Staphylococcus aureus
3. Staphylococcus aureus

3.1 Introduction

Staphylococcus, a genus of Gram-positive bacteria derived its name from Greek ‘staphyle’ meaning ‘bunch of grapes’ and ‘kokkos’ meaning ‘granule’. When viewed under microscope the organisms exhibit grape-like appearance. In this genus, there are forty species, which includes S. aureus, S. intermedius, S. hyicus, S. epidermidis, and S. saprophyticus.

The symptoms of S. aureus infection vary among animals. S. aureus causes mastitis in cattle (turbid and bloody milk), dermatitis and abscess in rabbit and post surgical infection and sepsis in human. S. aureus also results in bumble foot in poultry leading to inflamed foot. Other than S. aureus the following species in Staphylococci cause adverse effects to living being. S. intermedius results in pyoderma in dogs and S. hyicus is the major source of infection in pig. S. epidermidis being natural flora of skin it results in severe infection in immune suppressed patients. S. saprophyticus the natural flora of vagina results in severe infections of genitourinary tract in young women under opportunistic condition.

3.1.1 Staphylococcus aureus and mastitis

More than 6000 publications are available on literature analysis of bovine mastitis. The analysis of the causative organisms revealed that among the different pathogens S. aureus occupies the leading role (Fig. 3.1). The pathogenicity of S. aureus in bovine mastitis has been widely studied since the infection is most difficult to treat and cure.
S. aureus is a facultative anaerobic, gram positive micro-organism, appears as grape like clusters of large, round and golden yellow colonies (Fig 3.2).

When grown on blood agar plates it is often seen with hemolysis. S. aureus lives within the udder and also on the external parts of the cow, mostly
found in high numbers in the teat skin. The toxin produced by the bacteria destroys the cell membrane and damages the milk producing tissues.

The catalase test used to identify S. aureus also differentiates enterococci and streptococci. When exposed, S. aureus is converts hydrogen peroxide (H₂O₂) to water and oxygen resulting in a positive catalase test. A small percentage of S. aureus can be differentiated from most other staphylococci by the coagulase test. S. aureus produces the enzyme “coagulase” that forms clot formation differentiating with most other Staphylococcus species that are coagulase-negative.

3.2 Review of Literature

3.3 Classification of Staphylococcs aureus

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: Staphylococcus

Species: aureus

Classification of microbes is on the basis of 16 S rRNA sequence homology and based on such method, Staphylococcal genus is categorized into the following species S. aureus, S. simiae, S. capitis, S. caprae, S. epidermidis, S. saccharolyticus, and S. aureus subsp.
3.2.2 Phenotypic identification

Gram positive and gram negative microorganisms are identified based on gram staining results. Microbes which develop violet colour on gram staining is said to be gram positive whereas those microbes turning pinkish or orange in colour is said to be gram negative. Staphylococcus aureus are gram positive cocci that appear primarily to be spherical cells arranged in grape like structure.

When cultured on sheep blood agar, S. aureus grows as large gray or white to yellow colonies and they show mostly beta hemolysis. They are found to be more robust when grown in aerobic condition than in anaerobic condition. There are both coagulase positive and negative S. aureus strains. Coagulase negative S. aureus (CNS) are usually gray to white in color and are non hemolytic when grown on sheep blood agar. S. aureus can be confirmed using Mannitol salt agar method where coagulase positive microbe gives color change of media.

3.2.3 Biochemical test

Detection of causative pathogens in the milk sample is very crucial and thus, using the right method for its identification is necessary. Certain microorganisms are nonpathogenic and their elimination can be done using general sterilization methods like pasteurization. The pathogenic micro organism containing milk containers need to be discarded. Following are certain tests that can be used to identify pathogenic Staphylococcus aureus in the milk sample.

3.2.3.1 Catalase test

The catalase test is used to detect the presence of catalase enzyme by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is formed by some bacteria as an oxidative end product of the aerobic
breakdown of sugars. Hydrogen peroxide being highly toxic should be eliminated from bacteria or else it will result in death of the cell. Catalase usually degrades hydrogen peroxide and does not show any effect on other peroxides. This test is useful in distinguishing Staphylococci from Enterococci and Streptococci.

\[
\text{Catalase} \quad 2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2
\]

3.2.3.2 Coagulase test

There are two methods for identifying S. aureus by coagulase test. One is tube coagulase test and other is slide coagulase test. Slide test is also known as latex agglutination test. Coagulase is an enzyme which can clot blood plasma and convert into gel like consistency. On this basis, micro organisms can be classified as coagulase positive or coagulase negative. It is known that certain strains of Staphylococcus aureus can produce coagulase thus showing positive result for the coagulase test. Usually such strains can produce two types of coagulase, free and bound. As the name suggests, free is secreted extracellularly whereas bound is associated with cell wall associated protein.

Slide Coagulase method

This test is performed for bound coagulase. As bound coagulase helps in cross linking of $\alpha$ and $\beta$ chain of fibrinogen and formation of clot, it is also known as clumping factor.

On a clean glass slide, test micro organisms are applied at two ends of the slide and a drop of plasma is added on to it. Clot formation observed were noted within 5-10 secs is considered to be positive for coagulase.
Tube Coagulase method

This test is performed for detecting free Coagulase which helps in formation of thrombin and aids in clotting of plasma by converting fibrinogen to fibrin. Within a clean test tube containing 0.5ml of 1 in 10 diluted rabbit plasma, 0.1ml of test sample and 0.1ml of sterile broth is added individually. Gelling of plasma was observed after incubation for 4 hat 37°C. If plasma failed to clot and become jelly like, overnight incubation is preferred.

3.2.3.3 Oxidative Fermentation test

Every microorganism undergoes metabolism but determining it to be fermentative or oxidative is important. In 1953, Scientists first developed the oxidative fermentation test based on the ability of microorganism to degrade or metabolize sugar either by oxidation or fermentation process. During fermentation, pyruvate is converted into different acids and at higher concentration, these acids will turn the bromothymol blue indicator from green to yellow in the presence or absence of oxygen. Other microorganisms found which are non-fermentative, degrade glucose in presence of oxygen producing small amount of weak acids which can again be detected using bromothymol blue indicator. Such microorganisms are termed as oxidative.

Oxidative positive bacteria, otherwise known as nonfermenting bacteria converts glucose into small amount of acid due to reaction with atmospheric oxygen as it is the ultimate hydrogen acceptor resulting in yellow colouration of oxidative fermentation (OF) media. In case of fermentative microbes, the hydrogen acceptor is other than oxygen like sulphur, thus, the microbes do not require oxygen for metabolizing glucose. Hence fermentative microbes show reaction under aerobic as well as anaerobic environment.
3.2.3.4 Aurease test

Aurease test commonly known as RAPIDEC system is used for rapid and sensitive identification of S. aureus infection in blood cultures. The test works on the principle of aurease enzyme activity. Aurease is an enzyme present in S. aureus for coagulation and reacts with prothrombin and forms staphylothrombin. The product is then used to cleave florescence molecule tagged with the peptide in the test thus releasing the peptide and the florescent molecule.

3.2.3.5 Enzyme Linked Immuno Sorbant Assay (ELISA)

Mastitis can be detected using immunological methods where anti S. aureus Ig present in milk is measured by ELISA method. Milk samples of mastitis affected cows are analyzed for Staph-ab titre and simultaneously for bacterial count using microbiological methods. Antibody titre of milk sample was expressed as percentages of control with known S. aureus antibody titre. Sample showing antibody titre percentage more than positive control is said to be from S. aureus IMI, whereas those showing between 85 to 100% is said to be obtained from cows suspected to be infected with S. aureus and those with below 85% is said to be obtained from S. aureus free cattle.

3.2.3.6 Culture method

3.2.3.6.1 DNase test agar method

This method is used to confirm Staphylococcus aureus which also shows positive result for Coagulase test. This method helps to identify only those microorganisms which can degrade Deoxyribonucleic acid (DNA) in the medium thus, showing clear zone around the colonies showing DNase activity.
3.2.3.6.2 Hemolytic test

Hemolytic test otherwise known as Blood Agar culturing method is used for identification of forms of hemolysis from pathogenic microorganisms. Generally pathogenic microbes secrete an enzyme known as “Hemolysin”, an exotoxin by nature and disrupt membrane of the host likely erythrocyes. The mechanism of action of hemolysin is that it disrupts RBC’s and increases the content of free iron and is also involved in dermonecrosis and vasoconstriction.

Growth of Staphylococcus species onto the Blood agar medium differs highly depending on the source of blood in the media. S. aureus produces yellow colonies showing clear zone around them whereas S. epidermidis produces white colonies with no zone of clearance around them.

3.2.3.6.3 Mannitol Salt Agar

Mannitol Salt Agar is a selective medium for differentiation of S. aureus from other Staphylococcal species. It contains major nutritive composition such as peptone and beef extract, which provides essential growth factors like carbon, nitrogen, sulfur and trace nutrients. Inhibition of other microbes is due to the presence of sodium chloride in the medium. Fermentation of sugar such as Mannitol results in colour change of phenol red indicator provided in the medium and helps in differentiation between Staphylococcal species.

Table 3.1: Typical morphology of colonies observed on Mannitol Salt Agar plate

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>Small to large with yellow zones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococci other than S. aureus</td>
<td>Small to large with red zones</td>
</tr>
<tr>
<td>Streptococci</td>
<td>No growth to trace growth</td>
</tr>
<tr>
<td>Micrococci</td>
<td>Large, white to orange</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>No growth to trace growth.</td>
</tr>
</tbody>
</table>

3.2.3.6.4 Milk Agar

Milk Agar is recommended for the enumeration of microorganisms in milk, milk products, water, ice-cream, etc. by the plate count test. The nutrient agar containing powdered milk is used to screen an organism’s ability to secrete extracellular proteases that catalyze the hydrolysis of the milk protein casein. A clear zone around a colony indicates casein hydrolysis. The incubated plates were observed for positive results after 18-24 hr and expressed as colonies/mL (CFU).

3.2.3.6.5 Baird Parker Medium

Baird Park test is used for enumeration of S. aureus in biological samples. Enzymatic Digest of Casein and Beef Extract are the carbon and nitrogen sources in Baird Parker Agar. Yeast Extract supplies B-complex vitamins that stimulate bacterial growth. Glycine and Sodium Pyruvate stimulate growth of staphylococci. The selectivity of the medium is due to Lithium Chloride and a 1% Potassium Tellurite Solution, suppressing growth of organisms other than staphylococci. The differentiation of coagulase-positive staphylococci is based on Potassium Tellurite and Egg Yolk Emulsion. Staphylococci that contain lecithinase break down the Egg Yolk and cause clear zones around the colonies. An opaque zone of precipitation may form due to lipase activity. Reduction of Potassium Tellurite is a characteristic of coagulase-positive staphylococci, and causes blackening of colonies. Agar is the solidifying agent.

Coagulase-positive staphylococci produce black, shiny, convex colonies with entire margins and clear zones, with or without an opaque zone. Coagulase-
negative staphylococci produce poor or no growth. If growth occurs, colonies are black; clear or opaque zones are rare. The majority of other organisms is inhibited or grows poorly. If growth appears, colonies are light to brown-black, with no clear or opaque zones.

3.2.3.7 Molecular Screening

3.2.3.7.1 16S rRNA Sequence Homology

Molecular methods like 16s rRNA sequencing in bacterial phylogeny and taxonomy has long been in use. This most common housekeeping genetic marker is preferred since it is present in almost all bacteria, often existing as a multigene family, or operons. It has been demonstrated that 16S rRNA gene sequence data of an individual strain with a nearest neighbor exhibiting a similarity score of <97% represents a new species. The function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and evolution and the 16S rRNA gene with approximate 1.5 kbp is large enough for informatics purposes.

3.2.3.7.2 Evaluation of Methicillin resistance by mec A and fem A PCR

Infections caused by methicillin-resistant S. aureus (MRSA) strains, result in increased morbidity, mortality in cattle mastitis. Treatment of S. aureus infections has become problematic due to development of methicillin resistance. Methods to detect MRSA isolated from milk samples should have high sensitivity and specificity and most importantly confirmation of MRSA should be available within a short time. Various methods have been developed for the early detection of methicillin resistance in Staphylococci spp but the optional methods of detection remains controversial. In a country like India with larger
dairy industry, detection and early control of MRSA is of prime importance. MRSA strains harbor the mecA gene, which encodes a modified penicillin binding protein (PBP2a or PBP2') with low binding affinity for methicillin and β-lactum antibiotics (Brakstad and Maeland, 1997; Chambers, 1997). Since MRSA is resistant to all β-lactum antibiotics, the therapeutic options are limited.

Another gene fem A (factor essential for methicillin resistance) belongs to the fem AB operon. It is a housekeeping gene and is found in all S. aureus strains, both sensitive and resistant ones. Like all fem factors fem A is involved in specific step of cell wall synthesis. The fem A gene might be responsible for the addition of the second and third glycine residues to the intra-peptide bridge. This long and flexible intra-peptide bridge allows the extremely high cross-linking between the individual peptide moieties typical for S. aureus cell wall. Although the addition of each glycine residue requires an identical chemical reaction, these genes are necessary to proceed from one step to the next, namely fem X, fem A and fem B. Despite the significant similarity of their gene product, they cannot complement each other. femA mutants are more susceptible for β-lactum antibiotics than the wild type strain. In a non-MRSA background fem A mutants are hyper-susceptible to β-lactums. The potential use of femA as a target for DNA based detection test for Methicillin-resistant Staphylococci requires investigation.

3.2.4 S. aureus Pathogenesis in Mastitis

The in vitro studies of affected bovine mammary cells showed that unencapsulated S. aureus adhered more readily than the encapsulated variants, suggesting that the bacterial cell wall has a high affinity for extra cellular matrix proteins and for damaged cells and that this affinity is blocked by the exopolysaccharide capsule (Almeida et al., 1996; Cifrian et al., 1994).
Bacteria adapt and multiply in the milk, gaining access to the upper part of the gland. It has been proposed that bacteria adhere to the ductular and alveolar epithelium in the gland and begin production of toxins (Fig 3.3). These adhered bacteria trigger macrophage activation and neutrophil migration from the blood into the milk (a situation resulting in an increase in the somatic cell count), inflammation of the mammary gland, impairment of the host immune system, and epithelial cell damage (Paape et al., 2000). As a result, bacteria reach the basal subepithelial cell layers, bind fibrinogen and other host receptor proteins, and finally establish an infection. Such chronic infections are associated with a bacterial growth in the form of adherent colonies surrounded by a large exopolysaccharide matrix, constituting a biofilm. Because of their aggregate size, biofilm are not susceptible to macrophage phagocytosis and become resistant to some antibiotics (Cucarella et al., 2004a).
3.2.7 Transmission

Infected udders, teat canals, and teat lesions, and also teat skin, muzzles, nostrils and vagina act as major reservoirs of the micro-organisms causing mastitis and the bacteria tend to spread to uninfected parts by teat cup liners, milkers’ hands, wash cloths and flies (Brody et al., 2008; Madsen et al., 1991; Rund et al., 1986; Yeruham et al., 1996). At times even aerosols play a role in transmission of bacteria to uninfected parts. Usually Staphylococci do not colonize on healthy teat skin, but readily colonize teat canal if there are lesions present. The organisms multiply in infected lesions and enter into the udder causing the eradication procedure to be more severe.

3.2.8 Strategies in S. aureus mastitis eradication

The following control strategy should be developed for S. aureus mastitis control

- Test and Cull strategy

Culling could be the most efficient way to remove the chronic S. aureus cows and to reduce the pressure on healthy cows.

- Control Program based on fomites control

Fomites control can be achieved through proper milking hygiene which include use of disposable gloves by the milker, use of single – service towels to clean the teat, fore stripping and use of post milking teat disinfectants of known efficacy.

- Control based on segregation

All cows that are recently calved are sampled 7 and 14 days after enrollment. Purchased cows are sampled 7 and 14 days after entry into the herd. Cows with
S. aureus mastitis are segregated and are not sampled again until they had calved (Hoblet and Miller, 1991). In a study, segregation or separate milking of cows that were positive for S aureus reduced prevalence from 29.5 to 16.3% and bulk tank SCC from 6,00,000 to 345,000 / ml (Fenlon et al., 1995; Wilson et al., 1995b).

The complete control (eradication) of S. aureus IMI implies that consistent measures of biosafety and monitoring should be applied, once the program has been completed. Practitioner should advise farmers to adopt a consistent control program based on segregation, efficient hygienic and management procedures and rational therapeutical protocols.
3.3 Materials and Methods

3.3.1 Standard cultures of S. aureus

Control culture of *Staphylococcus aureus* MTCC 3160 and *Staphylococcus*, was obtained from MTCC, Chandigarh, India. Culture was qualified by the standard methods like DNase plate, mannitol salt agar plate. No special characteristics were reported for this strain.

3.3.2 Media

3.3.2.1 Nutrient Agar (1 L)

<table>
<thead>
<tr>
<th>Composition</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2</td>
</tr>
<tr>
<td>Peptone</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

3.3.2.2 Mannitol Salt Agar media (1 L)

<table>
<thead>
<tr>
<th>Composition</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest of Casein</td>
<td>5</td>
</tr>
<tr>
<td>Enzymatic Digest of Animal Tissue</td>
<td>5</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1</td>
</tr>
</tbody>
</table>
D-Mannitol 10
Sodium chloride 75
Phenol Red 0.025
Agar 15

For the above media, the final pH was adjusted to 7.4 ± 0.2 at 25°C and sterilization was carried out by autoclaving at 121°C for 15 min.

3.3.2.3 Stains

Gram staining- Crystal violet, Safranin

3.3.3 Methods

Milk samples collected were evaluated for their infection status by culturing the micro organisms, identification of the colonies by morphology analysis and biochemical assays. Every milk sample was analyzed in triplicate. Of the identified organisms, S. aureus, S. epidermidis and E. coli were specifically analyzed for their antibiogram and gene expression.

3.3.3.1 Isolation of micro organisms and characterization of S. aureus

Nutrient agar plates were prepared by adding 10 to 15 ml of autoclaved nutrient agar (2.8 gm of nutrient agar in 100 ml of distilled water and autoclave) into sterile petri plates and allowed to cool. From the normal and mastitis milk samples, an aliquot of 100 µl was spread on a nutrient agar plate and incubated at 37°C overnight. The number of individual colonies grown was counted. The colonies of same phenotype from one milk sample were grouped and classified
to their specific microbial species. Each species from one milk sample was
given an identification number. The number of isolates represents the number of
milk samples and the cows.

Species specific colonies were picked up using sterile nichrome loop,
dispensed into nutrient broth then incubated at 37°C for 48 hrs. Morphologically
identified Staphylococcus aureus colonies were confirmed by catalase and
coagulase test along with gram staining.

3.3.3.2 Gram staining for identification

Gram staining is the preliminary step in identifying gram positive
bacterial classification of Staphylococcus spp. The distinct colonies formed in
the nutrient agar were selected and grown in nutrient broth. From a loop full of
culture taken from the nutrient broth a smear was prepared in a fresh sterile
slide. The smear preparations were subjected to gram staining to classify the
bacteria depending on their gram staining properties. The slide was fixed by
heating for 2-3 min and crystal violet stain was added onto it. The slide was
incubated for 1 min at 37°C. The slide was washed with running water for 1 min
to which iodine was added and incubated for 1 min. The treated slide was rinsed
with tap water thoroughly and 95% ethanol was added for discoloration. After
incubation for 10 sec, safranin was added and incubated for 30 sec. The slide
was rinsed properly and evaluated under light microscope with 45X lens.

3.3.3.3. Catalase test

Catalase test was performed to identify the microbes that are able to
convert hydrogen peroxide (H₂O₂) to water and oxygen. This test was useful in
distinguishing Staphylococci from Enterococci and Streptococci. In this simple
test, the colonies produced effervescence when a loop full of culture was
immersed in 3% hydrogen peroxide (H₂O₂) solution were categorized as catalase positive.

3.3.3.4 Coagulase test

Coagulase test distinguishes *S. aureus* and other *Staphylococcus* spp (Capurro et al., 1999; Roberson et al., 1992). *S. aureus* forms clot when treated with blood whereas *S. epidermidis* does not show any clot formation. A loop of culture was transferred onto a sterile glass slide to which fresh blood plasma was layered on it. Incubate the slide at 37°C for 3-15 min. Observed for clot formation against a dark background. The coagulase positive cultures are then further proceeded for mannitol salt agar plate, muller hinton agar plate and congo red plate.

3.3.3.5 Mannitol Salt Agar plate

The final confirmation of *Staphylococcus aureus* was obtained from screening on Mannitol Salt agar (Han et al., 2007; Sharp and Searcy, 2006). A loop full of culture from nutrient broth was streaked onto the Mannitol salt agar plate and incubated at 37°C for 24-48 h. *S. aureus* is capable of surviving under sodium chloride and fermenting Mannitol. This results change in the pH which turns phenol red indicator into yellow color. This selective medium generated yellow colonies of *S. aureus* with clear zone in reaction with D-mannitol salt.

3.3.3.6 Molecular confirmation of *S. aureus* mec A and fem A genes

Over night cultures of *Staphylococcus* spp grown on nutrient agar was subjected to DNA extraction. A single colony of *Staphylococcus aureus* was picked up by the tip of a sterile toothpick and inoculated into 25µl of distilled autoclaved water. This inoculum was exposed to heat shock at 95°C for 5 min in
a thermal cycler. Heat shock leads to disruption of bacterial cell and bacterial DNA is released into the suspension.

Two set of primers were used for the detection of mecA P1F (5’-CGATGGTA AAGGTTGGCAAAAAG-3’) and mecA P1R (5’-CCACCCAATTGTCTGCCCAGT T-3’) yielding 568bp amplicon and mecA P2F (5’-GTAGAAATGACTGAAC GTCCGATAA-3’) and mecA P2R (5’CCAATTCCACATTGTTTCGTCTAA-3’) yielding 310bp amplicon. femA was detected by primers femAF (5’-AAAAAAGCACATAACAAGCG-3’) and femAR (5’GATAAAAGAAAGACGA GCAG-3’) generating 132bp amplicon. Whereas icaD gene was amplified with icaDF (5’-CTTCGATGTCGAAAATAAACTC-3’) and icaDR (5’GCTTCTGGAATGAGT TTGCT-3’) yielding 238bp amplicon. PCR was carried out in a sterile 200μl thin walled tube. The 25μl PCR reaction mixture comprised of 10x Buffer 5μl, 200 mM dNTP mix, 10 pmoles of each primer, 1U Taq polymerase, and 1μl of DNA suspension (colony lysis). Cycling parameters were as follows: 95°C for 5 min, 40 cycles of 94°C for 1 min, 55.5°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min. The amplified PCR fragments were analyzed by 2% agarose gel electrophoresis with 100 bp DNA ladder as marker. The resultant bands were visualized by UV transilluminator (Bio-rad, USA).
3.4 Results

3.4.1 Screening and Gram Positive Identification of Pathogens from Milk Samples

The milk samples from normal and mastitis cows were screened for microorganisms on nutrient agar and the bacterial cultures obtained were grouped based on Gram staining. The results are presented in Table 3.2 and Fig 3.4. Almost all the milk samples from mastitis group (93% to 100%) exhibited bacterial colonies. About 20% to 33% of the normal milk samples showed colonies on nutrient agar. The density of the colonies from the mastitis samples was high and to pick up single colonies, the cultures were repeated in triplicate after dilution. The colonies obtained from the normal samples were scattered and morphologically most of them belonged to normal flora like E. coli. From every milk sample, morphologically similar colonies were grouped and assigned with specific identification number.

These colonies were further evaluated by Gram staining. The incidence of gram positive cocci (GPC) for clinical and subclinical mastitis was found to be 80% and 65% in HF, 79% and 64% in Jersey and 82% and 67% in Kangayam, respectively. In comparison, the percentage of GPC was higher in clinical mastitis than subclinical mastitis. The severity of the disease could be attributed to S. aureus pathogenicity. With regard to colonies from normal milk samples, GPC was found to be within 14% with HF and Jersey and only one animal in Kangayam group.
A. Preliminary screening on Nutrient agar plate

Legend: Milk samples from mastitis and normal cows were screened for infection in a sequential manner as described under materials and methods. The colonies obtained were morphologically evaluated and grouped based on gram staining.

Fig. 3.4: Screening of pathogens from mastitis and normal milk samples
Table 3.2: Screening and Gram positive identification of pathogens in milk samples from mastitis and normal cows

<table>
<thead>
<tr>
<th>Group</th>
<th>Breed</th>
<th>Diagnosis</th>
<th>No. of animals</th>
<th>Nutrient Agar</th>
<th>Gram Staining +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n*</td>
</tr>
<tr>
<td>Mastitis</td>
<td>HF</td>
<td>Clinical</td>
<td>138</td>
<td>135</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>51</td>
<td>49</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>Jersey</td>
<td>Clinical</td>
<td>79</td>
<td>78</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>15</td>
<td>14</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>Kangayam</td>
<td>Clinical</td>
<td>34</td>
<td>33</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>3</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Normal</td>
<td>HF</td>
<td></td>
<td>67</td>
<td>22</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>Jersey</td>
<td></td>
<td>58</td>
<td>16</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>Kangayam</td>
<td></td>
<td>15</td>
<td>3</td>
<td>20%</td>
</tr>
</tbody>
</table>

* - Number of samples out of nutrient agar positive

3.4.2 Sequential Confirmation of S. aureus by Catalase, Coagulase and Mannitol Salt Agar Tests

The group of colonies classified as GPC was further subjected to sequential confirmation by three culture assays wide catalase, coagulase and mannitol salt agar. The results of positive samples at every stage were compared with that of GPC (Table 3.3). At first level catalase test distinguished
Staphylococci from Enterococci and Streptococci. Out of GPC positive samples, 67% to 90% with exception in Jersey subclinical category of 100% were found to belong to Staphylococci spp. The conclusion on S. aureus was obtained by the biochemical assays based on coagulation and mannitol fermentation. While more than 70% of the colonies were confirmed to be S. aureus, in case of Jersey subclinical mastitis, it dropped to 50%.

Table 3.3: Sequential Confirmation of S. aureus by Catalase, Coagulase and Mannitol Salt Agar Tests

<table>
<thead>
<tr>
<th>Group</th>
<th>Breed</th>
<th>Diagnosis</th>
<th>No. of animals*</th>
<th>Catalase Test</th>
<th>Coagulase Test</th>
<th>Mannitol Salt Agar Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Mastitis HF</td>
<td></td>
<td>Clinical</td>
<td>108</td>
<td>93</td>
<td>86%</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>32</td>
<td>27</td>
<td>84%</td>
<td>24</td>
</tr>
<tr>
<td>Jersey</td>
<td></td>
<td>Clinical</td>
<td>62</td>
<td>56</td>
<td>90%</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>9</td>
<td>6</td>
<td>67%</td>
<td>5</td>
</tr>
<tr>
<td>Kangayam</td>
<td></td>
<td>Clinical</td>
<td>27</td>
<td>23</td>
<td>85%</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>2</td>
<td>2</td>
<td>100%</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>HF</td>
<td>3</td>
<td>2</td>
<td>67%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jersey</td>
<td>2</td>
<td>1</td>
<td>50%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kangayam</td>
<td>1</td>
<td>1</td>
<td>100%</td>
<td>1</td>
</tr>
</tbody>
</table>

* - Number of animals is based on the GPC as described in Table 4.
Legend: The colonies which were positive for *S. aureus* through the preliminary evaluation of the milk samples were further analyzed by catalase, coagulase and mannitol salt agar tests as described under materials and methods (3.3).

Fig 3.5: Confirmation of *S.aureus* by Catalase, Coagulase and Mannitol Salt Agar Tests
3.4.3 Molecular confirmation of S. aureus mec A and fem A genes

The colonies confirmed phenotypically and biochemically for S. aureus were taken up for molecular analysis. The presence of mecA and femA genes confirmed not only the species but also the pathogenic i.e MRSA condition of the organisms (Table 3.4 and Fig 3.6) (Brakstad and Maeland, 1997; Chambers, 1997). PCR designed to detect femA gene (Factor Essential for Methicillin resistance) revealed the presence of this gene in almost all (92% to 100%) the isolates regardless of their susceptibility profile. The mecA gene conferring methicillin resistance was present above 60% in clinical mastitis group and around 40% in subclinical mastitis group. This is in concordance with the available literature. The clinical mastitis group samples with higher MRSA incidence were associated with sudden episode and resistant to antibiotic therapy.
Table 3.4: Molecular confirmation of S. aureus mecA and femA genes

<table>
<thead>
<tr>
<th>Group</th>
<th>Breed</th>
<th>Diagnosis</th>
<th>S. aureus +ve samples*</th>
<th>mecA n</th>
<th>%</th>
<th>femA n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastitis HF</td>
<td></td>
<td>Clinical</td>
<td>82</td>
<td>52</td>
<td>63%</td>
<td>82</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>24</td>
<td>9</td>
<td>38%</td>
<td>22</td>
<td>92%</td>
</tr>
<tr>
<td>Jersey</td>
<td></td>
<td>Clinical</td>
<td>48</td>
<td>31</td>
<td>65%</td>
<td>46</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>5</td>
<td>2</td>
<td>40%</td>
<td>5</td>
<td>100%</td>
</tr>
<tr>
<td>Kangayam</td>
<td></td>
<td>Clinical</td>
<td>19</td>
<td>12</td>
<td>63%</td>
<td>18</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>2</td>
<td>0</td>
<td>0%</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>Normal HF</td>
<td></td>
<td>Clinical</td>
<td>2</td>
<td>1</td>
<td>50%</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jersey</td>
<td>1</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kangayam</td>
<td>1</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>100%</td>
</tr>
</tbody>
</table>

* - Number of samples positive for S. aureus as described in Table 3.3.
Fig 3.6: Molecular confirmation of *S. aureus* mec A and fem A genes

**Legend:**

A. DNA of confirmed *S. aureus* isolates from standard and milk samples were amplified with mec A (two set of primers) and fem A as detailed under materials and methods (3.3) B. The *S. aureus* colonies were lysed and subjected to multiplex colony PCR employing the primers for mec A and fem A together.

A: Lane1 - Negative control, Lane2 - mec A primer set 1, Lanes 2 &3: mec A primer set 2 Lane4 - fem A primer, Lane5 - DNA ladder
B: Lane1 - Positive control, Lanes 2 to 6 - *S. aureus* colonies, Lane7 - DNA ladder
3.5 Discussion

The etiology of mastitis is multifactorial involving host, pathogens and carriers. There is a wide range of microbes involved in the infection process including S. aureus, Str. agalactiae, S. epidermidis, Brucella melitensis, Corynebacterium bovis, Mycoplasma, Pseudomonas aeruginosa, E. coli and Klebsiella pneumonia of which S. aureus is the predominant organism (Almaw et al., 2008; Halasa et al., 2009; Madsen et al., 1992). Some of the causative pathogens generally present as part of normal flora and are opportunistic; spread through contaminated area like towels, bedding material, feed, milker’s hand and also to some extent through flies.

Three infection patterns are observed in mastitis. Firstly, infections due to Str. agalactiae, S. aureus and Str. dysgalactiae show subclinical symptoms and control of infected quarter of the udder takes several years. The second type shows symptoms of acute clinical mastitis which can be easily controlled by quarantining the cattle and upgrading the housing area to a clean environment. Third infection pattern is associated with non-lactating cows, a common incidence during early dry period. This last type of infection pattern can be easily controlled by drying off therapy and fly control.

The normal and mastitis milk samples were cultured in triplicate by the National standards and the colonies appeared were evaluated. While all the mastitis samples were positive for infection, fewer normal milk samples showed microbial growth in gram staining, catalase and coagulase tests. Out of 320 mastitis samples, 240 (75%) were positive for gram-positive Cocci of which, 207 (65%) belonged to Staphylococcus spp based on the catalase test (Fig. 3.5.). Coagulase and mannitol salt agar tests differentiated 180 (56%) S. aureus
isolates with other Staphylococcus spp. Since S. aureus has been qualified as the predominant pathogen causing mastitis, it was subjected to a detailed study.

Methicillin-resistant Staphylococcus aureus (MRSA) strains have posed many problems in diagnosis of mastitis (Moon et al., 2007; Turutoglu et al., 2009). The incidence varies depending on geographical distribution of the strains and the methods used to isolate them. Such problems mostly arise from their hetero-resistance where within a given methicillin-resistant strain, only a certain proportion of cells are able to express the resistance trait under normal conditions. Although "over identification" of methicillin-susceptible isolates as methicillin resistant is more common than usually believed, the lack of special precautions aimed at favoring an increased expression of the resistance (such as salt addition to the test medium, the use of a larger inoculum, or plate incubation at a lower temperature or for a longer time) may lead to "under identification" of MRSA isolates. S. aureus resistance to methicillin has generally been regarded as a typical example of intrinsic resistance. MRSA strains harbor the mec A gene, which encodes a modified penicillin binding protein (PBP2a or PBP2') with low binding affinity for methicillin and β-lactam antibiotics (Chambers, 1997; Rahman et al., 2005). Since MRSA and MR-CoNS is resistant to all β-lactam antibiotics, the therapeutic options are limited.

Another gene femA (factor essential for methicillin resistance) belongs to the femAB operon (Muraki et al., 1993; Yamashita et al., 1994). It is a housekeeping gene and found in all both sensitive and resistant S. aureus strains. Like all fem factors femA is involved in specific step of cell wall synthesis. The femA gene might be responsible for the addition of the second and third glycine residues to the intra-peptide bridge. This long and flexible intra-peptide bridge allows the extremely high cross-linking between the individual peptide moieties
typical for S. aureus cell wall. Although the addition of each glycine residue requires an identical chemical reaction, these genes are necessary to proceed from one step to the next, namely femX, femA and femB. Despite the significant similarity of their gene product, they cannot complement each other. femA mutants are more susceptible for β-lactum antibiotics than the wild type strain. In a non-MRSA background femA mutants are hyper-susceptible to β-lactums. The potential use of femA as a target for DNA based detection test for MRSA requires investigation.

In this study of the 320 milk samples, 106 (33%) were found to be positive for MRSA strains by mecA PCR. This constitutes 59% (106 / 180) of the S. aureus isolates biochemically confirmed. The emergence of MRSA has left us with very few therapeutic alternatives available to treat Staphylococcal infections. Rapid, specific and sensitive diagnosis and confirmation of intrinsic resistance, hetero-resistance, and methicillin resistance from milk samples is therefore considered to be very crucial for proper infection control and management of the animal. Delay of appropriate antimicrobial therapy could lead to loss of the quarter of the udder, length of treatment and other veterinary costs.

The multiplex-colony PCR approach can be beneficial adjunct to conventional microbiological methods for rapid (<24h) and specific identifications of pathogens and resistance patterns. In this study as substantiated by the observations made, multiplex-colony PCR could specifically detect MRSA, taking less diagnosis time (<24h) against the conventional methods which take 36-72 h. As this method is less time consuming, specific and sensitive, it could be employed in the routine diagnosis
and lowering the cost of treatment. The major requirement is the need of trained personnel and appropriate facility.