy industry worldwide.

The focus of attention in the present study was to evaluate the involvement of *Staphylococcus* as well as *S. aureus* in bovine clinical and subclinical mastitis and to identify the Methicillin resistant *Staphylococcus* genotypically along with antibiotics resistance profile. Primarily these organisms were prioritized because of the emergence of multidrug resistant *Staphylococcus* as a result of indiscriminate use of antibiotics in veterinary as well as human medicine which is a major public health concern.

The first MRSA was reported in England in 1961, soon after the introduction of β-lactamase resistant Penicillin-Methicillin in human medicine (Livermore, 2000). Since then, the frequency of reporting of MRSA has increased significantly every year, worldwide (Pinho *et al.,* 2001; Grundmann *et al*., 2006). The reports of occurrence of MRSA also raised the concern on the role of animals particularly livestock in MRSA epidemiology. The spatial distribution of MRSA showed the interspecies transmission and colonization in different populations. There are several reports which revealed that this pathogen is worldwide prevalent in cattle, dogs, cats, pigs, horses and poultry (Boag *et al*., 2004; Leonard and Markey, 2008; Morgan, 2008).

Coagulase negative staphylococci (CoNS) have often been considered as minor udder pathogens, causing relatively negligible udder health problems. However, CoNS infections may cause substantial herd problems due to high prevalence of sub-clinical and/or clinical mastitis (Wilson *et al.*, 1997). CoNS are now, a frequent cause of mastitis in modern dairy herds in many countries. They have even become the predominant pathogen isolated from milk samples from cows with mastitis. CoNS are most frequently isolated from quarters with subclinical or clinical mastitis with only mild clinical signs. In a nationwide survey in Finland, CoNS were isolated from 17 per cent of the quarters (Pitkala *et al*., 2004).

In present study, 200 bovine milk samples collected from different clinical and subclinical mastitic animals, based on presence of clinical signs and finding of onsite screening of animals with CMT, respectively. All the 200 clinical and subclinical mastitic milk samples collected, comprises of 50 samples each from the four districts of two agroclimatic zone of Bihar viz., Gaya, Patna, Vaishali and Sitamarhi. The samples collected from Gaya district comprised of 16 clinical and 34 subclinical mastitic milk samples. Similarly, samples from Patna district comprised of 14 clinical and 36 subclinical, Vaishali district comprised of 16 clinical and 34 subclinical, and Patna district comprised of 17 clinical and 33 subclinical mastitic milk (Table-1).

All samples were processed for isolation of *Staphylococus* by conventional method, including enrichment in BHI broth and selective plating on MS agar with 18-24 h incubation at 37^{0}C followed by biochemical and molecular confirmation targeting genus specific *tstaG* gene PCR. Out of 200 samples processed, 86.50 % of clinical and subclinical milk samples were found to harbor *Staphylococcus*. The two districts of Agroclimatic zone I of Bihar showed the distribution of *Staphylococcus* among 80.00% and 88.00% samples, respectively from Gaya and Patna district. While the district of Agroclimatic zone II showed a distribution of *Staphylococcus* among 82.00% and 96.00%, samples of Sitamarhi and Vaishali district, respectively. This finding indicates the *Staphylococcus* as a major cause of clinical & subclinical mastitis in the region (Table-4, Fig.-4 & 8).

Among *Staphylococcus* isolates from Gaya and Patna district (Agroclimatic zone 1), 40.00% and 31.81%, respectively belonged to clinical mastitis while 60.00% and 68.18 % from subclinical mastitis. Similarly, from Sitamarhi and Vaishali (Agroclimatic zone II), 41.46% and 33.33% as well as 58.54% and 66.67% of isolates belonged to clinical and subclinical mastitis, respectively (Table-4, Fig.-4 & 8). The present findings indicate occurrence of *Staphylococcus* in sub-clinical mastitis cases are more as compared to clinical mastitis which may predisposes animals for clinical mastitis. The lower percentage of *Staphylococcus* in clinical mastitis cases may also be due to use of antibiotic for the treatment of overt cases of mastitis in animals, where as in subclinical mastitis cases no therapy is being given to animals, due to lack of overt signs and symptoms which probably predisposes the animals to develop clinical mastitis.

The finding of the present study was in concordance with the finding of Ayano *et al*., (2013) who performed a cross-sectional study to determine the prevalence of subclinical mastitis in lactating dairy cows in Holeta district, Ethiopia. They examined 224 subclinical mastitis milk samples and reported that 81.7% of the samples were positive for bacterial isolation with predominance (13.8%) of *S. aureus*. Various other workers reported quite lower prevalence (70% to 43%) of *Staphylococcus* among clinical mastitic and sub clinical mastitic animals from a range of 70% to 43% including 60.90% (Datta and Rangenkar, 2001),58.55% (Mehmeti et al., 2016) 54.00% **(**Sharma and Prasad, 2003;Thakur *et al*., 2018),45% (Krishnamoorthy *et al*., 2017) as well as 42.55% (Verma *et al*., 2018) from different part of India and world.

In this study, we screened all isolates of *Staphylococcus* by tube coagulase test using human plasma to determine the distribution of coagulase positive and negative staphylococci as indicated in Table-4. The district wise distribution of *S. aureus* is also indicated in the Table-4 and section 4.5 and 4.6 of the result. The finding of the present study is in accordance with the finding of study performed in

South India by Preethirani *et al*., (2015). They screened 190 milk sample and isolated the coagulase-negative staphylococci (64.8%) and *S. aureus* (7.3%). The higher percent of *S. aureus* in the present study may be due to consideration of clinical and subclinical mastitis cases only in the present study. Mausam *et al*., (2016) reported that the prevalence of *S. aureus* in 56.67% in bovine milk samples in Bihar. However, in contrast to the present finding a lower prevalence of 26% to 38.66 % coagulase positive *Staphylococcus* have been reported by Pankaj *et al*., (2012) in Hisar, Mehmeti *et al.,* (2016) in Kosovo, Thakur et al., (2018) in Bihar.

The overall occurrence and district wise distribution of *S. aureus* by PCR targeting *16S rRNA* is indicated in the Table 4 and section 4.6 of the result. The results of the present study about the occurrence of higher percentage of CoPS indicates the major role of coagulase positive S*taphylococcus aureus* in subclinical mastitis. Such findings about involvement of S*taphylococcus aureus* in subclinical and clinical mastitis were reported by Mausam *et al.* 2016 indicating prevalence of *S. aureus* as 56.67%. In contrast to the finding of present study lower prevalence of 7.3 to 47.10% was also reported by various workers (Mehmeti *et al*., 2016; Palanival *et al*., 2008; Barrett *et al*., 2005; Preethirani *et al*., 2015; Hamid *et al*., 2017; Tel *et al*., 2009; Sevinti and Şahin, 2009; Haque *et al*., 2013; Mekibib *et al.*, 2010; Khan and Muhammad, 2005**)** while slight higher prevalence of 58% was reported by Harini and Sumathi (2011).

It is well established mechanism that Methicillin resistance in *Staphylococcus* is due expression of PBP2a, encoded by *mec*A gene, which are highly conserved in *Staphylococcal* species, so it is possible to detect methicillin resistance by detection of these genes (Hartman and Tomasz, 1984). In the present study PCR based detection of MRS targeting *mecA* gene was standardized. The distribution of *mecA* gene was found in 38.73% of *Staphylococcus* isolates (Table 4). The occurrence of higher percentage of mecA gene in the district under study indicates indiscriminate use of antibiotics resulting into development of resistant organism.

Most of the study were performed by various workers to determine the distribution of *mecA* gene among *S. aureus* however, this study was performed to detect *mecA* distribution among *Staphylococcus* isolates from clinical and subclinical mastitic milk samples. Hamid *et al.,* (2017) reported the occurrence of 16.6% Methicillin-resistant *S. aureus* (MRSA) in bovine mastitis in Jammu and Kashmir. Vanderhaeghen *et al.,* (2010) reported a distribution of 9.3 % MRSA in clinical and subclinical mastitis in Belgian cows. Mausam *et al.,* (2016) reported 29.33% occurrence of MRSA in bovine milk samples of Bihar which corroborate with the present findings indicating emergence of MRS as an important agent in mastitis. In our study we found *Staphylococcus* which were oxacillin susceptible and *mecA* positive, now called as OS-MRS. The overall occurrence of OS-MRS in the district under the study was 10.40% while the district wise distribution of OS-MRS show 9.7% to 12.5% occurrence (Table-13, Fig-21). Our finding on OS-MRS is in concordance to the report of Mahato *et al.,* (2017) which reported OS-MRS from Telangana & Tamilnadu (India) for the first time (11.9% in India). However, such isolates were reported for the first time from China (Li *et al.,* 2015) causing bovine mastitis. The current finding of occurrence of OS-MRS in clinical/subclinical mastitis indicates the need to include both the phenotypic and genetic test to screen the MRS accurately. Further, such isolates may result in the treatment failure with beta lactam antibiotics and can develop into more resistant population. (Mahto *et al*., 2017)

The presence of MRSA & MRS in animals and humans is of veterinary and public health concern in many countries. MRS & MRSA infected cattle may act as reservoir and later transmit the infections to other animals and humans (Manian *et al.*, 2003; Spoor *et al.*, 2013). It has been reported as transmissible diseases of zoonotic and of humanotic importance. Animal to human transmission occurs through direct contact, environmental contamination and through handling of infected animal's product (Nunang and Young, 2007) whereas human to animal transmission is still unclear (Weese, 2010). However, some authors have reported bidirectional transmission of *Staphylococcus, S. aureus* & MRSA (Juhasz-Kaszanyitzky, 2007; Price *et al.*, 2012). *Staphylococcus, S. aureus* & MRSA colonization in cattle may be an occupational risk to the people in close contact with veterinarians, farmers, milkers and people working at slaughter houses (Juhasz-Kaszanyitzky, 2007; Paterson *et al.*, 2012). Transmission of animal *Staphylococcus, S. aureus* & MRSA to occupational workers personnel have been reported and it is more common among large animal handler than small animal (O'Mahony, 2005; Hanselman *et al.*, 2006; Wulf *et al.*, 2008).

Antibiotic resistance pattern provides vital information that can be used in evolving a strategy for prevention and treatment of mastitis in cattle and buffaloes. In a particular geographical area antibiotic-susceptibility/resistance in bacterial isolates varies due to different strategies and policies adopted in containment programs, availability and usage patterns of antibiotics to treat mastitis. Therefore, isolates of *Staphylococcus* spp. were analyzed to find the status or information on the resistance patterns among the strains. The antibiogram provided the status of resistance in isolates. Higher resistance to the antimicrobials regularly used in veterinary practices are observed very frequently by various workers. (Sabour *et al.*, 2004; Moon *et al*., 2007; Ghatak *et al.* 2013; Bandyopadhyay *et al.* 2015; Sivakumar *et al.* 2019;). Susceptibility patterns can therefore be expected to return when the microbes are no longer exposed to the given antimicrobials.

In the present study of antibiotic susceptibility profile of all isolates of *Staphylococcus* were observed by disc diffusion method using 9 antibiotic discs viz Oxacillin, Ciprofloxacin, Teicoplanin, Cefoxitin, Erythromycin, Rifampicin, Clindamycin, Gentamicin and Tetracycline. These antibiotics were selected in the present study as they are used commonly for treatment of animals under field condition however teicoplanin was selected to monitor the sensitivity of the drug which is used as the last choice of drug for gram positive organisms in animals and human (Vimberg *et al*., 2017; Nakipoglu *et al*., 2016). The antibiogram study of isolates reveals the high resistance of *Staphylococcus* to Oxacillin (62.42%), Cefoxitin (53.76%), Rifampicin (47.4%) and Erythromycin (38.15) while moderate resistance observed against Gentamycin (31.79%) & Clindamycin (29.48%) and low resistance against Ciprofloxacin (13.29%), Teicoplanin (7.32%), Tetracycline (1.73%).

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Gaya district showed maximum resistance (72.5%) to Cefoxitin followed by a resistance of 55% to Oxacillin & Erythromycin, 50% to Gentamicin, and 35% to Rifampicin, while 20 to 28% resistant to Ciprofloxacin, Clindamycin and Tetracycline. However, none of the isolates were found resistant to Teicoplanin.

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Patna district showed maximum resistant (84.09%) to Rifampicin followed by 75%, 70.45%, 45.45% & 36.36% to Oxacillin, Cefoxitin, Clindamycin, Erythromycin respectively while 18.18%, 6.81% & 2.27% to Gentamicin, Ciprofloxacin and Tetracycline and none of the isolates were resistant to Teicoplanin (Fig.-14, Table-9 & 10).

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical bovine mastitic milk from Vaishali district showed maximum resistance of 62.50% to Oxacillin followed by 43.75% 41.66%, 39.58%, 33.33% & 33.33% to Erythromycin, Clindamycin, Rifampicin, Cefoxitin and Gentamicin respectively, while less than 14.58% to Ciprofloxacin and Tetracycline. and none of the isolates were resistant to Teicoplanin (Fig.-17, Table-9 &10).

The antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Sitamarhi district showed maximum resistance of 58.53% to Oxacillin followed by a resistance of 43.90%, 31.75% and 26.82% to Cefoxitin, Rifampicin and Gentamicin respectively while other showed less than 19.51% resistance to Erythromycin, Tetracycline, Teicoplanin, Ciprofloxacin and Clindamycin. The important finding in this region was the resistance of 7.31% isolates against Teicoplanin (Fig.-18 Table-11 & 12).

The antibiotic resistance profiling studied by other researches also showed a wide range of susceptibility profile. A study performed by Mahato *et al*. (2017)

reported a high resistance to Oxacillin (85.5%) and Cefoxitin (83.9%) and moderate resistance to Rifampicin (37.1%), Clindamycin (32.3%), Erythromycin (25.8%), and Tetracycline (20.9%). They also found that all strains were susceptible to Vancomycin, Teicoplanin and Linezolid. In other study Kumar *et al* (2011) reported a susceptibility of MRSA of 29.9% to Gentamicin, 26.2% to Ciprofloxacin while 22.22% to Clindamycin, Gentamicin, and Tetracycline. A study performed by Sahai *et al.,* (2014) showed that *S. aureus* isolates produced a susceptibility pattern of antibiotics showing that 100% sensitivity to Vancomycin. In contrast to earlier report by Mahato *et al*. (2017). The present study also demonstrates occurrence of oxacillin susceptible and *mecA* positive *Staphylococcus* which is supported by similar findings earlier report Mahato *et al*. (2017) from India and Li *et al.* (2015) from China. We also found occurrence of isolate such as OS-MRSA in our study which is the second report from mastitic animals in India. The study also demonstrated resistance to Rifampicin and Teicoplanin (Table-13, Fig-21). Teicoplanin is a glycopeptide antibiotic which is used as a last resort of antibiotic (Vimberg, *et al*., 2017; Nakipoglu *et al*., 2016). Therefore, occurrence of isolates resistant to Teicoplanin indicates alarming condition of antibiotic resistance in the state or the country. Although we have not studied the factors associated with occurrence of such resistant *Staphylococcus,* this may occur due to overuse of antibiotics or use of glycopeptide (avoparcin) as feed additive, resulting in development of Teicoplanin resistance (Wright, 2010; Nakipoglu *et al*., 2016)

Penicillin has been reported as the most common resistant antibiotic from various countries. Around one third to two third of bovine *S. aureus* isolates from different countries were reported resistance against penicillin (Devriese *et al*.,1972; Gentilini *et al*., 2002; De Oliveira *et al*., 2000; Erskine *et al*., 2002; Gianneechini *et al.*, 2002; Makovec and Ruegg, 2003; Tenhagen *et al*., 2006; Rabello *et al*., 2005). This has necessitated the use of penicillinase resistant antibiotic for the control of *Staphylococcus* in animals. In the present study we have observed low resistance of isolates against Tetracycline which may be due to the fact that being irritant nature of tetracycline it is not being used for treatment of mastitis (Makovec and Ruegg 2003) In addition, antimicrobials available for treating *S. aureus* mastitis in different countries likely to affect the panel of the tested substances, which tends to vary among studies. The observations of the present investigation are comparable with the earlier Indian studies (Parmar *et al*., 2006; Joshi and Gokhale, 2006; Awandkar *et al*., 2009).

Staphylococcus isolates resistant to more than two groups of antibiotics are considered as multidrug resistant (MDR) *staphylococcus* (Mahato *et al.,* 2017; Anderson *et al.* 2006; Cosgrove *et al*. 2003; Roberts *et al*. 2009; Ibrahim *et al*. 2000). On the basis of antibiogram study performed the occurrence of multidrug resistant (MDR) *staphylococcus* was 53.76% (93/173). The district wise distribution of MDR *Staphylococcus* among *Staphylococcus* isolates are indicated in Table 15. The mastitis milk samples of Gaya district showed a distribution of MDR *Staphylococcus* in 60.00% (24/40) isolates of *Staphylococcus*. Similarly, in Patna, Vaishali and Sitamarhi district MDR *Staphylococcus* were found in 65.91% (29/44), 50.00% (24/48) and 39.02% (16/41) isolates of mastitic milk samples, respectively. (Fig.-22). Previous studies shown variation in the resistance when compared with strains of different geographical regions. Such variations in antibiotic-resistance patterns in isolates of different regions shown could be due to diverse antibiotic policies and conditions (Gentilini *et al*., 2002; Erskine *et al*., 2002; Moon *et al*., 2007; Wang *et al*., 2008; Ochoa-Zarzosa *et al*., 2008; Turutoglu *et al*., 2009). Thus, from the present study it could be concluded that multidrug resistant *Staphylococcus* and *S. aureus* is widely prevalent in clinical and subclinical mastitic animals, that might become a serious threat.

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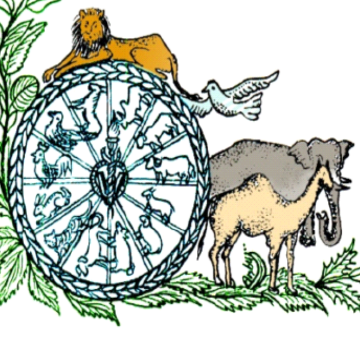
Summary and Conclusions



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SUMMARY AND CONCLUSIONS

This study was conducted to determine the occurrence and molecular characterization of *Staphylococcus, S. aureus,* MRS from clinical and subclinical mastitic milk samples and their antimicrobial susceptibility in Bihar. A total 200 milk samples from clinical and subclinical mastitic animals were collected *Staphylococcus, S. aureus* were identified by conventional bacterial culture techniques and polymerase chain reaction *tstaG*. which were further screened for *16S rRNA* and *mecA* gene by polymerase chain reaction (PCR). The isolates were further analyzed for the presence of *mecA* gene by PCR. The antimicrobial susceptibility profiling was performed by disc diffusion method. The occurrence of *Staphylococcus* in the current study was 80.00% (40/50) from Gaya district, 88.00% (44/50) from Patna district, 96.00% (48/50) from Vaishali district and 82.00% (41/50) from Sitamarhi district, among isolates from clinical and subclinical mastitic milk samples of Gaya district, 40% (16/40) and 60% (24/40) belonged to clinical and subclinical, respectively. Similarly, 31.81% (14/44) and 68.18 % (30/44), 33.33% (16/48) and 66.67% (32/48), as well as 41.46% (17/41) and 58.54% (24/41) isolates from Patna, Vaishali and Sitamarhi district were isolated from clinical and subclinical milk samples, respectively. The PCR could detect *tstaG* gene in 86.50% as *Staphylococcus* isolates. The PCR could detected*16S rRNA* gene, occurrence of 56.64% *S. aureus* among *Staphylococcus* spp. The occurrence of *S. aureus* among 105 coagulase positive Staphylococci showed that 93.33% (98/105) isolates were coagulase positive *S. aureus.* Gaya district showed the distribution of *S. aureus* species specific *16S rRNA* gene in 57.50% (23/40) isolates out of which 47.83% (11/23) and 52.17% (12/23) were from clinical and subclinical milk samples respectively. Similarly, Patna district showed the distribution of *S. aureus* species specific *16S rRNA* gene in 52.27% (23/44) isolates, out of which 43.48% (10/23) and 56.52% (13/23) were from clinical and subclinical mastitic milk samples, respectively. Vaishali district showed the distribution of *S. aureus* species specific *16S rRNA* gene in 50.00% (24/48), out of which 45.83% (11/24) were from clinical and 54.17% (13/24) were from subclinical milk samples. Similarly, *Staphylococcus* isolates from Sitamarhi district showed the distribution of *S. aureus* species specific *16S rRNA* gene in 68.29% (28/41), out of which 46.43% (13/28) were from clinical and 53.57% (15/28) isolates were from subclinical milk samples. Antibiogram study of *Staphylococcus* isolates of clinical and subclinical mastitic milk from Gaya district showed maximum resistance of 72.5% to Cefoxitin followed by a resistance of 35 to 55.00% to Oxacillin Erythromycin, Gentamicin, and Rifampicin, while 20 to 28% resistant to Ciprofloxacin, Clindamycin and Tetracycline. None of the isolates were found resistant to Teicoplanin. Similarly, from Patna district showed maximum resistant 84.09% to Rifampicin followed by 36.36 to 75.00% to Oxacillin, Cefoxitin, Clindamycin, Erythromycin, while less than 18.18% to Gentamicin, Ciprofloxacin and Tetracycline. Isolates from Vaishali district showed maximum resistance of 62.50% to Oxacillin followed by 33.33% to 43.75% to Erythromycin, Clindamycin, Rifampicin, Cefoxitin and Gentamicin, while less than 14.58% to Ciprofloxacin and Tetracycline. Whereas from Sitamarhi district showed maximum resistance of 58.53% to Oxacillin followed by a resistance of 26.82 to 43.90% to Cefoxitin, Rifampicin and Gentamicin while other showed less than 19.51% resistance to Erythromycin, Tetracycline, Teicoplanin, Ciprofloxacin and Clindamycin. Clinical and subclinical mastitic milk samples were identified as a potential carrier of *Staphylococcus* in Bihar.

From the present study it can be concluded that *Staphylococcus* were found in more than 80% of mastitic milk sample *and* were present higher in subclinical cases than clinical cases. The study reported the occurrence of OS- MRSA in mastitic animals from India for the second time that indicates emergence of such *Staphylococcus* in India. We also reported an isolate resistant to Teicoplanin which last drug for treatment against *Staphylococcus* which is an alarming situation for antimicrobial resistance in the country. A way forward to this study is to understand the genetic mechanisms of emergence of such strain to enhance our understanding and insight to design new treatment or diagnostic approaches.

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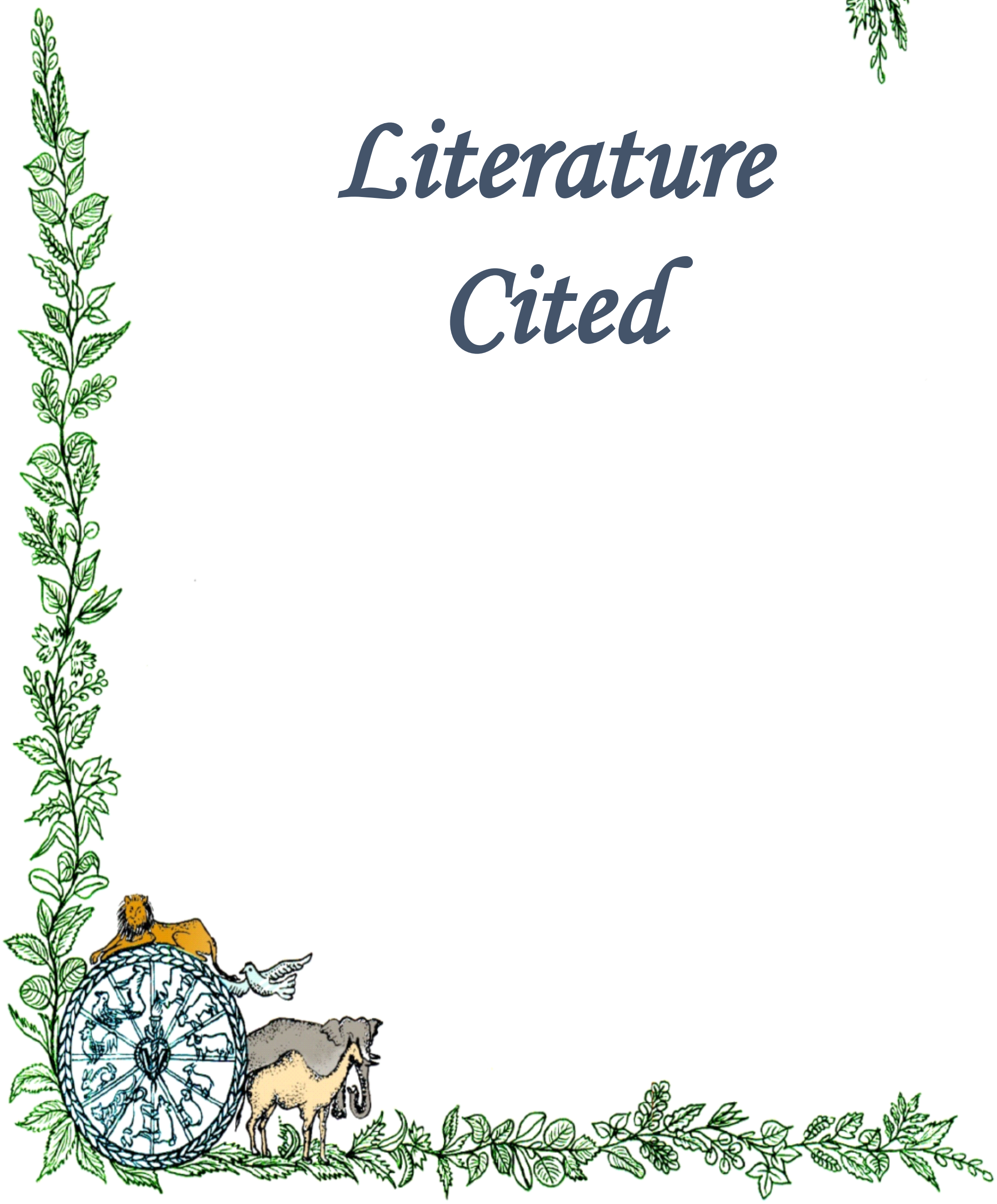

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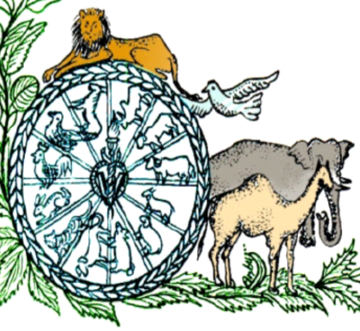
Appendix



8 Appendix

COMPOSITION OF MEDIAS AND GENERAL REAGENTS USED:

APPENDIX-A

Nutrient broth

Constituent	Concentration (g/L)
Peptone	5.0
Meat extract	1.0
Yeast extract	2.0
Sodium chloride	5.0
pH	7.0
Peptone	5.0

Brain heart infusion broth

Constituent	Concentration (g/L)
Peptic digested of animal tissue	10.0
Calf brain infusion	12.5
Beef heart infusion	5.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH	7.0

Mannitol Salt Agar

Constituent	Concentration (g/L)
NaCl	75.0
D-Mannitol	10.0
Pancreatic Digest of Caesin	5.0
Peptic Digest of Animal Tissue	5.0
Beef Extract	1.0
Phenol Red	0.025
Agar	15.0

Mueller-Hinton agar

Constituent	Concentration (g/L)
Beef infusion	3.0
Starch	1.5
Casein hydrolysate	17.5
Agar	17.0

California Mastitis Test (CMT)

Constituent	Concentration (g/L)
Sodium lauryl sulfate	3.0g
Warm distilled water	100 ml
NaOH	to adjusted to 8.0.
Bromocresol purple at the ratio	1:10,000

APPENDIX-B

Composition of chemicals and reagents

Gram Stain Crystal Violet

Solution A

Crystal Violet	2.0g (90% dye content)
Ethyl Alcohol	20.0m1 (95%)

Solution B

Ammonium Oxalate	0.8g
Distilled Water	80.0m1

(Note: Mix Solutions A and B)

Gram's Iodine

Iodine	1.0g
Potassium Iodide	2.0g
Distilled Water	300.0 ml

Ethyl Alcohol (95%)

Ethyl Alcohol (100%)	95.0 ml
Distilled Water	5.0ml

Safranin

Safranin	0.25ml
Ethyl Alcohol (95%)	10.0ml
Distilled Water	100.0ml

Catalase Test reagent

Hydrogen Peroxide	3% (H202)

Agarose Gel Loading Buffer (6X)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30% (w/v)

Ethidium Bromide (1%)

Stock Solution:

Ethidium bromide	10 mg
Distilled water	1.0 ml

Working Solution:

Make a 0.5 µg/ml working solution, (Add 10 µl of 10 mg/ml ethidium bromide solution to 200 ml ddi water)

Electrophoresis Buffer (TBE 10x)

Tris base	108.0 g
Boric acid	55.0 g
EDTA disodium salt	8.3 g
Double distilled water up to	1000.0 mL

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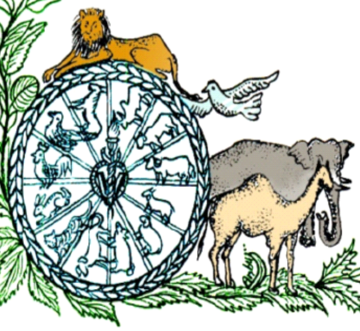
Resume



9 Brief Resume of the student

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Sr No	Name of the exam/degree	School/College	Board/University	Year of Passing	Percentage
1.	10th Class	R. S. R High School, Bariyarpur, Sitamarhi	B.S.E.B. Patna	2003	68%
2.	12th Class	R. N. T. S. College, Morsand, Sitamarhi	B. I. E. C. Patna	2006	65.22%
3.	B. V. Sc. & A.H.	Bihar Veterinary College, Patna	Bihar Agriculture University, Sabour	2016	77.11%
4.	M. V. Sc.	Bihar Veterinary College, Patna	Bihar Animal Sciences University	2019	82.69%

Title of the thesis : "**Study on occurrence and molecular characterization of *Staphylococcus* from clinical and subclinical mastitic milk samples and their antimicrobial susceptibility in Bihar**".

Membership in professional societies:

1. Member of Veterinary Council of India.
2. Member of Bihar State Veterinary Council.