1. INTRODUCTION

India is the highest milk producer in the world, but the per capita availability of milk still remains half of the world average, demanding strategic intervention. Although per capita availability of milk has increased from 128 gram/day in 1980-81 to 267 gram/day in 2010-11, it is far below the requirement of 280 gram/day. By the end of 12th plan, demand for milk is expected to increase to 141 million tons as against present production of 121.8 million tons. One of the reason for low productivity is mastitis which is single largest problem in dairy animals in terms of economic losses in India there by all over the world (Sharma et al., 2010). India losses Rs.7165.51 crores on account of mastitis and it stands second to FMD as a most challenging disease to high yielding dairy animals in India (Bansal and Gupta, 2009).

Mastitis is a general term which refers to the inflammation of the parenchyma of the mammary gland, regardless of the cause. It is a multiple etiological disease complex, being most prevalent in high yielding dairy cattle, buffaloes, goats and sheep throughout the world. Mastitis is accompanied by abnormal alterations in physical, chemical and bacteriological characteristics of milk. Of the two forms of mastitis, subclinical mastitis (SCM) is subtle, causes huge economic losses and is difficult to detect as the cow appears healthy, the udder and the milk appears normal. However, micro-organisms and somatic cells are found in elevated numbers in the milk (Hase et al., 2013).

Different kinds of microorganisms e.g. Staphylococcal species (spp.), Streptococcal spp., Escherichia coli, Klebsiella spp., Enterobacter spp., Corynebacterium bovis, Corynebacterium pyogenes, Bacillus spp., Mycoplasma bovis, Micrococcus spp., etc. are common etiological agents of mastitis in animals, however, the major part of microbial mastitis has been reported due to Staphylococci and Streptococci (Lee et al., 2008). Amongst these causative agents, Staphylococcus aureus (hereafter referred to as S. aureus) has been recognized as the most common cause of intra-mammary infection in milch animal species, which often leads towards the damage and sometimes even complete loss of the gland. Staphylococcal mastitis has been
reported as 25-30% of the entire mastitic infections and milk losses, which has been reported to vary between 10 to 25% (Sutra and Poutrel, 1994).

Bacteriological culture of milk is reckoned as a useful tool for accurate diagnosis of mastitis and identification of causative organism (Dhanda and Sethi, 1962). Standard bacteriological procedures were used for isolation and identification of Staphylococcal intra-mammary infection (imi) (Kivaria et al., 2007). The bacteriological identification of mastitis pathogen is important because control and eradication procedures depend on the kind of infection prevalent in the herd.

In case of *S. aureus* there is variability in expression of phenotypic characters by strains of bovine origin. There is lack of proper phenotypic tests for identification between *S. aureus*, *S. intermedius* and *S. psuedintermedius*. Therefore, accurate identification of *S. aureus* by genotypic methods is advocated by various workers. The nuclease (*nuc*) gene encodes the thermonuclease (Tnase) production which has species-specific sequences and amplification of the *nuc* gene has a potential for the rapid diagnosis of *S. aureus* (Brakstad et al., 1992).

The bacteriological cure rates for the treatment of Staphylococcal mastitis with either intramammary infusion or parenteral antimicrobial administration are notoriously less than satisfactory, particularly in the lactating cow, also, insufficient and improper therapy of mastitis results in development of resistant strains of bacteria. Further, incomplete treatment or continuous use of antimicrobial agents may lead to availability of their residues in the milk (Jeykumar et al., 2013). Thus, there is need to find out suitable therapeutic agents to treat or control the Staphylococcal mastitis in cattle.

Aloe vera belongs to Liliacea family, it posses anti-inflammatory, antibacterial, antiviral and antidiabetic properties. Aloe vera gel is used topically for its anti-inflammatory and wound-healing properties, but it has also been used internally as a general tonic. The topical application of the aloe vera quickly heals the tissues (Karreman, 2007).
Keeping in view the above facts the present study is proposed with the following objectives.

**OBJECTIVES**

1. To study the occurrence of subclinical mastitis in cattle using modified California mastitis test and intensity of infection using somatic cell count.

2. Isolation and identification of *Staphylococcus* species in milk samples positive for subclinical mastitis.

3. Identification of *nuc* gene in isolates of *Staphylococcus* species.

4. To evolve the most effective therapeutic regimen against Staphylococcal subclinical mastitis.
2. REVIEW OF LITERATURE

Mastitis is internationally recognized as one of the most important disease of economic importance to the dairy industry due to its high morbidity, loss of milk production, high cost of treatment and adverse effects on the quality of milk. Detection of mastitis at the subclinical stage is relatively difficult task but it is equally desirable to carry it to avoid the subsequent heavy economic losses to the dairy farmers (Harmon, 2001).

Subclinical mastitis

1. Occurrence

Almaw et al. (2008) screened 351 lactating cattle of small holder farms in Ethiopia to determine prevalence of subclinical mastitis which was reported as 34.4% (animal wise) and 17.9% (quarter wise).

Getahun et al. (2008) reported overall prevalence of clinical mastitis and subclinical mastitis as 2.6% and 22.3%, respectively, in small dairy farms of Central Ethiopia.

Hashemi et al. (2011) screened 6180 quarters from 1545 dairy cows by clinical examination and California mastitis test (CMT) from different dairy farms in the central region of Fars province, in which, 4714 (76.28%) quarters were healthy, 1335 (21.6%) quarters were positive by results of CMT for SCM, 44 (0.71%) quarters showed clinical mastitic signs and 87 (1.41%) quarters were blind. The clinical and subclinical mastitis prevalence on cow basis was 2.2 and 42.5%, respectively.

Islam et al. (2011) found the prevalence of subclinical mastitis in lactating dairy cows of Bangladesh Agricultural University dairy farm (BAUDF) and rural areas of Tangail sadar upazila. A total of 200 milk samples (40 from BAUDF and 160 from Tangail sadar upazila) were collected. Overall prevalence of SCM in lactating dairy cows found was 29%.

Dar et al. (2014) analyzed 800 individual quarter milk samples by CMT, 413 samples of 130 cows (74.61% crossbred and 25.38% native) were found positive for subclinical mastitis.
Mir et al. (2014) reported that the prevalence of subclinical mastitis was 57.80 % and 30.73% on animal and quarter basis respectively.

2. **Individual quarter wise**

Awasthi and Upadhyay (2006) recorded SCM in 33.88% of the animals. The right hind quarter was found to be most affected (37.09%) followed by the left hind (27.74%) and the left fore quarter (22.25%), while, right fore quarter (19.35%) was least affected.

Ramprabhu and Rajeshwar (2006) collected 432 mid stream milk samples from 108 cows of different dairy farms of Kanyakumari (Tamilnadu) and reported that rate of infection was higher in rear quarters (54.54%) as compared to fore quarters (45.46%).

Awale et al. (2012) observed that the hind quarters were more affected (56.52%) than the fore quarters (43.47%).

3. **Breed wise**

Islam et al. (2011) detected higher prevalence of SCM in milch crossbred cows (36.36%) in comparison to non-descript cows (24.61%).

Dar et al. (2014) reported that out of 250 cows tested, 130 cows had subclinical mastitis which included 97 crossbred cows and 33 native cows with percentage of 38.80% and 13.20%.

4. **Age wise**

Mahajan et al. (2011) conducted a study in 4133 cattle including cross bred and non descript cattle. The higher risk of mastitis was found to be between the age of 4-6 years, followed by age group between 2-4 years, with the least occurrence between 6-8 years of age. It was also noted that crossbred animals were 2.5 times more susceptible to mastitis than non descript ones.

Tiwari et al. (2013) demonstrated that occurrence of mastitis in infected quarters increases with age in cows being the highest at 7 years of age. This may be due to increased cellular response to intra-mammary infection or due to permanent udder tissue damage resulting from the primary infection. Efficient innate host defense mechanism of the younger animals is one possibility that makes them less susceptible to infection.
Dar et al. (2014) analyzed 800 individual quarter milk samples by California mastitis test, 413 samples of 130 cows (74.61% crossbred and 25.38% native) were found positive for subclinical mastitis. Whereas, 16 (81.25% crossbred and 18.75% native) of them were in the age group of 2-4 years. 71.53% cows within age group of 5-7 years had subclinical mastitis and only 16.15% cows within age of 7 years and above had subclinical mastitis.

5. Parity wise

Shukla et al. (2005) reported that the prevalence of SCM in crossbred and Sahiwal cows were highest during third lactation.

Joshi and Gokhale (2006) studied on 250 animals for incidence of SCM in improved and peri urban farms in Pune, Maharashtra and reported that the incidence of SCM increased with higher lactation number and animals in 4th-5th month of lactation were found to be more susceptible (59.49%).

Nauriyal and Verma (2009) carried out studies to find out prevalence of SCM in indigenous (Kankrej) cows maintained at Livestock Research Station (LRS), Anand and observed the highest incidence of SCM in second lactation.

Islam et al. (2011) collected 200 milk samples from lactating dairy cows of BAUDF and rural areas of Tangail sadar upazila (40 from BAUDF and 160 from Tangail sadar upazila). Overall prevalence of SCM in lactating dairy cows was found to be 29%. The highest prevalence of SCM was recorded during the early lactation stage in both the local breed cows (30.0%) and cows of BAUDF (45.83%) in comparison to their respective mid and late stages of lactation. The prevalence of SCM was highest in lactating cows having third lactation and high yielding (cows produced >10 liter milk per day) both in local and crossbred cows.

Ayano et al. (2013) examined 546 lactating cows by MCMT, 224 were found positive for subclinical mastitis. Highest prevalence was seen in animals at mid lactation stage (50%), followed by animals at late lactation (47.2%) and least in early lactation stage (2.8%).
6. Organized and unorganized dairy farms wise

Tiwari et al. (2000) reported the incidence of subclinical mastitis in unorganized and organized dairy farms as 26.68% and 20.36%, respectively.

Clinical examination of animals and their udder

Reddy et al. (2001) collected 135 milk samples from 35 apparently healthy cross bred cows maintained at organized dairy farms in and around Tiruparti and observed that no apparent symptoms appear in SCM.

Chakrabarti (2004) observed that SCM denotes absence of gross abnormalities in the mammary gland but with the recovery of bacteriological pathogens from the milk and only symptoms available were less milk production.

Saravanan et al. (2009) studied a total of 1376 udder samples from 344 cows from private farms (control), 1104 samples from 276 cows with SCM and 232 samples from 58 cows from dairy farms in Tamilnadu, India, and reported that the milk samples from all the positive cases of SCM were found to have normal colour, odour and consistency.

Radostits et al. (2010) documented that the SCM is indicated by a high somatic cell count in the milk without any visible abnormality of the milk and udder.

Suresh et al. (2010) investigated a total of 24 quarter milk samples from 6 Jersey cross bred cows aged between 4-6 year brought to the Madras Veterinary College Hospital, which are collected aseptically and examined. The cows affected with SCM were not exhibiting any clinical signs except reduced milk. The colour, consistency and odour of milk were found to be normal.

Modified California mastitis test (MCMT)

Shukla (1980) suggested that California mastitis test can be adopted as a routine test on all medium and large sized dairy farms for detection of subclinical mastitis, this also gives very good indication about the beginning of infection in the udder tissues and also found that California
A mastitis test was the most sensitive test among the indirect tests used in his study.

Bastan et al. (2008) investigated the incidence of SCM in private dairy farms in Turkey and found that CMT was the most reliable diagnostic test for detection of SCM in cows.

Sharma et al. (2009) studied a total of 24 quarter milk samples which were collected aseptically from 6 Jersey cross bred cows aged between 4-6 year brought to the Madras veterinary College Hospital and found that CMT was the most accurate and reliable diagnostic method in field condition after culture.

Sharma et al. (2010) compared indirect screening tests for detection of subclinical mastitis in dairy cows like California mastitis (CMT), sodium lauryl sulphate (SLST) and somatic cell count (SCC) tests. CMT was concluded to be the most accurate test for diagnosis of subclinical mastitis in field condition.

According to Ayano et al. (2013) CMT is good diagnostic tool in the detection of subclinical mastitis; hence it could be most reliable test to investigate subclinical mastitis in the dairy farms.

Saidi et al. (2013) investigated the reliability of a test for early diagnosis of mastitis in cattle and after conducting CMT, quarter-based milk samples were collected from 108 cows. Based on CMT, 29.20% of quarters and 29.62% of cows had subclinical mastitis; the sensitivity of CMT to infections with all bacteria was 96%. *Staphylococcus aureus* was the most common pathogens (40%). On the basis of results of the current study, CMT has very acceptable and sensitive in diagnosis.

Kayesh et al. (2014) concluded that out of total 200 cows, 57 (28.50%) were positive to CMT and of 800 active quarters, 209 (26.13%) were positive to CMT and out of 57 CMT positive cases, 15 were strongly positive (score value 3+), 9 were distinctly positive (score value 2+) and 33 were weakly positive (score value 1+).
Rahman et al. (2014) collected 113 milk samples from crossbred dairy cows and 40 samples from Red Chittagong cows. From the 113 samples 95 were positive i.e. 84% and from rest 40 samples from Red Chittagong cows, only 5 were positive that is 12.5%. Bacteriological examination for isolation of causative organism was done using those samples which showed strong positive result (+++) in CMT test.

**Somatic cell count (SCC)**

According to Harmon (2001) somatic cells always present in milk and they increase due to mammary gland infections. When udders are healthy the somatic cell count (SCC) in milk is between 50,000 and 100,000 cells/ml. If the SCC is greater than 200,000 cells/ml, it is assumed to be a threshold distinguishing a healthy udder from a diseased udder. High SCC in milk reduces the quality of both milk and dairy products.

Maiti et al. (2003) examined 64 lactating cows (209 quarters) and reported that SCC count in milk of SCM quarter was $8.75 \times 10^5$ (mean) /ml where as that of healthy quarter was $1.13 \times 10^5$ (mean)/ml.

As per Sharma et al. (2011) SCC is a useful predictor of intramammary infection. An elevated SCC in milk has a negative influence on the quality of raw milk. Subclinical mastitis is always related to low milk production, changes to milk consistency (density), reduced possibility of adequate milk processing, low protein and high risk for milk hygiene since it may even contain pathogenic organisms.

Bhandari and Garg (2012) observed that when milk samples were collected after calving, on 10th and 40th day for analysis of SCC, cows affected by SCM showed SCC in the range of 1.56 to 4.90 ($x10$/ml milk) and 1.39 to 4.11 ($x10^5$/ml of milk), on 10th and 40th day, respectively which was significantly higher than the normal animals.

De and Mukherjee (2013) reported a group of cows positive for mastitis, screened on the basis of CMT and somatic cell count (more than 0.11 million cells/ml of milk). They found that the somatic cell count was reduced significantly to 53.79%, 65.42% and 81.99% on the days 3, 7 and 15 post treatment with intramammary antibiotic.
Langer and Nauriyal (2013) tested 796 quarters milk, the mean somatic cell count of healthy quarter was $155.59 \times 10^3$/ml whereas, that of infected quarter was $1949.48 \times 10^3$/ml ($P<0.01$).

Hase et al. (2014) observed that somatic cell counts of affected quarters were higher than threshold (3, 50,000 cells/ml of milk) for subclinical mastitis and were identified to be affected with subclinical mastitis. The SCC ($X10^5$ cells/ml) of control group T1 was $6.01 \pm 4.03$ and treatment group T2 was $6.29 \pm 3.33$ at day 0. The SCC was significantly ($P<0.01$) reduced ($4.01 \times 10^5 \pm 2.06$) in treatment group T2 on 5th day post treatment with mastilep gel as compared to untreated control, where rise in SCC ($6.69 \pm 3.52$ on day 5 and $7.16 \pm 0.32$ on day 10) was observed.

**pH of milk**

Bansal and Randhawa (2003) stated that mastitis caused a rise in milk pH and this was primarily due to leakage of blood bicarbonates into milk following damage to mammary epithelium.

Vijaykumar (2003) documented that normal milk pH varies between 6.4-6.8 slightly on to the acidic side.

Samanta et al. (2006) observed that pH value of milk from healthy, mildly, moderately and severely affected Karan Swiss cross bred cows were 6.65, 6.84, 6.87 and 7.0, respectively. The corresponding figures in the case of Karan Fries crosses averaged 6.67, 6.80, 6.86 and 6.98, respectively. The milk pH tented to increase with the severity of infection but varied significantly.

Batavani et al. (2007) collected milk samples from quarters of 35 cows with SCM as well as from 37 healthy controls. Milk from quarters with SCM showed elevated pH (6.69 Vs 6.59). These changes in pH of milk show the presence of tissue damage provoked by SCM.

Shahid et al. (2011) collected milk samples from 125 animals (25 buffaloes, 30 crossbred cows, 15 Sahiwal and 55 Achai breed) and
suggested that the pH level more then 6.8 indicated the incidence of subclinical mastitis.

**Bacterial isolation and characterization of Staphylococcus spp. in milk samples**

Pitkala *et al.* (2001) reported that coagulase negative Staphylococci (CNS) (49.6%) was most commonly isolated bacterial group in bovine mastitis followed by *Corynebacterium bovis* (34.4%), *S. aureus* and *Strep. agalactiae* (0.1%).

Tenhagen *et al.* (2009) reported the prevalence of coagulase positive *S. aureus* was 4% and CNS was 46.8% in Germany on the basis of bacterial culture.

Sori *et al.* (2011) collected 218 milk samples aseptically and CMT was carried out to identify subclinical mastitis in dairy cows in Ethiopia. 164 CMT high scored milk samples were cultured of which 86 (52.4%) of pure strains of *S. aureus* were isolated.

As per Ayano *et al.* (2013) culture method may be used to confirm the infection from specific bacteria and helpful in proper treatment.

**Staphylococcal SCM**

1. **Occurrence**

Coagulase negative Staphylococci have traditionally been considered to be minor mastitis pathogens, especially in comparison with major pathogens such as *S. aureus*, Streptococci and Coliforms. The main reason for this is that mastitis caused by CNS is very mild and usually remains subclinical (Taponen *et al.*, 2009).

Sharma and Sindhu (2007) reported 38.81% prevalence of *Staphylococcus* spp. in Haryana.

In the United States, 46.7% incidence of coagulase negative *S. aureus* (CNS) was detected in post calving milk samples (Pantoja *et al.*, 2009).
Saravanajayam et al. (2015) recorded the prevalence of microbes associated with mastitis. *Staphylococcus* spp. (46.32%) were found most predominant species among all the isolates.

Sharma et al. (2015) collected 2695 quarter milk samples from 770 cows for bacteriological examination from July 2014 to June 2015, of which 1416 (52.54%) samples were found culturally positive. Out of 1416 isolates obtained from infected quarters of cows 1010 samples were found positive for *Staphylococci* suggesting incidence of 71.32%.

2. **Individual quarter wise**

Khanal and Pandit (2013) reported highest occurrence of Staphylococcal mastitis in right hind and left fore quarter, respectively.

3. **Breed wise**

Sirohi and Sirohi (2001) reported incidence rate of Staphylococcal SCM about 50.47%, 30.74% and 7.71% for crossbred, indigenous cattle and buffaloes, respectively.

4. **Age wise**

Garedew et al. (2015) reported that the risk of *Staphylococcus* associated clinical and subclinical mastitis increases significantly with age and parity of the cow.

5. **Parity wise**

Zadoks et al. (2001) and Osteras et al. (2006) reported high incidence of *S. aureus* intramammary infection (IMM) in early stage than late stage of lactation.

**Molecular detection**

Brakstad et al. (1992) used synthetic oligonucleotide primers of 21 and 24 bases, respectively, in the polymerase chain reaction (PCR) to amplify a sequence of the nuc gene, which encodes the thermostable nuclease of *S. aureus*. A DNA fragment of approximately 270 bp was amplified from lysed *S. aureus* cells or isolated DNA. The PCR product was detected by agarose gel electrophoresis or Southern blot analysis. The
primers recognized 90 of 90 reference or clinical \textit{S. aureus} strains. Hence, suggested that the PCR for amplification of the \textit{nuc} gene has potential for the rapid diagnosis of \textit{S. aureus} infections.

Eswaran \textit{et al.} (2011) analyzed 15 strains of coagulase-positive \textit{S. aureus} recovered from subclinical bovine mastitis by PCR amplification with a pair of primers specific for the \textit{nuc} gene of \textit{S. aureus} which encodes the thermo stable nuclease. The \textit{nuc} primer set amplified an expected PCR product, amplicon of 270 bp in all 15 coagulase-positive isolates of \textit{S. aureus}. Further the amplified DNA was analyzed by restricted fragment length polymorphism using HaeIII restriction enzyme. The RFLP yielded two bands of 180 and 90 bp.

Rusenova \textit{et al.} (2013) compared three identification methods that are routinely used for the detection of \textit{S. aureus} as bovine mastitis agent, the conventional biochemical method, commercial identification system and amplification of species-specific gene (\textit{nuc}) by polymerase chain reaction (PCR) and suggested that a routine approach using a combination of phenotypic and molecular detection systems could improve \textit{S. aureus} detection in milk.

\textbf{Antibiogram of \textit{Staphylococcus} spp. isolates}

Roychodhury and Dutta (2009) determined the prevalence of causative bacterial agents of clinical and subclinical bovine mastitis and evaluated the resistance or susceptibility of the mastitis causing bacteria against the commonly used antibacterials. It was observed that \textit{S. aureus} was the major pathogen which contributed 80.56\% in bovine mastitis. The isolated bacteria were found sensitive to antibacterial agents in descending order: gentamicin, enrofloxacin, streptomycin, tetracyclin, amikacin, norfloxacine, cloxacinil, carbenicillin and kanamycin. The isolates exhibited high level of resistance against tetracycline, carbenicillin, clindamycin, oxacillin, cephataxime, cloxacinil, norfloxacine, cotrimazine, amoxicillin and cotrimoxazole. It is suggested that it is necessary to evaluate the indiscriminate use of antibiotics in the treatment of mastitis.
Jain and Joseph (2013) studied mastitis pathogens and their antibiogram. They isolated bacteria and fungi and identified them on the basis of morphology, staining and biochemical characteristics. *In vitro* antibiogram of isolates was done with different antimicrobials *viz.*, enrofloxacin (10 μg), amoxycillin (30 μg), ampicillin (25 μg), cloxacillin (6 μg), penicillin (10 μg), tetracycline (30 μg) streptomycin (25 μg) and gentamicin (10 μg), by single disc diffusion method. Higher incidences were observed for *Staphylococcus* infection (53.34%). *In vitro* sensitivity reflected maximum sensitivity against enrofloxacin (100%).

Jeykumar *et al.* (2013) perform the antibiogram of mastitis pathogens in the milk of crossbred cows in Namakkal district, Tamil Nadu. Out of 72 samples, 32 (44.44%) were found positive for *Staphylococcus* spp. Enrofloxacin was found to be most effective antibiotic among all the tested antibiotics.

Chandrasekaran *et al.* (2014) performed *in vitro* antibiotic sensitivity test against *S. aureus* and found that all the isolates were most sensitive to enrofloxacin (79.8%) followed by gentamicin (71.2%), amoxicillin and sulbactam (69.2%) and ceftriazone (69.2%).

Didugu *et al.* (2015) performed antibiogram on mastitis pathogens in buffalo. Results revealed that most common pathogen in mastitis was *S. aureus*. Most effective antimicrobial agent was chloramphenicol (95%) followed by tetracycline (92%), gentamicin (89%), ciprofloxacin (86%) and enrofloxacin (80%).

Saravanajayam *et al.* (2015) investigated the prevalence of microbes associated with clinical mastitis and their pattern of antibacterial resistance. *Staphylococcus* spp. were found most common isolates. *In-vitro* antibiogram revealed that ceftriaxone (53.47%) was most sensitive followed by amoxicillin (19.18%), gentamicin (11.72%) and enrofloxacin (7.89%).

Sharma *et al.* (2015) reported the prevalence of *S. aureus* among bovines used for milk production in Mathura, India. The results revealed that the incidence of *S. aureus* in clinical as well as sub-clinical mastitis was higher in cattle in comparison to buffaloes. Drug sensitivity
revealed the 100% resistance against penicillin followed by vancomycin (88.89%), nalidixic acid (77.78%), cefixime, methicillin, novobiocin (66.67% each), amoxiclav, colistin, pipemidic acid (55.56% each), ofloxacin, streptomycin, sulphamethizole (44.44% each), ampicillin/sulbactam, cefalexin, cefazolin, cefoperazone, enrofloxacin, floxidin, meropenem (33.33% each), cefuroxim, ciprofloxacin, clindamycin, gentamicin, levofloxacin, norfloxacin, tetracycline (22.22% each). Thus, the findings are useful for formulating specific control programme for bovine mastitis caused by *S. aureus* in this region.

Shende *et al.* (2015) determined the antibiogram profile of mastitis pathogens in cows for effective therapeutic management. Out of 57 cows with subclinical mastitis (30) and clinical mastitis (27), 47 were found positive for *S. aureus*. On antibiogram all the isolates were found sensitive to enrofloxacin (100%).

**Therapy for Staphylococcal subclinical mastitis**

Aloe vera gel is used as a teat-dip in lactating cows, by intramammary administration for (adjuvant) treatment of mastitis or high somatic cell counts (The European agency for the evaluation of medicinal products, 1999).

Urch (1999) described the use of aloe vera in treatment of bovine mastitis. He recommended the aloe vera gel locally on affected quarter twice daily, also orally to affected cow, 250 ml either into the food or as a drench, until symptoms resolved.

McCrory (2001) advocated that aloe vera has the ability to override cortizol (fight or flight mechanism) so that the immune system does not get compromised in times of stress, therefore, aloe vera can be given orally at a dose of 300 cc twice a day for three days. Aloe vera is an immune booster and will help the cow fight off the infection.

Agarry *et al.* (2005) in their *in vitro* research regarding the comparison of anti microorganism activity of gel and extract from the leaves of *Aloe barbadenis*, showed that both forms posses significant inhibitory properties in relation to Staphylococci.
Dettloff (2007) mentioned that if somatic cells are high because of staphylococcal mastitis, organic or conventional treatment such as with aloe vera is not effective.

Sudhan and Sharma in 2010 and Bansal (2013) concluded that enrofloxacin shows high in vitro sensitivity and is pharmacologically considered to distribute well in the udder, clinically proved to be less efficacious against Staphylococcal mastitis because of its inability to kill intracellular organisms.

Suresh et al. (2010) studied on clinical efficacy of long acting enrofloxacin in bovine subclinical mastitis in six crossbred cows. Hundred per cent quarter cure was observed and the clinical cases recovered completely from SCM on the fourth day.

Kasravi et al. (2011) evaluated the efficacy of intra-mammary administered cefquinome for the treatment of sub-clinical mastitis in lactating dairy cows. Seventy-three Holstein dairy cows from a single farm with 150 infected quarters were enrolled in the study. Most intramammary infection (IMI) were caused by coagulase-negative Staphylococci, Streptococci and Coliforms. The overall bacteriological cure (BC) rates for subclinical IMI was 84.61%. Results of this study indicate that cefquinome therapy was effective in reducing SCC and eliminating sub-clinical IMI in lactating dairy cows.

Steinka and Kukulowicz (2011) studied in vitro efficacy of aloe vera gel on S. aureus and found effective against S. aureus, because of anthraquinones present in it.

Crişan et al. (2012) studied on milking cows raised under semi-intensive conditions, showing signs of mastitis in one or more quarters of the udder. Microbiological results indicated prevalence of Gram positive rods, but only one strain of S. aureus was identified (6.6%), the rest of the strains being classified as S. sciuri, S. xylosus and S. lentus. Bacteriological extracts of Echinacea angustifolia, Hippophae rhamnoides, Sylibum marianum, Aloe barbadensis and Thymus vulgaris were tested in vitro for their restoring potential of adaptive cell mediated immunity. The most effective extracts found were Thymus vulgaris and Aloe vera with a significant stimulating effect over the untreated control culture. The tested vegetal extracts could be of help in increasing systemic immunity.
Aloe vera is an important source of phytochemicals and increases the absorption of vitamins C and E and hence it can be used to improve the production performance in animals as well as in promoting recovery from any disease ailments (Mekala and Arivuchelvan, 2012)

Reddy et al. (2015) selected 32 buffaloes with acute mastitis for the study. Cultural examination of milk samples revealed *Staphylococcus* spp. and *E. coli* and samples were sensitive to enrofloxacin. Buffaloes were treated with long acting enrofloxacin along with supportive therapy. Out of 32 buffaloes 21 showed improvement by 1st dose and improvement in 7 buffaloes were noticed after 2nd dose of therapy. In the present study 87.5 per cent of buffaloes were cured from mastitis after 6 days of therapy.

Shende et al. (2015) performed *in vitro* and *in vivo* study in cows for effective therapeutic management. Out of 30 cows with subclinical mastitis 24 were found positive for *S. aureus*. On antibiogram all the isolates were found sensitive to enrofloxacin (100%). Five cows were randomly selected for treatment. Enrofloxacin @ 5 mg/kg b.wt were injected intramuscularly daily for 5 days. The efficacy of drug was judged on the basis of significant decrease in SCC and negativity of milk samples with MCMT. Recovery of predominant bacteria was also recorded. The post treatment mean SCC was found significantly decreased. With MCMT 3 cases showed trace reaction while 2 were found negative with MCMT. After treatment none of the milk samples were detected positive for *Staphylococcus* on culutural examination, suggesting 100% efficacy against *Staphylococcus*. 
3. MATERIALS AND METHODS

Place of work

The work was conducted in the Department of Veterinary Medicine, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur (M.P).

Duration of work

The study was conducted for a period of more than one year i.e. from February 2015 to February 2016.

Animals

For this study, a total of 550 lactating cattle belonging to non descript, cross breed and exotic breeds were screened over a period of more than a year i.e. from February 2015 to February 2016. The cattle belongs to the dairy farms in and around Jabalpur viz. Livestock Farm, Adhartal, N.D.V.S.U., Dayodaya gaushala, central jail gaushala and Rani Avantibai gaushala, Gadarkheda, Barella, nearby villages of Jabalpur like Nuniakala (Panagar) and Noni (Shahpura) and different private dairy farms of Jabalpur, Madhya Pradesh (M.P.). The details are presented in table 01 and plate 01.

Table 01: Sources of animals screened under the study

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Place</th>
<th>No. of animals</th>
<th>Positive animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Military dairy farm, Gaura bazaar</td>
<td>226</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Dayodaya gaushala, Tilwara</td>
<td>50</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Live Stock Farm, Adhartal</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Nuniakala, Panagar</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Noni, Shahpura</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>Kanchan dairy, Pariyat</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Ram dairy, Pariyat</td>
<td>25</td>
<td>08</td>
</tr>
<tr>
<td>8</td>
<td>Yadav dairy, Pariyat</td>
<td>15</td>
<td>06</td>
</tr>
<tr>
<td>9</td>
<td>Yadav dairy, Kanchghar</td>
<td>22</td>
<td>07</td>
</tr>
<tr>
<td>10</td>
<td>Central Jail gaushala, Civil lines</td>
<td>25</td>
<td>05</td>
</tr>
<tr>
<td>11</td>
<td>Rani Avantibai gaushala, Barella</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>550</strong></td>
<td><strong>153</strong></td>
</tr>
</tbody>
</table>
Different parameters, about individual animals i.e. breed, age, lactation number, stage of lactation, herd size and number of quarters affected were recorded.

**Clinical examination of the udder/ milk**

The udder was subjected to clinical examination by manual palpation of each individual teat and was clinically observed for atrophy, consistency or variation in the size of teat and teat position. The glands were palpated for indurations and asymmetry. Teat ends were observed for alterations such as scars, wounds, patent teat orifice and ease of milking. The udder was examined to ascertain the abnormality (unilateral or bilateral).

Milk was examined for quantity which includes reduction in milk and watery consistency of milk and also for the quality i.e. discoloration, clots or flakes, pus and blood staining.

**Testing of milk samples**

**Modified California mastitis test (MCMT)**

The MCMT was performed as per the method adopted by Shukla (1980) on day 0 (pre treatment) and on day 3, 7 and 15 (post treatment). The reagent was prepared by adding 2 ml stock solution B (Bromocresol purple reagent) to make volume 100 ml by adding remaining volume of stock solution A (Sodium lauryl sulphate reagent).

**MCMT grading**

Equal quantity of milk and MCMT reagent was added in a plastic paddle, giving gentle swirling motion in a horizontal plane with minimum agitation (plate 02). The reaction was graded by intensity of gel formation and colour change as follows:
<table>
<thead>
<tr>
<th>MCMT grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>No change</td>
</tr>
<tr>
<td>Trace (T)</td>
<td>Slime formation which disappeared with continuous movement of paddle</td>
</tr>
<tr>
<td>1+</td>
<td>Distinct slime but no gel formation</td>
</tr>
<tr>
<td>2+</td>
<td>Viscous with gel formation which adherent to the margin of the cup</td>
</tr>
<tr>
<td>3+</td>
<td>The gel formation with convex projection, the gel did not dislodge after swirling movement of the paddle</td>
</tr>
</tbody>
</table>

(Radostits et al., 2010)

Collection of milk sample

The milk sample was collected from each teat of cattle indicating MCMT positive for subclinical mastitis (plate 03). The udder and teats were cleaned and washed with potassium permanganate 0.01% then wiped with clean cloth. First few streams of foremilk was discarded and then about 8 ml of milk from each affected quarter was collected in fresh, sterile, labeled screw cap test tubes and brought to the department in ice for further examination.

Laboratory tests

1. Somatic cell count (SCC)

   The leukocyte count in the subclinical mastitic milk was performed to assess the degree of infection using modified Newman’s stain as per the procedure described by Harmon (2001) on day 0 (pre treatment) and on day 3, 7 and 15 (post treatment).

 Preparation of milk smear

   The smear of milk for SCC was prepared within one hour of it’s collection to minimize disintegration of leukocyte. Each milk sample was uniformly mixed by gentle shaking of the vials and the milk (0.01 ml) was spread with sterilized bacteriological loop, over one cm rectangular area on a clean microslide. The milk smear from the test sample was stained by modified Newman’s stain. A total of 30 fields were counted under oil immersion lens and average number of cells per field was worked out. The average number of cells was multiplied by the multiplication factor of the microscope i.e. 497512 to obtain the number of cells per ml of the milk.
Microscopic factor determination

The diameter of the field of 10x eye piece was predetermined by using stage micrometer. The lowest division of micrometer scale was 0.01 mm. accordingly the diameter was measured and obtained as 0.016 cm. The area of the microscopic field was determined by the formula $\pi r^2$. The calculations were made as under:

\[
\begin{align*}
\text{Diameter} &= 0.016 \text{ cm.} \\
\text{Radius} &= 0.008 \text{ cm} \\
\text{Area} &= 3.14 \times (0.008)^2 \\
&= 0.000201 \\
\text{Microscopic factor} &= 100 \times \left(\frac{1}{\text{Area}}\right) \\
&= 100 \times \left(\frac{1}{0.000201}\right) \\
&= 497512.43 \text{ or } 497512
\end{align*}
\]

The same calibrated research microscope was used throughout the course of study (Shukla, 1980).

2. Milk pH

It was estimated by the digital pH meter. The pH reading of the normal and mastitic milk sample was recorded on day 0 (pre treatment) and on day 3, 7 and 15 (post treatment).

Bacterial isolation and characterization of *Staphylococcus* spp. in positive milk samples (MCMT positive)

1. Isolation of *Staphylococcus* spp.

Pre enrichment using 6.5 per cent sodium chloride

The culture media for isolation of bacteria was prepared as per the procedure of Markey *et al.* (2012). Bacterial examination was done on the MCMT-positive milk samples. Pre enrichment of *Staphylococcus* spp. was done on Muller Hinton broth with 6.5 per cent sodium chloride (plate 04).
**Isolation of *Staphylococcus* spp. on selective agar**

Pre enriched samples were cultured bacteriologically to isolate the *Staphylococcus* spp. on Mannitol salt agar. These isolates were incubated aerobically at 37°C for 24 hours. The yellow or pink colony on Mannitol salt agar were indicating the presence of *Staphylococcus* (Markey *et al.*, 2012).

**Gram's staining**

Clean slide was prepared then a loopful of water was placed in the center of the slide. With the inoculating needle, aseptically very small amount of culture was picked up and mixed into the drop of water and was spread to 1/2-inch area. Slide was air dried and passed through a Bunsen burner flame three times to heat-fixed and to kill the bacteria. The heat fixed smear was placed on a staining receptacle and slide was covered with crystal violet solution for one minute and then washed off briefly with tap water and drained. The smear was then treated with few drop of Gram's Iodine and allowed to act for a minute. The slide was again washed in water and then decolorized in absolute ethyl alcohol. After the smear is decolourized, it was washed in water without any delay. The smear was finally treated with few drops of counter stain safranin and washed off with tap water. Excess water was removed using a blotting paper, dried in air and examined microscopically under the oil immersion lens. Gram positive cocci (violet colour) arranged in grape-like clusters were seen (Markey *et al.*, 2012).

The individual bacterial isolates were serially numbered as 1, 2, 3 and so on. Then, these isolates were preserved after characterization.

2. **Identification of *Staphylococcus* spp.**

**Biochemical characterization**

Each bacterial isolate was further characterized by various biochemical tests.
Catalase test

A loopful of 24 hours old culture from slant was mixed with a drop of 3% hydrogen peroxide solution on a clean microslide. A catalase positive strain showed effervescence (Barrow and Feltham, 1993).

Coagulase test

It was performed to identify the pathogenic Staphylococci as per Collee et al. (1996).

Slide coagulase test

Two drops of saline were put on the labeled slide and emulsified with the test organism using wooden stick. A drop of rabbit plasma was added to the one of inoculated saline drop mixed well and then the slide was rocked gently for about 10 seconds. In positive results macroscopic clumping was observed in the plasma within 10 seconds.

Tube coagulase test

For this test 0.1 ml of broth culture was added to 0.5 ml of EDTA rabbit plasma diluted with normal saline solution (1:10) and incubated at 37°C. Diluted rabbit plasma without culture was used as control. The tubes were observed for coagulase production at 1, 3 and 6 hour interval and in negative cases the tubes were left overnight at room temperature and were then re-examined. All strains producing coagulase upto 6 hours and overnight at room temperature were taken as coagulase positive. No clotting of plasma was observed in negative case.

Haemolysis on blood agar

All the isolates were streaked on blood agar plates and incubated at 37°C for 24 hours. Thereafter, the blood agar plates were examined for haemolysis. Clear zone of haemolysis in immediate surroundings of the colony was indicator of alpha haemolysis, wider and hazy zone of haemolysis around the colony was regarded as beta haemolysis which clears off after 24 hours of refrigeration (Markey et al., 2012).
DNase test

DNase activity was used to identify potentially pathogenic Staphylococci as per MacFaddin (1985). For this test plates were inoculated by spotting a inoculum of test organism from the BHI broth approximately 5 mm in diameter. Plates were Incubated at 37 °C for 18 – 24 hours, then plates were flooded with 1 N HCl. Clear zone around the spot indicated DNase activity.

Molecular detection

Extraction of DNA from culture:

The procedure of DNA extraction was performed by chelex based extraction of DNA using Insta Gene Matrix (Bio-Rad laboratories, India Pvt. Ltd.) as described by Giraffa et al. (2000).

Procedure

For extraction of DNA, 1.5 ml of broth culture was vortexed for 10 seconds and centrifuged for 1 minute at 10,000–12,000 rpm. The supernatant was removed and 200 micro liters (µl) of Insta Gene matrix was added to the pellet. The suspension was incubated at 56°C for 15–30 minutes and again vortexed at high speed for 10 seconds. The cell suspension was heated in a boiling water bath for 8 minutes and again vortexed at high speed for 10 seconds and spun at 10,000–12,000 rpm for 2–3 minutes. Then 10 µl of the resulting supernatant was used per 25 µl PCR reaction and the remainder supernatant was stored at -20°C for further use.

Nuc gene amplification using polymerase chain reaction (PCR)

Reconstitution of oligonucleotide primers

The oligonucleotide primers were synthesized and supplied in lyophilized form by Integrated DNA Technologies, Avantor Performance Materials India Limited, Faridabad. They were reconstituted in sterile nuclease free water (NFW) as per the manufacturer’s recommendation.
**Oligonucleotide sequence and PCR cycling condition**

The primer (synthesized by Integrated DNA Technology) used in the study for amplification of *nuc* gene from 24 hour old BHI broth culture is listed below:

**Details of the primer used in the study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease (Nuc)</td>
<td>Forward</td>
<td>GCG ATT GAT GGT GAT ACG GTT</td>
<td>Brakstad <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGC CAA GCC TTG ACG AAC TAA AGC</td>
<td></td>
</tr>
</tbody>
</table>

**PCR Conditions for detection of *nuc* gene**

The PCR was carried out in 25 µl of reaction mixture which was composed of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR mastermix</td>
<td>12 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sample DNA</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The cycling conditions used for amplification of *nuc* gene were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Temperature/Timers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>37 cycles of denaturation</td>
<td>94°C for 1 min</td>
</tr>
<tr>
<td>Step 2</td>
<td>Annealing</td>
<td>52°C for 0.5 min</td>
</tr>
<tr>
<td>Step 3</td>
<td>Extension</td>
<td>72°C for 1.5 min</td>
</tr>
<tr>
<td>Step 4</td>
<td>Final extension</td>
<td>72°C for 3.5 min</td>
</tr>
</tbody>
</table>

The PCR products were stored in the cycler at 4°C until they were collected (Brakstad *et al.*, 1992). The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1.5% agarose gel and viewed in UV transilluminator system.
Detection of PCR product by gel electrophoresis

Agarose gel electrophoresis

Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1 X TBE buffer. After cooling for about 2 min, ethidium bromide was added to the agarose solution to a final concentration of 0.5 μg/ml. The molten agarose was then poured into the tray and comb was kept undisturbed till gel solidified. The comb was then taken out carefully and the tray containing gel was then placed in a submarine horizontal electrophoresis unit (GENE™ minipack-250, Bangalore) filled with 1 X TBE buffer upto level of 1 mm above the gel surface.

Five μl of each PCR product was loaded into each well. A 100 bp DNA ladder was used as marker. Gels were run in 1 x TBE buffer at 80 V for 2 hours. After sufficient migration, the gel was observed under UV transilluminator to visualize the bands. The size of PCR product was determined by comparing with a standard molecular weight marker, and was photographed by gel documentation system (Alpha Imager™ 1220, Documentation and Analysis, Alpha Innotech Corporation, USA) (details are presented in plate 05).

Antibiogram of Staphylococcus spp. isolates

Drug sensitivity test was done by using antibiotic discs. The antibiotic discs (Himedia) viz., cefuroxime (30 mcg), enrofloxacin (10 mcg), cefepime (30 mcg), cefoparazone (75 mcg), ampicillin+ sulbactum (10/10 mcg) and piperacillin+ tazobactum (100/10 mcg) were placed on the surface of charged agar plates aseptically at equidistant from each other. The plates were incubated at 37ºC for 18-24 hours. The susceptibility of organisms to different drugs was observed by measuring the zone of inhibition (Bauer et al., 1966 and CLSI, 2013).

Experimental design

To study the efficacy of different drugs for the treatment of Staphylococcal SCM, a total of 32 cows positive for Staphylococcus spp.,
were placed into four groups i.e. B – E, each group comprised of 8 animals. Eight clinically healthy cattle and negative for MCMT and culture were selected to serve as healthy control (Group A). The details of therapeutic trial are presented in Table 02 and plate 06.

Table 02: Specific therapies for various groups of cattle under study

<table>
<thead>
<tr>
<th>Group</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Healthy control</td>
</tr>
<tr>
<td>B</td>
<td>Inj- Enrofloxacin LA @ 7.5 mg/kg b. wt. S/C once on day 0 of treatment</td>
</tr>
<tr>
<td>C</td>
<td>Inj- Enrofloxacin LA @ 7.5 mg/kg b. wt. S/C once on day 0 of treatment+ oint- Cefquinome @ 75 mg/per quarter IMM, q12h for 3 successive milking</td>
</tr>
<tr>
<td>D</td>
<td>Inj- Enrofloxacin LA @ 7.5 mg/kg b. wt. S/C once on day 0 of treatment+ oint- Cefquinome @ 75 mg/per quarter IMM, q12h for 3 successive milking + liq- Aloe vera PO @ 1 ml/kg b. wt. q12h for 3 days + Aloe vera gel topical q12h for 3 days</td>
</tr>
<tr>
<td>E</td>
<td>Liq- Aloe vera PO @ 1 ml/kg b. wt. q12h for 3 days + Aloe vera gel topical q12h for 3 days</td>
</tr>
</tbody>
</table>

LA- Long acting, I/MM- Intra Mammary, S/C- Subcutaneous q_h- After every hour, PO- Per Os, b. wt.- body weight

**Therapeutic response evaluation**

The response of therapeutic study was evaluated on the basis of MCMT, SCC, milk pH after the completion of treatment i.e. on day 3, 7 and 15 post treatment. In addition, for evaluation of therapeutic response the bacteriological culture examination on post treatment days was also recorded under the study.
**Statistical analysis**

Analysis of data of occurrence studies was done by using Chi square test. The data obtained for SCC and pH were analyzed statistically for estimating level of significance by applying Z test. The alterations in different treatment groups at different intervals were analyzed using hierarchical design of ANOVA and means were compared using Duncan’s multiple range test (Snedecor and Cochran, 1994).
4. RESULTS

Subclinical mastitis is most commonly seen in dairy animals causing huge economic losses to the farmers and dairy industry.

The study consisted of 8 apparently healthy animals and 32 field cases of Staphylococcal subclinical mastitis. Epidemiological study, clinical examination of animal and their udder/milk and phenotypic and genotypic characterization of *Staphylococcus* spp. was carried out. The field cases of Staphylococcal SCM were randomly divided into 4 groups. The data obtained were statistically analyzed and presented.

**Occurrence of subclinical mastitis (SCM)**

The present survey was undertaken to assess and find out the occurrence of SCM in cattle. For this purpose lactating cattle belonging to different private dairy farms and livestock farm, Adhartal, N.D.V.S.U., Jabalpur were screened for SCM. A total of 550 lactating cattle were tested by using modified California mastitis test (MCMT) and somatic cell count (SCC). The overall occurrence of infected animal was found to be 27.81 per cent (153/550) on animal basis and 10.13 per cent (212/2092) on quarter basis, respectively. The results are shown in table 03, figure 01 and 02.

**Table 03: Occurrence of SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Particulars</th>
<th>Number Screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total number of animals</td>
<td>550</td>
<td>153</td>
<td>27.81</td>
</tr>
<tr>
<td>2.</td>
<td>Total number of quarters</td>
<td>2092 (108 blind teats)</td>
<td>212</td>
<td>10.13</td>
</tr>
</tbody>
</table>

**Individual quarter wise**

The occurrence of SCM in different quarters of udder in cattle is illustrated in table 04 and figure 03. The occurrence was highest in right hind quarter i.e. 13.35 per cent (70 out of 524 quarters) followed by 9.88 per cent
(53 out of 536 quarters) in left hind quarter, 9.09 per cent (47 out of 517 quarters) in right fore quarter and lowest occurrence of 8.15 per cent (42 out of 515 quarters) in left fore quarter was found in cattle with SCM. The occurrence was higher in hind quarters i.e. 11.60 per cent (123 out of 1060 quarters) as compared to fore quarters of 8.62 per cent (89 out of 1032 quarters), right side of 11.23 per cent (117 out of 1041 quarters) as compared to left side of 9.03 per cent (95 out of 1051 quarters). The quarter wise occurrence revealed a non-significant variation.

**Table 04: Individual quarter afflicted with SCM in cattle**

<table>
<thead>
<tr>
<th>Quarter's position</th>
<th>No. screened</th>
<th>No. positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fore</td>
<td>517</td>
<td>47</td>
<td>9.09</td>
</tr>
<tr>
<td>Hind</td>
<td>524</td>
<td>70</td>
<td>13.35</td>
</tr>
<tr>
<td>Total</td>
<td>1041</td>
<td>117</td>
<td>11.23</td>
</tr>
<tr>
<td>Left</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fore</td>
<td>515</td>
<td>42</td>
<td>8.15</td>
</tr>
<tr>
<td>Hind</td>
<td>536</td>
<td>53</td>
<td>9.88</td>
</tr>
<tr>
<td>Total</td>
<td>1051</td>
<td>95</td>
<td>9.03</td>
</tr>
</tbody>
</table>

χ² = 7.1211     df = 03      P = 0.06813

**Breed wise**

The breed wise occurrence study of SCM in lactating cattle revealed a highest occurrence of 36.43 per cent (94 out of 258 cattle) in cross bred cattle followed by occurrence of 22.06 per cent in non-descript cattle (32 out of 145 cattle) and lowest occurrence of 18.36 per cent (27 out of 147 cattle) in H.F. cattle. Breed wise occurrence revealed the significant (P<0.05) effect of different breeds on occurrence of SCM (Table 05 and Figure 04).
Table 05: Breed wise occurrence of SCM

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Breed type</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ND</td>
<td>145</td>
<td>32</td>
<td>22.06</td>
</tr>
<tr>
<td>2.</td>
<td>H.F.</td>
<td>147</td>
<td>27</td>
<td>18.36</td>
</tr>
<tr>
<td>3.</td>
<td>Cross bred</td>
<td>258</td>
<td>94</td>
<td>36.43</td>
</tr>
</tbody>
</table>

$\chi^2 = 10.4452 \quad df = 02 \quad P = 0.005392$

Age wise

The age wise occurrence of SCM in lactating cattle revealed highest occurrence i.e. 36.36 per cent (104 out of 286 cattle) in the cattle of 5-7 years of age followed by 19.64 per cent (22 out of 112 cattle) occurrence in cattle of 7 years and above age and lowest occurrence of 17.76 per cent in cattle of 3 to 4 years of age (27 out of 152 cattle). The age wise occurrence revealed a significant variation ($P < 0.01$) among various age groups. The details are outlined in table 06 and figure 05.

Table 06: Age wise occurrence of SCM in cattle

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Age group</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3-4 years</td>
<td>152</td>
<td>27</td>
<td>17.76</td>
</tr>
<tr>
<td>2.</td>
<td>5-7 years</td>
<td>286</td>
<td>104</td>
<td>36.36</td>
</tr>
<tr>
<td>3.</td>
<td>7 years and above</td>
<td>112</td>
<td>22</td>
<td>19.64</td>
</tr>
</tbody>
</table>

$\chi^2 = 12.4458 \quad df = 02 \quad P = 0.001983$

Parity wise

Parity wise occurrence of SCM was also recorded. The parity number was taken from 1$^{st}$ to 7$^{th}$ and more parity number. The parity wise occurrence revealed a significant variation ($P < 0.01$) among various parity groups. The highest occurrence of SCM was observed in 3$^{rd}$ parity i.e. 41.83
per cent (41 out of 98 cattle) followed by 34.54 per cent in 4\textsuperscript{th} parity (38 out of 110 cattle), 32.05 per cent in 5\textsuperscript{th} parity (25 out of 78 cattle), 22.91 per cent in 6\textsuperscript{th} parity (11 out of 48 cattle), 18.18 per cent in 2\textsuperscript{nd} parity (16 out of 88 cattle), while, lowest occurrence was observed in 1\textsuperscript{st} and 7\textsuperscript{th} and more parity i.e. 17.18 per cent (11 out of 64 cattle). The results are outlined in table 07 and figure 06.

**Table 07: Parity wise occurrence of SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parity number</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1\textsuperscript{st}</td>
<td>64</td>
<td>11</td>
<td>17.18</td>
</tr>
<tr>
<td>2.</td>
<td>2\textsuperscript{nd}</td>
<td>88</td>
<td>16</td>
<td>18.18</td>
</tr>
<tr>
<td>3.</td>
<td>3\textsuperscript{rd}</td>
<td>98</td>
<td>41</td>
<td>41.83</td>
</tr>
<tr>
<td>4.</td>
<td>4\textsuperscript{th}</td>
<td>110</td>
<td>38</td>
<td>34.54</td>
</tr>
<tr>
<td>5.</td>
<td>5\textsuperscript{th}</td>
<td>78</td>
<td>25</td>
<td>32.05</td>
</tr>
<tr>
<td>6.</td>
<td>6\textsuperscript{th}</td>
<td>48</td>
<td>11</td>
<td>22.91</td>
</tr>
<tr>
<td>7.</td>
<td>7\textsuperscript{th} and more</td>
<td>64</td>
<td>11</td>
<td>17.18</td>
</tr>
</tbody>
</table>

χ\textsuperscript{2} = 13.852     df = 06     P = 0.0089

**Lactation stage wise**

The lactation stage was divided in 3 classes i.e. early lactation, 1 to 3 months, mid lactation, 3 to 6 months and late lactation, 6 months and onwards till drying off. The overall occurrence of SCM according to lactation stage was observed as 47.46 per cent (75 out of 158 cattle), 24.10 per cent (54 out of 224 cattle) and 14.28 per cent (24 out of 168 cattle) in early, mid and late lactation stage, respectively (Table 08 and figure 07). The lactation stage wise occurrence of SCM showed significant variation (P<0.01) among different lactation stage.
Table 08: Lactation stage wise occurrence of SCM in cattle

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Lactation stage</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Early (1-3 months)</td>
<td>158</td>
<td>75</td>
<td>47.46</td>
</tr>
<tr>
<td>2.</td>
<td>Mid (3-6 months)</td>
<td>224</td>
<td>54</td>
<td>24.10</td>
</tr>
<tr>
<td>3.</td>
<td>Late (&gt; 6 months)</td>
<td>168</td>
<td>24</td>
<td>14.28</td>
</tr>
</tbody>
</table>

χ² = 25.4423  df = 02  P = 0.00001

Organized and unorganized dairy farms wise

No significant variation was noticed in the occurrence with respect to rearing pattern of cows in dairy farms, however, occurrence of SCM in unorganized dairy farms was observed higher i.e. 36.92 per cent (24 out of 65 cattle) than the occurrence in organized sector of dairy farms i.e. 26.59 per cent (129 out of 485 cattle). Results are represented in table 09 and figure 08.

Table 09: Occurrence of SCM in organized and unorganized dairy farms

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sector/ Rearing Pattern</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Organized dairy farms</td>
<td>485</td>
<td>129</td>
<td>26.59</td>
</tr>
<tr>
<td>2.</td>
<td>Unorganized dairy farms</td>
<td>65</td>
<td>24</td>
<td>36.92</td>
</tr>
</tbody>
</table>

χ² = 1.6197  df = 01  P = 0.203128

Clinical examination of the udder/ milk

Clinical examination of udder and milk revealed that they were apparently normal in cattle with SCM, also colour, consistency and odour of milk samples from all positive cases of SCM were found to be apparently normal however, reduction in milk yield was recorded in SCM.
Modified California mastitis test (MCMT)

In the present study 550 lactating cattle were screened for SCM and 27.81 per cent (153 out of 550 cows) showed positive result by formation of gel or viscous mass on plastic paddle.

- **MCMT grading**

MCMT score in cattle with SCM are given in table 10, figure 09 and plate 02. A score of 1+, 2+ and 3+ in MCMT was noticed in 57.55 per cent (122 out of 212 quarters), 31.60 per cent (67 out of 212 quarters) and 10.85 per cent (23 out of 212 quarters) in cattle afflicted with SCM, respectively.

**Table 10: MCMT grading in SCM**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Score</th>
<th>No. of positive quarters for SCM (n= 212)</th>
<th>Per cent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1+</td>
<td>122</td>
<td>57.55</td>
</tr>
<tr>
<td>2.</td>
<td>2+</td>
<td>67</td>
<td>31.60</td>
</tr>
<tr>
<td>3.</td>
<td>3+</td>
<td>23</td>
<td>10.85</td>
</tr>
</tbody>
</table>

Somatic cell count (SCC)

Apparently healthy cattle had a mean value of SCC 1.26 ± 0.90 X 10^5 cells/ml. The mean SCC in SCM (15.55 ± 1.20 X 10^5 cells/ml) was significantly increased as compared to control (Table 11 and plate 07).

**Milk pH**

Apparently healthy cattle had a mean value of milk pH 6.43 ± 0.08. Mean milk pH in SCM (7.30 ± 0.09) significantly increased as compared to control (Table 11 and plate 07).

**Table 11: Milk profile in SCM**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Apparently healthy control (n= 8)</th>
<th>Subclinical mastitis (n=212)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SCC (10^5 cells/ml)</td>
<td>1.26 ± 0.90</td>
<td>15.55 ± 1.20**</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>6.43 ± 0.08</td>
<td>7.30 ± 0.09**</td>
</tr>
</tbody>
</table>

**- Differ significantly (P< 0.01)**

n= number of quarters
Bacterial isolation and characterization of *Staphylococcus* spp. in milk samples found positive for SCM

A total of 2092 milk samples from functional quarters of lactating cattle were screened for udder health status. Milk samples were collected for bacterial isolation and identification on primary and selective media, respectively, from 212 quarters found positive on MCMT. As many as 127 samples were found positive for *Staphylococcus* spp. as identified on the basis of characteristic colour changes on Mannitol salt agar (yellow or pink colour colony) (plate 08), colony morphology (Gram positive violet colour cocci arranged in grape-like clusters), positive catalase test (plate 09), haemolysis pattern on blood agar (alpha and beta haemolysis) and DNase activity on DNase agar (plate 10).

Out of 212 milk samples collected from mastitic quarters, 31 (14.62 per cent) isolates were found coagulase positive Staphylococci (CPS) as revealed by coagulase test (plate 10) and 96 (45.28 per cent) isolates were found to be coagulase negative Staphylococci (CNS). The details are shown in Table 12, figure 10 and plate 10.

**Table 12: Occurrence of coagulase positive Staphylococci based on bacterial culture in quarter milk samples**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Pathogen</th>
<th>Number examined</th>
<th>Number positive</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Coagulase positive Staphylococci (CPS)</td>
<td>212</td>
<td>31</td>
<td>14.62</td>
</tr>
<tr>
<td>2.</td>
<td>Coagulase negative Staphylococci (CNS)</td>
<td>212</td>
<td>96</td>
<td>45.28</td>
</tr>
</tbody>
</table>

Occurrence of Staphylococcal SCM

The overall occurrence of *Staphylococcus* spp. in subclinical mastitis from February 2015 to February 2016 was 16.36 per cent (90 out of 550 cattle) in lactating cattle. However, the occurrence among the cattle
suffering from subclinical mastitis was reported to be 58.82 per cent (90 out of 153 SCM cattle). The quarter wise occurrence of *Staphylococcus* spp. in subclinical mastitis was 6.07 per cent (127 out of 2092 quarters) and occurrence among subclinical mastitis was found to be 59.90 per cent (127 out of 212 quarters). Significant variation (P< 0.01) was observed in occurrence of staphylococcal SCM in lactating cattle. Results are depicted in table 13 and 14; figure 11 and 12.

**Table 13: Animal wise Occurrence of Staphylococcal SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Cows</th>
<th>Number examined</th>
<th>Number positive</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Screened</td>
<td>550</td>
<td>90</td>
<td>16.36</td>
</tr>
<tr>
<td>2.</td>
<td>Subclinical mastitis</td>
<td>153</td>
<td>90</td>
<td>58.82</td>
</tr>
</tbody>
</table>

χ² = 37.281       df = 01   P = 0.00001

**Table 14: Quarter wise occurrence of Staphylococcal SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Quarters</th>
<th>Number examined</th>
<th>Number positive</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total no. of functional quarter</td>
<td>2092</td>
<td>127</td>
<td>06.07</td>
</tr>
<tr>
<td>2.</td>
<td>Subclinical mastitis</td>
<td>212</td>
<td>127</td>
<td>59.90</td>
</tr>
</tbody>
</table>

χ² = 331.2459       df = 01   P = 0.0000.1

**Individual quarter wise**

The quarter wise occurrence of Staphylococcal SCM showed significant variation (P< 0.05). The occurrence was highest in right hind quarter i.e. 8.77 per cent (46 out of 524 quarters) followed by 5.60 per cent (29 out of 517 quarters) in right fore quarter, 5.59 per cent (30 out of 536 quarters) in left hind quarter and lowest occurrence of 4.27 per cent (22 out of 515 quarters) in left fore quarter. The occurrence was also found higher in
hind quarters i.e. 7.16 per cent (76 out of 1060 quarters) as compared to fore quarters of 4.94 per cent (51 out of 1032 quarters), right side of 7.20 per cent (75 out of 1041 quarters) as compared to left side of 4.94 per cent (52 out of 1051 quarters). The details are outlined in table 14 and figure 13.

**Table 15: Individual quarter afflicted with Staphylococcal SCM in cattle**

<table>
<thead>
<tr>
<th>Quarter's position</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fore</td>
<td>517</td>
<td>29</td>
<td>5.60</td>
</tr>
<tr>
<td>Hind</td>
<td>524</td>
<td>46</td>
<td>8.77</td>
</tr>
<tr>
<td>Total</td>
<td>1041</td>
<td>75</td>
<td>7.20</td>
</tr>
<tr>
<td>Left</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fore</td>
<td>515</td>
<td>22</td>
<td>4.27</td>
</tr>
<tr>
<td>Hind</td>
<td>536</td>
<td>30</td>
<td>5.59</td>
</tr>
<tr>
<td>Total</td>
<td>1051</td>
<td>52</td>
<td>4.94</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 8.8105 \quad df = 03 \quad P = 0.031919 \]

**Breed wise**

The breed wise occurrence of *Staphylococcus* SCM in lactating cattle revealed a highest occurrence of 22.48 per cent (58 out of 258 cattle) in cross bred followed by 12.41 per cent in non-descript cattle (18 out of 145 cattle) and lowest occurrence of 9.52 per cent (14 out of 147 cattle) in H.F. cattle. The breed wise occurrence of Staphylococcal SCM showed significant variation (P<0.05) among various breeds of cattle. Results are outlined in table 16 and figure 14.

**Table 16: Breed wise occurrence of Staphylococcal SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Breed</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ND</td>
<td>145</td>
<td>18</td>
<td>12.41</td>
</tr>
<tr>
<td>2.</td>
<td>H.F.</td>
<td>147</td>
<td>14</td>
<td>09.52</td>
</tr>
<tr>
<td>3.</td>
<td>Cross bred</td>
<td>258</td>
<td>58</td>
<td>22.48</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 9.8837 \quad df = 02 \quad P = 0.007141 \]
**Age wise**

The age wise occurrence of Staphylococcal SCM in lactating cattle revealed highest occurrence i.e. 23.77 per cent (68 out of 286 cattle) in age group of 5-7 years followed by 10.71 per cent occurrence (12 out of 112 cattle) in 7 years and above age group and lowest occurrence i.e. 6.57 per cent in 3 to 4 years of age group (10 out of 152 cattle). The age wise occurrence revealed a significant variation (P< 0.01) among various age groups. The details are outlined in table 17 and figure 15.

**Table 17: Age wise occurrence of Staphylococcal SCM in cattle**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Age group</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3-4 years</td>
<td>152</td>
<td>10</td>
<td>6.57</td>
</tr>
<tr>
<td>2.</td>
<td>5-7 years</td>
<td>286</td>
<td>68</td>
<td>23.77</td>
</tr>
<tr>
<td>3.</td>
<td>7 years and above</td>
<td>112</td>
<td>12</td>
<td>10.71</td>
</tr>
</tbody>
</table>

χ² = 17.4069  df = 02  P = 0.000166

**Parity wise**

The high occurrence of staphylococcal SCM was observed in 3rd 4th and 5th parity i.e. 23.46 per cent (23 out of 98 cattle), 22.72 per cent (25 out of 110 cattle) and 25.64 per cent (20 out of 78 cattle), respectively. This was followed by 12.50 per cent in 6th parity (06 out of 48 cattle), 9.37 per cent in 7th and more parity (06 out of 64 cows), 6.81 per cent in 2nd parity (06 out of 88 cattle) and lowest occurrence was observed in 1st parity i.e. 6.25 per cent (04 out of 64 cattle). Parity wise occurrence revealed significant variation (P< 0.05). Results are outlined in table 18 and figure 16.
Table 18: Parity wise occurrence of Staphylococcal SCM in cattle

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parity number</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>64</td>
<td>04</td>
<td>6.25</td>
</tr>
<tr>
<td>2.</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>88</td>
<td>06</td>
<td>6.81</td>
</tr>
<tr>
<td>3.</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>98</td>
<td>23</td>
<td>23.46</td>
</tr>
<tr>
<td>4.</td>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>110</td>
<td>25</td>
<td>22.72</td>
</tr>
<tr>
<td>5.</td>
<td>5&lt;sup&gt;th&lt;/sup&gt;</td>
<td>78</td>
<td>20</td>
<td>25.64</td>
</tr>
<tr>
<td>6.</td>
<td>6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>48</td>
<td>06</td>
<td>12.50</td>
</tr>
<tr>
<td>7.</td>
<td>7&lt;sup&gt;th&lt;/sup&gt; and more</td>
<td>64</td>
<td>06</td>
<td>9.37</td>
</tr>
</tbody>
</table>

\[\chi^2 = 18.4249 \quad df = 06 \quad P = 0.018\]

Lactation stage wise

The overall occurrence of Staphylococcal SCM according to lactation stage was observed as 31.64 per cent (50 out of 158 cattle), 10.71 per cent (24 out of 224 cattle) and 9.52 per cent (16 out of 168 cattle) in early, mid and late lactation stage, respectively. The lactation stage wise occurrence of staphylococcal SCM showed significant variation (P<0.01) among different lactation stage. The details are outlined in table 19 and figure 17.

Table 19: Lactation stage wise occurrence of Staphylococcal SCM in cattle

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Lactation stage</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Early (1-3 months)</td>
<td>158</td>
<td>50</td>
<td>31.64</td>
</tr>
<tr>
<td>2.</td>
<td>Mid (3-6 months)</td>
<td>224</td>
<td>24</td>
<td>10.71</td>
</tr>
<tr>
<td>3.</td>
<td>Late (&gt;6 months)</td>
<td>168</td>
<td>16</td>
<td>9.52</td>
</tr>
</tbody>
</table>

\[\chi^2 = 25.4603 \quad df = 02 \quad P = 0.00001\]
Organized and unorganized dairy farms wise

Occurrence of Staphylococcal SCM in organized and unorganized dairy farms was observed as 16.49 per cent (80 out of 485 cattle) and 15.62 per cent (10 out of 65 cattle). No significant variation was noticed in the occurrence with respect to rearing pattern of cows in dairy farms. Results are represented in table 20 and figure 18.

Table 20: Occurrence of Staphylococcal SCM in organized and unorganized dairy farms

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sector/ Rearing Pattern</th>
<th>Number screened</th>
<th>Number positive</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Organized dairy farms</td>
<td>485</td>
<td>80</td>
<td>16.49</td>
</tr>
<tr>
<td>2.</td>
<td>Unorganized dairy farms</td>
<td>65</td>
<td>10</td>
<td>15.62</td>
</tr>
</tbody>
</table>

χ^2 = 0.0374  df = 1  P = 0.8467

Molecular detection

Amplification of nuclease (nuc) gene using PCR

Agarose gel electrophoresis showing amplification of 270 bp fragment specific for *S. aureus* with M: 100 bp molecular marker; Lane 1-6 shows positive bacterial culture; Lane 7-11 shows negative bacterial culture. The occurrence recorded using PCR for detection of nuc gene was 14.62 per cent (31 isolates were found positive for nuc gene). The results are shown in table 21 plate 11.

Table 21: Detection of nuc gene based on PCR method of identification in bacterial culture positive for *Staphylococcus* spp.

<table>
<thead>
<tr>
<th>Total no. of samples</th>
<th>Bacterial culture positive for <em>Staphylococcus</em> spp.</th>
<th>PCR for nuc gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>212</td>
<td>127</td>
<td>31 (14.62 per cent)</td>
</tr>
</tbody>
</table>
Antibiogram of *Staphylococcus* spp. isolates

The *in vitro* antibiogram of *Staphylococcus* spp. from 32 cattle under therapeutic trial was studied. Results of antibiogram revealed that most effective antimicrobial agent was enrofloxacin (100 per cent) followed by piperacillin + tazobactum (87.80 per cent), cefoparazone (82.92 per cent), ampicillin + sulbactum (82.92 per cent) and cefuroxime (82.92 per cent). All the isolates showed resistance to cefepime (100 per cent) as shown in table 22 and plate 12.

**Table 22: Sensitivity of *Staphylococcus* spp. to different antimicrobials**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Antibiotic</th>
<th>Sensitivity (Per cent)</th>
<th>Intermediate (Per cent)</th>
<th>Resistant (Per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cefuroxime (30 mcg)</td>
<td>82.92</td>
<td>4.88</td>
<td>12.20</td>
</tr>
<tr>
<td>2.</td>
<td>Enrofloxacin (10 mcg)</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>3.</td>
<td>Cefepime (30 mcg)</td>
<td>0.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>4.</td>
<td>Cefoparazone (75 mcg)</td>
<td>82.92</td>
<td>12.20</td>
<td>4.88</td>
</tr>
<tr>
<td>5.</td>
<td>Ampicillin+ sulbactum (10/10 mcg)</td>
<td>82.92</td>
<td>12.20</td>
<td>4.88</td>
</tr>
<tr>
<td>6.</td>
<td>Piperacillin+ tazobactum (100/10 mcg)</td>
<td>87.80</td>
<td>12.20</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Evolving the suitable therapy for *Staphylococcal* subclinical mastitis

Thirty two cattle with *Staphylococcal* SCM were randomly grouped into 4 with 8 animals each. Pre treatment and post treatment (on day 3, 7 and 15) values were recorded to judge the efficacy of drugs and compared with healthy cattle.

**Clinical examination of the udder/ milk**

Clinical examination of udder and milk revealed no changes following therapy in all the treatment groups. The colour, consistency and odour of milk samples from all positive cases of *Staphylococcal* SCM were found normal however, reduction in milk yield was recorded, which was increased after instillation of therapy.
Somatic cell count (SCC) (10^5 cells/ml) in response to different therapies

Somatic cell count (SCC) of all the 32 cattle under therapeutic trial was recorded on day 0 (pre-treatment), 3, 7 and 15 (post treatment) and compared with the healthy cattle.

The results revealed that SCC on day 0 pre-treatment was significantly higher in all treatment groups than healthy control group i.e. cattle of treatment groups B, C, D and E showed mean SCC 14.72±1.05 x 10^5 cells/ml, 15.85±0.40 x 10^5 cells/ml, 15.48±0.54 x 10^5 cells/ml, 14.84±0.71 x 10^5 cells/ml, respectively and 1.26±0.09 x 10^5 cells/ml in cows of healthy control group (group A). After treatment, the mean SCC on day 3 in cattle of treatment group B, C, D and E was reduced to 10.05±1.10 x 10^5 cells/ml, 6.21±1.00 x 10^5 cells/ml, 5.59 ±0.70 x 10^5 cells/ml and 7.93±0.86 x 10^5 cells/ml, respectively. On day 7 post treatment, the mean SCC of treatment groups B, C, D and E was 8.14±1.10 x 10^5 cells/ml, 2.99±0.50 x 10^5 cells/ml, 2.46±0.20 x 10^5 cells/ml and 4.72±0.69 x 10^5 cells/ml, respectively. On day 15 post treatment, the mean SCC of treatment groups B, C, D and E was 7.77±1.01 x 10^5 cells/ml, 2.31±0.47 x 10^5 cells/ml, 1.58±0.08 x 10^5 cells/ml and 3.33±0.64 x 10^5 cells/ml, respectively.

A significant decrease in the SCC was noticed in all treatment groups on days 3 post treatment. However, the mean SCC on day 7 and 15 post treatment in treatment groups C and D were essentially similar to healthy cattle. The SCC on day 7 and 15 post treatment in treatment group E were reduced significantly as compared to day 0 (pre-treatment) but not as significantly as compared to treatment groups C and D. SCC of treatment group B on day 7 and 15 were reduced but not significantly as compared to other treatment groups as well as with in treatment group and did not return to normal level. The detailed variation in SCC in different treatment groups at different intervals are outlined in table 23 and figure 19.
Table 23: Mean somatic cell count (SCC) (10^5 cells/ml) in different treatment groups at different intervals

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>Pre treatment</th>
<th>Post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>1.</td>
<td>A</td>
<td>1.26^{Ab}±0.09</td>
<td>1.33^{Ac}±0.07</td>
</tr>
<tr>
<td>2.</td>
<td>B</td>
<td>14.72^{Aa}±1.05</td>
<td>10.05^{Ba}±1.10</td>
</tr>
<tr>
<td>3.</td>
<td>C</td>
<td>15.85^{Aa}±0.40</td>
<td>6.21^{Bb}±1.00</td>
</tr>
<tr>
<td>4.</td>
<td>D</td>
<td>15.48^{Aa}±0.54</td>
<td>5.59^{Bb}±0.70</td>
</tr>
<tr>
<td>5.</td>
<td>E</td>
<td>14.84^{Aa}±0.71</td>
<td>7.93^{Bb}±0.86</td>
</tr>
</tbody>
</table>

Values with different superscript within the treatment group (ABC: p< 0.01) and between the group differ significantly (abc: p< 0.01)

Milk pH

Milk pH of all the cattle under therapeutic trial were recorded on days 0 pre-treatment, 3, 7 and 15th day post treatment and compared with healthy cattle.

The results revealed that milk pH of cattle on day 0 pre treatment was significantly higher in all the treatment groups than the healthy control group i.e. animals of treatment groups B, C, D and E showed mean pH of 7.43±0.05, 7.51±0.06, 7.40±0.09, 7.40±0.09, respectively and 6.43±0.08, in cattle of healthy control group (group A). After being treated, the mean pH on day 3 in cattle of treatment groups B, C, D and E were 7.19 ±0.10, 6.60±0.10, 6.63±0.17 and 6.90±0.08, respectively. On day 7 post treatment, the mean pH in treatment groups B, C, D and E were 7.10±0.12, 6.60±0.10, 6.50±0.15, and 6.80±0.08, respectively. On day 15 post treatment, the mean pH in treatment groups B, C, D and E were 7.03±0.11, 6.50±0.12, 6.43±0.17, and 6.70±0.10, respectively.

A significant declining trend in the pH was noticed in treatment groups C, D and E on days 3, 7 and 15 post treatment when compared to day 0 (pre-treatment). However, the mean pH of day 7 and 15 post treatment in cattle of treatment groups C and D and the mean pH on day 15 post treatment
in treatment group E were essentially similar to healthy cattle. In treatment group B the mean pH value did not return to normal level as compared to other treatment groups. The detailed variation in mean pH value in different treatment groups at different intervals are presented in table 24 and figure 20.

**Table 24: Mean milk pH in different treatment groups at different intervals**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>Pre treatment</th>
<th>Post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>1.</td>
<td>A</td>
<td>6.43$^{Ab}$±0.08</td>
<td>6.43$^{Ab}$±0.08</td>
</tr>
<tr>
<td>2.</td>
<td>B</td>
<td>7.43$^{Aa}$±0.05</td>
<td>7.19$^{ABA}$±0.10</td>
</tr>
<tr>
<td>3.</td>
<td>C</td>
<td>7.51$^{Aa}$±0.06</td>
<td>6.60$^{Bb}$±0.10</td>
</tr>
<tr>
<td>4.</td>
<td>D</td>
<td>7.40$^{Aa}$±0.09</td>
<td>6.63$^{Bb}$±0.17</td>
</tr>
<tr>
<td>5.</td>
<td>E</td>
<td>7.40$^{Aa}$±0.09</td>
<td>6.90$^{Bab}$±0.08</td>
</tr>
</tbody>
</table>

Values with different superscript within the treatment group (ABC: p< 0.01) and between the group differ significantly (abc: p< 0.01)

**Comparative therapeutic efficacy**

Bacteriological culture was performed for confirmation of *Staphylococcus* spp. in milk samples of all the 32 cattle under different therapeutic regimen on day 0 pre-treatment. Evaluation of different therapies was done by performing culturing of milk sample from all the afflicted cattle on days 3, 7 and 15 (post treatment). The post treatment bacteriological culture is depicted in plate 13 and 14. Comparative therapeutic efficacy of different treatment groups are outlined in table 25.

**Group B (Inj- Enrofloxacin LA, S/C)**

Out of 8 enrofloxacin LA treated cattle, bacteriological cure was noticed as 37.50 per cent (3 out of 8 cattle) and 36.36 per cent (4 out of 11 quarters) animal wise and quarter wise, respectively.
Group C (Inj- Enrofloxacin LA, S/C + Oint- Cefquinome, IMM)

In treatment group “C”, bacteriological cure was noticed as 75 per cent (6 out of 8 cattle) and 70 per cent (7 out of 10 quarters) animal wise and quarter wise, respectively.

Group D (Inj- Enrofloxacin LA, S/C + Oint- Cefquinome, IMM + Liq- Aloe vera PO + Aloe vera gel topical)

When Staphylococcal SCM cattle treated with group “D” drug, bacteriological cure was noticed as 87.55 per cent (7 out of 8 animals) and 80 per cent (8 out of 10 quarters) animal wise and quarter wise, respectively.

Group E (Liq- Aloe vera PO + Aloe vera gel topical)

In treatment group “E”, bacteriological cure was noticed as 50 per cent (4 out of 8 cows) and 50 per cent (5 out of 10 quarters) animal wise and quarter wise, respectively.

Table 25: Comparative therapeutic efficacy on the basis of MCMT, SCC, pH of milk and post treatment bacterial culture

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment group</th>
<th>Animal cure rate in per cent</th>
<th>Quarter cure rate in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>A</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>1.</td>
<td>B</td>
<td>37.50</td>
<td>36.36</td>
</tr>
<tr>
<td>2.</td>
<td>C</td>
<td>75.00</td>
<td>70.00</td>
</tr>
<tr>
<td>3.</td>
<td>D</td>
<td>87.50</td>
<td>80.00</td>
</tr>
<tr>
<td>4.</td>
<td>E</td>
<td>50.00</td>
<td>50.00</td>
</tr>
</tbody>
</table>

Sample size in each group comprises of 8 animals
5. DISCUSSION

An investigation about a disease helps to understand the distribution and epidemiology of the disease status. Mastitis is an economically important disease of dairy animals caused by variety of microorganisms. It is a disease predominantly of milking cattle and buffalo. Many workers had pointed out the Staphylococcal infections predominant in mastitis but a meagre work regarding establishment of therapy of staphylococcal subclinical mastitis (SCM) in cattle in Madhya Pradesh has been carried out. Hence, results of occurrence of SCM as well as Staphylococcal SCM in cattle, detection of *Staphylococcus* spp. in milk samples of cattle by phenotypic and genotypic characterization and a suitable therapy for Staphylococcal SCM have been discussed here.

**Occurrence of SCM**

During the present investigation a total of 550 lactating cattle from different dairy farms and Livestock Farm, Adhartal, N.D.V.S.U., Jabalpur were screened for SCM. The overall occurrence of SCM was found to be 27.81 per cent (153 out of 550 cattle) on animal basis which is very close to the findings of Getahun *et al.* (2008) and Islam *et al.* (2011) who have reported 22.3% and 29% prevalence of SCM, respectively. However, the higher occurrence of SCM was reported by Hashemi *et al.* (2011); Dar *et al.* (2014) and Mir *et al.* (2014) who have recorded 42.5%, 65% and 57.80% prevalence of SCM, respectively.

This variation in the occurrence of SCM in cattle might be attributed to the selection of animals, season, breed, managerial practices. The factors like herd size, agroclimatic conditions of the region, variations in socio-cultural practices, milk marketing, literacy level of the animal owner and system of feeding were found important affecting the occurrence of subclinical mastitis (Ergun *et al*., 2004).

The overall occurrence of SCM was found to be 10.13 per cent on quarter basis. Contrary to our observations Hashemi *et al.* (2011) and Mir *et al.* (2014) recorded 21.60% and 30.73% occurrence quarter wise, respectively. The reason of low occurrence during the study was due to large
number of quarters (2098) screened under the study belonging to the Livestock Farm, Adhartal and other dairy farms. However, the observations were in close conformity to the findings of Almaw et al. (2008) who have recorded 17.9% occurrence quarter wise in Ethiopia.

**Occurrence of Staphylococcal SCM**

A significant variation (P<0.01) was observed in occurrence of Staphylococcal SCM. The overall occurrence of Staphylococcal SCM was reported as 16.36 per cent (90 out of 550 cattle) on animal basis and 6.07 per cent (127 out of 2092 quarters) on quarter basis. However, the occurrence among the cattle suffering from subclinical mastitis was 58.82 per cent animal wise (90 out of 153 mastitic cattle) and 59.90 per cent (127 out of 212 quarters) quarter wise. The occurrence during the study period was recorded by identifying the presence of *Staphylococcus* spp. in the milk samples. Similarly, Sahu (2009) also reported the prevalence of Staphylococcal mastitis as 22.02% and 8.64% animal wise and quarter wise, respectively. Staphylococci are the most important and prevalent mastitis causing organism globally including India. It is an opportunistic pathogen which survives on skin of udder and can infect udder via teat canal or any wound (Saravanajayam et al., 2015).

**Individual quarter wise**

During the present investigation on screening the cattle for SCM using MCMT, it was found that the hind quarters (11.60%) were found to be more susceptible to SCM when compared to fore quarters (8.62%) which are in agreement with the reports of Ramprabhu and Rajeshwar (2006) and Awale et al. (2012). Similarly, the occurrence of Staphylococcal SCM was higher in the hind quarters (7.16%) when compared to fore quarters (4.94%) which are in agreement with the reports of Khanal and Pandit (2013). The high chance of getting faecal and environmental contamination (Bansal et al., 1995) furthermore, this could be attributed to the high production capacity of hind quarters (Radostits et al., 2010).

The right side quarters were found to be more susceptible (11.23%) to SCM than the left side quarters (9.03%). These observations are
in conformity with those of Qadri (2015) who have reported the occurrence as 32.25% and 27.25% in right and left side quarters, respectively. Similarly, the occurrence of Staphylococcal SCM was higher in right side quarters (7.20%) as compared to left side quarters (4.94%). Because the animals adopted right side sitting posture which causes widening of teat canal due to pressure exerted to right side quarters hence, it provides exposure to entrance of organism.

Further, the study revealed that right hind quarter (13.35%) was found to be more susceptible than the left hind quarter (9.88%). Occurrence of Staphylococcal SCM was also found higher in right hind quarter (8.77%) as compared to left hind quarter (5.59%). The higher occurrence of right hind quarter could be further justified as common practice adopted by the milkers during milking the animals. So, there may be probability of infection because of the handling of right hind quarter at first by the milkman while milking the animal.

**Breed wise**

Analysis of data of occurrence with respect to breed showed significant variation (P<0.05) among various breeds of cattle. Occurrence study of SCM in lactating cattle revealed a highest occurrence (36.43%) in cross bred cattle, 22.06 per cent in non-descript cattle and lowest (18.36%) occurrence in H.F. cattle.

The breed wise occurrence study of Staphylococcal SCM in lactating cattle revealed a highest occurrence of 22.48 per cent in cross bred cattle followed by occurrence of 12.41 per cent in non-descript cattle and lowest occurrence of 9.52 per cent in H.F cattle.

Islam et al. (2011) and Dar et al. (2014) detected higher prevalence of SCM in milch crossbred cows as 36.36 per cent and 38.80 per cent. The significant difference between the breeds may be associated with their high milk yield. Radostits et al. (2010) stated that high yielding cattle are more susceptible to mastitis than low yielding ones might be because of more resistance to disease.
Age wise

The age wise occurrence of SCM revealed a significant variation (P<0.01) among various age groups. Highest occurrence (36.36%) was observed in the cattle of 5-7 years of age group followed by 19.64 per cent occurrence in cattle of 7 years and above age group and lowest occurrence of 17.76 per cent in cattle of 3 to 4 years of age group. Similar to these findings Mahajan et al. (2011) and Dar et al. (2014) also showed that cattle of 5-7 years of age were more susceptible to SCM as compared to other age groups. During 5-7 years of age the animals were approximately in 3rd to 5th lactation, had a highest milk yield, thus remained under stress and more prone to infection. Tiwari et al. (2013) demonstrated that occurrence of mastitis in infected quarters increases with age in cows being the highest at 7 years of age. This might be due to increase cellular response to intra-mammary infection. Efficient innate host defense mechanism of the younger animals are one possibility that makes them less susceptible to infection, while in older animals there is reduction in milk yield, less milking practice, meager handling of the animals etc. may reduce the chance of infection.

Similarly, the age wise occurrence of Staphylococcal SCM also revealed a significant variation (P<0.01) among various age groups with highest occurrence of 23.77 per cent in cattle of 5-7 years age group followed by 10.71 per cent in cattle of 7 years and above age group and lowest occurrence i.e. 6.57 per cent in cattle of 3 to 4 years of age group.

In the current study, the occurrence of Staphylococcus spp. associated SCM was found to be increasing as the age of the cattle increases. This finding agreed with Garedew et al. (2015), who reported that the risk of Staphylococcus associated clinical and subclinical mastitis increases significantly with age and parity of the cattle. The increasing occurrence of mastitis along with age and parity could be partially explained by the possibility of exposure to the infectious agents with increasing age and parity.
**Parity wise**

The parity wise occurrence of SCM exhibited significant variation (P<0.01) among various groups and it was highest during the 3\textsuperscript{rd} parity (41.83%) followed by 4\textsuperscript{th} (34.54) and 5\textsuperscript{th} parity (32.05) in cattle. Similar observations were recorded by Shukla \textit{et al.} (2005) and Islam \textit{et al.} (2011). Whereas, Nauriyal and Verma (2009) recorded highest incidence of SCM in 2\textsuperscript{nd} parity. Occurrence of staphylococcal SCM also depicted significant variation (P<0.05) among various groups and it was observed higher in 3\textsuperscript{rd} (23.46%), 4\textsuperscript{th} (22.72%) and 5\textsuperscript{th} parity (25.64%) as compared to other parity. Busato \textit{et al.} (2000) studied the incidence of sub clinical mastitis and its effect on parity number. Increased occurrence may be the result of increased incidence of new IMI or increased duration of Staphylococcal SCM infection (Sampimon \textit{et al.}, 2009).

**Lactation stage wise**

The occurrence of SCM according to lactation stage was observed highest in early lactation stage (47.46%). Islam \textit{et al.} (2011) also reported highest prevalence of SCM during the early lactation stage. Similarly, the occurrence of Staphylococcal SCM was also found highest in early lactation stage (31.64%). Similar to present findings, Zadoks \textit{et al.} (2001) and Osteras \textit{et al.} (2006) also reported high incidence of \textit{S. aureus} IMI in early stage than late stage of lactation. High incidence of udder infection and mastitis in early lactation might be due to the rapid physiological changes which take place in the mammary tissues post partum, resulting in reduced udder resistance (Oliver \textit{et al.}, 1956). Increased incidence of mastitis during early or peak lactation may be a result of negative energy balance (Suriyasathaporn \textit{et al.}, 2000). Significant variation was observed with respect to different lactation stage.

**Occurrence in organized and unorganized dairy farms**

In the present study, no significant variation was noticed in the occurrence with respect to rearing pattern of cattle however, occurrence of SCM in unorganized dairy farms was reported to be higher (36.92%) than in
organized sector (26.59%). Similarly, the occurrence of Staphylococcal SCM in unorganized (15.62%) and organized dairy farms (16.49%) does not differ significantly. Tiwari et al. (2000) reported the incidence of subclinical mastitis in unorganized dairies and organized dairies to be 26.68 and 20.36%, respectively. The occurrence of SCM was found to be higher in unorganized dairy farms as compared to organized dairy farms might be due to poor hygienic management, awareness, method of milking, the type of housing, bedding and weather.

Clinical examination of animals and their udder

During the present investigation the cattle afflicted with SCM did not exhibit any clinical signs except reduced milk yield. Similar observations were recorded by Reddy et al. (2001); Chakrabarti (2004) and Radostits et al. (2010) who have reported that no clinical signs associated with SCM as well as no physical abnormalities were found in the milk.

In the present study the colour, consistency and odour of milk were found to be normal however, reduction in milk yield was recorded in SCM. The present observations were correlated by the findings of Saravanan et al. (2009) and Suresh et al. (2010).

Modified California mastitis test (MCMT)

In the present study, screening for detection of SCM, MCMT was employed as a standard scientific test; because among all presently available tests, MCMT is simple, cheap, quick, reliable and applicable under the field condition. The above findings were similar to the Shukla (1980); Bastan et al. (2008); Sharma et al. (2009); Sharma et al. (2010) and Ayano et al. (2013), who also stated that MCMT was the most accurate and reliable diagnostic test in the field conditions.

This test had specificity for leukocytes in milk. The positive reaction was indicated by formation of gel or viscous mass, when SCM milk of high cell counts was brought in contact with sodium laurel sulphate. The surface surfactant ruptures milk leukocytes releasing deoxyribonucleic acid (DNA) from the nuclei which were further unfolded DNA molecules unites with the reagent causing them to form precipitate or gel formation. The reaction
precedes best in alkaline pH. Therefore, MCMT showed specific positive reaction in SCM as mastitis milk is always alkaline and contained huge number of leukocytes. Hence, among all available indirect tests, MCMT is considered to be more immediate and efficient field test for detection of SCM.

**MCMT grading**

MCMT scores in Staphylococcal SCM varied from 1+ to 3+ in various treatment groups of cattle. After treatment, milk was restored to normal in treatment groups C and D. These observations were indicative of slow return of biochemical changes in Staphylococcal SCM affected milk. The changes in leukogram also supported this interpretation.

**Somatic cell count (SCC)**

During the present investigation the mean milk SCC of apparently healthy animals was $1.26 \pm 0.90 \times 10^5$ cells/ml.

There was a significant increase ($15.55 \pm 1.20 \times 10^5$ cells/ml) in the mean value of SCC in the animals afflicted with SCM. These observations were in agreement with the reports of Langer and Nauriyal (2013) who have tested 796 quarters milk and noticed that the mean somatic cell count of healthy quarter was $1.55 \times 10^5$/ml whereas, that of infected quarter was $19.49 \times 10^5$/ml (P<0.01). The increase in SCC indicated the inflammatory reaction that might be due to shift of leukocytes to the udder after entry of infection in the mammary gland and as a protective mechanism against infection and increase in SCC which was found to be directly proportional to the severity of infection.

**Milk pH**

During the present observation the mean milk pH in apparently healthy cattle was $6.43 \pm 0.08$. The mean milk pH in cattle afflicted with SCM was significantly higher ($7.30 \pm 0.09$) as compared to healthy control group. The mastitic milk is associated with low citric acid levels and alkaline pH (pH>7) favouring bacterial growth. The elevation of milk pH was mainly due to the leakage of blood bicarbonate in to the milk through damaged epithelium of mammary gland (Bansal and Randhawa, 2003).
Bacterial isolation and characterization of *Staphylococcus* spp. in milk samples found positive for SCM

Quarter milk samples from cases of IMI in cattle showed 59.90% (127 out of 212 quarter milk samples) isolates of *Staphylococcus* spp. Out of which 14.62% isolates were found CPS whereas 45.28% were identified as CNS by coagulase test. The proportion of CPS was found lesser than CNS in Staphyloccocal SCM in cattle. Tenhagen *et al.* (2009) observed 4.0% *S. aureus* and 46.80% *Staphylococcus* spp. in a milk samples submitted for microbiological examination. Contrary to this, Sindhu *et al.* (2010) reported higher proportion (53.78%) of CPS and lesser proportion (11.76%) of CNS in quarter milk samples from buffaloes in Haryana. Also, Nirwan (2006) reported higher rate of *S. aureus* (26.07%) and lesser rate (13.5%) of other Staphylococci in milk samples collected from cases of bovine mastitis in IVRI dairy farm, Bareilly. Ahmadi *et al.* (2010) reported 14% of the milk samples from cow was positive for *S. aureus* on the basis of bacteriological culture examination in industrial dairy herds in Iran.

**Molecular detection**

*S. aureus* is the most common cause of contagious mastitis in cattle and it is most significant bacterial pathogen associated with bovine mastitis. It generally causes chronic and sub clinical mastitis in lactating dairy animals. As the organism is intra cellular in nature, the resistance to antibiotics makes this pathogen to be one of the most difficult entities to treat.

Nuclease gene is one of the virulence factor which decides virulence of *S. aureus*. The nuclease (*nuc*) gene encodes the thermonuclease (Tnase) production. The *nuc* gene polymorphism is frequently applied for epidemiological investigations of bovine *S. aureus* mastitis (Rusenova *et al.* 2013). A total of 212 MCMT positive milk samples were collected aseptically for bacterial isolation and identification using phenotypic test, then PCR was performed for amplification of *nuc* gene. The *nuc* gene of *S. aureus* yielded a PCR product of 270 bp on amplification. Brakstad *et al.* (1992), Eswaran *et al.* (2011) and Rusenova *et al.* (2013) have also amplified the *nuc* gene to detect the *S. aureus*. The occurrence of *nuc* gene was recorded in 14.62% (31
isolates were found positive for nuc gene) in subclinical mastitis. Rusenova et al. (2013) suggested that routine approach using a combination of phenotypic and molecular detection systems could improve S. aureus detection in milk.

**Antibiogram of Staphylococcus spp. isolates**

A good amount of literature is available on the antibiogram. While considering overall sensitivity, all the strains of Staphylococci were found sensitive to enrofloxacin (100%) among the battery of antibiotics used in the *in vitro* which was in accordance to Jain and Joseph (2013); Jeykumar et al. (2013); Chandrasekaran et al. (2014) and Shende et al. (2015). In contrast to the present findings, Didugu et al. (2015) and Saravanajayam et al. (2015) reported antibiotics other than enrofloxacin as most effective drug from the studies done in various parts of country. Isolates in the present study showed moderate sensitivity to piperacillin + tazobactum (87.80 per cent), cefoparazone (82.92 per cent), ampicillin + sulbactum (82.92 per cent) and cefuroxime (82.92 per cent), while all the isolates were found resistance to cefepime (100%).

It was interesting to note that Staphylococci isolates revealed 100 percent sensitivity towards enrofloxacin, while, *in vivo* it was found least efficacious. Roychoudhury and Dutta (2009) and also Sharma et al. (2015) have showed increased resistance towards different traditional and newly introduced antibiotics. In contrast to these studies, the antibiogram obtained in the current study indicated high sensitivity towards newer and older antibiotics, showing rational use of these antibiotics at farms under study. Antibiotic resistance patterns vary among different farms, regions, states and countries depending upon the type of organisms and use of antibiotics in a particular area; therefore, antimicrobial sensitivity is suggested before institution of treatment. The information obtained by this study will also be of useful to the dairy industry and individual farmers. It will be helpful in prioritizing mastitis control efforts.
Therapy for Staphylococcal subclinical mastitis

To evaluate the therapeutic efficacy of various combinations, a total of 32 cattle afflicted with Staphylococcal SCM were randomly divided into 4 groups and treated with different combinations of drugs by different routes using enrofloxacin, cefquinome and aloe vera.

Clinical examination of animals and their udder

In the present investigation clinical examination of animals and udder revealed no significant change following therapy in all treatment groups of cattle. The colour, consistency and odour of milk from cattle with Staphylococcal SCM varied insignificantly during pre and post treatment.

Somatic cell count (SCC)

During the present investigation the overall SCC in milk were found to be decreased on post treatment at all the intervals. Whereas, marked decreased values have been obtained in the cattle treated under treatment groups D and C which is again an indicative of more efficacy of the drug used in treatment groups D and C.

Milk pH

The mean pH value during the study was found to be gradually decreased on 3rd, 7th and 15th day post treatment in all the treatment groups. However, in cattle treated under treatment group D and C there was significant decrease in the post treatment pH values at all the intervals, justifying the maximum efficacy of combined drug administration, as compared to treatment groups B and E.

Comparative therapeutic efficacy

Evaluation of different therapies was done by performing culturing of milk sample for Staphylococcus spp. from all the afflicted cattle on days 3, 7 and 15 (post treatment).

In treatment group “B” bacteriological cure was noticed in 37.5 per cent (3 out of 8 cattle) and 36.36 per cent (4 out of 11 quarters) animal
wise and quarter wise, respectively. As per Sudhan and Sharma (2010) and Bansal (2013) enrofloxacin shows high in vitro sensitivity and is pharmacologically considered to distribute well in the udder, clinically proved to be less efficacious against Staphylococcal mastitis because of its inability to kill intracellular organisms. According to Radostits et al. (2010) after parenteral therapy bacteriological cure of the affected glands is seldom achieved because of the relatively poor diffusion of the antimicrobial from the blood into the milk. However, the results are contradictory to Suresh et al. (2010); Shende et al. (2015) and Reddy et al. (2015) who have reported excellent recovery after treatment with long acting enrofloxacin as 100%, 100% and 87.5%, respectively.

In treatment group “C”, 75 per cent (6 out of 8 cattle) animal wise and 70 per cent (7 out of 10 quarters) quarter wise bacteriological cure was noticed. As per Radostits et al. (2010) the combined parenteral and intramammary therapy is more effective than intramammary/parenteral therapy alone. Cefquinome is a fourth-generation cephalosporin and is resistant to beta-lactamase. Chemically, its zwitterionic structure can facilitate rapid penetration across biological membranes, including porins of bacterial cell walls, also, it has a higher affinity to target penicillin-binding proteins. The reactive site is a beta-lactam nucleus, it acts by inhibition of the cell wall synthesis. Manimaran et al. (2014) indicated that drug resistance appears to be less prevalent in cephalosporin group.

Out of 8 cows in treatment group “D”, bacteriological cure was noticed in 87.55 per cent (7 out of 8 animals) and 80.00 per cent (8 out of 10 quarters) animal wise and quarter wise. Again in this group highest efficacy is achieved because of combination of drugs and their synergistic effect. Aloe vera is an important source of phytochemicals and it has antioxidant and immunomodulating property, hence, it can be used in promoting recovery from any disease ailments (Mekala and Arivuchelvan, 2012). Cefquinome should be effective against a wide range of contagious and environmental mastitis pathogens. The most common route of administration of antimicrobials in mastitis is the intramammary route. The advantages of this
route are high concentrations of the substance is achieved in the milk and low consumption of the antimicrobial as the drug is directly infused into the diseased quarter (Pyorala, 2009). Intramammary therapy of antibiotic is the first line of treatment of mastitis to get rapid response (De and Mukherjee, 2013). Early detection and cure of Staphylococcal subclinical mastitis cases may be beneficial in the individual as cure may reduce duration of infection and prevent subsequent clinical episodes.

In treatment group “E”, bacteriological cure was noticed in 50 per cent (4 out of 8 cattle) and 50 per cent (5 out of 10 quarters) animal wise and quarter wise, post treatment. Similar to our findings Dettloff (2007) suggests that if somatic cells are high because of staphylococcal mastitis, organic or conventional treatment such as with aloe vera is not effective. However the results are contradictory to Urch (1999); McCrory (2001); Agarry et al. (2005); Steinka and Kukulowicz (2011) and Crişan et al. (2012) who have showed efficacy of aloe vera in the treatment of mastitis and Staphylococcus spp.

The overall findings of the present investigation indicated that the combination of enrofloxacin, cefquinome and aloe vera induced an excellent response by producing stronger effects against the teat skin opportunistic organisms (Saravanajayam et al., 2015) followed by combination of enrofloxacin and cefquinome, aloe vera alone oral and topical and lastly enrofloxacin alone.

In view of the above study it is concluded that as the Staphylococcal SCM occurs frequently in cattle characterized by normal gland and apperance of milk with increased SCC and pH.

So, the internal changes are only detected by using screening test at an early stage and by quantitative estimation of SCC and pH. It could be well managed by routine testing of animals using MCMT, culturing of bacteria and adopting good managemental conditions at the farms.
6. SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

Subclinical mastitis (SCM) in cattle is a complex disease with inapparent clinical manifestations, difficult to diagnose and of major economic issue. Very scarce data are available on the occurrence of pathogenic Staphylococcal intra-mammary infections, forms and severity of disease. Treatment of mastitis is much costlier due to its multiple etiological agents. Insufficient and improper therapy of mastitis results in development of resistant strains of bacteria. Thus, the present work was aimed to study the occurrence, phenotypic and genotypic detection of *Staphylococcus* spp. and to evolve the suitable therapy against Staphylococcal SCM in cattle.

In this study, a total of 550 lactating cattle from LSF and different dairy farms in and around Jabalpur were screened from February 2015 to 2016 by MCMT and SCC, the overall occurrence of SCM was reported to be 27.81% (153/550) on animal basis and 10.13% (212/2092) on quarter basis. During the study highest occurrence of SCM was observed in right hind quarter (13.35%) followed by left hind quarter (9.88%), right fore quarter (9.09%) and left fore quarter (8.15%). The occurrence was higher in hind quarters (11.60%) as compared to fore quarters (8.62%), right side (11.23%) as compared to left side (9.03%). Breed wise occurrence revealed the significant (P<0.05) effect of different breeds on occurrence of SCM. Highest occurrence of 36.43 per cent in cross bred cattle followed by occurrence of 22.06 per cent in non-descript cattle and lowest occurrence of 18.36 per cent in H.F. cattle were observed. Significant variation among age wise occurrence of SCM (P<0.01) was noticed. It was highest (36.36%) in 5-7 years of age followed by 7 years and above age (19.64%) and 3 to 4 years (17.76%) of age. The parity wise occurrence revealed a significant variation (P< 0.01) among various parity groups. The highest occurrence of SCM was observed in 3rd parity (41.83%) and in early lactation stage (47.46%) of each parity. Occurrence in organized and unorganized dairy farms does not differ significantly (P>0.05). However, it was higher in unorganized dairy farms (36.92%) as compared to organized sector of dairy farms (26.59%).

58
A total of 212 MCMT positive quarter milk samples were processed for bacteriological examination and molecular identification of *S. aureus* by *nuc* gene amplification. Thirty one isolates (14.62%) were found CPS as revealed by coagulase test and 96 (45.28%) were found to be CNS.

The overall occurrence of Staphylococcal SCM in lactating cattle was 16.36 per cent animal wise and 6.07 per cent quarter wise. However, the occurrence among the cattle suffering from subclinical mastitis was reported to be 58.82 per cent and 59.90 per cent animal wise and quarter wise, respectively. Significant variation (P< 0.01) was observed in occurrence of staphylococcal SCM in lactating cattle. Individual quarter wise occurrence of Staphylococcal SCM showed significant variation (P< 0.05). The occurrence was highest in right hind quarter (8.77%) followed by right fore quarter (5.60%), left hind quarter (5.59%) and lowest occurrence in left fore quarter (4.27%) were observed. The occurrence was also found higher in hind quarters (7.16%) as compared to fore quarters (4.94%), right side (7.20%) as compared to left side (4.94%). The occurrence of Staphylococcal SCM showed significant variation (P<0.05) among various breeds. Cross bred cattle showed highest occurrence (22.48%), followed by 12.41 per cent in non-descript cattle and lowest occurrence in H.F. cattle (9.52%). The age wise occurrence of Staphylococcal SCM reported a significant variation (P<0.01) among various groups. Highest occurrence was noticed in cattle of 5-7 years age group (23.77%) followed by 10.71 per cent in cattle of 7 years and above age group and lowest occurrence (6.57%) in cattle of 3 to 4 years of age group. The parity wise occurrence revealed a significant variation (P< 0.05) among various parity groups. The highest occurrence of was observed in 5th parity (25.64%) and in early lactation stage (31.64%) of each parity. The occurrence of Staphylococcal SCM in organized and unorganized dairy farms does not differ significantly.

Amplification of *nuc* gene yielded a single PCR product of 270 bp in culture found positive for *Staphylococcus* spp. when visualized in UV transiluminator gel documentation system after amplification and electrophoresis in 1.5% agarose. The occurrence recorded using PCR for identification of *nuc* was 14.62%.
Results of antibiogram revealed that most effective antimicrobial agent was enrofloxacin (100%) followed by piperacillin + tazobactum (87.80%), cefoparazone (82.92%), ampicillin + sulbactum (82.92%) and cefuroxime (82.92%) against Staphylococcus spp. All the isolates showed resistance to cefepime (100%).

For therapeutic study, eight healthy animals were taken as control group. A total of 32 cattle with staphylococcal SCM were subjected to clinical examination of udder, physical examination of milk, milk pH, somatic cell count and culturing of bacteria before and after treatment, to judge the efficacy of drug through various routes.

Staphylococcal SCM affected animals were divided in to four treatment groups each consisted of eight animals. The “A” group was kept as control. Group “B” was treated with enrofloxacin long acting @ 7.5mg/kg of b.wt. S/C. Group “C” was treated with combination of enrofloxacin long acting (@ 7.5mg/kg of b.wt. S/C) and cefquinome (@ 75 mg/per quarter IMM). Group “D” was treated with combination of enrofloxacin long acting (@ 7.5mg/kg of b.wt. S/C), cefquinome (@ 75 mg/per quarter IMM), aloe vera (@1 ml/kg b. wt. PO) and aloe vera gel (topical). Group “E” was treated with aloe vera liquid (@ 1 ml/kg b. wt. PO) and aloe vera gel (topical).

The physical examination of milk did not show appreciable change in colour, consistency and odour, whereas, chemical examination of milk indicated significant increase in pH, SCC and positive scores for MCMT in affected animals before treatment. The mean values of milk pH, SCC and positive scores for MCMT reached towards normal after treatment. Bacteriological culture examination on post treatment days was also recorded under the study.

On the basis of MCMT, SCC, pH and post treatment bacterial culture animals of group D showed highest recovery. Thus, combination of enrofloxacin, cefquinome and aloe vera was found most efficacious followed by combination of enrofloxacin and cefquinome, aloe vera alone oral and topical and lastly enrofloxacin alone.
6.2 Conclusions

- The overall occurrence of subclinical mastitis was 27.81 per cent and 10.13 per cent animal wise and quarter wise, respectively. Occurrence of subclinical mastitis was found to be the highest in 5-7 years of age group, in right hind quarter, between third parity and early lactation stage in cattle.

- On the basis of culturing of bacteria, overall occurrence of Staphylococcal SCM was reported 16.36% animal wise and 6.07% quarter wise. However, In subclinical mastitis the occurrence was reported as 58.82% animal wise and 59.90% quarter wise. The proportion of CPS was lesser than CNS. Occurrence of Staphylococcal SCM was found to be highest in age group of 5-7 years, in right hind quarter, in 5\textsuperscript{th} parity and in early lactation period.

- Occurrence of \textit{nuc} gene was reported to be 14.62%.

- \textit{In vitro} most effective antimicrobial agent was enrofloxacin (100%). Post treatment values revealed that the combination of enrofloxacin, cefquinome and aloe vera was most efficacious against Staphylococcal SCM in cattle.
6.3 Suggestions for further work

- Greater sample size from different regions of M.P. would be included so as to increase the accuracy of the study.

- The \textit{in vitro} studies on aloe vera can be undertaken to know its efficacy.

- Different permutation and combination of aloe vera as such or its active ingredients can be tested in future.

- Intensive research on molecular epidemiology is required to fully understand risk factors associated with the disease.
7. REFERENCES


