EVALUATION OF ANTIBIOTIC RESISTANT MASTITIS IN DAIRY COWS

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DEPARTMENT OF VETERINARY CLINICAL MEDICINE, ETHICS AND JURISPRUDENCE
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CHENNAI – 600 051

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Thesis submitted in partial fulfillment of the requirements for the degree of

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2013

Dedicated
To my wife
and to my Son

CERTIFICATE

This is to certify that the thesis entitled **"EVALUATION OF ANTIBIOTIC RESISTANT MASTITIS IN DAIRY COWS"** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **VETERINARY CLINICAL MEDICINE, ETHICS AND JURISPRUDENCE** to the Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 051, is a record of a bonafide research work carried out by Mr**. D.CHANDRASEKARAN,** under my supervision and guidance and that no part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

Title	:	**Evaluation of Antibiotic resistant Mastitis in dairy cows**
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The aim of the study was to study the prevalence of drug resistant mastitis, clinico-pathological changes and their pattern of antibiotic resistance in dairy cows. Comparative evaluation of different therapeutic protocols for clinical management of resistant mastitis and assessment of the economic impact of drug resistant mastitis were undertaken for the study.

Twenty apparently healthy cows were taken as healthy control group. Based on culture and antibiotic sensitivity tests, two hundred and thirty five cases of resistant mastitis were selected and grouped as group I *E.coli* (n=119), group II *Staphylococcus aureus* (n=104) and group III methicillin resistant *Staphylococcus aureus* (MRSA)(n=12) and subjected to clinical examination, haematology, serum biochemistry and minimum inhibitory concentration (MIC).

The incidence of clinical mastitis at Madras Veterinary College Teaching Hospital was 9.57 per cent, of which 1.90 per cent was acute mastitis and incidence in organized farm was 16.56 per cent, of which 11.08 per cent was acute mastitis. The incidence of resistant mastitis was 56.l per cent.

The predominant resistant causative pathogen was *E.coli* (50.64 per cent) followed by *S.aureus* (44.25 per cent) and MRSA (5.11 per cent). Highest incidence was observed in early stage of third lactation and hind quarters. Haemato biochemical changes were reduced Hb, PCV, TEC, leukocytosis with neutrophilia, lymphopenia, hypoalbuminemia and hyperglobulinemia. A significant increase in ALP and AST were observed in early lactation which might reflect the negative energy balance and fatty liver.

In vitro antibiotic sensitivity test and MIC breakpoints, *E.coli, S.aureus* and MRSA organisms showed more sensitivity to enrofloxacin, amoxicillin+sulbactam, gentamicin and ceftriaxone and had highest resistant to penicillin followed by amoxicillin, oxytetracycline and methicillin. Most of MRSA isolates were found to be multi-drug resistant whereas *E.coli* and *S.aureus* isolates were found to be resistant. Nitrocefin and MRSA alert kit was found to be useful in preliminary screening of β lactamase production and methicillin resistant organisms in clinical mastitis. Targeting the specific genes of *E.coli*, *S.aureus* and MRSA isolates and performing multiplex PCR were useful in the confirmation.

Highly significant increase in pH and SCC and a significant decrease in electrical conductivity were noticed in all clinical mastitis. In *E.coli* and *S.aureus* mastitis treated with amoxycillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed uniform improvement in clinical mastitis. In MRSA mastitis, enrofloxacin was found to be highly effective in comparison to amoxicillin+sulbcactam. Similar economic impact was observed in *E.coli, S.aureus* and MRSA mastitis.

Key words: Acute resistant mastitis- culture- *E.coli* - *S.aureus* – MRSA – *in vitro* sensitivity test – MIC – PCR target gene – genatamicin – enrofloxacin – ceftriaxone – amoxicillin+sulbactam

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ABBREVIATIONS

%	Per cent
*	Significant
**	Highly significant
µl	Microlitre
ABST	Antibiotic sensitivity test
AMR	Antimicrobial resistance
AST	Aspartate transaminase
BHI	Brain heart infusion
bp	Base pair
BTM	Bulk tank milk
BTSCC	Bulk tank somatic cell count
CFP	Cefopeazone
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
cm	Centimeter
CM	Clinical mastitis
Cmm	Cubic milimeter
CMT	California mastitis test
CNS	Coagulase negative *Staphylococcus*
CoNS	Coagulase negative *Staphylococcus*
CoPS	Coagulase positive *Staphylococcus*
DC	Differential count
DIM	Days in milk
dl	desilitre
DNA	Deoxyribonucleic acid
E. coli	*E scherichia coli*
EC	Electrical conductivity
EDTA	Ethylene diamine tetra acetate
FP	Forward primer
g	gram
GGT	Gamma gluatmyl transferase

H	Healthy
Hb	Haemoglobin
IFCC	International Federation of Clinical Chemistry
IMI	Intramammary infection
IMM	Intramammary
kg	Kilogram
LFQ	Left fore quarter
LHQ	Left hind quarter
LDH	Lactate deghydrogenase
MDR	Multidrug-resistant
mg	milli gram
MIC	Minimum inhibitory concentration
MIC_{50}	Concentration at which ≥ 50 per cent of isolates inhibited
MIC_{90}	Concentration at which ≥ 90 per cent of isolates inhibited
ml	milliliter
m-PCR	Multiplex Polymerase Chain Reaction
MR	Methicillin Resistance
MRSA	Methicillin resistant *Staphylococcus aureus*
mS	Milli Siemens
MSA	Mannitol salt agar
N.S	Statistically non significant
NAGase	NAGase enzyme
NEFA	Non esterified fatty acid
^0C	Degree centigrade
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PCV	Packed cell volume
PMN	Polymorho nuclear cell
RFQ	Right fore quarter
RHQ	Right hind quarter
RP	Reverse primer
rRNA	Ribosomal ribo nucleic Acid

S. aureus	*Staphylococcus aureus*
SAP	Serum alkaline phosphatase
SCC	Somatic cell count
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCM	Subclinical mastitis
SID	*semel in die*
SIR	Susceptible – intermediate - resistant
Spp.	Species
SPSS	Statistical package software solution
T	Trace
TEC	Total erythrocyte count
TLC	Total leukocyte count
TP	Total protein
U	Units
WBC	White Blood Corpuscles

CHAPTER I

INTRODUCTION

Mastitis (inflammation of mammary gland) is a most devastating disease condition in terms of economic losses, occurring throughout the world (Kumar *et al.,* 2010). Due to the involvement of multiple aetiological agents it always remains as a challenge to veterinarians all over the globe.

Mastitis is the most economically important disease of dairy cows. Mastitis negatively affects the quality of milk, milk production, farm economics and animal welfare. Calculations of economic losses resulting from mastitis vary among countries. In India, the overall economic loss due to mastitis is estimated to be Rs.7165.51 crores (Bansal and Gupta, 2009).

Mastitis results when pathogenic bacteria are able to gain entry into the udder, overcome the cow's immune defences, establish an infection and produce inflammation of udder secretary tissue. Variety of factors like mechanical trauma, thermal injury and chemical insults predispose the gland to intramammary infection. Occurrence of mastitis depends on the interplay of host, agent and environmental factors (Zhao and Lacasse, 2007).

Bovine mastitis is generally classified into clinical and subclinical mastitis. Clinical mastitis is characterized by local (e.g. swelling of the udder, heat and pain) or systemic (e.g. fever, anorexia, depression) symptoms with milk abnormalities (e.g. milk clots, flakes, watery secretions, blood), whereas subclinical mastitis is marked by high Somatic Cell Count (SCC), milk production losses and lowered milk quality (Gruet *et al.,* 2001).

The classical mastitis pathogen is classified as contagious and/or environmental. The contagious pathogens are considered as organisms adapted to survive within the host, particularly in the mammary glands. They are capable of producing sub clinical infection which is typically manifested by elevation of SCC in the milk from the affected quarter. The organisms usually spread from cow to cow, around or at the time of milking. In contrast, the environmental pathogens are best

described as ubiquitous in nature and opportunistic invaders of mammary gland. These organisms are not adapted to survive in the host but produce clinical manifestations and are rapidly eliminated (Bradley, 2002).

Of the 135 infectious agents associated with clinical mastitis episodes in dairy cattle, the most commonly isolated are *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae,* and *Escherichia coli* (Bramley *et al.,* 1996; Watts, 1988). *S. aureus* and *E. coli* are the most frequent causes of contagious and environmental clinical mastitis in dairy cattle, respectively (Barkema *et al.,* 1998 and Olde Riekerink *et al.,* 2008).

Coliforms cause environmental mastitis in dairy cattle mostly in early lactation with local and more often severe systemic signs than gram-positive mastitis. The majority of these coliforms are *E. coli* that originate from the cow's environment and infect udder *via* the teat canal.

Staphylococci are the predominant mastitis pathogens causing heifer mastitis and *Staphylococcus aureus* can represent a substantial percentage of these infections. *S.aureus* mastitis in dairy heifers could persist through the prepartum period and into the first lactation.

The *S.aureus* is one of the most frequently (45-60 per cent) isolated pathogens and causes clinical or subclinical or chronic bovine mastitis.

Staphylococci are often classified diagnostically based on their ability to coagulate plasma, as either coagulase-positive *Staphylococci* (CoPS) or coagulase-negative *Staphylococci* (CNS). *S.aureus* is the best known coagulase-positive *Staphylococcus* species and is considered a contagious pathogen. Other coagulase positive *Staphylococci* include *S.hyicus* and *S.intermedius*. The term coagulase-negative *Staphylococci* (CNS) includes most *Staphylococci* isolated from bovine milk other than *S. aureus* (National Mastitis Council, 1999).

Acquired antimicrobial resistance in bacteria is an increasing threat in human as well as in veterinary medicine. Hence, monitoring antimicrobial susceptibility in pathogenic as well as in commensal bacteria in animals is recommended by OIE

(Acar and Rostel, 2001). Such monitoring generates data of importance for therapeutic decisions and provides information on trends in resistance.

The selective pressure enforced by the use of antibiotics might cause an increase in the prevalence of antimicrobial resistance and regular monitoring of susceptibility of udder pathogens is therefore warranted (Bengtsson *et al.,* 2009).

The treatment of mastitis necessitates extensive use of antibiotics in dairy herds. But increasing public concern over food safety, the governing authorities are making efforts to minimize antibiotic residues in milk. Moreover, the presence of *S.aureus* in raw milk used by dairy industries also poses threat to public health.

The antibiotic-resistance of *S.aureus* strains is another serious concern besides the pathogenicity. The emergence of antibiotic-resistance in *S. aureus* mastitic dairy animals has been shown in recent years. Strains of *S.aureus* are reported to show resistance against multiple antimicrobials. Among the various antibiotic-resistant isolates, methicillin-resistant *S. aureus* (MRSA) is a serious cause of concern in both human and animals.

In many cases, colonization or infection by MRSA in animal species appears to be due to zoonotic transfer from human owners or caretakers. MRSA infections are not easy to be treated. These genomic components enable *S. aureus* to reside for a long time inside the host and herd environment, and often create hurdles in treatment.

Due to limitations of culture methods, molecular approaches using polymerase chain reaction (PCR) have been developed to identify pathogens causing mastitis.

A definitive and sensitive test for the rapid identification of bacteria associated with bovine mastitis would be clinically and epidemiologically very important. Different PCR based methods have been developed for specific and sensitive detection of mastitis causing pathogens in milk.

Hence the present study on antibiotic resistant mastitis in cows was taken up with the following objectives.

1. To study the prevalence of drug resistant mastitis and their pattern of antibiotic resistance in dairy cows
2. To study the clinical presentation of drug resistant mastitis and its clinico-pathological changes
3. Comparative evaluation of different therapeutic protocols for clinical management of resistant mastitis
4. Assessment of the economic impact of drug resistant mastitis.

CHAPTER II

REVIEW OF LITERATURE

Mastitis is a multi etiological complex disease with inflammation of mammary gland parenchyma, representing significant economic losses to dairy producers because of high morbidity, discarded milk, treatment costs and reduced milk production (Dobbins, 1977).

Radostits *et al.* (2008) defined mastitis as an inflammation of the parenchyma of the mammary gland, characterized by physical, chemical and usually bacteriological changes in the milk and pathological changes in the glandular tissue.

2.1 Clinical mastitis (CM)

In clinical mastitis there are visible changes to the normal appearance of milk, which can include change in colour, consistency or the presence of flakes, clots, and occasionally blood. In severe cases, physical changes of the udder ranging from warmth, diffuse swelling, and pain to gangrene are seen. Fibrosis and atrophy of mammary tissue may result in chronic mastitis (Radostits *et al.,* 2000).

In mild or moderate case of mastitis, local signs are evident. In severe mastitis, systemic signs such as fever, anorexia, and shock are seen as a result of inflammatory response (Erskine, 2011).

2.2 Prevalence

The prevalence of mastitis in cows ranged from 29.34 to 78.54 per cent (Sharma and Rai, 1977; Ebrahimi *et al.*, 2007 and Sharma and Maiti, 2010).

Sachin kumar (2007) reported that the incidence of subclinical mastitis in cows and buffaloes were 2.67 per cent and 2.03 per cent, respectively.

In Indian crossbred cattle, cases of mastitis have been reported as 36.7 per cent (Sharma *et al.*, 2006) and 66 per cent (Kumar *et al.,* 2010). De and Mukharjee (2009)

recorded higher prevalence of subclinical mastitis (42.9 per cent) as compared to clinical mastitis (15.2 per cent).

Kumar *et al*. (2010) recorded the incidence of mastitis in Kolar district of Karnataka, India was highest in cross bred (9.3 per cent) as compared to indigenous cows (3.6 per cent) and buffaloes (4.1 per cent).

Mohana sundhari (2010) reported that clinical mastitis was detected in 28.2 per cent and sub clinical mastitis in 51.2 per cent in dairy cows of Tamil Nadu.

2.2.1 Age

Shukla *et al.* (2005) reported that cross bred animals aged between four to six years and Sahiwal cows aged between six to eight years old were susceptible to mastitis.

Older cows (> 10 years) are at more risk (44.6 per cent) particularly for sub clinical mastitis (38.6 per cent), than younger cows (23.6 per cent) in which clinical mastitis was predominant (Biffa *et al*., 2005).

Sachin Kumar (2007) reported that the incidence of subclinical mastitis in cows and buffaloes were higher in the age group of 4 to 6 years and 8 years respectively

Incidence of infected quarters increases with the age of dairy cows (Sharma *et al.,* 2007 and Sharma and Maiti, 2010).

2.2.2 Breed

Occurrence rate of mastitis differs from breed to breed, crossbred animals have been shown to be more prone to mastitis as compared to native breeds (Joshi and Shrestha, 1995).

Low Incidence of mastitis was recorded in Holstein-Friesian cows when compared to Red and White (Elbers *et al.,* 1998).

Mastitis rate was observed to be lower in Jersey than in Holstein cattle (Washburn *et al.*, 2002)

Subedi and Dhakal (2003) reported that Holstein Friesian cross (65 per cent) and local buffaloes (52 per cent) were found to be most susceptible with clinical mastitis.

Joshi and Gokhale (2006) reported that the incidence was highest in pure breds and lowest in indigenous cattle and buffaloes.

Sharma and Maiti (2010) observed that Holstein-Friesian and Jersey cross bred cows were at higher risk (94.95 per cent) for mastitis than local Zebu cows (31.25 per cent) in India.

Mohana sundhari (2010) reported that prevalence of mastitis was found to be high in Jersey cross (89.6 per cent) than Holstein Friesian cross (89.2 per cent) followed by Sindhi (83.3 per cent) and non-descript (53.6 per cent).

2.2.3 Lactation

Mastitis is more prevalent in older than in young animals, and also more at the 1st to the 3rd stage of lactation (Olugbemi, 2002).

In cattle Subedi and Dhakal (2003) reported that most of the breeds were susceptible to clinical mastitis at 2nd calving.

First-parity cows have been shown to have higher incidence of udder disorders in early lactation as compared to older cows (Valde *et al.*, 2004).

Swedish Holstein cows for the first, second and third lactation, showed that incidence of mastitis increased with parity, with mean values of 10, 12 and 15 per cent, respectively (Carlen *et al.*, 2005). Similarly, rise in mastitis occurrence has been reported significantly with the increase in parity orders in Holstein-Friesian cattle (Breen *et al.*, 2009).

Sachin Kumar (2007) observed that sub clinical mastitis (SCM) was higher during third lactation in cows and buffaloes.

An Indian herd of Sahiwal cattle revealed that the effect of parity on mastitis and the incidences were 20.9, 24.2 and 41.6 per cent in first, second and sixth lactation, respectively (Khate and Yadav, 2010).

2.2.4 Stage of Lactation

The incidence of CM in heifers was higher in the first few days postpartum than in cows (Barkema *et al.,* 1998 and McDougall *et al.,* 2007).

Subedi and Dhakal (2003) reported that clinical mastitis was highest within the first month of lactation.

Corbett (2009) observed that the highest number of clinical mastitis cases occured during the first week of lactation and that the lactating cows were more likely to develop clinical mastitis during the first three months of lactation than the remaining lactating period.

Fadlelmula *et al.* (2009) reported that the first month of lactation displayed the highest incidence of mastitis (62.7 per cent), while the last stage of lactation showed the lowest incidence (11.2 per cent).

Mohana sundhari (2010) reported that the prevalence of mastitis was more in early stage of lactation and it has highly significant when compared to mid and late lactation.

Sharma *et al.* (2011) reported that dairy cows seemed to have more oxidative stress and low antioxidant defence during early lactation or just after parturition than advanced pregnant cows and this appears to be the reason for their increased susceptibility to production diseases (*e.g.*mastitis, metritis, retention of fetal membrane etc) and other health problems.

2.2.5 Quarter affected

Singh and Baxi (1982) recorded higher infection rate in the hind quarters which was attributed to dung and urine contamination.

Joshi and Gokhale (2006) reported that hind quarters were more affected (56.52 per cent) than fore quarters (43.47 per cent).

Sachin Kumar (2007) reported that the incidence was high in right hind quarter followed by left hind, right fore and left fore in cows and buffaloes

Mohana sundhari (2010) observed that all the four quarters were equally affected.

2.3 Management

The incidence of mastitis is influenced by managerial and environmental factors, such as housing of cows, milking equipment, feeding regimen, hygienic quality of feed and water, cleanliness of cows, implementation of preventive measures, and general practices related to, for instance, drying-off (Elbers *et al.,* 1998; Barkema *et al.,* 1999; Peeler *et al.,* 2000; Schreiner and Ruegg, 2003 and Nyman *et al.,* 2007).

Heat and humidity increased the pathogen load in the environment resulting in a greater incidence of mastitis in warm weather (Godden *et al*., 2003).

The teat canal may remain partially open for 1-2 hour after milking and during this period the pathogens may freely enter into the teat canal (Jones, 2006).

Shathele (2009) reported that the incidence of mastitis decreased with increasing ambient temperature but increased with decreasing ambient temperature.

2.4 Housing

Schukken *et al.* (1990) found poor stall hygiene correlated with both the rate of CM and the cleanliness scores of cow for herds with low BTSCC. Poor stall hygiene facilitates exposure to micro-organisms as teats are in close contact with stall surfaces or bedding (Elbers *et al.,* 1998) thereby increasing the chances for pathogens to infiltrate the udder and cause CM; this is particularly true of environmental pathogens such as *Escherichia coli* (Schukken *et al.,* 1991 and Elbers *et al.,* 1998) and contagious pathogens such as *Staphylococcus aureus* (Elbers *et al.,* 1998).

Housing has been identified as a risk factor for clinical mastitis and is thought to be related to an increase in exposure to environmental pathogens (Barkema *et al.,* 1999). Poorly designed facilities can contribute to increased incidence of environmental mastitis.

Tie stall housing was associated with higher incidence rates of CM when compared to free-stall housing systems (Fadlelmoula *et al.,* 2007 and Olde Riekerink *et al.,* 2008). This may result from particular management strategies used in each housing system. However, Getahun *et al.,* (2008) reported higher prevalence in cows living in poor housing system.

Heifers with dirty udders have a higher risk of mastitis (Compton *et al.,* 2007), and managing heifers in the same pasture areas as cows increases the risk of CM (Parker *et al.,* 2007). Heifers with teats closer to the ground were more likely to have dirty teats, and as a potential consequence, had an increased risk of subclinical mastitis (Compton *et al.,* 2007). Sawdust or wood shavings in the calving pen was related to poor udder health in heifers (Nyman *et al.,* 2009).

2.5 Clinical Signs

Heyneman and Burvenich (1992) reported that the clinical symptoms of severe mastitis were a swollen and painful udder, a strongly diminished or lost secretory function of the infected quarter, discoloured milk containing fibrin clots and rumen stasis.

Manifestations of mastitis included decreased milk production, compositional changes in the milk, abnormal milk appearance, swelling and pain in the udder, elevated rectal temperature, depression and decreased feed intake (Harmon, 1994).

Radostits *et al.* (2000) reported that the clinical signs in mastitis included abnormalities of secretion, abnormalities of the size, consistency and temperature of the mammary gland and a systemic reaction.

Peeler *et al.* (2002) reported that abnormalities in milk and udder were the clinical signs in mild clinical mastitis cases while abnormalities in the milk, systemic signs of lethargy, depression, inappetance were the clinical signs in severe cases.

The abnormalities of udder generally observed in acute cases were diffuse swelling, warmth, pain and gangrene, whereas in chronic infections local fibrosis and atrophy were commonly observed (Pyorala, 2003).

Mifflin (2004) reported that in *S.aureus* mastitis, clinical signs varied with the severity of the disease and generally included pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase.

The general signs in acute cases of clinical mastitis were anorexia, toxaemia, dehydration, fever, tachycardia, ruminal stasis, recumbency and death in severe cases. Other signs observed in clinical cases included abnormalities in milk (discoloration, clots, flakes, pus etc.) and physical abnormalities of udder (Galdhar *et al.,* 2005).

Milk that is watery, thick or ropy means an active case of mastitis has progressed enough to be recognized as clinical mastitis. However, such clinical cases make up only 20 per cent of mastitis in dairy herds. Infected quarters usually produce normal appearing milk and infections may persist for weeks before the abnormal milk or soreness of the udder is noticed (Robert *et al*., 2006).

The host immune response elicited by the invading bacteria is variable, however, clinical signs can fluctuate in severity depending on the bacterial strain (Petzl *et al.,* 2008).

Detailed examination of the teat and teat orifices gives an idea about inflammation and loss of function. Physical examination of the udder after milking has been reported and was informative in assessing size, shape and consistency (Viguier *et al*., 2009).

2.6 MILK PROFILE

2.6.1 pH of milk

Barta *et al.* (1990) reported that the pH value of milk was between 6.8 and 7.4 in samples from quarters with clinical mastitis.

Harmon (1994) stated that the pH of the mastitis milk may increase

from a normal range of 6.6 to 6.9 or higher because of movement of blood components into milk.

Prajakta *et al.* (2000) observed that the pH of normal milk was 6.57±0.03 with 0.133 per unit increase in CMT score.

The normal pH of milk is 6.4 to 6.8 and isotonic with blood plasma. In mastitis, during late lactation and dry period, the concentration of lactose and casein in the milk is reduced, whereas sodium chloride and sodium bicarbonate enter in to the alveoli from plasma to maintain isotonicity and milk becomes alkaline (Galdhar *et al.,* 2005).

Sachin Kumar (2007) observed that the mean milk pH value of apparently healthy cow and buffaloes were 6.48±0.40 and 6.50±0.52, respectively and the mean value was significantly higher in sub clinical mastitis.

2.6.2 California mastitis test (CMT)

Ishikawa *et al.* (1982) reported that the severity of mastitis was detected by CMT and the reaction was expressed as scores 0, trace (T), 1, 2 and 3 with mean leucocyte counts of 88,000, 3,50,000, 9,21,000, 20,73,000 and 36,77,000/ml, respectively.

Shukla and Superkar (1983) reported that of indirect tests available for diagnosis of SCM and CMT proved to be the most sensitive and easy to be performed and the results could also be read by the farmer himself.

Patgiri *et al.* (1987) estimated that 39.65 per cent milk samples were positive by CMT and regarded this as a standard, cheap, rapid and easy test to perform on the spot itself.

Ramachandraiah *et al.* (1990) showed that 63.7 per cent cows were found to be positive for CMT when correlated with cultural examination.

Patil *et al.* (1995) suggested that the CMT was easy to perform, interpret, rapid and economical than other indirect tests.

Shitandi and Kihumbu (2004) suggested that the CMT may be a useful indicator of udder infection in farms that should however be used alongside bacteriological culture.

Jain *et al.* (2009) reported that indirect test like CMT which was a simple, inexpensive and rapid screening test, that could be used in combination with SCC for selecting cows with intramammary infections for subsequent bacteriological sampling.

2.6.3 Electrical conductivity

Milner *et al.* (1996) reported that clinical mastitis was detectable by changes in electrical conductivity of fore milk and 90 per cent of cases were detectable when clots first appeared in milk and 55 per cent of cases were detectable upto two milkings prior to the appearance of clots.

Hamann and Zecconi (1998) suggested that when the mammary epithelium was damaged as a result of mastitis the electrical conductivity of the milk changed because of altered sodium, potassium and chloride ions. These changes tended to occur before the development of visible clinical signs and could therefore help to detect mastitis early.

Electrical resistance of milk from quarters infected with major pathogens was 256 ± 46 units, minor pathogens 313 ± 31 units and uninfected quarters 301 ± 36 units. The mean electrical resistance of infected quarters was significantly lower than that of uninfected quarters and it offered potential, simple and rapid test for diagnosis of subclinical mastitis. So use of hand-held electronic detectors has been promoted for earlier detection which permits earlier treatment (Seguya and Mansell, 2000).

In healthy cows and cows with sub clinical mastitis the electrical resistance were 288± 24.0 units and 254±49 units, respectively (Ahmed *et al*., 2008).

2.6.4 Somatic cell count (SCC)

Sheldrake and Hoare (1981) reported that SCC more accurately detected the infectious status of quarters than electrical conductivity.

Ball *et al.* (1991) reported that cell counts above 400 X 10^3 cells /ml were regarded as positive for subclinical mastitis.

Emanuelson *et al.* (1988) reported that SCC was more effective than NAGase in discriminating between quarters with no organism and those with minor or major pathogens.

Schukken *et al.* (1989) opined that the somatic cells in the milk played an important role in the innate immunity of uninfected mammary gland. A complete absence of cells would put cows at risk for disease and a very low concentration of somatic cells increases the risk of clinical mastitis.

The majority of cells consisted of macrophages (60 per cent), lymphocytes approximately 30 per cent and polymorpho nuclear cells 10 per cent and the epithelial cells two per cent of the population (Paape *et al*., 1991; Burvenich *et al*., 1994 and Zaman *et al*., 2009).

Tuteja *et al.* (1993) reported that milk samples with SCC of more than 500,000 per ml was found to be culturally positive.

The somatic cell count penalty limits for saleable milk differs between countries, with the European Union, Australia and New Zealand at 400,000, Canada at 500,000 and the United States at 750,000 per ml of milk (Paape and Contreras, 1997).

Schepers *et al*. (1997) studied that somatic cell count of *S.aureus* was greater than *Corynebacterium bovis* infection.

Malinowski *et al.* (2006) reported that, SCC of Coagulase-negative staphylococci (CNS), *S. aureus* and *Streptococcus* spp. were 2,00,000-20,00,000 /ml. The highest SCC (≥10 million/ml) in intramammary infections by *Arcanobacterium*

pyogenes (95.5 per cent), *Streptococcus agalactiae* (57.6 per cent) and Gram-negative organisms (46.5 per cent). Very high SCC (≥5 million/ml) was connected with infections caused by *Prototheca* spp. (64.5 per cent), Yeast-like fungi (60.2 per cent) and *Streptococcu*s spp. (55.1 per cent). *S. aureus* (76.2 per cent), CNS (84.2 per cent), Gram-positive bacilli (72.4 per cent) and *Corynebacterium* spp. (83.2 per cent) caused an increase in SCC that was smaller than 5 million/ml.

High sensitivity and specificity of SCC (69.49 per cent and 87.58 per cent) and CMT (61.02 per cent and 89.44 per cent) were observed by Jain *et al*., (2009) and they reported that the CMT and SCC could be used as a preferential screening test for selecting cows with intramammary infections for subsequent bacteriological sampling.

The average somatic cell count for normal milk, subclinical and clinical mastitis in cows were 155.60 ±3.88 x 10^4, 251.6 ± 42.94 X 10^4 and 443.62± 20.75 x 10^4**,** respectively (Mohana sundhari, 2010).

The somatic cell count (SCC) in the MRSA infected quarters fluctuated between 300 and 6000 cells / µl, which was not in parallel with the shedding of MRSA (Pilla *et al*., 2012).

2.6.5 Factors affecting SCC of milk

2.6.5.1 Infectious status

Sheldrake and Hoare (1981) stated that the major pathogens which cause greatest increase in SCC included *S. aureus, Streptococcus agalactiae, Coliforms* and *Streptococcus* spp. other than *Strep. dysgalactiae.*

Kitchen (1981) stated that minor pathogens (Coagulase negative *Staphylococci* and *Corynebacterium bovis*) elicited a mild increase (504 X 10^3 cells /ml) with marginal tissue damage. While major pathogens (*S.aureus, Str.agalactiae, Str.dysgalactuiae* and *Str.uberis*) caused more severe tissue damage and increased SCC.

Haggard *et al*. (1983) observed that somatic cell count was above 500 X 10^3 in 56 per cent of *Staphylococcus* infection when compared with 39 per cent for other infections.

2.6.5.2 Physiological factors

Kitchen (1981) stated that stress, nutritional imbalance, climatic conditions and other illness such as ephemeral fever may also influence cell count.

Hassan *et al.* (1984) stated that the total cell count increased from one lactation to the next lactation and was mainly due to an increase in the number of polymorphs.

Berning *et al.* (1987) recorded that isolation and heat stress were associated with increased milk somatic cell count.

2.7 HAEMATOLOGY

Jackson and Bramley (1983) reported that the multiplication of *E.coli* within the gland rapidly stimulated massive neutrophilia in cows from mid to late lactation.

Heyneman and Burvenich (1992) observed that at the onset of mastitis, a reduction in neutrophil numbers and concomitant shift to left in severe and moderate responders were evident. In moderate responders this initial neutropenia was followed by a return to basal levels after two or three days and one week after infection, whereas severe responders developed neutrophilia from the initial stages.

Piccinini *et al.* (1999) reported that proportions of PMN cells viability and NAGase activity were significantly higher in infected quarters.

Wenz *et al.* (2001) stated that leucopenia, specifically neutropenia has been well documented in cows with acute clinical mastitis and cows with more systemic disease signs had more profound neutropenia.

Cows with gram-negative mastitis had significantly lower blood leukocyte, segmented neutrophil, monocyte and lymphocyte counts and had higher blood

hemoglobin concentrations and hematocrits than the cows with gram-positive mastitis (Smith *et al.,* 2001).

Neutrophil population in milk is a useful indicator in the evaluation of mammary gland infection as whenever the inflammatory process starts, the neutrophil number increases. (Moroni *et al*., 2006; Piepers *et al*., 2009 and Zaman *et al*., 2009). However, it was observed that lymphocyte, monocyte and macrophage population significantly decreased in the milk of infected animals (Schukken *et al*., 2003 and Gargouri *et al*., 2008).

2.8 SERUM BIOCHEMISTRY

2.8.1 Aspartate transaminase (AST)

Manston and Allen (1981) reported that AST has been found to be a sensitive enzyme and relatively inexpensive enzyme for screening purposes.

Margolles *et al*. (1987) recorded increased AST values after parturition and various authors observed the increased AST levels were observed in mastitic cows (Margolles *et al.,* 1987; Lothammer *et al.,* 1988; Jovanoic *et al.,* 1990; Ezhilmaran, 1995 and Rajeev, 2000)

Janosi *et al.* (2003) observed no significant association between serum AST at 1 to 3 DIM and mastitis outbreak within the first 28 DIM.

The activity of AST and GGT enzymes showed occasional irregular, changes during pregnancy and early lactation (Stojevic *et al.,* 2005).

Babaei *et al.* (2007) observed a non-significant difference in the AST levels of milk samples from mastitic and healthy cattle. The increased levels of various enzymes in milk occur mainly due to increased permeability of microcirculatory vessels in inflamed areas along with leakage from degenerated/necrotic parenchymal cells and leukocytes.

Moyes *et al.* (2009) showed a significant positive relationship between circulating AST and the development of clinical mastitis in CM cows during early lactation with no differences observed between H and SM cows.

2.8.2 Serum alkaline phosphatase (SAP)

Soldatov *et al.* (1991) reported that levels of the alkaline phosphatase activity was significantly lower in cows with chronic form of mastitis than healthy cows.

Risvani *et al.* (1999) opined that no significant change was observed in the concentration of alkaline phosphatase in cows with clinical mastitis.

Wada *et al.* (2001) reported that serum alkaline phosphatase value was significantly higher in gram negative mastitis.

Increased serum alkaline phosphatase in mastitic cows, suggest that this enzyme plays a role in the pathogenesis of the disease (Vangroenweghe, 2004).

Significant elevation of ALP in SCM milk might be due to both mammary epithelial damage and a breach in the blood-milk barrier, selectively damaged by bacterial toxins. The elevated LDH and ALP activity was from leukocyte and mammary epithelial and interstitial cells damaged during inflammation, particularly from disintegrated leukocytes (Katsoulos *et al.,* 2010).

2.8.3 Serum total protein, Albumin and Globulin

Rowlands *et al.* (1980) opined that concentrations of serum globulin declined steadily during five weeks before calving and followed by an increase in concentration during the first three weeks after calving.

Ezhilmaran (1995) stated that serum globulin value was significantly higher in clinical mastitis.

Risvani *et al.* (1999) observed that serum albumin, total bilirubin and glucose concentrations were increased in animals with clinical mastitis.

Rajeev (2000) reported that serum albumin values were decreased while serum globulin value was increased in clinical mastitis.

Increased proteins and globulin in the blood of cows indicate an activation of immune response following infection of the mammary gland. These proteins are mainly serum albumin and immunoglobulins that are implicated in udder defence mechanisms (Tsenkova *et al.,* 2001).

Immunoglobulin plays an important role in host immunity and inflammation, and there is a correlation between total serum protein (globulins and albumin) and somatic cell count in milk (Pandey et al., 2005).

Total serum protein (9.14±2.74 g/dl) and serum globulin (5.76 ±1.82 g/dl) from subclinically mastitic cows were higher compared to healthy cows. Increased protein and globulin in the blood of cows indicated an activation of immune response following infection of the mammary gland (Matei *et al.,* 2010).

2.9 CLASSIFICATION OF MASTITIS PATHOGENS

Batra and McAllister (1983) grouped *S.aureus, Streptococcus* spp., *E.coli* and *Klebsiella* spp. as primary pathogens while non-haemolytic *Staphylococcus* and other bacteria as secondary pathogens.

Bansal *et al.* (1990) reported that *S.aureus, Streptococcus agalactiae, E.coli* and *Candida* spp. were the most common aetiological agents in clinical mastitis.

Harmon (1994) categorized *S.aureus, Streptococcus agalactiae, Coliforms* and *Enterococci* as major pathogens and coagulase negative *Staphylococci* and *Corynebacterium bovis* as minor pathogens.

Staphylococci are often classified diagnostically based on their ability to coagulate plasma mastitis pathogens as either *S.aureus* (coagulase-positive) or coagulase-negative Staphylococci. *S.aureus* is the best known coagulase-positive Staphylococcus species and is considered as a contagious pathogen. Other coagulase positive staphylococci included *S.hyicus* and *S.intermedius*. The term coagulase-negative *Staphylococci* (CNS) includes most *Staphylococci* isolated from bovine milk other than *S.aureus* (National Mastitis Council, 1999).

Owens *et al.* (2001) opined that *Staphylococci* were the predominant mastitis pathogens causing heifer mastitis and *S. aureus* represented a substantial percentage of these infections. *S.aureus* mastitis in dairy heifers persisted throughout the prepartum period and into the first lactation.

Wenz *et al.* (2001) suggested that acute coliform mastitis typified by *E.coli* intramammary infection, has become the predominant form of mastitis in herds in which contagious mastitis has been effectively controlled.

Singh *et al.* (2002) reported that S.*aureus* mastitis was an important cause for clinical mastitis and *S.aureus* infection had the highest prevalence in herds with poor udder health management.

Bhattacharya (2002) observed a higher incidence of *Staphylococci* (44.44 per cent) followed by gram negative bacteria (25 per cent), *Bacillus* Spp. (15.27 per cent) and *Candida* spp. (2.77 per cent) in mastitis milk samples from West Tripura district.

Karthikeyan (2003) reported that the gram positive organisms were predominant in all the clinical groups when compared with gram negative organisms.

Sharma and Prasad (2003) isolated a total of 11 bacterial isolates from 107 quarters while 12 quarters did not yield any microbial growth. The predominant isolates in their study were *Staphylococcus* spp. (54.05 percent) followed by *Streptococcus* spp. (14.41per cent), *E.coli* (11.71 per cent), *Bacillus* spp. (9.01 per cent) and *Corynebacteriu*m spp. (7.21 per cent).

Sachin Kumar (2007) reported that bacterial culture of mastitis milk revealed *Staphylococcus* spp., *Streptococcus* spp. and *Bacillus* spp. as the gram positive organisms and *E.coli* as the major gram negative organism.

2.9.1 *Escherichia coli*

Smith *et al.* (1985) reported that approximately 65 per cent of clinical cases caused by Coliforms that occurred in the first two months of lactation, originated during the dry period. Coliform bacteria need iron to survive inside the mammary gland. During mammary involution high levels of lactoferrin present in mammary

secretions bind to iron and iron becomes a limiting nutritional factor for bacterial growth.

Short peaks of increased somatic cells in milk are observed after clinical mastitis caused by *E.coli* and somatic cells usually return to pre-infection levels about 3-4 weeks after infection (Pyorala *et al*., 1994 and Haas *et al.,* 2002).

Intramammary infections caused by Coliforms occur most often at calving and during early lactation (Burvenich *et al.,* 2003 and Grohn *et al*., 2005) and decrease as days in milk increase (Hogan and Smith, 2003). While risk factors vary among herds, the dry period is often the period of greatest susceptibility for acquisition of an infection caused by Coliforms, especially during the first or last two weeks (Hogan and Smith, 2003).

The severity of clinical disease has been positively correlated with peak number of Coliform bacteria in mammary secretions. Recurrent cases of clinical mastitis caused by Coliforms have been reported as a result of reinfection from the environment or persistence of the organism within the mammary gland. Older cows usually have a greater rate of clinical mastitis caused by Coliform bacteria compared to primiparous cows. Mastitis caused by Coliform bacteria tend to have a relatively short duration. The severity of the clinical cases can range from mild local signs to severe systemic involvement (Hogan and Smith, 2003).

About 10-13 per cent of clinical mastitis cases caused by Coliforms are estimated to result in severe clinical signs (Bradley and Green, 2001 and Burvenich *et al.,* 2003).

2.9.2 *Staphylococcus aureus*

Lafi *et al.* (1994) reported that *S.aureus* occurred predominantly in both clinical and sub clinical bovine mastitis.

Kaya *et al.* (1998) examined 141 milk samples collected from cows with clinical mastitis for pathogenic bacteria. The authors isolated *S.aureus* (57 per cent),

Streptococcus spp. (8 per cent), *E.coli* (5 per cent), *Lactobacillus* spp. (5 per cent), *Klebsiella pneumoniae* (5 per cent), *C. pyogenes* (4 per cent) and *Pseudomonas aeruginosa* (3 per cent).

S.aureus is regarded as a contagious mastitis pathogen because it is commonly spread from infected to non-infected cows during milking (Sears and McCarthy, 2003).

Balakrishnan *et al.* (2004) obtained 40 bacterial isolates from 65 milk samples. The spectrum of different bacteria comprised *S.aureus* (35 per cent), *E. coli* (27.5 per cent), *S. agalactaie* (17.5 per cent), *Pseudomonas aeruginosa* (12.5 per cent), *S.dysgalactaie* (2.5 per cent), *Pasteurella haemolytica* (2.5 per cent) and *Actinobacillus capsulatus* (2.5 per cent).

Palanivel *et al.* (2008) obtained 12 bacterial isolates from 80 mastitis positive milk samples and the bacterial agents isolated were *S.aureus, S.uberis, S.dysgalectiae, E. coli, Corynebacterium* and *Pseudomonas* spp.

S. aureus (Anderson *et al.,* 2006), *Streptococcus agalactiae* and *Streptococcus dysgalactiae* are among the most frequent and prevalent microorganisms, partly due to the contagious mode of transmission. *S. aureus* is able to produce a variety of extra cellular toxins and virulence factors in the host (Compton *et al.,* 2007).

S.aureus is one of the most prevalent and contagious pathogens of intramammary infections in dairy cattle. The antibiotic resistance in *S. aureus* strains is a serious cause of concern in dairy animals (Wang *et al.,* 2008).

Rajeev *et al.* (2009) reported that *S.aureus* followed by *E.coli* were the most predominant pathogens causing both clinical and subclinical mastitis. The prevalence of *S.aureus* was high in subclinical mastitis (24.36 per cent) when compared to clinical mastitis (13.95 per cent), whereas prevalence of *E.coli* was marginally high in clinical mastitis (15.7 per cent) as compared to subclinical mastitis (14.09 per cent) in dairy cows.

2.9.3 Methicillin resistant *Staphylococcus aureus* (MRSA)

MRSA has been considered comparatively rare in cows, and those *S. aureus* found to be resistant to methicillin tended to lack SCC*mec* (Staphylococcal cassette chromosome *mec)*, gaining resistance from the production of β-lactamases (De Oliveira *et al.,* 2000).

Zero to low occurrence of MRSA among *S.aureus* isolates from bovine milk—0.6 per cent of 846 *S. aureus* isolates in Michigan (Erskine *et al.,* 2002), 1.8 per cent of 2,132 *S. aureus* isolates in Wisconsin (Makovec and Ruegg, 2003) and 0 per cent of 357 *S. aureus* isolates in North Carolina and Virginia (Anderson *et al.,* 2006).

S.aureus isolates are often resistant to other classes of antibiotics (through different mechanisms) (CLSI, 2005 and 2006) making treatment options limited. Such type of isolates are designated as Methicillin-resistant *S. aureus* (MRSA).

MRSA produces a specific penicillin binding protein PBP2′ that possesses reduced affinities for binding to β-lactam. The PBP2′ is encoded by the *mec*A gene carried by a large mobile genetic element, i.e Staphylococcal cassette chromosome mec (Kwon *et al*., 2005).

Methicillin is not generally used in treatment of cows, MRSA is increasing in its significance as a cause of bovine mastitis (Bernabe *et al.,* 2005 and Turutoglu *et al.,* 2006).

The use of antimicrobials for mastitis treatment can promote emergence or survival of MRSA and other methicillin resistant staphylococci in dairy cattle (Moon *et al.,* 2007 and Garcia-Alvarez *et al.,* 2009).

Methicillin-resistant *S.aureus* (MRSA) strains in intra-mammary dissemination often produce incurable severe intra-herd infections (Moon *et al.,* 2007 and Kumar *et al.,* 2010).

In Korea, higher levels of both methicillin resistance and *mecA* carriers were found in mastitis-related *S.aureus* and CNS (Moon *et al.,* 2007). The presence of

methicillin resistance in CNS isolates is of concern, because horizontal *mec*A gene transfer among different *Staphylococcal* spp. has previously been reported (Archer *et al.,* 1994).

MRSA in milk was associated with higher somatic cell counts than methicillin-sensitive *S. aureus* (Juhasz-Kaszanyitzky *et al.,* 2007; Van den broek *et al.,* 2009; Spohr *et al.,* 2010 and Febler *et al.,* 2010).

Antibiotic susceptibility tests such as the agar screen test, disk diffusion test or MIC determination can also be used to identify MRSA (Lee, 2003; Van Duijkeren *et al.,* 2004 and CDC, 2005).

The *mec*A gene was detected in eleven (9.3 per cent) of the 118 *S.aureus* isolates, indicating that nearly 10 per cent of the Belgian farms suffering from *S. aureus* mastitis have MRSA problem (Vanderhaeghen *et al.,* 2010)

Very low prevalence rates of MRSA in either individual cow milk or bulk tank milk (BTM) have been reported internationally. In Hungary, a prevalence of 70 per cent was reported for *S. aureus* on farms (14 of 20 farms), no MRSA isolates were detected from BTM. Similarly, in Korea, a prevalence of 5.6 per cent was reported for *S. aureus* from milk samples from individual cow udders (835 of 14,688 samples). However, only 2.8 per cent of these isolates were identified as MRSA (Moon *et al.,* 2007).

Methicillin-resistant *S.aureus* (MRSA) isolates are frequently multidrug resistant and has been associated with infections in human and animals (Witte *et al.,* 2007).

Acquisition of MRSA may occur through direct contact with humans: one outbreak of mastitis has been attributed to contact with a farm worker (Devriese *et al.,* 1986; Fox *et al.,* 1991 and Roberson *et al.,* 1994). However, this is slightly contentious as evidence to the contrary suggests that transmission between humans and cows is rare (Lopes *et al.,* 1990 and Kapur *et al.,* 1995) due to the host-specificity of *S. aureus* clones (Smith *et al.,*2005).

The transmission of bovine MRSA to humans is possible and may contribute to outbreaks in animal and human populations (Lee, 2003). Hence, it is necessary to know which endemic strains of *S.aureus* in dairy cattle populations are highly pathogenic and methicillin-resistant.

Methods used to detect *mec*A gene or the presence of PBP2' protein are considered reference methods for confirmation of methicillin-resistance in *S.aureus* isolates (CLSI, 2008 and Cattoir and Leclercq, 2010).

The bovine and human MRSA strains are epidemiologically related, which indicates transmission from either cow to human or human to cow. This strain is negative for the PVL genes, which differentiates it from community-associated MRSA ST 1, which is positive for PVL genes (Vandenesch *et al.,* 2003).

Sporadic reports of MRSA transmission between other types of cattle and humans, including cases where cows with mastitis and human handlers shared the same isolate (Juhász-Kaszanyitzky *et al.,* 2007 and Febler *et al.,* 2010).

Many MRSA strains causing mastitis in cattle seem to be of human origin, although bovine-associated strains have been suggested and CC398 has also been identified (Juhász-Kaszanyitzky *et al.,* 2007; Van den Broek *et al.,* 2009 and Febler *et al.,* 2010).

Some strains of *S.aureus* carry Panton- Valentine leucocidin (PVL), a two-component, pore-forming cytotoxin that can cause tissue necrosis, leukocyte destruction and severe inflammation (Van Duijkeren *et al.,* 2005 and Holmes *et al.,* 2005). The PVL genes have usually been associated with community-acquired rather than hospital-linked human MRSA strains (Van Duijkeren *et al*., 2005; Boucher *et al.,* 2010 and Blaine *et al.,* 2010)

PVL has been linked to skin, soft tissue infections and severe necrotizing pneumonia, and some authors have also suggested that the PVL gene is associated with increased virulence in general (Holmes *et al.,* 2005; Van Duijkeren *et al.,* 2005 and Blaine *et al.,* 2010).

2.10 Zoonotic Importance

Methicillin resistant *S. aureus* has been reported in human medicine as a cause of nosocomial and community associated infections (Otter and French, 2010). In veterinary medicine, MRSA strains have been identified in a wide range of animals and diseases (Leonard and Markey, 2008; Huber *et al.,* 2010 and Turkyilmaz, 2010), thus it is considered an emerging threat with a high zoonotic potential (Juhasz-Kaszanyitzky *et al.,* 2007).

MRSA from colonized or infected animals can be transmitted to humans, as well as to other animals (Catry *et al.,* 2010 and Cuny *et al.,* 2010).

2.11 *In vitro* antibiotic sensitivity test

Mackie *et al.* (1988) reported that cloxacillin was highly effective against *S.aureus* infection (100 per cent) followed by neomycin (97 per cent) erythromycin (90 per cent) and furazolidone which were highly effective against Coliform isolates.

Chanda *et al.* (1989) stated that most of the isolated mastitis pathogens were sensitive to gentamicin followed in order by amplicillin, tetracycline, chloramphenicol, kanamycin, oxytetracycline, nitrofurantoin, doxycycline, penicillin and streptomycin and none of the isolates were sensitive to erythromycin, sulphadimidine and sulfadiazine.

Owens *et al.* (1997) stated that *Staphylococcus* was highly susceptible to tetracycline and cloxacillin (100 per cent) followed by enrofloxacin (95 per cent) and erythromycin (90 per cent), *Streptococcus* was highly susceptible to cloxacillin (100 per cent) followed by erythromycin (92 per cent) and ampicillin (85 per cent).

Thornsberry *et al.* (1997) opined that novobiocin was active against all *Staphylococcal* spp. except *S.xylosus* and based on the MIC and disk diffusion, penicillin and novobiocin combination was slightly more active than individual compound against both the *Staphylococci* and *Streptococci* spp.

Dhote *et al.* (1999) tested the bacterial isolates for in vitro sensitivity to antibiotics and found that *Staphylococcus* spp. and *Streptococci* spp. the most

commonly isolated organisms, were sensitive to ciprofloxacin and least sensitive to amoxicillin and penicillin.

Bhalerao *et al.* (2000) conducted antibiotic sensitivity test on milk samples and found that bacterial isolates were sensitive to gentamicin (93.18 per cent), tetracycline (15.91 per cent) cloxacillin (13.64 per cent) and amoxycillin (45.55 per cent).

Oliveria *et al.* (2000) reported that the combination of penicillin and novobiocin demonstrated rapid bactericidal and synergistic action against mastitis strains of *S.aureus* as well as active against β-lactamase positive strains.

De Oliveira *et al.* (2000) reported that penicillin-clavulanate, penicillin-novobiocin, erythromycin, pirlimycin demonstrated good activity against *S.aureus* strains.

Ross *et al.* (2001) stated that *S.aureus* was highly susceptible to chloramphenicol, gentamicin while cloxacillin and the Coliform organisms were found to be susceptible to chloramphenicol, gentamicin and ciprofloxacin in the order mentioned.

Vijayalakshmi *et al.* (2001) stated that the most sensitive antimicrobial agent against *S.aureus* was found to be ciprofloxacin which showed a 100 per cent sensitivity followed by enrofloxacin (90.9 per cent) and erythromycin (81.8 per cent).

Erskine *et al.* (2002) stated that the proportion of susceptible of Coliform isolates ranged from 91 to 100 percent, 6 to 87 percent and 24 to 66 percent for gentamicin, cephalothin and tetracycline, respectively.

The most sensitive antimicrobial agent against gram negative pathogens was found to be enrofloxacin (100 per cent) followed by ciprofloxacin and gentamicin and sensitive antimicrobial agent against gram positive pathogens were found to be gentamicin followed by ciprofloxacin and enrofloxacin (Karthikeyan, 2003).

Guler *et al.* (2010) reported that *S. aureus* strains were found to be susceptible to oxacillin. Methicillin resistance, which is important for human isolates, is not a

problem in bovine isolates (De Oliveria *et al*., 2000; Gentilini *et al*., 2000; Calvinho *et al*., 2002 and Erskine *et al*., 2004).

Moroni *et al.* (2006) reported that amoxicillin and clavulanate (augumentin) were both highly effective against *S.aureus.*

Sachin Kumar (2007) observed the antibiotic sensitivity in cattle with sub clinical mastitis in the descending order was ceftriaxone, enrofloxacin, gentamicin, chloramphenicol, cloxacillin, ampicillin and amoxicillin.

Chandrashekhar *et al.* (2010) observed that *E.coli and S.aureus* isolates showed highest sensitivity against norfloxacin and ofloxacin (93.33 per cent each), amikacin, ciprofloxacin and chloramphenicol (100 per cent each); enrofloxacin (91.4 per cent) and amikacin (100 per cent), respectively.

2.12 MINIMUM INHIBITORY CONCENTRATION (MIC)

Statistics such as MIC_{50} (the median MIC for all isolates) and MIC_{90} (the MIC value that exceeds or equals the MIC for 90 per cent of the isolates) are frequently used to summarize population data. The MIC distribution for each antibiotic should be depicted in a figure to document the degree of skewness or bimodality in the population (Anonymous, 2000).

Antimicrobial susceptibility data may be categorized as susceptible–intermediate–resistant (SIR) or may be expressed as the MIC. The MIC is the lowest concentration of antimicrobial agent that completely inhibits bacterial growth (Prescott *et al.,* 2000 and Clinical and Laboratory Standards Institute, 2002). The susceptible–intermediate– resistant categories are based on MIC breakpoints defined by CLSI (Clinical and Laboratory Standards Institute, 2002).

Examining antimicrobial susceptibility of *S.aureus* isolated from bovine mastitis have used either the disk diffusion method (Watts and Salmon, 1997 and Aarestrup and Jensen, 1998), which is a qualitative method, or broth microdilution test (quantitative method) using commercially available test panels that have a fixed and standardized range of antimicrobial concentrations.

Comparison of results of antimicrobial susceptibility testing among studies is difficult because of differences in methodology. Moreover, based on CLSI, (2008), only a few antimicrobials have a valid veterinary breakpoint for bovine mastitis (ceftiofur, penicillin/novobiocin and pirlimycin). The lack of interpretative criteria specific to most mastitis therapeutics (Watts *et al.,*1994) complicates comparisons among studies.

The interpretative criteria for ampicillin, cephalothin, erythromycin, oxacillin, penicillin, sulfadimethoxine and tetracycline were based on human data. The interpretative criterion for enrofloxacin was based on cattle respiratory disease (CLSI, 2008). No recommendation is currently available from the CLSI, (2008) for spiramycin; therefore, the breakpoint used in Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM, 2003) was considered.

Broth microdilution test was used for determination of antimicrobial susceptibility; however, used a custom-designed panel with a greater number of antimicrobials and broader range of dilutions developed by Apparao *et al.* (2009).

Gentamicin was very active against *S. aureus*, and MIC_{90} ranged from 0.016 μg/ml to 8 μg/ml. The recommended breakpoint for gentamicin is 16 μg/ml (NCCLS, 1997).

The level of resistance for penicillin was low regardless of country, the MIC_{90} for penicillin were within 0.25 to 0.5 μg/mL and $\leq$0.06 μg/mL in Norway (De Oliveira *et al.,* 2000).

Minimum inhibitory concentrations in micrograms per milliliters that inhibit 90 per cent of the strains tested were: 1.5, 0.5, 0.75, 1.50, 0.75, 1.0 and 0.125 for penicillin, oxacillin, cephalothin, gentamicin, erythromycin, clindamycin and ampicillin-sulbactam, respectively. Resistance was detected in 83 (40.3 per cent), 24 (11.6 per cent), 16 (7.7 per cent) and 7 (3.4 per cent) *S. aureus* isolates for penicillin, erythromycin, pirlimycin and gentamicin respectively. Results indicated that *S. aureus* isolates in Argentina exhibited high resistance to penicillin of all antimicrobial agents tested (Gentilini *et al.,* 2000).

The antimicrobial agents tested were penicillin, ampicillin, oxacillin, cephalothin, ceftiofur, amoxicillin + clavulanate, penicillin + novobiocin, enrofloxacin, premafloxacin, erythromycin, clindamycin, lincomycin, pirlimycin, neomycin, lincomycin + neomycin, and sulfamethazine. The MIC_{90} for these antimicrobial agents for all strains were 0.5, 1.0, 1.0, 0.5, 1.0, ≤0.06, 0.125, 0.125, ≤0.0078, 0.5, 1.0, 16.0, 1.0, 2.0, 0.5, and 4.0 μg/ml, respectively. MIC_{90} for AMOX+CLAV against the *S.aureus* strains tested ranged from ≤0.06 to 0.125 μg/ ml, which was substantially lower than the MIC_{90} observed for ampicillin alone (De Oliveira *et al.,* 2000).

The concentration in micrograms per milliliters that inhibits 90 per cent (MIC_{90}) of *S.aureus* strains tested were >8, 8, ≤0.5, ≤4, ≤1, ≤0.5, >64, ≤0.25, 0.5, ≤1 and ≤1 to penicillin, ampicillin, oxacillin, cephalotin, gentamicin, erythromycin, oxytetracycline, enrofloxacin, trimethoprim/sulfamethoxazole, neomycin and clindamycin respectively (Gianneechini *et al.,* 2002).

Minimum inhibitory concentrations recorded show that only certain β-lactamase–resistant penicillins (specifically cloxacillin) or penicillin combinations (amoxicillin + clavulanate) were consistently effective against *S.aureus,* Thus, β-lactamase–resistant penicillins are to be considered the antimicrobial agents of choice for treatment of bovine mastitis resulting from infection by *S.aureus.* The β-lactamase–resistant cloxacillin and amoxicillin + clavulanate (a widely used β- lactamase inhibitor) were both highly effective, with MIC_{50} of 0.25 and 1 to 0.5 μg /mL and MIC_{90} of 0.5 and 8 to 4 μg/mL, respectively (Moroni *et al.,* 2006).

The MIC that inhibited 90 per cent of the isolates tested (MIC_{90}) of penicillin, oxacillin, cefazolin, gentamicin, sulfamethoxazole/trimethoprim, oxytetracycline and enrofloxacin were 4, 0.5, 1, 1, 0.25, 0.25, and 0.06 μg /mL for *S.aureus* and ≥64, 8, 1, 32, ≥64, ≥64 and 0.06 μg/mL respectively for *S. epidermidis*. All *S.aureus* isolates showed susceptibility to oxacillin, cefazolin, gentamicin, sulphamethoxazole/trimethoprim and enrofloxacin (Nunes *et al*., 2007).

The MIC values for ciprofloxacin or enrofloxacin of *E.coli* isolates from mastitis are low and resistance of *E.coli* to fluoroquinolones is rare (FINRES-Vet, 2007).

2.13 Testing of β lactamase and methicillin resistance

Selepak and Witebsky, (1985) reported that MRSA alert kit test was found useful in the detection of methicillin resistance in β lactamase resistance *S.aureus*.

Nitrocefin discs were used for the detection for β- lastamase production by Staphylococcus isolated from bovine IMI (Watts and Salmon, 1997 and Gentilini *et al.,* 2002).

Penicillin resistant isolates were tested for β-lactamase production using a chromogenic cephalosporin disk method (Dry Slide Nitrocefin, Difco Laboratories, Detroit, USA) following induction of β-lactamase production (Soloaga *et al.,* 2004).

Clinical and Laboratory Standards Institute (CLSI), recommends cefoxitin or oxacillin disc screen test for the detection of MRS as standard method along with PCR for the detection of *mec*A gene responsible for encoding PBP2a in *Staphylococcus* Spp. (CLSI, 2005).

Guidelines for prudent use of antimicrobials in the treatment of animal infections, including bovine mastitis, to control antimicrobial resistance, have been published in Finland (Anon, 2003). In these guidelines it was recommended that treatment of mastitis should be based on testing of the antimicrobial susceptibility of the causal agent. In routine diagnostics this was normally limited to testing of β-lactamase production of staphylococci by a nitrocefin test (Pitkala *et al.,* 2007).

Production of β-lactamase was tested using nitrocefin discs, as recommended by the manufacturer (Sigma Aldrich). Nitrocefin (chromogenic cephalosporin) discs (Fluka 49862) were used for the detection *mec*A mediated resistance of MRSA. The change in colour of disc from blue to maroon or red was recorded and considered as positive (Watts and Salmon, 1997 and Broekema *et al.,* 2009).

Nirocefin disc were also applied directly to mastitis milk samples to assess the presence of MRSA (Azeez *et al.,* 2012).

2.14 Antimicrobial Resistance

Various national and international bodies have therefore recommended coordinated ongoing surveillance of AMR in pathogens and potential pathogens in human and veterinary medicine (Nicholls *et al*., 2001 and WHO, 2001).

Overuse and misuse of antibacterial agents have been incriminated as the major selective forces encouraging the development of resistance in bacteria (WHO, 2002).

Antimicrobial resistance (AMR) was potentially one of the reasons for treatment failures (Barkema *et al*., 2006), hence antimicrobial susceptibility testing of udder pathogens were an important step in defining appropriate farm–level treatment protocols.

Penicillin and ampicillin resistance are more widespread due to the frequent use of these antibiotics in treating intramammary infections (Gentilini *et al.,* 2000; Pengov and Ceru, 2003 and Guler *et al.,* 2010).

In Canada, Sabour *et al.* (2004) conducted a study to determine AMR in 288 *S.aureus* isolates from clinical mastitis cases on 58 Eastern Canadian dairy farms in three provinces (Ontario, Québec and Prince Edward Island). 25 percent of isolates were resistant to one or more antimicrobials tested (penicillin, pirlimycin, tetracycline, ceftiofur, tilmicosin, erythromycin, penicillin-novobiocin combination, cephalothin, oxacillin, and sulfadimethoxine). Resistance to penicillin (10.0 per cent) was most common followed by resistance to sulfadimethoxine (8.0 per cent). Multi-drug resistance was rare.

Many studies from different countries have showed that among *S.aureus* strains isolated from bovine mastitis cases, the highest resistance was observed against penicillin, a β-lactam antibiotic, and resistance to other antimicrobials was usually low (Aarestrup *et al.,* 1995; Erskine *et al.,* 2002 and 2004; Vintov *et al.,* 2003 and Sabour *et al.,* 2004).

In case of bovine mastitis Coliforms, resistance proportions ranged from 5.0 to 37.0 per cent for tetracycline (FINRES-Vet, 2007 and Makovec and Ruegg, 2003), 7.0 to 34.0 per cent for sulfisoxazole (FINRES-Vet, 2007 and Srinivasan *et al.,* 2007), 0 to 5.0 per cent for ceftiofur (Erskine *et al.,* 2002 and FINRES-Vet, 2007) and 7.0 to 21.0 per cent for ampicillin (Lanz *et al.,* 2003 and Lehtolainen *et al.,* 2003) across various studies worldwide.

Multi-drug resistance was common in bovine coliform mastitis. In general, resistance to various antimicrobials is frequently seen in bovine mastitis isolates (Watts and Salmon, 1997, Makovec and Ruegg, 2003 and Guler *et al*., 2010).

Antimicrobial use in humans and animals were considered a primary cause of antimicrobial resistance (AMR) in bacteria, which was a public health hazard (Levy and Marshall, 2004). Concern was growing about the use of antimicrobials in food-animal production systems and its potential role in creating reservoirs of AMR determinants that can be transferred from animal to human populations along the food chain (White and McDermott, 2001 and Tikofsky *et al.,* 2003).

Antimicrobial resistance can occur *via* an assortment of mechanisms including the organism being intrinsically resistant to one or more antimicrobials, by spontaneous mutation, or by transfer of genes encoding for resistance from one bacterial host to a new bacterial host via conjugation (sexual transfer of DNA), transduction (bacteriophage transfer), or transformation (acquisition and incorporation of DNA released into the bacteria's environment by lysis of other bacteria) (Cohn and Middleton, 2010).

Antimicrobials are commonly used in livestock production for treatment, prophylaxis, and to improve production. When an antimicrobial drug is used, AMR is promoted either because there is a competitive advantage for inherently resistant bacterial strains to proliferate in the population or use of the antimicrobial facilitates movement of resistance genes from one bacterial host to a new bacterial host (Call *et al*., 2008).

MRSA strains have been observed to be multi-drug resistant, such as aminoglycosides, macrolides, lincosamides, streptomycin, tetracyclines etc., which are often used in the treatment of mastitis (Wang *et al.,* 2008 and Kumar *et al.,* 2010).

In a recent study of MRSA isolated from milk samples from mastitic cows, 100 per cent of isolates were resistant to erythromycin, clindamycin, chloramphenicol and gentamicin (Turkyilmaz *et al.,* 2010). In another study in Switzerland, two MRSA isolates from 142 milk samples from mastitic cows were resistant to ampicillin, ceftiofur, clindamycin, erythromycin, oxacillin, penicillin and tetracycline (Huber *et al.,* 2010). While Vanderhaeghen *et al.,* (2010) reported resistance to tetracycline, macrolides, lincosamides and aminoglycosides, in MRSA from milk from mastitic cows.

Appropriate therapy of β-lactam resistant staphylococcal infection requires the knowledge of antimicrobial resistant profile (Asfour and Darwish, 2011).

2.15 Polymerase Chain Reaction (PCR)

Modifications of sample preparations have allowed PCR analysis to be performed on clinical specimens, considerably reducing the time required for bacterial identification (Rapley *et al*., 1992).

Riffon *et al.* (2001) reported a rapid and sensitive PCR assay for identification of major pathogens in bovine mastitis like *E.coli, S.aureus, S.agalactiae, S.dysgalactiae* and *S.uberis*.

S. aureus is not difficult to cultivate and easily identified, but still, need for a rapid and sensitive DNA-based assay specific for detecting *S.aureus* (Saei *et al.,* 2010). Most recent studies used polymerase chain reaction (PCR) techniques to identify *S.aureus* and for genotyping (Martineau, 2000 and Ghorbanpoor, 2007).

Methods that use PCR, based on the 16S–23S rRNA region sequences have been applied successfully for the identification of many bacteria (Phuektes *et al.,* 2001; Riffon *et al.,* 2001 and Phuektes *et al.*, 2003).

Compared with bacterial culture methods, PCR based detection from mastitic milk samples were less time consuming (Amin *et al.,* 2011). Another main advantage of PCR assay was the direct detection of DNA and thus no matter of live or dead organisms which was crucial point for culture based detection but disadvantage was that PCR detect lower number of organisms compared to culture methods (Yamagishi *et al.,* 2007).

Mohana sundhari (2010) reported that all the three primer (*Staphylococcus* Spp., *E.coli* and *Streptococcus* Spp.) sets showed 100 per cent sensitivity and specificity with known positive culture isolates.

2.16 Multiplex PCR

Multiplex polymerase chain reaction is a modification of polymerase chain reaction in order to rapidly detect deletions or duplications in a large gene. This process amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler (Chamberlain *et al*.,1988)

Multiplex PCR has also been used for diagnosis of multiple pathogens in bovine mastitis milk samples (Phuektes *et al*., 2001and Amin *et al*., 2011).

Pheuktes *et al.* (2001) reported that the multiplex PCR had a detection limit of 10^6 CFU/m1 for *S.aureus, S.agalactiae, S.dysgalactiae* and *S.uberis* when DNA was extracted directly from milk with phenol: chloroform method. Multiplex real time PCR assay correctly identified 96.4 per cent of all quarter milk, 91.7 per cent *S. aureus*, 98 per cent of *Str. agalactiae* and 100 per cent of *Streptococcus uberis* with a sensitivity of 95.5 per cent and specificity of 99.6 per cent in direct milk samples.

Multiplex PCR methods targeting the 16S–23S rRNA spacer regions were developed to detect *S. aureus* and *Streptococcus* Spp., *S. agalactiae, S. dysgalactiae,* and *S.uberis* (Phuektes *et al.,* 2001 and 2003).

Riffon *et al.* (2001) reported that the major advantage of PCR lay in the possibility of using only nanogram of nucleic acid samples, allowing the elimination of culture, rapidity, and easy analysis.

Lee *et al.* (2008) reported that the detection limit of the multiplex PCR for the identification of the seven mastitis pathogens like *S.aureus, S.agalactiae, S.dysgalactiae* and *S.uberis, Corynebacteriurn bovis* and *Mycoplasma bovis* was found to be in the range of 10^3— 10^5 CFU/ml .

Ramdass and Meerarani (2008) reported that in multiplex PCR, two or more primer pairs specific for different targets were included in the same amplification reaction and this technique was used to conserve the reagent and template and reduction in preparation and analysis time required to identify multiple target sites in one assay, as compared to running separate analysis for each product.

2.17 MRSA PCR

The detection of *mec*A gene by PCR is now a gold standard for the detection of methicillin resistance (Boubaker *et al.,* 2004; Van Duijkeren *et al.,* 2004; CDC, 2005 and Loeffler and Lloyd, 2010).

Mechanism of β- lactam resistance in *Staphylococci* include production of β lactamases was encoded by structural *bla*Z gene and / or production of altered form of low affinity penicillin binding protein (PBP) termed PBP2 or PBP2a, is encoded by *mec*A gene designated as Methicillin resistance (MR), preclude therapy with any of the currently available β- lactams among all *Staphylococci* (De Oliveira *et al.,* 2000; Fuda *et al*., 2005; Aarestrup, 2006; Kilic *et al.,* 2006 and Olsen *et al*., 2006).

The genome of *S.aureus* is well known, as well as the significance of some genes, such as the *mec*A gene which encodes resistance to all β-lactam antibiotics and *blaZ* gene which encodes resistance to penicillin (Haveri, 2008).

2.18 Treatment of Mastitis

Cure rates for subclinical mastitis caused by *S.aureus* have been shown to decrease with age (from 81 per cent for cows $\leq$ 48 months of age to 55 per cent for cows $\geq$96 months), the number of infected quarters (from 73 per cent for one infected quarter to 56 per cent for 4 infected quarters) and increasing SCC (Sol *et al.,* 1997).

To date, the only treatment that has shown promise as an adjunctive therapy for Coliform mastitis is the use of non-steroidal anti-inflammatory drugs (Vangroenweghe *et al.,* 2005). Non steroidal anti-inflammatory drugs function by inhibiting cyclooxygenase (COX), the enzyme responsible for the oxidation and subsequent conversion of arachidonic acid to prostaglandins, a class of compounds that are well known mediators of pain and inflammation (Soberman and Christmas, 2003).

Effective treatment for clinical mastitis depends on different factors related to the cow, the pathogen and the drug used for treatment. Factors associated with treatment efficacy included age, stage of lactation, effectiveness of the cow's immune response, somatic cell count, number of infected quarters, chronicity and severity of the cases. Pathogen factors included inherent characteristics of the pathogen, duration of the infection, and pathogen response to antimicrobial therapy (Constable and Morin, 2003 and Bradley and Green, 2009).

Systemic antimicrobial therapy was preferred in cases of bacteremia potentially due to Coliform mastitis (Wagner and Erskine, 2006) or when the udder was swollen thereby indicating that the milk duct system was swollen, compressed or blocked by inflammatory debris and that the site of infection was inaccessible to an antimicrobial agent (Gruet *et al*., 2001). However, the rate of passage of an antimicrobial into milk after parental administration depends upon degree of ionization, lipid solubility and extent of plasma-protein binding (Baggot, 2006).

Only the lipid-soluble, non-ionized and plasma protein unbound fraction of an antimicrobial can penetrate the blood-milk barrier to enter into milk and diffuse into transcellullar fluid. On the contrary, the intramammary route of administration has the potential to provide higher and persistent drug concentration than systemic

administration (Walker and Giguere, 2006), thereby enabling smaller amounts of an antimicrobial to be used.

Antimicrobial therapy is one of the basis of control programs for mastitis caused by *S.aureus* organism; however, several factors such as cow, pathogen and antibiotic treatment levels affect the probability of cure in *S.aureus* IMI (Zecconi *et al.,* 2006). Therefore, the appropriate selection of an antimicrobial agent for treatment of bovine mastitis should not only include knowledge about pharmacokinetics, but also about local susceptibility patterns of the main pathogens. Furthermore, surveys on in vitro susceptibility patterns of *S. aureus*, performed by quantitative methods are required for the detection of emerging resistance worldwide (Salmon *et al.,*1998 and De Oliveria *et al*., 2000).

The use of oxytocin and frequent milking is often recommended as adjunct therapy for sub acute and acute coliform mastitis (Leininger *et al.,* 2003).

Antimicrobials such as oxytetracycline, sulfadimethoxine, ceftiofur, ampicillin and amoxicillin have an appropriate spectrum of activity against *E.coli* and *Klebsiella* species isolates (Wagner and Erskine, 2006).

Treatment of cows infected with *S. aureus* may be successful when infections are of short duration (<2 weeks) in young cows and in early lactation. The use of extended duration of intramammary therapy (8 days) may further improve cure rates (Deluyker *et al.,* 2001 and Ruegg and Araujo, 2002).

Systemic administration of antimicrobials was recommended for the treatment of severe coliform mastitis because of the risk of bacteremia (Cebra *et al.,* 1996 and Wenz *et al.,* 2001). Few antimicrobial substances were recommended for systemic treatment of coliform mastitis (Constable *et al.,* 2008).

According to the Finnish recommendations for the use of antimicrobials in treatment of severe *E. coli* mastitis in dairy cows, fluoroquinolones are the first choice and in high doses of trimethoprim sulfonamides the second-choice (Evira, 2009).

Parenteral administration of antimicrobials is recommended for the treatment of severe coliform mastitis (Cebra *et al.,* 1996 and Wenz *et al.,* 2001), mainly because

of the risk of bacteremia. Enrofloxacin is one of the antimicrobial drugs recommended for treatment of coliform mastitis because of its favorable pharmacokinetic and pharmacodynamic properties (Constable *et al.,* 2008).

Enrofloxacin is a bactericidal, concentration-dependent fluoroquinolone antimicrobial approved for dairy cattle in the European Union. High concentrations of its active metabolite ciprofloxacin, are reached and maintained in blood and milk (Kaartinen *et al.,* 1995 and Rantala *et al.,* 2002). Milk does not significantly interfere with the antimicrobial activity of enrofloxacin in vitro (Fang and Pyorala, 1996). Fluoroquinolones were shown to have positive immunomodulatory effects by increasing the killing ability of neutrophils, which may contribute to therapeutic effect (Hoeben *et al.,* 1997).

Gentamicin is already known for being one the most efficient antibiotics for treatment of bacterium-borne bovine mastitis (Olson *et al.,* 2002).

Tufani *et al.* (2012) conducted therapeutic trial with gentamicin at a dose rate of 2.5 mg/kg intramuscular twice daily for 3.32 days (average) and found 84.21 per cent clinical recovery. The highest clinical recovery rate was recorded in animals treated with gentamicin (84.21 per cent), followed by enrofloxacin (80.77 per cent), ciprofloxacin and ofloxacin (80 per cent) each, amoxicillin-cloxacillin (75 per cent) and ampicillin-cloxacillin (50 per cent), respectively within 3.32, 3.46, 4.00, 4.50, 4.50 and 4.75 days (average).

Akhtar *et al.* (2004) conducted therapeutic trials with Enrofloxacin (QuinIntas) at a dose rate of 15 ml intramuscular once daily for five consecutive days and found 84.62 per cent quarters were cured.

Trailovic *et al.* (1992) conducted clinical trials in which a single administration of 250 mg of ceftriaxone intramammary per quarter resulted in bacteriological cure in 34 of 36 quarters (9 cows) in 14 days.

Mathur (2004) conducted therapeutic trials with Ceftriaxone (Intacef) at a dose rate of 5 mg per kg body weight intramuscular once daily for three consecutive days

along with mammitel intramammary as conventional therapy and found all cases recovered uneventfully between 3 to 5 days.

Ramprabhu *et al.* (2004) conducted clinical trial with three different antibiotics and found cent per cent recovery rate in ceftriaxone group followed by 80 per cent ciprofloxacin and 70 per cent gentamicin groups.

Cefoperazone (CFP) is a third-generation cephalosporin that can be used for IMM due to its broad spectrum of activity (both gram-negative and gram positive bacteria), non irritant properties, and high persistence in treated quarters after single administration (Barragry, 1994 and Prescott, 2006).

Amoxicillin and clavulanate, separately or together, possess immune stimulating and antibacterial enhancing activity against a wide range of pathogens (Pascual *et al.,* 1989; Gomez-Lus *et al.,* 1997 and Cuffini *et al.,*1998). Further, these antimicrobials increase phagocytosis and the intracellular killing capacity of polymorphonuclear cells in immune suppressive diseases where impairment of phagocytosis is an important defect (Finlay *et al.,* 2003 and Cuffini *et al.,* 2001). It has been observed that sulbactam increases chemotaxis, respiratory burst and microbicidal activity of leukocytes in humans during bacterial infection and enhancing the bactericidal function of leukocytes is considered as a secondary antibacterial mechanism of action of sulbactam (Kazmierczak *et al.,* 1989 and Santos and Arbo, 1989).

Amoxicillin alone or in combination with β-lactamase inhibitors is potentially useful for the treatment of mastitis caused by pathogenic organisms (Wilson *et al.,* 1999 and De-Oliveira *et al.,* 2000). A very effective clinical recovery of bovine mastitis after intramammary infusion of amoxicillin plus sulbactam was reported by Sharma *et al.,* (2010) and Tufani *et al.,* (2010).

De Oliveira *et al.* (2000) reported that the specific β-lactam combinations of penicillin-clavulanate and penicillin-novobiocin were very active against *S.aureus* strains.

The most common method available for treating mastitis is IMM infusion of antibiotics. The current FDA approved antibiotic classes include β-lactams, macrolides (erythromycin), coumarines (novobiocin), and lincosamides (pirlimycin). Of these four classes, β-lactams are the most common antibiotics used for IMM treatment of mastitis in conventional dairy farms (Pol and Ruegg, 2007).

The clinical mastitis were treated with intramammary infusions containing penicillin, aminopenicillins or cephalosporins. Each cow also received drying off therapy with cloxacillin or cephalosporins. The use of antimicrobial drugs may have contributed to the emergence of MRSA in dairy farm (Juhasz Kaszanyitzky *et al.,*2007).

Cephalosporins, penicillins, penicillin combinations, lincosamides, and macrolides are most commonly administered intramammarily either during the lactation or dry period for treatment and prevention of mastitis in dairy cattle (Saini *et al.,* 2012).

Use of antiseptics post milking and at the opening of teat also reduces the chances of microbial entry, thus considered as an effective management practice for prevention of contagious mastitis (Olde Riekerink *et al.,* 2012)

Bovine *S.aureus* mastitis can be prevented and controlled by the use of effective post-milking teat drying off, culling animals with chronic infections, treatment of clinical mastitis during lactation and proper use of functioning milking machines (Capurro, 2009).

2.19 MRSA TREATMENT

For *S.aureus* infection caused by conventionally resistant strains, fluroquinolone (Riddle *et al.,* 2000) is a control alternative to penicillin resistance shown by β-lactamic and MRSA strains. In veterinary practice, enrofloxacin is a broad spectrum antibiotic used for treatment of localized and systemic infections (Owens *et al.,* 1997 and Sol *et al.,* 2000).

Most veterinary isolates of MRSA are usually susceptible to routine antibacterials, including potentiated sulphonamides, tetracyclines, fusic acid and mupirocin, although these are not all licensed for use in animals (Hunter *et al.,* 2010).

Imran *et al.* (2010) found that enrofloxacin was the most effective antibiotic against methicillin resistance followed by norfloxacin and chloramphenicol.

Enrofloaxacin was found to be most effective against MRSA (Ganiere *et al.,* 2001; Kenar *et al.,* 2012 and Azeez *et al.,* 2012). All MRS were resistant to members of the penicillin family, such as ampicillin, oxacillin and penicillin.

2.20 ECONOMIC IMPORTANCE OF MASTITIS

Clinical mastitis caused by pathogens of environmental origin can cause substantial losses to producers in terms of milk quality, milk production and survivalof dairy cattle (Hoblet *et al.,* 1991; Miller *et al.,* 1993; Rodrigues *et al*., 2005 and Bar *et al.,* 2008).

Economic consequences of mastitis that have not received much study which included short term mortality, reductions in feed intake, effect of disease on body weight and reproduction. Middle and long term costs included decreased milk production, and increased risk of premature culling and fatality. Persistent decrease in milk production is the main detrimental effect that contributes to the economic impact of mastitis (Seegers *et al.,* 2003).

Mastitis caused considerable economic loss for dairy producers, mainly from reduced milk production, discarded milk, lower milk quality premiums (Poso and Mantysaari, 1995; Seegers *et al.,* 2003 and Halasa *et al.,* 2007) increased cost for replacement of animals (Kalorey, 2000 and Seegers *et al.,* 2003), elevated labor costs (Seegers *et al.,* 2003 and Halasa *et al.,* 2007) and veterinary services (Rubb and Biochard,1999 and Halasa *et al.,*2007).

The cost of clinical mastitis varies greatly for individual cows, depending on the milk yield, the lactation number, the stage of lactation, pregnancy status, current price of milk and supplies and breeding and replacement options (Bar *et al.,* 2008).

A large proportion of the cost of clinical mastitis is associated with milk discarded after treatment. The number of days the milk is discarded depends on the severity of the case, the treatment protocol and the withholding time for the product used for treatment. The proportion of total cost of mastitis treatment associated with milk loss has been reported to be 50 per cent and 64 per cent by Rodrigues *et al*. (2005) and Bar *et al.* (2008), respectively.

CHAPTER III

MATERIALS AND METHODS

The study on "Evaluation of antibiotic resistance mastitis in dairy cows" was conducted at the Centre of Advanced Faculty Training in Veterinary Clinical Medicine, Ethics and Jurisprudence, Madras Veterinary College, Chennai over a period of five semesters during the year 2011-2013.

3.1 Design of Study

Clinical study was done in cows with clinical signs suggestive of mastitis enrolled in Large Animal Clinics, Medical Outpatient Unit, Madras Veterinary College Teaching Hospital, Chennai. Animals available in dairy farms in Coimbatore district of Tamil Nadu were also utilized for the study.

Twenty apparently healthy cows presented for general check up were selected as control animals.

Cows with clinical signs suggestive of acute mastitis were subjected to general clinical examination. Milk was collected from affected quarters subjected to culture and antibiotic sensitivity test. Based on antibiogram, organism resistant to one and more than one antibiotics were considered as resistant mastitis. All resistant mastitis cows were subjected to detailed milk and laboratory examination.

Based on culture, isolation and sensitivity tests, cows with resistant mastitis were grouped as follows,

Group I

Escherichia coli (n=119)

Group II

Staphylococcus aureus (n=104)

Group III

Methicillin resistant staphylococcal aureus (n=12)

Group IV

Apparently healthy cows (n=20)

Based on Antibiotic sensitivity tests, treatment trials were conducted using the following antibiotics. The post treatment evaluation was performed after 7 days of treatment.

1. Gentamicin

2. Ceftriaxone

3.Enrofloxacin

4.Amoxycillin + Sulbactam

3.2 Parameters under study

1. **History and clinical signs**
2. **Milk profile**

California mastitis test (CMT)

pH of milk

Electrical conductivity test (EC)

Somatic cell count (SCC)

Antibiotic sensitivity test (ABST)

Bacterial culture

Minimum inhibitory concentration (MIC)

3. **Haematology**

Haemoglobin (Hb)

Packed cell volume (PCV)

Total Erythrocyte Count (TEC),

Total Leukocyte Count (TLC)

Differential count (DC)

4. **Serum Biochemistry**

Aspartate Transaminase (AST)

Serum Alkaline Phosphatase (SAP)

Total protein (TP)

Albumin

Globulin

5. **Polymerase Chain Reaction (PCR)**

3.3 Evaluation of various parameters

3.3.1 History and clinical signs

The cows selected for the present study were subjected to detailed history. Clinical examination was carried out as described by Boddie (2000).

Under management history, adoption of scientific practices, and parameters like flooring, type of feed, type of milking, udder washing, teat dipping after milking and feeding after milking were studied.

3.3.2 Haematology

Blood was collected in a dry vial containing 10 per cent ethylene diamine tetra acetate and haematological analysis was done as per Chauhan (2003) using automated haematology analyser (Mindray-bc-2800 vet) and parameters such as haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC) and differential count (DC) were estimated and recorded.

3.3.3 Serum biochemical profile

Quantitative estimation of Serum aspartate transaminase (AST) by IFCC (International Federation of Clinical Chemistry) method, serum alkaline phosphatase (SAP) by IFCC method, serum total protein and albumin by modified Biuret and Dumas method.

3.3.4 Collection and analysis of milk samples

3.3.4.1 Collection of milk samples

The udder and teats of the animals were thoroughly cleaned with water and wiped with towel. After letting out first few strips, milk samples of about 10 ml were collected separately from each quarter in sterile culture tubes which were labelled as left fore, left hind, right fore and right hind.

The relevant information of each animal with regard to the breed, age, lactation number, stage of lactation and milk yield were recorded.

3.3.4.2 Analysis of milk samples

The collected milk samples were examined for colour immediately as suggested by Rosenberger (1979) and was further subjected to pH, Electrical conductivity, California mastitis test, somatic cell count, culture and antibiotic sensitivity test. The milk samples were stored in a deep freezer at -20° C until further use for polymerase chain reaction (PCR).

3.3.4.2.1 California Mastitis Test

California mastitis test was performed as per the procedure described by Schalm *et al.* (1971).

3.3.4.2.2 pH

The pH of milk was estimated immediately after collection with the help of pH strips.

3.3.4.2.3 Electrical conductivity test

Electrical conductivity of mastitis milk was determined by a hand held DRAMINSKI mastitis detector (Plate 1) adapting the procedure of Hillerton and Walton (1991). Milk sample was poured till the brim marked in the sampling cup of the instrument. Electrical conductivity of less than 250 units were taken as criteria to declare the milk as clinically mastitic (Plate 2).

3.3.4.2.4 Somatic cell count

Somatic Cell Count was estimated using Cell Counter DCC (De Laval, Denmark) (Plate 3). 1 µl of milk sample was aspirated into the cassette (Plate 4) and then inserted into the cell counter to obtain SCC values.

Somatic cell counter operates on the principle of optical fluorescence, propidium iodide was used to stain nuclear DNA to estimate the SCC in milk (Viguier *et al*., 2009)

The SCC value > 5,00,000 cells / ml of milk with clinical signs were taken as criteria to declare the milk / animal as clinically mastitic.

3.3.5 Isolation and identification of causative agents

Milk samples were mixed well and two to three loopfuls of milk was inoculated into nutrient broth and incubated at 37°C for 24 hours. After overnight incubation one to two loopfuls of the broth culture was streaked on to blood agar and Mac Conkey's agar plates and incubated at 37°C for 24-48 hours. A minimum of five colony forming units were recorded as causative agent and growth of more than one type of colonies was determined as mixed growth. The bacteria grown on the plates were identified up to the genus level based on the culture characters, staining and biochemical reactions as per the methods described in Manual of Clinical Microbiology (2003).

3.3.6 Isolation on selective agar and staining

The causative agent was initially identified by their growth and formation of typical colonies in selective agar, followed by Gram's staining. Gram's staining kit, which contained crystal violet, gram's iodine, decolourizer and safranin was procured from M/s Hi-Media, Mumbai. Staining of all the culture isolates was carried out as per the instructions mentioned in the kit.

For identification of *E. coli* about 0.1 ml of milk was cultured on blood agar (6-10 per cent sheep blood). The organisms were identified as *E. coli* by colony morphology, gram-stain and typical growth on eosin methylene blue (EMB) agar (Plate 5) (Hogan *et al*., 1999).

For identification of *Staphylococcus* spp., about 0.1 ml of milk sample was initially enriched in 3 ml of Brain heart infusion (BHI) broth for 8 hrs at 37^0 C, then Streaked on to Mannitol salt agar (MSA) (plate 6) and incubated at 37^0 C for 24 hrs. After reading the colony morphology on MSA, the cultures were streaked onto BHI agar for further identification procedures. Purity of the culture was checked by Gram's staining and a panel of biochemical tests.

3.3.7 Biochemical characterization

3.3.7.1 Catalase test

Ebullition of gas bubbles after the addition of 24 hr culture to 3 per cent hydrogen peroxide (Plate 7) indicated positive reaction. Absence of ebullition of gas bubbles indicated negative reaction. Only catalase positive cultures were considered for further characterization (Collee *et al*., 1989).

3.3.7.2 Coagulase test

All the *Staphylococcal* isolates obtained, were first subjected to tube coagulase test. About 0.3 ml of 18 hr old *Staphylococcus* culture in BHI broth was mixed with 0.5ml of diluted rabbit plasma (1:4 in PBS) and incubated overnight at 37°C. The result was recorded at 1 hr, 4 hr and after overnight incubation. Formation of clot/ stiff gel (Plate 8) which remained in place when tube was tilted through 90º angle or inverted was considered as positive for coagulase production. The tubes were

read as negative when plasma remained liquid (Plate 9) or showed only a flocculent or ropy precipitate even after overnight incubation (Collee *et al.,* 1989). Based on the results of coagulase tests, the isolates were further categorized as either Coagulase positive (CPS) or Coagulase negative (CNS).

3.3.8 Testing for β-lactamase production

Production of β-lactamase was tested using nitrocefin discs, as recommended by the manufacturer (Sigma Aldrich). Nitrocefin (chromogenic cephalosporin) discs (Fluka 49862) were used for the detection of *mec*A mediated resistance of MRSA (Watts and Salmon, 1997 and Broekema *et al.,* 2009) or β-lactamase production of *Staphylococci* (Pitkala *et al.,* 2007). The change in colour of disc from yellowish to red was considered as positive (Plate 10).

3.3.9 Methicillin resistant *Staphylococcus aureus* (MRSA)

1. K058 MRSA Alert Kit (w/o swabs) (M/s Hi-media Laboratories Ltd., Mumbai) was used to determine the presence of MRSA strains.

2. Atleast 4-5 well isolated colonies from mannitol salt agar of similar type was inoculated into 3 ml sterile nutrient broth and incubated overnight at 37 ° C.

3. The plasma tube (MRSA Alert ^{TM} tube) provided in the kit was rehydrated with 0.6 ml of the diluent to the specific marking and dissolved for 60 seconds to produce a red coloured (Plate 11), slightly cloudy liquid.

4. 50 µl of overnight incubated nutrient broth sample was inoculated into the dissolved plasma. After adding the nutrient broth sample to the plasma, the MSRA Alert ^{TM} was incubated at 35^{o}c without shaking.

5. The MRSA Alert ^{TM} tubes were tilted to look for change in the colour and clot formation after 24 hours.

6. A clot and color change from red to yellow (Plate 11,12) was a positive test which was indicative of Methicillin resistant *S. aureus.*

3.4 Antibiogram of Isolated Organisms

Antimicrobial susceptibility testing was carried out in accordance with the guidelines published by the Clinical and Laboratory Standard Institute (CLSI, 2008). The interpretation of zone of diameter was carried out according to manufacturer's protocol. The organisms were reported as 'sensitive' and 'resistance' to the antimicrobial agents tested.

3.4.1 Preparation of the inoculums

The inoculums was prepared from material picked up with sterile loop from atleast 4-5 well isolated similar type colonies and inoculated into 5 ml sterile nutrient broth. The inoculated broth was incubated at 37°C until slight visible turbidity appeared usually within 2 to 4 hours.

3.4.2 Medium for antimicrobial susceptibility test

Muller Hinton Agar, the recommended medium for disc diffusion test was used in this study. The prepared medium was autoclaved and was immediately placed in a water bath maintained at 45- 50°C. When cooled, it was poured into a sterile petri dish on a leveled surface to give a uniform depth of about 4 mm (25-30 ml for 10 mm plates) and allowed to cool to room temperature. Freshly prepared plates were kept in the incubator overnight to check for sterility and stored at 4°C. The plates were pre-warmed to 37°C for 30 min before use.

3.4.3 Inoculation procedure

A sterile cotton swab was dipped in the incubated broth, rotated several times and gently pressed on the inside wall of the tube level to remove excess inoculums from the swab. The swab was then streaked over the entire surface of the Muller Hinton agar plates three times, with the plate rotated approximately 60° each time to ensure even distribution of inoculum. The following antibiotic discs were transferred aseptically on the inoculum.

The results were observed and recorded after 24 h of incubation on the basis of zone of inhibition. On the basis of zone of inhibition, isolates were categorized as susceptible or resistant.

Resistant isolates were categorised as (1) exhibiting *in vitro* susceptibility to all antimicrobials tested were classified as pansusceptible. (2) exhibiting *in vitro* resistance to 1 or 2 classes of antimicrobials were classified as resistant and (3) exhibiting *in vitro* resistance to 3 or more classes of antimicrobials were classified as multidrug-resistant (Tenover, 2006 and Schwarz *et al.,* 2010).

Sl.No.	**Antibacterials**	**Code**	**Concentration**
1	Gentamicin	GEN	10 mcg/disc
2	Ceftriaxone	CTR	30 mcg/disc
3	Oxytetracycline	TE	30 mcg/disc
4	Penicillin G	P	10 units/disc
5	Amoxycillin	AMX	10 mcg/disc
6	Enrofloxacin	EX	10 mcg/disc
7	Methicillin	MET	5 mcg/disc
8	Amoxycillin + Sulbactam	AMS	15 mcg/disc

3.5 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of different antibiotics for *S.aureus*, *E.coli* and MRSA isolates were determined by modified resazurin assay microdilution technique (Sarker *et al.,* 2007).

3.5.1 Preparation of 0.5 Mcfarland standard

To standardize the inoculums density for a susceptibility test, a barium sulphate turbidity standard equivalent to 0.5 McFarland standard was used (NCCLS, 2003). It was prepared by adding 0.5 ml of 0.048 molar $Bacl_2$ (1.17 per cent w/v $Bacl_2$) to 99.5 ml 0.18 gm H_2SO_4 (1 per cent w/v) with constant stirring. This turbidity is equivalent to $1x10^8$ Colony Forming Units (CFU)/ ml. The standards were transferred to the test tubes closed with ebonite screw caps and stored in dark at room

temperature. Before using, it was vigorously agitated in a mechanical vortex mixer to achieve uniform turbidity.

3.5.2 Preparation of Resazurin dye solution

The Resazurin solution was prepared by dissolving 27 mg of Resazurin dye in 4 ml of distilled water. A vortex mixer was used to ensure that it was a well dissolved and homogenous solution.

3.5.3 Preparation of stock solution of drug

Reference powders of amoxicillin, ceftriaxone, enrofloxacin, gentamicin, pencillin, oxytetracycline, amoxicillin + sulbactam and oxacillin were obtained commercially from Himedia® (Himedia Laboratories., Mumbai, India) to prepare antimicrobial stock solutions. All reference powders were stored as recommended by the manufacturers. The stock solutions were prepared to contain drug concentrations at four times the final concentrations of the highest concentration on the MIC panel.

Ten mg of different antibiotics were initially dissolved in 500 µl of 0.1 N HCL, which was further diluted with sterile distilled water to produce 0.1 per cent solution (1mg/ml). A stock solution of the drug tested, was prepared at a concentration of 1mg / ml.

3.5.4 Preparation of pure bacterial suspension

Three to five isolated colonies of the same morphological type were selected from an agar plate culture and transferred to a tube containing 4-5 ml of nutrient broth. The broth culture was incubated at 35 ^{o}C until the turbidity to or more than 0.5 McFarland standard was achieved. The turbidity of the actively growing broth culture was adjusted with sterile normal saline to obtain turbidity comparable to that of the 0.5 McFarland standard. The inoculum tube and McFarland standard were visually matched against a paper with white background and contrasting black lines. After adjusting the inoculums to 0.5 McFarland standard, it was diluted 100 fold with sterile normal saline so as to reduce the concentration of the organism to 10^6 CFU/ml. It was further diluted 200 fold using sterile Muller-Hinton broth to get a suspension containing 0.5 x 10^4 CFU/ml. This suspension was used within 30 minutes of preparation.

A pure culture of a bacteria to be tested was prepared by pulling out a single colony and allow for log phase multiplication (by transferring it into nutrient broth solution) just before the test, the bacterial concentration was adjusted to 1 x 10^{8} CFU/ ml (0.5 ml $Bacl_2$ 0.048 gm $Bacl_2$ solution added to 99.5 ml of 0.18 gm sulphuric acid) – McFarland standard.

3.5.5 Serial dilution of the drug

A 96 - well U bottom microplate was used and labeled appropriately for the drug and bacteria incubated to be tested. To the first well 100 µg of drug solution was added. From the 2^{nd} well to the twentieth well 50 µl of nutrient broth was added to each well. After thorough mixing, 50 µl from the first well was transferred to the 2^{nd} well. The contents of second well were mixed thoroughly and then 50 µl was transferred to the 3^{rd} well. This process was repeated till the 20^{th} well. 50 µl was taken from the 20^{th} well and discarded. Antibiotic solutions were prepared with a two - fold higher concentration than the final dilution range required so as to compensate for the addition of an equal volume of inoculums. This step ensured that all wells contained 50 µl while the drug concentration was half of that of the previous well. 10 µl of $1/20^{th}$ of 0.5 McFarland standard bacterial suspension was added to all wells. Then 10 µl of Resazurin dyes was added to all the wells. Finally 30 µl of sterile nutrient broth was added to all the wells. A growth control was maintained in the 21^{st} well by adding 10 µl of bacteria, 10 µl of dye and 30 µl of nutrient broth. A sterile well was maintained in the 22^{nd} well by adding 100 µl of nutrient broth. The plate was incubated at 37 ^{0}C for 24 hrs.

3.5.6 Result and Interpretation

Resazurin dye imparts a purple colour to all the wells. If bacteria was viable, the oxido - reductive process converted resazurin dye to pink colour. After incubation the wells were observed for any change in colour. The well containing lowest concentration with reflection of purple colour was identified and the concentration in that well was the MIC of the drug of plate. The lowest concentration of antibiotic that resulted in complete inhibition of visible growth and did not produce any turbidity was taken as the MIC endpoint.

3.6 Polymerase Chain Reaction (PCR)

The organisms identified by their growth pattern on selective agar followed by their biochemical characteristics were also confirmed for their identity by performing PCR on the bacterial pellet targeting the specific gene for the different mastitis pathogens. The identity of the PCR amplified targets from the different strains were confirmed by sequencing the PCR amplicons.

3.6.1 PCR for identification of the mastitis causing bacteria

The nutrient broth inoculated with the milk samples and incubated overnight was used as source for performing the PCR mediated confirmation. About 0.1 ml of the bacterial suspension was centrifuged to pellet the bacteria, resuspended in 20µl of nuclease free water and 2-3µl of the suspension was used in the PCR directly. The following primers were used in the PCR reaction for identification of the different mastitis causing bacteria

Primer	**Primer sequence**	**Organism targeted and Reference**
SU-F	5’ TTC GTA CCA GCC AGA GGT GGA 3’	*S. aureus* (bp229); (Pradhan *et al.,* 2011)
SU-R	5’ TCT TCA GCG CAT CAC CAA TGC C 3’	
Eco 2083	5’ GCT TGA CAC TGA ACA TTG AG 3’	*E. coli* (662bp) ; (Riffon *et al.,* 2001)
Eco 2745	5’ GCA CTT ATC TCT TCC GCA TT 3’	
mecA	5’ AAA ATC GAT GGT AAA GGT TGG C 3′ 5’AGT TCT GCA GTA CCG GAT TTG C 3’	Methicillin resistant S.*aureus* (MRSA) (533 bp); (Lee, 2003)
blaZ	5’ ACT TCA ACA CCT GCT GCT TTC 3’ 5’ TGA CCA CTT TTA TCA GCA ACC 3’	Methicillin resistant *S. aureus* (MRSA) (173 bp); (Martineau *et al*., 2000)

3.6.2 PCR reaction mixture

Polymerase Chain Reaction was performed in an Eppendorf thermal cycler. All the reactions were carried out in volume of 25 µl in 0.2 ml PCR tubes

Materials	**Volume (µl)**
PCR master mix	12.5
Forward primer (FP)	1.0
Reverse primer (RP)	1.0
Nuclease free distilled water	7.5
Template DNA (Bacterial suspension)	3.0

3.6.3 PCR cyclic condition

Cycle conditions	***E.coli***	**S.*aureus***	**MRSA**
Initial denaturation	95° C -10 min	94° C -10 min	95° C -10 min
Denaturation	95° C -45 sec	95°C-1 sec	95° C -30 sec
Annealing	57° C- 1 min	55° C -30 sec	60° C -30 sec
Extension	72° C -2 min	72° C -3 sec	72° C -30 sec
No. of cycles	35	35	35
Final Extension	72° C-10 min	72° C -10 min	72° C-10 min
Hold			

3.6.4 Optimization of Multiplex PCR for the detection of mastitis pathogens

In order to save the time required to screen field milk samples, a common cyclic conditions was standardized for performing the PCR assay with all the three primer sets. For optimization of the PCR cyclic condition, gradient PCR was carried out in the gradient thermal cycler. The PCR amplification was carried out in an automated thermal cycler according to cyclic condition mentioned in above. The reaction mixture was prepared as below:

Multiplex PCR reaction mixture

Materials	Volume (µl)
PCR master mix	12.5
Forward primer (FP) (*E.coli, S. aureus* and MRSA each @1 µl)	3.0
Reverse primer (RP) (*E.coli, S. aureus* and MRSA each @1 µl)	3.0
Nuclease free distilled water	3.5
Template DNA	3.0
Total	25.0

Amplification was performed using Eppendorf - Mastercycler PCR system (Thermalcycler). An initial activation step at 95°C for 10 min, denaturation at 95°C for 45 sec was followed by 35 cycles of amplification, annealing at 57° C for 1 min and extension at 72° C for 2 min and a final extension at 72° C for 10 min.

3.6.5 Agarose Gel Electrophoresis

The PCR products were electrophoresed in 1.5 per cent agarose gel using 1X Tris acetate EDTA buffer along with standard DNA marker (100 bp ladder, Genei, Bangalore) and stained with ethidium bromide. The agarose gel was viewed under transilluminator (Fotodyne, USA) and the results were documented.

3.6.6 Sequence confirmation of the mastitis causing bacteria

The amplification observed in the PCR targeting the specific genes of the different strains were also confirmed by gel purification of the specific amplicons by cyclic sequencing of the amplicons at Department of Animal Biotechnology, Madras Veterinary College, Chennai. The sequences obtained from the different samples were subjected for BLAST analysis with the NCBI database to confirm their identity.

3.7 Therapeutic Trials

Cows positive for mastitis during the study were used for drug trials. Based on culture, antibiotic sensitivity test they were allotted the following treatment trial.

1. Gentamicin

Dose rate of 4mg / kg body weight intramuscular once daily for 7 days in *E.coli* (n=31) group (Saini *et al*., 2011)

Dose rate of 4mg / kg body weight intramuscular once daily as well as 100 mg per quarter intramammary infusion once daily for 7 days in *S. aureus* (n=29) group.

2. Ceftriaxone

Dose rate of 5 mg / kg body weight intramuscular once daily for 7 days in *E.coli* (n=31) group (Sachin Kumar, 2007).

Dose rate of 5 mg / kg body weight intramuscular once daily for as well as Ceftriaxone 250 mg per quarter intramammary infusion once daily for 7 days in *S.aureus* (n=23) group.

3. Enrofloxacin

Dose rate of 5 mg / kg body weight intramuscular once daily for 7 days in *E.coli* (n=30) group (Evira.,2009).

Dose rate of 5 mg / kg body weight intramuscular once daily as well as 100 mg per quarter intramammary infusion once daily for 7 days in *S.aureus* (n=29) and Methicillin resistant *S.aureus* (MRSA) (n=6) group.

4. Amoxycillin + Sulbactam

Dose rate of 10 mg / kg body weight intramuscular twice daily for 7 days in *E.coli* (n=27) group (Sharma *et al*., 2010 and Tufani *et al*., 2010)

Dose rate of 10 mg / kg body weight intramuscular twice daily as well as 300 mg per quarter intramammary infusion once daily for 7 days in *S.aureus* (n=23) and Methicillin resistant *S.aureus* (MRSA) (n=6) group.

Depending on the severity animals in all groups were treated with non-steroidal anti-inflammatory drug meloxicam either intravenously or intramuscularly at 0.5 mg/kg body wt daily for 1-5 days and chlorpheniramine maleate at 0.5 mg /kg body wt daily for 5 days.

In *E.coli* group Normal Saline was administered @10 ml/kg body weight intravenously daily for 1-5 days depending on the severity.

3.7.1 Post Treatment Assessment

Post treatment assessment was carried out after 7 days, based on milk pH, electrical conductivity, california mastitis test, somatic cell count and clinical improvement.

3.8 Economic Impact

Economic impact of mastitis was assessed by calculating production loss (milk yield loss and discarded milk) during mastitis, post treatment milk yield loss for 15 days and cost of treatment.

3.9 Statistical analysis

The results were statistically analyzed, utilizing SPSS – Version 14 statistical software package and discussed critically.

CHAPTER IV

RESULTS

In the present study, milk samples were collected from 401 lactating dairy cattle with acute clinical mastitis from Large Animal Clinics Outpatient Medical Unit, Madras Veterinary College, Chennai and organized dairy farms in Coimbatore districts.

The study of various parameters viz., milk profile, haematology, serum biochemistry were studied. The data obtained during this study were analysed statistically wherever applicable. The values of the control, clinical groups and treatment groups were analysed statistically.

4.1 Incidence of Clinical Mastitis

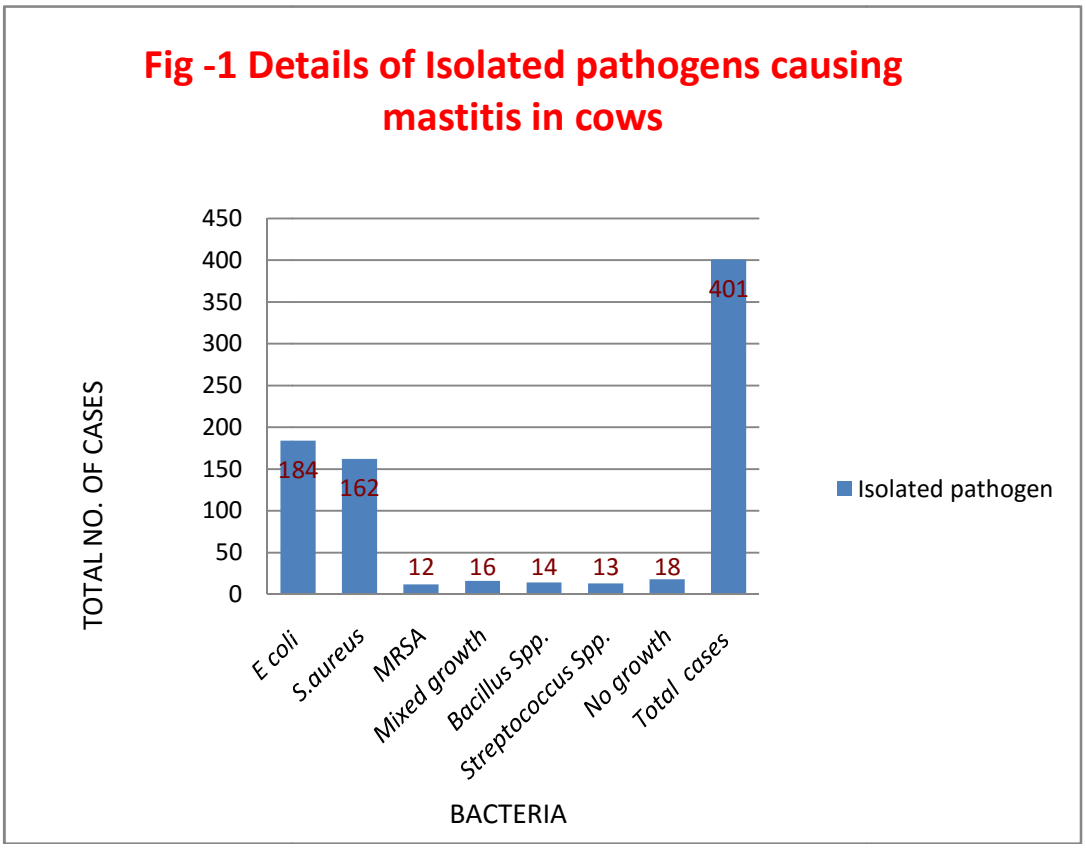
4.1.1 Overall Incidence

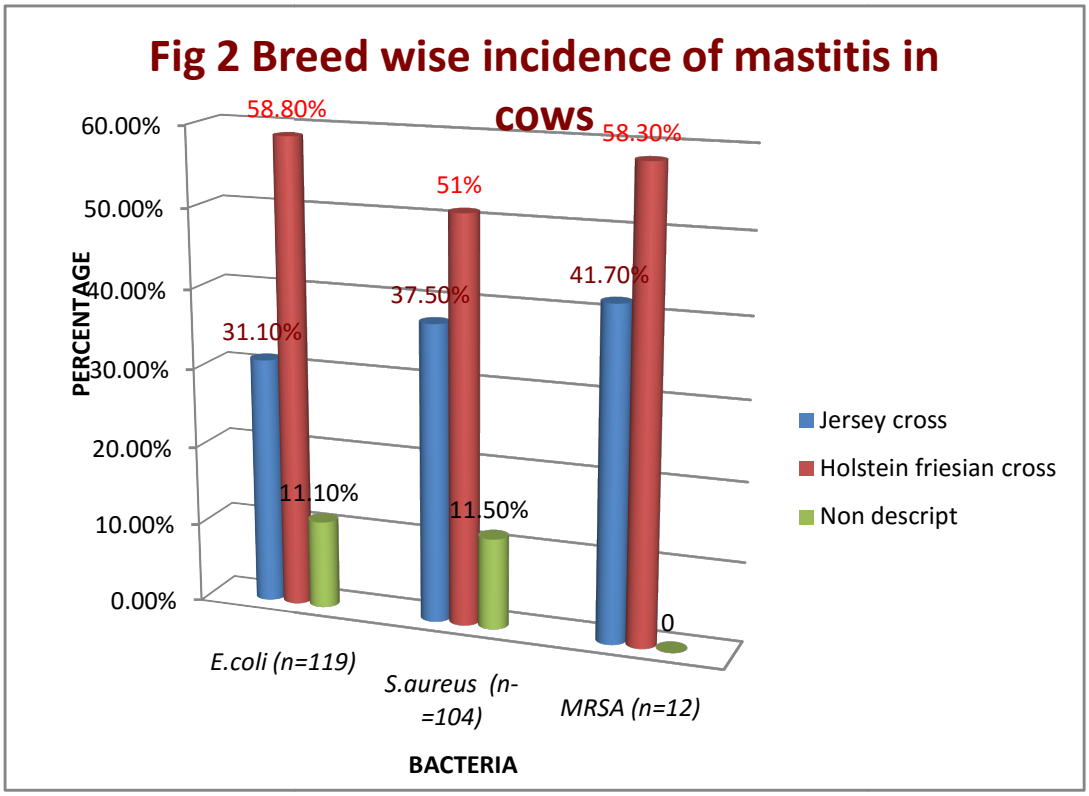
Out of 11,258 cows which attended the Large Animal Clinics Medical Outpatient unit of Madras Veterinary College Teaching hospital over a period of two and half year (2011-2013), 1078 cows (9.57 per cent) were found to be affected with clinical mastitis. Among these, 215 cows (1.90 per cent) were found with acute mastitis.

In Organized dairy farms of Coimbatore district, out of 1678 lactating dairy cattle, clinical mastitis was detected in 278 cows (16.56 per cent). Among these, 186 cows (11.08) were found with acute mastitis.

4.1.2 Pathogen wise incidence

Of the 401 acute mastitis samples subjected to bacterial isolation, 184 tested positive for *E.coli*, 162 positive for *S.aureus*, 14 positive for *Bacillus* spp., 13 positive for *Streptococcus* spp., 12 positive for MRSA and 16 samples showed mixed infection. However, in 18 cases no bacterial growth could be observed (Figure 1).





4.1.3 Incidence of Drug Resistant Mastitis

Drug resistant mastitis was detected in 235 out of 401 cows accounting to 56.1 per cent. Incidence of resistant mastitis in organized farm was 42.13 per cent, where as unorganized farm was 57.87 per cent. The predominant resistant causative pathogen was *E.coli* (50.64 per cent) followed by *S. aureus* (44.25 per cent) and MRSA (5.11 per cent) (Table 1).

4.1.4 Breed wise incidence

Breed wise incidence of resistant mastitis in cows are given in Fig-2.

In the case of *E.coli* group, the breed incidence was found to be high in Holstein Friesian cross (58.8 per cent) followed by Jersey cross (31.1 per cent) and non descript (11.1 per cent).

In the case of *S.aureus* group, the breed incidence was found to be high in Holstein Friesian cross (51 per cent) followed by Jersey cross (37.5 per cent) and non descript (11.5 per cent).

In the case of MRSA group, the breed incidence was found to be high in Holstein Friesian cross (58.3 per cent) and Jersey cross (41.7 per cent).

4.1.5 Lactation wise incidence

Lactation wise incidence of mastitis in cows are given in Table 2.

In *E.coli* group, incidence of mastitis in cows was higher in third lactation (35.29 per cent) followed by second lactation (31.93 per cent), first lactation (20.16 per cent), fourth lactation (5.88 per cent), fifth lactation (5.04 per cent) and least in sixth lactation (1.68 per cent).

In *S.aureus* group, incidence of mastitis in cows was higher in third lactation (32.69 per cent) followed by second lactation (28.84 per cent), first lactation (19.23 per cent), fourth lactation (13.46 per cent), fifth lactation (2.88 per cent) and sixth lactation (2.88 per cent).

TABLE -1

Details on the isolates of bacterial pathogens causing mastitis in cows

Sl.No.	Bacteria	Isolated pathogen	Resistant pathogen	% of Resistant pathogen
1	*E coli*	184	119	50.64
2	*Staphylococcus aureus*	162	104	44.25
3	MRSA	12	12	5.11
4	Mixed growth	16	-	-
5	*Bacillus* Spp.	14	-	-
6	*Streptococcus* Spp.	13	-	-
7	No growth	18	-	-
	Total cases	401	235	100

TABLE - 2

Lactation wise incidence of mastitis in cows

Lactation	*E.coli* (n=119)	*S.aureus* (n=104)	*MRSA* (n=12)	χ^2 test
1st	20.16%	19.23%	8.33%	7.21 NS
2nd	31.93%	28.84%	41.66%	
3rd	35.29%	32.69%	33.33%	
4th	5.88%	13.46%	16.66%	
5th	5.04%	2.88%	-	
6th	1.68%	2.88%	-	

NS – Non significant (P>0.05)

In MRSA group, incidence of mastitis in cows was higher in second lactation (41.66 per cent) followed by third lactation (33.33 per cent), fourth lactation (16.66 per cent) and first lactation (8.33 per cent).

4.1.6 Incidence in stages of lactation

Stage of lactation wise incidence of clinical mastitis is shown in Table 3**.**

In *E.coli* mastitis, incidence was high in the early stages of lactation (63.9 per cent) followed by mid lactation (31.1 per cent) and late lactation (5 per cent).

In *S.aureus* mastitis, incidence was high in the early stages of lactation (69.2 per cent) followed by mid lactation (25 per cent) and late lactation (5.8 per cent).

In MRSA mastitis, incidence of mastitis was high in the early stages of lactation (83.3 per cent) followed by mid lactation (16.7 per cent).

4.1.7 Quarter wise incidence

Quarter wise incidence of clinical mastitis is shown in Table 4**.**

In *E.coli* group, highest number of animals were infected in left hind quarter (28.36 per cent), followed by left fore (26.95 per cent), right hind (24.11 per cent) and right fore quarter (20.56 per cent).

Incidence in more than one quarter in the same animal was also observed. Incidence in two quarters was the highest (7.8 per cent) followed by three quarters (2.8 per cent) and all four quarters (0.7 per cent).

In S.*aureus* group, 30.28 per cent of the animals were infected in right hind quarter, followed by left hind (28.87 per cent), left fore (22.53 per cent), right fore quarter (18.30 per cent), two quarters (23.1 per cent), all four quarters (2.82 per cent) and three quarters (0.7 per cent).

In MRSA group, 50 per cent of the animals were infected in right hind quarter, followed by left hind (25 per cent), left fore (12.5 per cent), right fore quarters (12.5 per cent) and two quarters (25 per cent).

TABLE – 3

Stage of lactation wise incidence of mastitis in cows

Stage of Lactation	*E.coli* (n=119)	*S.aureus* (n=104)	*MRSA* (n=12)	χ^2 test
Early 1-3 Months	63.9%	69.2%	83.3%	2.73 NS
Mid 4-6 Months	31.1%	25%	16.7%	
Late 6-9 Months	5%	5.8%	-	

NS – Non significant (P>0.05)

TABLE – 4

Quarter wise incidence of mastitis in cows

Quarter	*E.coli* (n=141 Quarters)	*S.aureus* (n=142 Quarters)	*MRSA* (n=16 Quarters)
LFQ	26.95%	22.53%	12.5%
LHQ	28.36 %	28.87%	25%
RFQ	20.56%	18.30%	12.5%
RHQ	24.11%	30.28%	50%
2 Quarters	7.8%	23.1%	25%
3 Quarters	2.8%	0.7%	-
4 Quarters	0.7%	2.82%	-

4.1.8 Clinical signs

The clinical manifestations of mastitis in cows are shown in Table 5 and 6.

Clinical signs pertaining to *E.coli* mastitis included painful udder (99.1 per cent), hot udder (97.5 per cent), swelling of udder (95.8 per cent), pyrexia (63 per cent), reduced rumen motility (58 per cent), inappetance (31.9 per cent) and anorexia (21.8 per cent).

In *E.coli* mastitis, milk colour changes included dirty white (55.5 per cent), yellowish (39.5 per cent), flakes (37 per cent), serous / watery (8.4 per cent) and blood mixed (0.8 per cent).

Clinical signs relating to *S.aureus* mastitis included swelling of udder (98.1 per cent), hot udder (97.1 per cent), painful udder (97.1 per cent), inappetance (34.6 per cent), reduced rumen motility (34 per cent), anorexia (23.1 per cent) and pyrexia (22.1 per cent).

In *S.aureus* mastitis, milk colour changes included dirty white (60 per cent), yellowish (39.4 per cent), flakes (27.9 per cent), serous / watery (24 per cent) and blood mixed (1 per cent).

Clinical signs pertaining to MRSA mastitis included hot udder and painful udder (100 per cent each), swelling of udder (91.6 per cent), reduced rumen motility (39 per cent), pyrexia (25 per cent), inappetance and anorexia (16.6 per cent each).

In MRSA mastitis, milk colour changes included dirty white (66.7 per cent), yellowish (33.3 per cent), flakes (16.7 per cent) and serous / watery (16.7 percent).

4.1.9 Management and housing

In an organized farm 99 cows had concrete floor (Plate 13), 94 cows fed with commercial feed, 5 cows fed with own feed, 23 had hand milking, 76 had machine milking, 97 had udder washing and teat dip and 24 had after milking feeding practices followed.

In an unorganized farm 13 cows had concrete floor, 123 cows had mud floor, 73 cows drinking contaminated water (Plate 14), 136 cows fed with own feed, 136 hand milking and 29 had after milking feeding practices followed.

TABLE -5

Clinical signs of mastitis cows

Clinical signs	***E.coli* (n=119)**	***S.aureus* (n=104)**	***MRSA* (n=12)**
Inappetance	31.9%	34.6%	16..6%
Anorexia	21.8%	23.1%	-
Pyrexia	63%	22.1%	25%
Reduced rumen motility	58 %	34 %	39 %
Swelling of udder	95.8%	98.1%	91.6%
Hot udder	97.5%	97.1%	100%
Painful udder	99.1%	97.1%	100%

TABLE – 6

Milk examination in mastitis cows

Colour	***E.coli* (n=119)**	***S.aureus* (n=104)**	***MRSA* (n=12)**	**χ^2 test**
Dirty white	55.5%	60%	66.7%	14.64^{NS}
Yellow	39.5%	39.4%	33.3%	
Flakes	37%	27.9%	16.7%	
Serous watery	8.4%	24%	16.7%	
Blood	0.8%	1%	-	

NS – Non significant (P>0.05)

4.2 Diagnosis

4.2.1 MILK PROFILE

4.2.1.1 California mastitis test (CMT)

The California mastitis test (CMT) was found to be negative for all the animals in control group. The score of CMT in clinical mastitis are given in Table 7.

The CMT was found to be 2+ in *E.coli, S.aureus* group (43.7 per cent, 47.11 per cent) and 3+ in *E.coli, S.aureus* and MRSA groups (56.3 per cent, 52.89 and 100 per cent) respectively.

4.2.1.2 pH of milk

The mean ± S.E values of milk pH in control group, *E.coli, S.aureus* and MRSA groups were 6.45± 0.05, 7.63±0.04, 7.64±0.05 and 8.12±0.12 respectively (Table 8).

A highly significant increase in the pH was observed between control and clinical mastitis group. However, there was no significant difference in pH value between *E.coli* and *S.aureus* group. Highly significant difference in pH value in MRSA group was evident when compared to *E.coli* and *S.aureus* group.

4.2.1.3 Electrical conductivity

The mean ± S.E values of milk electrical conductivity (Units) in control group, *E.coli, S.aureus* and MRSA groups were 356±6.42, 243.27±1.68, 238.65±2.02 and 243.27±4.49 respectively (Table 8).

A highly significant decrease in the electrical conductivity was observed between control and clinical mastitis group. However, there was no significant difference in electrical conductivity between the clinical groups.

TABLE 7

CMT scores in mastitis cows

Treatment group	Scores	Pre treatment
E.coli (n=119)	+ 2	43.7 %
	+ 3	56.3 %
S.aureus (n=104)	+ 2	47.11%
	+ 3	52.89 %
MRSA (n=12)	+ 2	-
	+ 3	100 %

4.2.1.4 Somatic cell count

The mean ± S.E values of milk somatic cell count (Plate 5 and 6) in control group, *E.coli, S.aureus* and MRSA groups were 60.90 ±8.52, 2148.56±73.36, 2299.73±81.61 and 2489.50±120.36 lakhs respectively (Table 8).

A highly significant increase in the milk somatic cell count was observed between control and clinical mastitis group. However, there was no significant difference in SCC between clinical groups.

4.3 Haematology

The mean ± S.E values of haemoglobin (g/dl) in control group, *E.coli, S.aureus* and MRSA groups were 9.64±0.16, 7.99±0.13, 8.13±0.12 and 8.42±0.23 respectively (Table 9).

A highly significant decrease in the values of haemoglobin was observed between control and clinical mastitis groups. However, there was no significant difference in Hb value between clinical groups.

The mean ± S.E values of PCV (per cent) in control group, *E.coli, S.aureus* and MRSA groups were 30.73±0.71, 24.13±0.40, 25.04± 0.37 and 25.70±1.05 respectively (Table 9).

A highly significant reduction in the values of PCV was observed between control and clinical mastitis groups. However, there was no significant difference in PCV value between clinical groups.

The mean ± S.E values of TEC (million/cumm) in control group, *E.coli, S.aureus* and MRSA groups were 5.95±0.15, 5.38±0.12, 5.32±0.07 and 5.54± 0.19 respectively (Table 9).

A significant reduction in the values of TEC was observed between control and clinical mastitis groups. However, there was no significant difference in TEC value between clinical groups.

TABLE 8

pH, EC and SCC values in mastitis cows (Mean±S.E)

Sl.No.	Parameters	Control (n=20)	*E.coli* (n=119)	*S.aureus* (n=104)	*MRSA* (n=12)	F value
1	pH	$6.45^{a} \pm 0.05$	$7.63^{b} \pm 0.04$	$7.64^{b} \pm 0.05$	$8.12^{c} \pm 0.12$	53.847**
2	Electrical conductivity(Units)	$356.00^{a} \pm 6.42$	$243.27^{b} \pm 1.68$	$238.65^{b} \pm 2.02$	$243.27^{b} \pm 4.49$	202.089**
3	SCC x 10^3	$60.90^{a} \pm 8.52$	$2148.56^{b} \pm 73.36$	$2299.73^{b} \pm 81.61$	$2489.50^{b} \pm 120.36$	50.040**

Mean bearing the same superscript in the same row do not differ significantly.

** Highly significant (P<0.05)

TABLE – 9

Haematological values of mastitis cows (Mean±S.E)

Sl.No.	Parameters	Control (n=20)	*E.coli* (n=119)	*S.aureus* (n=104)	*MRSA* (n=12)	F value
1	HB (g/dL)	9.64^{b} ±0.16	7.99^{a} ± 0.13	8.13^{a} ±0.12	8.42^{a} ±0.23	12.054 **
2	PCV (%)	30.73^{b} ±0.71	24.13^{a} ± 0.40	25.04^{a} ±0.37	25.70^{a} ±1.05	19.171**
3	Total erythrocyte count(TEC) (10^{6}/cmm)	5.95^{b} ±0.15	5.38^{a} ± 0.12	5.32^{a} ±0.07	5.54^{a} ± 0.19	2.865*
4	Total leukocyte count (TLC)	5815^{a} ±176.8	10552^{b} ±471.9	9895^{b} ± 404.7	10087^{b} ±474.5	8.754**
5	Neutrophil	1860.80^{a} ± 95.31	6678.43^{b} ± 209.67	6256.74^{b} ± 324.74	6408.24^{b} ± 577.43	2.394**
6	Lymphocyte	3954.20^{a} ± 100.92	3873.57^{b} ± 342.55	3639.26^{b} ± 260.78	3678.76^{b} ± 478.22	2.507 **

Mean bearing the same superscript in the same row do not differ significantly.

* Significant (P<0.05)

** Highly significant (P<0.01)

4.4 Leukogram

4.4.1 Total leukocytes

The mean ± S.E values of total leukocytes (number/cumm) in control group, *E.coli, S.aureus* and MRSA groups were 5815±176.8, 10552±471.9, 9895 ± 404.7 and 10087±474.5 respectively (Table 9).

A highly significant increase in the values of total leukocytes was observed between control and clinical mastitis groups. However, there was no significant difference in total leukocytes between clinical groups.

4.4.2 Neutrophils

The mean ± S.E values of neutrophil (in percentage) of control group, *E.coli, S.aureus* and MRSA groups were 1860.80 ± 95.31, 6678.43 ± 209.67, 6256.74 ± 324.74 and 6408.24 ± 577.43 respectively(Table 9).

A highly significant increase in the values of neutrophils was observed between control and clinical mastitis groups. However, there was no significant difference in neutrophil between clinical groups.

4.4.3 Lymphocytes

The mean ± S.E values of lymphocyte (in percentage) of control group, *E.coli, S.aureus* and MRSA groups were 3954.20 ± 100.92, 3873.57 ± 342.55, 3639.26± 260.78 and 3678.76 ± 478.22 respectively (Table 9).

A highly significant decrease in the values of lymphocytes was observed between control and clinical mastitis groups. However, there was no significant difference in lymphocyte between clinical groups.

4.5 Serum Biochemical Profile

4.5.1 Serum Aspartate Transaminase (AST)

The mean ± S.E values of AST (g/dL) of control group, *E.coli, S.aureus* and MRSA groups were 65.05±2.51, 89.65±5.46, 95.38±4.29 and 94.50±6.44 respectively (Table 10).

TABLE 10

Serum biochemical values in mastitis cows (Mean±S.E)

Sl.No.	Parameters	Control (n=20)	*E.coli* (n=119)	*S.aureus* (n=104)	*MRSA* (n=12)	F value
1	SAP (U/L)	86.90 ^{a} ± 4.02	168.50 ^{b} ±5.34	154.90 ^{b} ±13.2	166.12 ^{b} ±14.45	4.555 **
2	AST (U/L)	65.05 ^{a} ±2.51	89.65 ^{b} ±5.46	95.38 ^{b} ±4.29	94.50 ^{b} ± 6.44	3.048*
3	Total Protein (g/dL)	6.98 ±0.05	6.82 ±0.04	6.92 ±0.04	6.76 ±0.03	1.867 ^{NS}
4	Albumin (g/dL)	3.66 ^{b} ±0.05	2.78 ^{a} ±0.02	2.75 ^{a} ±0.02	2.76 ^{a} ±0.04	86.646 **
5	Globulin (g/dL)	3.32 ^{a} ±0.06	4.03 ^{b} ±0.04	4.17 ^{b} ±0.04	4.00 ^{b} ±0.06	29.093**

Mean bearing the same superscript in the same row do not differ significantly.

* Significant (P<0.05)

** Highly significant (P<0.01)

NS – Non significant (P>0.05)

A significant increase in the AST values was observed in clinical groups when compared to control group. However, there was no significant difference in AST values between clinical groups.

4.5.2 Serum Alkaline phosphatase (SAP)

The mean ± S.E values of SAP (g/dL) of control group, *E.coli, S.aureus* and MRSA groups were 86.90±4.02, 168.50±5.34, 154.90±13.20 and 166.12+14.45 respectively (Table 10).

A highly significant increase in the SAP values was observed in the mastitis groups when compared to control group. However, there was no significant difference in SAP values between clinical groups.

4.5.3 Serum total protein, serum albumin and serum globulin

The mean ± S.E values of total protein (g/dL) of control group, *E.coli, S.aureus* and MRSA groups were 6.98±0.05, 6.82±0.04, 6.92±0.04 and 6.76±0.03 respectively (Table 10).

No significant difference in the total protein values was observed between control and clinical mastitis groups and among the clinical groups.

The mean ± S.E values of serum albumin (g/dL) of control group, *E.coli, S.aureus* and MRSA groups were 3.66±0.05, 2.78±0.02, 2.75±0.02 and 2.76± 0.04 respectively (Table 10).

A highly significant reduction in the serum albumin values was observed between control and clinical mastitis groups. However, there was no significant difference in serum albumin values between clinical groups.

The mean ± S.E values of serum globulin (g/dL) of control group, *E.coli, S.aureus* and MRSA groups were 3.32±0.06, 4.03±0.04, 4.17±+0.04 and 4.00 ± 0.06 respectively (Table 10).

A highly significant elevation in the serum globulin values was observed between control and clinical groups. However, there was no significant difference in serum globulin values between clinical groups.

4.5.4 Comparison of stage of lactation, SAP and AST

The mean ± S.E values of SAP and AST values are presented in Table 11.

The mean ± S.E values of SAP (U/L) for *E. coli* in early, mid and late lactation were 135.38 ± 46.50, 100.32 ± 43.34, 104.71 ± 36.20 respectively.

The mean ± S.E values of SAP (U/L) for *S. aureus* in early and mid lactation were 147.52 ± 13.48 and 126.38 ± 10.11 respectively.

The mean ± S.E values of SAP (U/L) for MRSA in early and mid lactation were 150.56 ± 9.45 and 87.00 ± 11.00 respectively.

The mean ± S.E values of AST (U/L) for *E. coli* in early, mid and late lactation were 88.89 ± 5.20, 61.58 ± 4.21 and 67.40 ± 9.21 respectively.

The mean ± S.E values of AST (U/L) for *S. aureus* in early, mid and late lactation were 100.39 ± 5.43, 66.83 ± 2.23 and 75.50 ± 5.18 respectively.

The mean ± S.E values of AST (U/L) for MRSA in early and mid lactation were 115.56 ± 9.78 and 83.00 ± 5.00 respectively.

A highly significant increase in SAP values was observed in early lactation when compared to mid and late lactation in *E.coli* group and mid lactation in *S.aureus* and MRSA group. However, there was no significant difference between mid and late lactation in *E.coli* group.

A highly significant increase in AST values was observed in early lactation when compared to mid and late lactation in *E.coli* and *S.aureus* groups and mid lactation in MRSA group. However, there was no significant difference between mid and late lactation in *E.coli* and *S.aureus* group.

TABLE 11

Comparison of stage of lactation, SAP and AST in mastitis cows

Stage of lactation	SAP			AST		
	E.coli	***S.aureus***	**MRSA**	***E.coli***	***S.aureus***	**MRSA**
Early 1-3 months	135.38 [b] ± 46.50	147.52 [b] ± 13.48	150.56 [b] ± 9.45	88.89 [b] ± 5.20	100.39 [b] ± 5.43	115.00 [b] ± 9.78
Mid 4-6 months	100.32 [a] ± 43.34	126.38 [a] ± 10.11	87.00 [a] ± 11.00	61.58 [a] ± 4.21	66.83 [a] ± 2.23	83.00 [a] ± 5.00
Late 7-9 months	104.71 [a] ± 36.20	-	-	67.40 [a] ± 9.21	75.50 [a] ± 5.18	-
'F' value	0.700**	0.923**	8.910**	2.564**	8.093 **	1.803**

** Highly significant (P<0.01)

4.6 *IN VITRO* ANTIBIOTIC SENSITIVITY TEST

The antibiogram of the isolated bacterial pathogens are depicted in Table 12. *E.coli* showed more sensitivity to enrofloxacin (79 per cent) followed by amoxicillin and sulbactam (74 per cent), gentamicin (73.1 per cent) and ceftriaxone (69 per cent). The isolates had highest resistance to penicillin (63 per cent) followed by amoxicillin (52.1 per cent), oxytetracycline (47.9 per cent) and methicillin (45.4 per cent) (Plate 15).

Most of the *E.coli* isolates (86.55) per cent were found to be resistant *i.e.,* resistant to one or two antibacterials used in this study. Few *E.coli* isolates (13.45 per cent) were found to be multi-drug resistant *i.e.,* resistant to more than three antibacterials used in the study (Fig - 3).

S.aureus isolates were most sensitive to enrofloxacin (79.8 per cent) followed by gentamicin (71.2 per cent), amoxicillin and sulbactam (69.2 per cent) and ceftriaxone (69.2 per cent). The isolates showed highest resistance to penicillin (63.5 per cent) followed by amoxicillin (61.5 per cent), oxytetracycline (49 per cent) and methicillin (52.9 per cent).

Most of the *S.aure*us isolates 80.77 per cent were found to be resistant (plate 16) *i.e.,* resistant to one or two antibacterials used in the study. Few *S.aureus* isolates 19.23 per cent were found to be multi-drug resistant i.e., resistant to more than three antibacterials used in the study (Fig -3).

MRSA showed maximum sensitivity to enrofloxacin (75 per cent) followed by amoxicillin and sulbactam (75 per cent), gentamicin (66.7 per cent) and ceftriaxone (58.3 per cent). The isolates showed highest resistance to methicillin (100 per cent), amoxicillin (91.7 per cent), followed by penicillin (83.3 per cent) and oxytetracycline (41.7 per cent) (Plate 17).

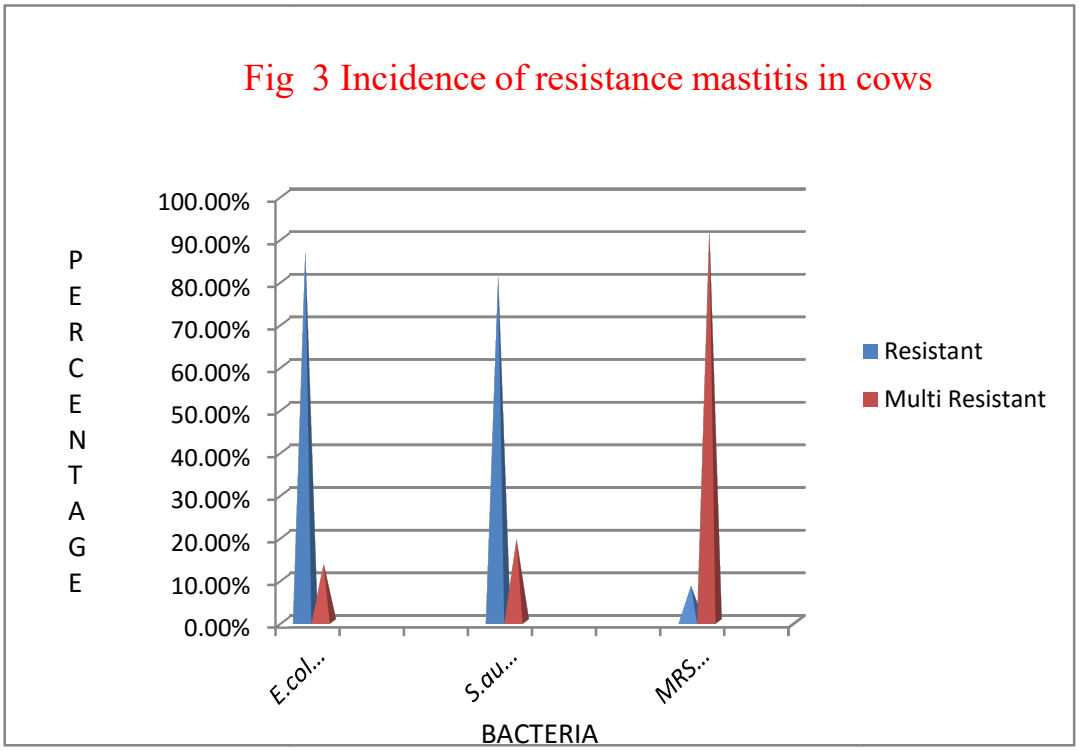
Few MRSA isolates 8.33 per cent were found to be resistant *i.e.,* resistant to one or two antibacterials used in the study. Most of the MRSA 91.67 per cent isolates were found to be multi-drug resistant i.e., resistant to more than three antibacterials used in the study (Fig -3).

TABLE 12

***In vitro* antibiogram of isolated pathogens in cows**

	***E.coli* (n=119)**		***S.aureus* (n=104)**		***MRSA* (n=12)**	
	Sensitive %	**Resistance %**	**Sensitive %**	**Resistance %**	**Sensitive %**	**Resistance %**
Enrofloxacin	79	21	79.8	20.2	75	25
Amoxicillin + Sulbactam	74	26	69.2	30.8	75	25
Gentamicin	73.1	26.9	71.2	28.8	66.7	33.3
Ceftriaxone	69	31	69.2	30.8	58.3	41.7
Oxytetracycline	52.1	47.9	51	49	58.3	41.7
Methicillin	54.6	45.4	47.1	52.9	-	100
Amoxicillin	47.9	52.1	38.5	61.5	8.3	91.7
Penicillin G	37	63	36.5	63.5	16.7	83.3

Fig 3 Incidence of resistance mastitis in cows



4.7 Biochemical Tests

4.7.1 Catalase test and coagulase test

All the Staphylococcal isolates were found to be positive for catalase test (Plate 8) and coagulase test (Plate 9).

4.8 Testing of β lactamase production and methicillin resistance

Among *S.aureus* organism, 64 out of 116 (55.17 per cent) showed positive for nitrocefin test (Plate 10).

In MRSA alert kit test (Plate 11, 12), 12 out of 116 *S.aureus (*10.35 per cent) showed positive for methicillin resistant *S. aureus*.

4.9 Minimum Inhibitory Concentration (MIC)

4.9.1 *E.coli*

Minimum inhibitory concentration of common antibiotics against *E.coli* are presented in Table 13.

Minimum inhibitory concentrations in micrograms per milliliters that inhibited 50 per cent (MIC_{50}) of the *E.coli* strains tested were: 1.95, 1.95, 1.95, 3.9, 31.25, 62.5, 62.5 and 31.25 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively (Plate 16).

Minimum inhibitory concentrations in micrograms per milliliters that inhibit 90 per cent (MIC_{90}) of the *E.coli* strains tested were: 62.5, 31.25, 62.5, 62.5, 125, 125, 125 and 62.5 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively.

4.9.2 *S.aureus*

Minimum inhibitory concentration of common antibiotics against *S.aureus* are presented in Table 14.

Minimum inhibitory concentrations in micrograms per milliliters that inhibited 50 per cent (MIC $_{50}$) of the *S.aureus* strains tested were: 3.9, 3.9, 3.9, 3.9, 31.25, 62.5, 62.5 and 31.25 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively (Plate 17)

Minimum inhibitory concentrations in micrograms per milliliters that inhibited 90 per cent (MIC $_{90}$) of the *S.aureus* strains tested were: 31.25, 31.25, 62.5, 62.5, 62.5, 125, 125 and 125 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively.

4.9.3 MRSA

Minimum inhibitory concentration of common antibiotics against MRSA are presented in Table 15.

Minimum inhibitory concentrations in micrograms per milliliters that inhibited 50 per cent (MIC $_{50}$) of the MRSA strains tested were: 15.62, 31.25, 31.25, 62.5, 62.5, 62.5, 62.5 and 125 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively (Plate 18, 19).

Minimum inhibitory concentrations in micrograms per milliliters that inhibited 90 per cent (MIC $_{90}$) of the MRSA strains tested were: 62.5, 62.5, 125, 62.5, 125, 125, 125 and 125 for enrofloxacin, amoxicillin+sulbactam, gentamicin, ceftriaxone, oxytetracycline, amoxicillin, pencillin G and oxacillin respectively.

4.10 Polymerase Chain Reaction (PCR)

4.10.1 Confirmation of the mastitis bacteria by targeting specific genes for different strains

In the present study, a total of 235 isolates were screened for PCR based detection targeting the specific genes for different strains as presented in Table 16.

Out of 235 milk samples, the specific target gene 16s-23s r RNA (*E.coli*) of 662 bp could be amplified from 119 isolates with a percentage of positivity as 50.64(119/235) (Plate 20).

Table 13

Minimum inhibitory concentrations (MIC) of drugs against *E.coli* (n=119) isolated from udder of cows

	MIC (µg/ml) (n=119)											
	125	**62.5**	**31.25**	**15.62**	**7.8**	**3.9**	**1.95**	**0.97**	**0.48**	**MIC Range**	**MIC $_{50}$**	**MIC $_{90}$**
Gentamicin	9.2	11	2.5	**3.4**	11.7	5.9	16	27.7	12.6	0.48-125	1.95	62.5
Oxytetracycline	17.7	25.2	15.1	5.9	**16**	6.7	8.4	5	-	0.97-125	31.25	125
Ceftriaxone	4.2	9.2	**20.2**	3.4	12.6	1.7	13.4	20.2	15.1	0.48-125	3.9	62.5
Enrofloxacin	8.4	3.4	12.6	4.2	11.7	3.4	**21.8**	18.5	16	0.48-125	1.95	62.5
Amoxicillin	22.7	30.3	6.7	**23.5**	6.7	2.5	7.6	-	-	1.95-125	62.5	125
Pencillin G	19.3	41.2	8.4	**12.6**	5.9	4.2	7.6	0.8	-	0.95-125	62.5	125
Amoxicillin + Sulbactam	-	0.8	31.1	**0.8**	8.4	8.4	31.1	12.6	6.7	0.48-125	1.95	31.25
Oxacillin	6.7	33.6	16	**17.7**	5	14.3	6.7	-	-	1.95-125	31.25	62.5

Numbers indicate percentage of isolates. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range and vertical line indicate clinical breakpoints, with the value to the right of the line being susceptible and those to the left being resistant.

MIC_{50} and MIC_{90} values are concentrations at which ≥ 50 % and ≥ 90 % of isolates are inhibited respectively

Table 14
Minimum inhibitory concentrations (MIC) of drugs against *S.aureus* (n=104) isolated from udder of cows

	MIC (µg/ml) (n=104)											
	125	**62.5**	**31.25**	**15.62**	**7.8**	**3.9**	**1.95**	**0.97**	**0.48**	**MIC Range**	**MIC $_{50}$**	**MIC $_{90}$**
Gentamicin	7.7	13.5	3.8	**2.9**	15.4	11.5	10.5	13.5	21.2	0.48-125	3.9	62.5
Oxytetracycline	19.2	24	12.6	**1.9**	13.5	11.5	7.7	9.6	-	0.97-125	31.25	62.5
Ceftriaxone	11.5	13.5	6.7	2.9	11.5	**9.6**	9.6	23.1	11.5	0.48-125	3.9	62.5
Enrofloxacin	8.6	9.6	5.8	3.8	22.1	**8.6**	7.7	12.6	21.2	0.48-125	3.9	31.25
Amoxicillin	22.1	34.6	7.7	5.8	11.5	6.7	11.5	-	-	1.95-125	62.5	125
Pencillin G	21.2	44.2	2.9	13.5	8.6	4.8	2.9	1.9	-	0.97-125	62.5	125
Amoxicillin + Sulbactam	-	4.8	32.7	2.9	**8.6**	14.4	14.4	18.3	3.8	0.48-62.5	3.9	31.25
Oxacillin	14.4	24	14.4	14.4	11.5	**17.3**	1.9	-	-	1.95-125	31.25	125

Numbers indicate percentage of isolates. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range and vertical line indicate clinical breakpoints, with the value to the right of the line being susceptible and those to the left being resistant.

MIC_{50} and MIC_{90} values are concentrations at which ≥ 50 % and ≥ 90 % of isolates are inhibited respectively.

Table 15

Minimum inhibitory concentrations (MIC) of drugs against MRSA (n=12) isolated from udder of cows

	MIC (µg/ml) (n=12)											
	125	**62.5**	**31.25**	**15.62**	**7.8**	**3.9**	**1.95**	**0.97**	**0.48**	**MIC Range**	**MIC $_{50}$**	**MIC $_{90}$**
Gentamicin	33.3	25	16.7	-	**8.3**	8.3	8.3	-	-	1.95-125	31.25	125
Oxytetracycline	25	41.7	16.7	-	-	8.3	8.3	-	-	1.95-125	62.25	125
Ceftriaxone	16.7	33.3	33.3	-	8.3	**8.3**	-	-	-	3.9-125	62.25	62.5
Enrofloxacin	-	16.7	16.7	16.7	8.3	25	8.3	-	**8.3**	0.48-62.5	15.62	62.5
Amoxicillin	33.3	50	16.7	-	-	-	-	-	-	31.25-125	62.5	125
Pencillin G	33.3	41.7	25	-	-	-	-	-	-	31.25-125	62.5	125
Amoxicillin + Sulbactam	8.3	25	16.7	-	-	**33.3**	16.7	-	-	1.95-125	31.25	62.5
Oxacillin	66.7	33.3	-	-	-	-	-	-	-	62.5-125	125	125

Numbers indicate percentage of isolates. Numbers in bold indicate percentage of isolates with MIC values greater than the highest concentration in the dilution range and vertical line indicate clinical breakpoints, with the value to the right of the line being susceptible and those to the left being resistant.

MIC_{50} and MIC_{90} values are concentrations at which ≥ 50 % and ≥ 90 % of isolates are inhibited respectively.

TABLE 16

Confirmation of the mastitis bacteria by targeting specific genes for different strains

Sl.No.	No. of Isolates Screened	Strain	Gene targeted							
			16 s-23 s r RNA (Riffon *et al.*, 2001)		*S.aureus*					
					16 s-23 s r RNA (Pradhan *et al.*, 2011)		*MRSA* (Soares *et al.*, 2012)			
							mec A gene		bla Z gene	
			Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
			662 bp	-	229 bp	-	513 bp	-	639 bp	-
1	235	*E.coli*	119	-	-	116	-	-	-	-
2	235	*S.aureus*	-	116	104	131	-	-	-	-
3	235	*MRSA*	-	116	-	-	12	233	12	233
4	235		-	116	-	-	12	233	12	233
	Percentage of Positive		50.64		44.26 *		10.34**		10.34**	

The prevalence of E.coli and Staphyloccus strains in the mastitis cows were almost equal

* Percentage of positive total *S.aureus*

** Percentage of positive MRSA from the *S.aureus* positive strains

Out of 235 milk samples, the specific target gene 16s-23s r RNA (*S.aureus*) of 229 bp could be amplified from 104 isolates with a percentage of positivity as 89.66 (104/116) (Plate 21).

Out of 235 milk samples, the specific target gene for both *mec*A (MRSA) of 513 bp & *blaZ*(MRSA) of 639 bp could be amplified from 12 isolates with a percentage of positivity as 10.34 (12/116) (Plate 22 and 23).

4.10.2 Sequencing of Polymerase Chain Reaction products

The details of isolates chosen / species/ genes for sequencing are shown in Figures 4, 5, 6 and 7. The sequence data obtained was subjected to BLAST analysis with NCBI GenBank data base to confirm the identity of the sequence data generated.

4.10.3 Screening of milk samples by Multiplex PCR

In this study, for multiplex PCR standardization, the 16S to 23S rRNA spacer region was targeted to detect *E.coli, S. aureus* and MRSA in a single tube. In the present study, a total of 235 clinical mastitis milk samples were screened by multiplex PCR. PCR detected the target DNA in 235 milk samples that were positive by culture.

Of the 235 positive milk samples, multiplex PCR resulted in single specific bands of *S. aureus* (370 bp), *E.coli* (662bp) and MRSA (*mec*A 513 bp and *blaZ* 639 bp) in 104, 119 and 12 samples respectively and two bands of *S. aureus* (370 bp) and *E.coli* (662 bp) (Plate 24), *S.aureus (370 bp)* and *MRSA* (513 and 639 bp for *mec*A and *blaZ* gene) in 2 and 12 samples respectively. Positive for three bands (Plate 25) of *E.coli* (662 bp), *S.aureus* (370 bp) and MRSA (*mec*A 513 bp and *blaZ* 639 bp) in 2 samples.

4.11 Comparison of different test for the identification of methicillin resistance in *S.aureus* mastitis cows

Comaparison of different test for the identification of methicillin resistance in *S.aureus* mastitis cows are given in Table 17

Out of 116 *S.aureus* organism, 62 isolates showed methicillin resistance (52.9 per cent) by ABST, 64 isolates were positive for nitrocefin test (55.17 per cent) and 12 were positive (10.35 per cent) for methicillin resistance by MRSA alert kit test and

TABLE 17

Comparison of different tests in the identification of methicillin resistance in *S.aureus* mastitis cows

	ABST (n=116)	**Nitrocefin test (n=116)**	**MRSA Alert kit (n=116)**	**PCR (*mec*A and *bla*Z gene) (n=116)**
Methicillin resistance	52.9 %	55.17 %	10.35 %	10.35 %

TABLE - 18

Post treatment CMT scores in mastitis cows

Pathogen	**Scores**	**Post treatment**
E.coli (n=119)	+ 2	4.2 %
	+ 3	25.21 %
S.aureus (n=104)	+ 2	4.8 %
	+ 3	26.9 %
MRSA (n=12)	+ 2	-
	+ 3	50 %

PCR target gene (*mec*A and *bla*Z) equally. Higher incidence of methicillin resisatnce was recorded by ABST and nitrocefin test. However, MRSA alert kit test and PCR target (*mec*A and *bla*Z) gene were found to be useful in the confirmation of methicillin resistance when compared to ABST and nitrrocefin test.

4.12 THERAPEUTIC EVALUATION

For the purpose of therapy, 235 cows with clinical mastitis were grouped into four groups based on results of in vitro antibiotic sensitivity test. They were subjected to antibiotic treatment trial with the following drugs Group 1.Amoxicillin+sulbactam, Group 2. Ceftriaxone, Group 3. Enrofloxacin and Group 4. Gentamicin. Therapeutic evaluation of different groups were done based on clinical signs, CMT, pH, EC and SCC.

4.12.1 Clinical signs

Complete remission of clinical signs was observed in 93.28 per cent in *E.coli*, 94.43 per cent in *S.aureus* and 94.43 per cent in MRSA.

In *E.coli* group, 74.1 per cent, 67.75 per cent, 76.67 per cent and 64.52 per cent of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively showed normal milk colour.

In *S.aureus* group, 65.25 per cent, 65.25 per cent, 72.43 per cent and 68.98 per cent of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively showed normal milk colour.

In MRSA group, 50 per cent, and 50 per cent of cases treated with amoxicillin+sulbactam and enrofloxacin respectively showed normal milk colour.

4.12.2 MILK PROFILE

4.12.2.1 California mastitis test (CMT)

The post treatment score of CMT 2+ was reduced to 4.2 per cent in *E.coli* and 4.8 per cent in *S.aureus* group. The score of CMT 3+ was reduced to 25.21 per cent in *E.coli,* 26.9 per cent in *S.aureus* and 50 per cent in MRSA groups respectively (Table 18).

4.12.2.2 pH of milk

The post treatment mean ± S.E values of milk pH in *E.coli, S.aureus* and MRSA groups are as given in Tables 19, 20 and 21**.**

In *E.coli* group, mean ± S.E values of milk pH in control group and amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 6.45± 0.05, 6.43±0.07, 6.48±0.07, 6.43 ±0.06 and 6.48±0.07 respectively.

In *E.coli* group, a highly significant decrease in post treatment milk pH values was observed in all treatment groups when compared to pre treatment group. Post treatment pH value of treatment groups showed lowering trend towards control group.

In *S.aureus* group, mean ± S.E values of milk pH in control group, amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 6.45±0.05, 6.50 ±0.06, 6.56±0.08, 6.53±0.08 and 6.53±0.07 respectively.

In *S.aureus* group, a highly significant decrease in post treatment milk pH values was observed in all treatment groups when compared to pre treatment values. Post treatment pH value of treatment groups showed lowering trend towards control group.

In MRSA group, mean ± S.E values of milk pH in control group, amoxicillin +sulbactam and enrofloxacin post treatment groups were 6.45 ±0.05, 6.83 ±0.10 and 6.50 ±0.18 respectively.

In MRSA group, a highly significant decrease in post treatment milk pH values was observed in all treatment groups when compared to pre treatment value. However, the post treatment pH value of enrofloxacin group was comparable to the control value. Whereas post treatment pH value of amoxicillin+sulbactam group was slightly above the control value.

TABLE 19

Comparison of post treatment values of pH, EC and SCC in *E.coli* mastitis among different groups of antibiotics

Sl.No.	Parameters	Control	Amoxicillin + sulbactam		Ceftriaxone		Enrofloxacin		Gentamicin		'F' value
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	
1	pH	6.45^{a} ± 0.05	7.61^{b} ±0.08	6.43^{a} ±0.07	7.59^{b} ±0.09	6.48^{a} ±0.07	7.60^{b} ±0.10	6.43^{a} ±0.06	7.70^{b} ±0.09	6.48^{a} ±0.07	56.976 **
2	Electrical conductivity (Units)	356.00^{b} ± 6.42	246.66^{c} ±2.72	334.00^{a} ±7.07	242.58^{c} ±3.65	320.32^{a} ±6.17	242.66^{c} ±3.45	330.74^{a} ±7.33	241.61^{c} ±3.53	321.25^{a} ±6.39	75.383**
3	SCC X 10^{3}	60.95^{a} ± 8.52	2038.22^{c} ±126.51	166.85^{b} ±23.71	2128.58^{c} ±166.16	201.16^{b} ±24.30	2216.53^{c} ±144.67	191.93^{b} ±22.09	2198.87^{c} ±146.30	199.64^{b} ±36.82	100.709**

Mean bearing the same superscript in the same row do not differ significantly.
** Highly significant (P<0.01)

TABLE 20

Comparison of post treatment values of pH, EC and SCC in *S.aureus* mastitis among different groups of antibiotics

Sl.No.	Parameters	Control	Amoxicillin + sulbactam		Ceftriaxone		Enrofloxacin		Gentamicin		'F' value
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	
1	pH	6.45^{a} ± 0.05	7.69^{b} ±0.10	6.50^{a} ±0.06	7.76^{b} ±0.13	6.56^{a} ±0.08	7.63^{b} ±0.04	6.53^{a} ±0.08	7.51^{b} ±0.09	6.53^{a} ±0.07	42.640**
2	Electrical conductivity (Units)	356.00^{c} ± 6.42	235.21^{a} ±3.71	322.41^{b} ±8.95	234.78^{a} ±3.96	319.56^{b} ±9.14	241.72^{a} ±3.94	322.06^{b} ±8.64	241.37^{a} ±4.31	291.73^{b} ±11.06	41.184**
3	SCC X 10^3	60.95^{a} ± 8.52	2316.21^{c} ±146.72	203.17^{b} ±24.33	2378.39^{c} ±222.62	223.44^{b} ±29.60	2346.48^{c} ±158.6	212.60^{b} ±32.44	2298.20^{c} ±126.41	218.27^{b} ±28.7	97.127**

Mean bearing the same superscript in the same row do not differ significantly.
** Highly significant (P<0.01)

TABLE 21

Comparison of post treatment values of pH, EC and SCC in *MRSA* mastitis among different groups of antibiotics

Sl.No.	Parameters	Control	Amoxicillin + sulbactam (n=6)		Enrofloxacin (n=6)		'F' value
			Pre	Post	Pre	Post	
1	pH	6.45^{a} ± 0.05	8.08^{c} ±0.08	6.83^{b} ±0.10	8.16^{c} ±0.10	6.50^{a} ±0.18	100.105**
2	Electrical conductivity (Units)	356.00^{b} ± 6.42	230.00^{a} ±3.65	247.34^{c} ±17.4	236.66^{a} ±2.10	352.66^{b} ±17.63	32.110**
3	SCC X 10^3	60.95^{a} ± 8.52	2394.50^{c} ±170.27	324.16^{e} ± 58.15	2434.16^{c} ±100.76	216.85^{d} ± 46.64	325.848**

Mean bearing the same superscript in the same row do not differ significantly.
** Highly significant (P<0.01)

4.12.2.3 Electrical conductivity

The post treatment mean ± S.E values of milk Electrical conductivity in *E.coli, S.aureus* and MRSA groups are as given in Tables 19, 20 and 21.

In *E.coli* group, mean ± S.E values of electrical conductivity in control group, amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 356.00 ± 6.42, 334.00 ±7.07, 320.32 ±6.17, 330.74±7.33 and 321.25±6.39 respectively.

In *E.coli* group, a highly significant increase in post treatment milk electrical conductivity values was observed in all treatment groups when compared to pre treatment values. However, there was no significant difference between different treatment groups in post treatment values. Post treatment electrical conductivity value of treatment groups showed increasing trend towards control group.

In *S.aureus* group, mean ± S.E values of electrical conductivity in control group, amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 356.00 ± 6.42, 322.41 ±8.95, 319.56±8.64, 322.06±8.64 and 291.73 ±11.06 respectively.

In *S.aureus* group, a highly significant increase in post treatment milk electrical conductivity values was observed in all treatment groups when compared to pre treatment values. However, there was no significant difference between different treatment groups in post treatment values. Post treatment electrical conductivity value of treatment groups showed increasing trend towards control group.

In MRSA group, mean ± S.E values of electrical conductivity in control group, amoxicillin +sulbactam and enrofloxacin post treatment groups were 356.00 ± 6.42, 247.34 ± 17.4 and 352.66 ±17.63 respectively.

In MRSA group, a highly significant increase in post treatment milk electrical conductivity values was observed in treatment groups when compared to pre treatment value. Post treatment electrical conductivity value of enrofloxacin group showed increasing trend when compared to control group. Even though significant

increase in post treatment electrical conductivity was observed in amoxicillin+sulbactam group, it did not reach the values in the control group.

4.12.2.4 Somatic cell count

The post treatment mean ± S.E values of milk SCC in *E.coli, S.aureus* and MRSA groups are as given in Tables 19, 20 and 21**.**

In *E.coli* group, mean ± S.E values of SCC in control group, amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 60.95 ± 8.52, 166.85 ±23.71, 201.16±24.30, 191.93 ±22.09 and 199.64±36.82 respectively.

In *E.coli* group, a highly significant decrease in post treatment milk SCC values was observed in all treatment groups when compared to pre treatment values. However, there was no significant difference between different treatment groups in post treatment value. Post treatment SCC value of treatment groups showed lowering trend towards control group.

In *S.aureus* group, mean ± S.E values of SCC in control group, amoxicillin +sulbactam, ceftriaxone, enrofloxacin and gentamicin post treatment groups were 60.95 ± 8.52, 203.17 ±24.33, 223.44±29.60, 212.60 ±32.44 and 218.27±28.7 respectively.

In *S.aureus* group, a highly significant decrease in post treatment milk SCC values was observed in all treatment groups when compared to pre treatment values. However, there was no significant difference between different treatment groups in post treatment values. Post treatment SCC value of treatment groups showed lowering trend towards control group.

In MRSA group, mean ± S.E values of SCC in control group, amoxicillin +sulbactam and enrofloxacin post treatment groups were 60.95 ±8.52, 324.16 ±58.15 and 216.85 ±46.64 respectively.

In MRSA group, a highly significant decrease in post treatment milk SCC values was observed in treatment groups when compared to pre treatment value. Post

treatment SCC value of enrofloxacin group showed lowering trend towards control group. Whereas post treatment SCC value of amoxicillin+sulbactam group was slightly above the control value.

4.13 Economic Impact of clinical mastitis

Economic impact of clinical mastitis in cows are depicted in Table.22.

The average milk yield loss (litres/day) in *E.coli, S.aureus* and *MRSA* groups were 4.56, 3.63 and 3.96 respectively. The average discarded milk yield (litres/day) in *E.coli, S.aureus* and *MRSA* groups were 5.62, 5.76 and 5.85 respectively. The production loss due to mastitis (Rs./day) in *E.coli, S.aureus* and *MRSA* groups were 254.5, 234.75 and 245.25 respectively. The cost of post treatment milk yield loss (Rs/ 15 days) for *E.coli, S.aureus* and *MRSA* groups were 1605.75, 1661.25 and 1716.75 respectively. The treatment cost (Rs./day) in *E.coli, S.aureus* and *MRSA* groups were 300, 300 and 300 respectively. The total economic loss (Rs./cow) in *E.coli, S.aureus* and *MRSA* groups were 5486.50, 5404.50 and 5556.75 respectively.

TABLE 22

Economic impact of mastitis cows

No.	Parameters	*E.coli*	*S.aureus*	*MRSA*
1	Average Milk yield loss / day (Litres)	4.56	3.63	3.96
2	Discarded milk / day (Litres)	5.62	5.76	5.85
3	Total milk yield loss (Litres/ day)	10.18	9.39	9.81
4	Production loss due to mastitis (Rs. / day)	254.5	234.75	245.25
5	Cost of milk yield loss for 7 days (Rs.)	1781.5	1643.25	1716.75
6	Post treatment milk yield loss / day (Litres)	4.28	4.43	4.64
7	Cost of post treatment milk yield loss for 15 days (Rs.)	1605	1661.25	1740
8	Treatment cost/ day (Rs.)	300	300	300
9	Total treatment cost / 7 days (Rs.)	2100	2100	2100
10	Total economic loss / cow (Rs.)	5486.50	5404.50	5556.75

CHAPTER V

DISCUSSION

Bovine mastitis is one of the most economically important diseases of dairy cattle resulting in a great deal of economic loss, mostly because of reduction in milk yield, decreased milk quality and higher production costs. Antimicrobial resistance (AMR) is potentially one of the reasons for treatment failures. Hence, antimicrobial susceptibility testing of udder pathogens is an important step in defining appropriate farm–level treatment protocols. Among the various antibiotic-resistant isolates, Methicillin resistant *Staphylococcus aureus* (MRSA) is a serious cause of concern in both human and animals (Turkyılmaz *et al*., 2010).

Hence, the present study was undertaken at Large Animal Clinic Medicine Unit of Madras Veterinary College Teaching Hospital and dairy farms in Coimbatore district with an attempt to study the prevalence of drug resistant mastitis, clinico-pathological changes and their pattern of antibiotic resistance in dairy cows. Comparative evaluation of different therapeutic protocols for clinical management of resistant mastitis and assessment of the economic impact of drug resistant mastitis are discussed in detail.

5.1 INCIDENCE OF MASTITIS

5.1.1 Overall incidence of mastitis

In the present study, incidence of clinical mastitis at Madras Veterinary College Teaching Hospital was 9.57 per cent, out of which 1.90 per cent was acute mastitis and the incidence in organized farm was 16.56 per cent, out of which 11.08 per cent was acute mastitis.

The present study is in partial agreement with De and Mukharjee (2009) who recorded prevalence of clinical mastitis at 15.2 per cent whereas Mohana sundhari, (2010) reported higher prevalence of 28.2 per cent of clinical mastitis in certain districts (Chennai, Salem, Dharmapuri and Krishnagiri) of Tamil Nadu.

In the present study, the incidence of drug resistant clinical mastitis at the Madras Veterinary College Teaching Hospital was comparatively lower than organized farms as well as studies at village level. The reasons may be attributed to the availability of large number of local veterinarians and treatment of cases by owners themselves.

5.1.2 Pathogen wise Incidence of clinical mastitis

Out of 401 clinical mastitis samples subjected to bacterial isolation, 184 (45.89 per cent) were positive for *E.coli*, 162 (40.4 per cent) were positive for *S.aureus*, 12 (2.99 per cent) were positive for MRSA, 14 (3.49 per cent) were positive for *Bacillus* spp. and 13(3.24 per cent) were positive for *Streptococcus* spp. and 16 (3.99 per cent) samples showed mixed infection. However, in 18 (4.49 per cent) cases no bacterial growth could be observed (Figure 1).

The prevalence of *E.coli* as a major pathogen along with *Streptococci* and *Staphylococci* has been reported by several workers (Schukken *et al.,* 1989; Ross *et al.,* 2001; Rajeev, 2006 and Botrel *et al.,* 2010). The prevalence reported by above said workers ranged from 13.2 to 24.13 per cent.

Busato *et al.* (2000) opined that the environment conditions were the major cause for the prevalence of the organisms causing mastitis.

Paape and Miller (1992) reported that lack of washing of hands, udder and teats before milking and hand milking as the cause for mastitis. Contamination of teat ends is a major predisposing risk factor in the development of environmental mastitis, due to the fact that environmental pathogens can survive and multiply in organic bedding materials and housing conditions that can influence teat contamination rates.

Zadoks and Fitzpatrick (2009) reported that control programs for contagious mastitis have been in place, resulting in a decrease in occurrence of *S.agalactiae* and *S.aureus* mastitis and an increase in the relative impact of *E.coli* mastitis.

Harmon (1994) reported that the high rate of isolation of *S. aureus* might be attributed to the fact that the principal reservoirs of *S. aureus* were the skin of the udder and milk of the infected gland. The high frequency of *Staphylococcal* mastitis was considered to be due to the existence of inadequate hygiene in the dairy industry,

poor animal health services and lack of proper attention to the health of the mammary gland.

Rajeev *et al*. (2009) reported that the *S.aureus* followed by *E.coli* were the most predominant pathogens causing both clinical and subclinical mastitis. The prevalence of *S. aureus* was 13.95 per cent and *E.coli* was 15.7 per cent. The above said studies are in partial agreement with the present study.

The differences in prevalence reports of mastitis in the present study and other reports could be attributed to difference in breeds of targeted cows, farm management practices, level of production and differences in study methods employed by the investigators.

5.1.3 Incidence of resistant mastitis

Out of 401 dairy cows, resistant mastitis was detected in 235 cows accounting to 56.1 per cent. In this study, the predominant resistant causative pathogen was *E.coli* (50.64 per cent) followed by *S.aureus* (44.25 per cent) and MRSA (5.11 per cent) (Table 1).

In the present study, no resistant mastitis pathogens were noticed in *Streptococcus* Spp. and *Bacillus* Spp. Antimicrobial resistance was most common among the Staphylococcal mastitis isolates with a much lower proportion of Streptococcal isolates exhibiting resistance (Call *et al.,* 2008).

Lehtolainen *et al.* (2003) reported that, 27 per cent of *E.coli* isolates were resistance to one or more tested antimicrobial agents. The antibiotic usage has directly contributed to an increased prevalence of resistance (Aarestrup, 1999; DANMAP, 2001). All antimicrobial use in the herd may affect the resistance of *E.coli* isolates by increasing the presence of these antimicrobial agents in the cow's environment. Findings of above said studies are in partial agreement with the present study.

Sori *et al.* (2011) reported that 52.4 per cent were resistant to 2 or > 2 antimicrobial agents against *S.aureus.*

S.aureus has developed multidrug resistance in many regions of the world (World Health Organization, 2000), although reported prevalence rates indicated that

wide variations existed regionally and even from herd to herd (Waage *et al.,* 2002). The high penicillin resistance amongst *S.aureus* was likely due to the wide use of intramammary preparations containing combinations and broad-spectrum antibiotics (Pitkala *et al.,* 2004).

Saini *et al.* (2012) reported that most studies have determined MIC values or resistance proportion estimates in (sub) clinical *S.aureus* (3.56 per cent) and *E. coli* (4.8 per cent) using broth microdilution test method.

In the present study, incidence of resistant *S.aureus* mastitis was higher which might be due to indiscriminate use of antibiotics and intramammary preparations and it is in agreement with the opinion of above authors.

In the present study, prevalence of MRSA accounted for 5.11 per cent. However, we found nearly 10.34 per cent of (12 out of 116) *S.aureus* strains to be MRSA. This was in agreement with Kumar *et al.,*(2010) who reported MRSA prevalence of 10 per cent by examining 128 *S.aureus* isolates from 280 animals of Karan Fries (Taurus × Zebu) with mastitis during a survey conducted between 2007 and 2008. The same research team (Kumar *et al.,* 2011) also reported MRSA prevalence of 13 per cent of *S.aureus* isolates (14/107) from cows with mastitis in a herd located in northwest India.

This high incidence in the present study might be due to indiscriminate use of antibiotics and consequent plasmid mediated antibiotic resistance and concurred with above authors.

Contrary to our findings, other countries have reported various rates of prevalence of MRSA in bovine milk: 1.4 per cent in Switzerland (Huber *et al.,* 2009), 1.5 per cent in Japan (Hata *et al.,* 2010) and 2.4 per cent in Korea (Moon *et al.,* 2007).

5.1.4 Breed wise Incidence

In the present study, incidence of *E.coli*, *S.aureus* and MRSA mastitis was high in Holstein Friesian cross breed followed by Jersey cross breed and non descript. Several authors reported that higher prevalence of mastitis in Holstein Friesian

(Washburn *et al*., 2002, Subedi *et al.,* 2003, Radostitis *et al.,* 2008 and Sharma and Maiti, 2010) (Table 4). These differences between breeds might be due to immune response to intra mammary infection between breeds (Sharma and Maiti, 2010).

5.1.5 Lactation wise incidence

In the present study, it was found that the animals in the third lactation were affected more and it was highly significant when compared to other lactation period animals (Table 3). Various authors also recorded higher incidence during third lactation (Shukla *et al*., 2005, Carlen *et al.*, 2005 and Sachin kumar, 2007).

Higher incidence in third lactation could be attributed to the negative energy balance with concurrent fatty liver syndrome and oxidative stress may be correlated to production performance of the animal.

5.1.6 Incidence in stage of lactation

In the present study the incidence of mastitis was higher in early stage of lactation when compared to mid and late lactation.

Radostits *et al.*(2000) suggested that, the mammary gland was more susceptible to new infection during the early and late dry period, which might be due to the absence of udder washing and teat dipping, which in turn may have increased the presence of potential pathogens on the skin of the teat.

This observation was in close agreement with Faldelmula *et al.* (2009) who reported that the highest incidence of mastitis (62.7 per cent) was in the first month of lactation. Various workers also recorded higher incidence rate in early stage of lactation (Premchand *et al.,* 1995, Sachin kumar, 2007, Corbett, 2009 and Mohana sundari, 2010).

Lairintluanga *et al.,* (2003) and Sharma *et al.,* (2011) opined that dairy cows seemed to have more oxidative stress and low antioxidants defence during early lactation and it could be due to stress of peak milk production during the early stage or just after parturition than in advanced pregnant cows.

Gowri (2010) recorded 11.5 per cent of mastitis in periparturient cows with moderate and severe fatty liver and further suggested that increase in NEFA concentration might be attributed to impairment of immune system in fatty liver cows.

5.1.7 Quarter wise prevalence

In the present study, incidence of mastitis was highest in hind quartets than fore quarters.

Various authors namely (viz.,) Kapur and Singh (1978), Joshi and Gokhale (2006) and Sharma *et al.* (2007) opined that dung, urine contamination and uterine discharges are the reason for more incidence of mastitis in the hind quarters.

5.2 Management and housing

In the present study, the incidence of resistant mastitis in both organized (42.13 per cent) and unorganized (57.87 per cent) dairy farms are similar and hence environmental contamination need not be the prime factor in causing clinical mastitis.

In organized farms all the cows raised on concrete floor, maintained on commercial feed, machine milking followed with regular udder washing and teat dipping were followed. In the present study, even with all these precautions high incidence of mastitis was recorded in organized farms. This might be due to negative energy balance, reduced immunity and oxidative stress may be correlated to production performance of the animal.

Fadlelmoula *et al.* (2007) and Olde Riekerink *et al.* (2008) recorded higher incidence in stall fed cows when compared to free ranging cows. This might be due to different management strategies used in each housing system. However, Getahun *et al.,*(2008) reported higher prevalence in cows living in poor housing system.

In unorganized farms, majority (90 per cent) of cows raised on mud floor, maintained on own feed, practiced hand milking for about 50 per cent cows and udder washing and teat dipping were not followed. This could lead to high incidence of clinical mastitis, since, stringent hygienic measures were not practised.

The incidence of mastitis is influenced by managerial and environmental factors, such as housing of cows, milking equipment, feeding regimen, hygienic

quality of feed and water, cleanliness of cows, implementation of preventive measures and general practices related to, for instance, drying-off (Elbers *et al.,*1998; Barkema *et al.,*1999; Peeler *et al.,*2000; Schreiner and Ruegg, 2003 and Nyman *et al.,* 2007).

5.3 CLINICAL SIGNS

In the present study, predominant clinical signs were painful, hot and swollen udder, reduced rumen motility and pyrexia in *E.coli, S.aureus* and MRSA mastitis. This is in agreement with the findings of various authors (Heyneman and Burvenich, 1992; Peeler *et al.,*2002; Pyorala, 2003 and Galdhar *et al.,* 2005).

5.4 DIAGNOSIS

5.4.1 Colour of milk

Predominant milk colour changes like dirty white, yellowish milk and milk with flakes were observed in this study. These findings concurred with the observations of several workers (Peeler *et al.,* 2002; Pyorala, 2003 and Galdhar *et al.,* 2005).

5.4.2 California mastitis test

CMT scores in cows affected with resistant mastitis ranged from +2 to +3 while major percentage of cows had +3 scores. The present observation is in accordance with Doxey *et al*., (1983) and Karthikeyan, (2003) who reported high CMT score in animals with clinical mastitis.

5.4.3 pH of milk

The mean± S.E pH values of milk of control animals in this study were in agreement with Vijayakumar (2003) and Sachin Kumar (2007). Vijayakumar (2003) who reported that the normal pH of milk was 6.40 and isotonic with blood plasma.

The mean ± S.E, pH values of milk in clinical cases were highly significant when compared to control group (Table 9). This was in agreement with Harmon (1994), Vijayakumar (2003) and Bansal and Randhawa (2002) who opined that the

pH of the mastitis milk may be increased from a normal range of 6.6 to 6.9 or higher because of movement of blood components into milk. The elevation of milk pH might be due to leakage of sodium and chloride and blood bicarbonate into milk through damaged epithelium of mammary gland to maintain isotonicity and the milk becomes alkaline (Batra *et al.,* 1990; Bansal and Randhawa, 2002; Galdhar *et al.,* 2005 and Blowey, 2010).

5.4.4 Electrical conductivity of milk

A highly significant decrease in the electrical conductivity were observed between control and clinical mastitis group.

The observed values were in agreement with Seguya and Mansell (2000) who stated that electrical resistance of milk from quarters infected with major pathogens (256 ± 46 units), minor pathogens (313 ± 31 units) and uninfected quarters (301 ± 36 units) and the mean electrical resistance of infected is significantly lower than that of uninfected quarters.

Hamann and Zecconi (1998) suggested that when the mammary epithelium was damaged in mastitis the electrical conductivity of the milk changed.

In the present study, the reduction of electrical resistance in clinical groups observed could be due to the altered concentration of Na+, K+ and Cl- in mastitis milk. This is in agreement with the findings of various authors (Kitchen, 1981; Hamann and Zecconi, 1998 and Seguya and Mansell, 2000).

5.4.5 Somatic cell count

Highly significant increase in milk SCC in clinical group when compared to control group was seen in the present study, which might be due to inflammation of udder that leads to high SCC in milk.

Dohoo and Meek (1982) also reported that *Staphylococcus* Spp., *Streptococcus* Spp. and *Coliform* organism were the major pathogens that caused greater increase in SCC in milk.

Pilla *et al*., (2012) stated that the somatic cell count (SCC) in the MRSA infected quarter fluctuated between 300 and 6000 cells / µl.

The result of present study concurred with the above workers.

5.5 HAEMATOLOGY

5.5.1 Haemogram

In the present study, a highly significant reduction in Hb, PCV and TEC values were recorded in the clinical groups when compared to control group.

This finding was in agreement with Cebra *et al*. (1996), Karthikeyan (2003) and Zaki *et al.* (2008) who observed a significant reduction in Hb, PCV and TEC levels in animals with clinical mastitis.

In severe clinical mastitis there may be marked changes in the leukocyte count and packed cell volume because of the effects of severe infection and toxaemia (Sischo *et al.,* 1997).

5.5.2 Total leukocytes Count

In the present study, a highly significant increase in TLC value of clinical groups were observed when compared to control group.

Elevated TLC levels in the present study, might be attributed to the stressful effect of mastitis infection which might force great number of white blood cells to build up natural resistance and leukocytosis takes place under the increased adrenal cortex function promoted by adrenocorticotropic hormone.

This finding is in agreement with elevated total leukocyte count observed by Cebra *et al.,* (1996) and Zaki *et al.,*(2008).

5.5.3 Neutrophils

In the present study, a highly significant increase in neutrophil count was observed in clinical groups when compared to control group. This might be due to acute stage of bacterial infections causing neutrophilia.

The present observation was in accordance with Smith et al., (2001) who opined that the neutrophils are the first line of defence mechanism in bacterial infections and thus neutrophilia in mastitis.

5.5.4 Lymphocytes

In the present study, reduction in lymphocyte count was highly significant in the clinical groups when compared to control group.

Ostensson (1993) stated that lymphocytes were the predominant cell type infiltrating infected mammary tissues. Smith *et al*.(2001) who reported that gram negative septicaemia and septic mastitis were the main cause of lymphopenia.

The decrease in lymphocyte value in this study could be due to gram negative septicaemia and septic mastitis and agree with the above authors.

5.6 SERUM BIOCHEMICAL PROFILE

5.6.1 Serum aspartate transaminase

In the present study, a significant increase in the AST values is observed in clinical groups compared to control group. The present study was in agreement with Lotthammer *et al.* (1988) and Jovanovic *et al.* (1990) who reported the elevated AST levels in clinical mastitis.

Elevation in AST values might be attributed to negative energy balance and concurrent fatty liver syndrome (Bogin *et al.,*1988 and Radostitis *et al*., 2000). Highly significant increase in AST values may be attributed to stressful conditions in mastitis (Karthikeyan, 2003; Zaki *et al.,* 2008 and Moyes *et al.,* 2009).

The increase in AST levels in the present study might be due to the negative energy balance and the resultant fatty liver. The finding of increased AST levels in early lactation in the present study also suggest the same, and it is in agreement with the opinions of above workers.

5.6.2 Serum alkaline phosphatase

A highly significant increase in the SAP values are observed in the clinical groups compared to control group.

Wada *et al.* (2001) reported that serum alkaline phosphatase value was significantly higher in gram negative mastitis. Vangroenweghe (2004) suggested that increased serum alkaline phosphatase levels in clinical mastitis indicated the role of this enzyme in the pathogenesis of the disease

The increased SAP value may be due to fatty liver or may be due to the udder inflammation as reported by earlier workers.

5.6.3 Serum total protein, serum albumin and serum globulin

No significant difference in the total protein values between control and clinical mastitis groups were observed. However, a highly significant decrease in the serum albumin values and highly significant increase in the serum globulin values were recorded in all clinical groups when compared to control group.

Several authors have observed that lower serum concentrations of albumin in cows with mastitis compared to healthy cows (Katholm *et al.,* 1992 and Risvani *et al.,*1999).

Murray *et al.* (2001) reported that albumin concentration decreased in acute inflammatory conditions. The report concurs with the findings of the present study.

Increased globulin levels in clinical mastitis might be attributed to the activation of the immune response following infection of the mammary gland. This was in concurrence with Mantson and Allen, (1981), Tsenkova *et al.,* (2001) and Matei *et al.,* (2010).

Pandey *et al.*(2005) reported that immunoglobulin plays an important role in host immunity and inflammation and there is a correlation between total serum protein (albumin and globulins) and somatic cells count in milk.

Total protein, albumin and globulin are altered in mastitis milk (Seleim *et al.,* 2002 and Karthikeyan, 2003).

Hyperglobulinemia observed in the present study is in agreement with the observations made by the above workers.

5.7. Comparison of stage of lactation, AST and SAP

In the present study, a highly significant increase in SAP and AST values were recorded in early lactation when compared to mid and late lactation in all clinical groups.

The elevation in AST and SAP levels in the present study might be attributed to negative energy balance and concurrent fatty liver syndrome (Bogin *et al.,* 1988 and Radostits *et al.,* 2000). The finding of increased levels of AST and SAP in the early lactation in the present study also suggest the same, and it is in agreement with the opinion of the above authors.

5.8 *IN VITRO* ANTIBIOTIC SENSITIVITY TEST

E.coli showed more sensitivity to enrofloxacin (79 per cent) followed by amoxicillin and sulbactam (74 per cent), gentamicin (73.1 per cent) and ceftriaxone (69 per cent). The isolates had highest resistance to penicillin (63 per cent) followed by amoxicillin (52.1 per cent), oxytetracycline (47.9 per cent) and methicillin (45.4 per cent).

Lairintlunage *et al.*(2003) and Karthikeyan (2003) who reported that gram negative pathogens were found to be more sensitive to enrofloxacin (100 per cent) followed by ciprofloxacin and gentamicin .

Lehtolainen *et al.* (2003) recorded that 27 per cent of *E.coli* isolates were resistant to one or more antimicrobial agents. Among resistant isolates highest resistance was against tetracycline, Dihydrostreptomycin (DHS) and ampicillin and 41 per cent were multiresistant. Multiresistant bacteria also tend to maintain their resistance to a particular antimicrobial even when that antimicrobial is absent from the environment if the other antimicrobials to which the resistance is linked are still present (Levy, 1992; Prescott, 2000; Galland *et al*., 2001).

The present observation was in agreement with Lairintluanga *et al.* (2003) and karthikeyan (2003) who reported that gram negative pathogens were more sensitive to enrofloxacin and gentamicin and less sensitive to ampicillin and penicillin.

In the present study, *S.aureus* isolates were most sensitive to enrofloxacin (79.8 per cent) followed by gentamicin (71.2 per cent), amoxicillin and sulbactam (69.2 per cent) and ceftriaxone (69.2 per cent). The isolates showed highest resistance to penicillin (63.5 per cent) followed by amoxicillin (61.5 per cent), oxytetracycline (49 per cent) and methicillin (30.8 per cent).

The resistance of *S.aureus* to penicillin and ampicillin may be attributed to the production of betalactamase, an enzyme that inactivates penicillin and closely related antibiotics (Green and Bradely, 2004) and wide use of intramammary preparations containing combinations and broad-spectrum antibiotics (Pitkala *et al.,* 2004). Gentilini, (2000) and Edward *et al.,* (2002) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials.

S.aureus has developed multidrug resistance in many regions of the world (World Health Organization 2002). Although reported prevalence rate indicates that wide variation regionally and even from herd to herd (Waage *et al*., 2002).

The present observation was in agreement with Vijayalakshmi *et al.*(2001), Lairintluanga *et al.* (2003) and Karthikeyan (2003) who reported that *Staphylococcus* Spp. was highly sensitive to enrofloxacin, gentamicin and least sensitive to ampicillin.

In the present study, MRSA showed maximum sensitivity to enrofloxacin and amoxicillin and sulbactam (75 per cent) each, gentamicin (66.7 per cent) and ceftriaxone (58.3 per cent). The isolates showed highest resistance to methicillin (100 per cent), amoxicillin (91.7 per cent), followed by penicillin (83.3 per cent) and oxytetracycline (41.7 per cent).

Strains of *S.aureus* resistant to β-lactam antibiotics are known as methicillin-resistant *S.aureus* (MRSA). These strains in intra-mammary dissemination often produce incurable severe intra-herd infections (Moon *et al.,* 2007; Kumar *et al.,*2010). MRSA strains have been observed to be multi-drug resistant, such as

aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, etc., which are often used in the treatment of mastitis (Wang *et al.,* 2008 and Kumar *et al.,*2010).

Kumar *et al.*(2011) recorded a high prevalence of MRSA (13.1 per cent). The isolates were also highly resistant to streptomycin, oxytetracycline, gentamicin and chloramphenicol, pristinomycin and ciprofloxacin.

The present observation was in agreement with Moon *et al., (*2007); Wang *et al.,* (2008); Turutoglu *et al.*, (2009) and Turkyılmaz *et al*., (2010). Kumar *et al*.,(2010) who reported that MRSA strains were multi-drug resistant which might be due to production of betalactamase and PBP2a (penicillin binding protein).

It is therefore, very important to implement a systematic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

5.9 Testing of β lactamase production and methicillin resistance

Out of 116 *S.aureus* organism, 64 (55.17 per cent) showed β lactamase production by positive nitrocefin test.

In this study, MRSA alert kit proved 10.34 per cent (12/116) of methicillin resistance *S. aureus*. In the present study, 55.17 per cent of *S.aureus* were positive for β lactamase by nitrocefin test and this could be used as a primary screening test for MRSA mastitis.

The present study concurred with Azeez *et al.*(2012) who reported that early detection of methicillin resistant *Staphylocoocus aureus* by nitrocefin test.

Azeez *et al.* (2012) reported that, there was higher incidence (34 per cent) of methicillin resistant carrying pathogens as detected through the nitrocefin test as compared to MRSA (16/52) and *S. epidermitidis* (4/16) combined involvement (29.4 per cent) in the mastitis milk samples. This suggest that the present study will help in the early detection of methicillin resistant status of milk from the infected animals. Antibiotic susceptibility findings would help the field veterinarians to treat the

animals with even good antibiotics to reduce the losses. Nitrocefin disc were also applied directly to mastitis milk samples to assess the presence of MRSA.

5.10 Minimum Inhibitory Concentration

The isolates were categorized as susceptible or resistant on the basis of CLSI and NARMS based MIC breakpoints (CLSI, 2008 and NARMS, 2011).

MIC_{50} and MIC_{90} are the lowest concentrations of antimicrobial agents that completely inhibits bacterial growth at ≥ 50 per cent and ≥ 90 per cent respectively. In the present study, MIC_{50} is taken for susceptibility consideration, since MIC_{90} values were so high that it would not be possible to achieve clinically.

The MIC_{50} of the *E.coli* isolates tested (MIC_{50}) for gentamicin and enrofloxacin were 2 and 0.25 μg /mL (San Martin *et al.,* 2012). The MIC_{50} of the *E.coli* isolates tested for amoxicillin, oxytetracycline, ceftriaxone, penicillin G, oxacillin and amoxicillin-clavulanate were 2, 4, 0.25, 2, 2 and 4 μg /mL (Saini e*t al.,* 2012).

The data for $MIC_{50,}$ clinical breakpoints of amoxicillin, penicillin G, oxacillin and amoxicillin+sulbactum against *E.coli* were not available. However, the clinical break point of ampicillin and amoxicillin-clavulanate were taken for the present study.

In the present study MIC_{50} of the *E.coli* strains tested were: 1.95, 1.95, 1.95, 3.9, 31.25, 31.25, 62.5 and 62.5 for gentamicin, enrofloxacin, amoxicillin+sulbactam, ceftriaxone, oxytetracycline, oxacillin, amoxicillin and pencillin G respectively.

The breakpoints for gentamicin and enrofloxacin were ≥16 and ≥2 µg/ ml for *E.coli* respectively(San Martin *et al.,* 2012). The breakpoints for amoxicillin, oxytetracycline, ceftriaxone, penicillin G, oxacillin and amoxicillin-clavulanate were 16, 8, 32, 16, 16 and 16 μg /mL for *E.coli* respectively (Saini e*t al.,* 2012). Similar MIC break points were observed in the present study.

Based on the breakpoints, the result indicated that *E.coli* was sensitive to gentamicin (56.3 per cent), enrofloxcain (56.3 per cent), amoxicillin+sulbactam (50.4 per cent), ceftriaxone (86.6 per cent) and resistant to amoxicillin (53 per cent),

oxytetracycline (58 per cent), penicillin G (60.5 per cent) and oxacillin (56.3 per cent) and this concur with the findings of San Martin *et al.,* (2012) and Saini *et al.,* (2012).

The MIC break point for pencillin G, oxytetracycline, amoxicillin and oxacillin in *E.coli* mastitis in the present study was 16, 8, 16 and 16 μg /mL. Hence, they are considered as resistance. This resistance could be attributed to the indiscriminate use of these drugs. It highlights the need for systematic study of resistance pattern before initiating antibiotic therapy.

The MIC_{50} of *S.aureus* isolates tested for oxacillin and tetracycline were ≤ 2 and ≤ 1 µg/ mL respectively(Saini e*t al.,* 2012). The MIC_{50} of the *S.aureus* isolates tested for penicillin, amoxicillin, amoxicillin-clavulanate, oxacillin, gentamicin, tetracycline and enrofloxacin were 0.25, 0.125, 2, 1, 2 and 0.125 µg/ mL respectively (San Martin e*t al.,* 2012).

The data for $MIC_{50,}$ clinical breakpoints of amoxicillin+sulbactum against *S.aureus* were not available. However, the clinical break point of amoxicillin-clavulanate were taken.

In the present study, MIC_{50} of the *S.aureus* strains tested were: 3.9, 3.9, 3.9, 3.9, 3.9, 31.25, 62.5, 62.5 and 3.9 for gentamicin, ceftriaxone, enrofloxacin, amoxicillin+sulbacatam, oxacillin, oxytetracycline, amoxicillin and pencillin G respectively.

The breakpoints for amoxicillin, amoxicillin+clavulanate, enrofloxacin, gentamicin, oxytetracycline, penicillin and oxacillin were ≥ 0.5, ≥ 8, ≥ 4, ≥ 16, ≥ 16, ≥ 0.25 and ≥ 4 µg/ ml for *S.aureus* respectively (San Martin *et al.,* 2012). The breakpoints for ceftriaxone were ≥ 4 µg/ ml for *S.aureus* (Saini *et al.,* 2012). Similar MIC break points were observed in the present study.

Based on the breakpoints the result indicated that *S.aureus* was sensitive to gentamicin (56.7 per cent), enrofloxcain (50.1 per cent), amoxicillin+sulbactam (50.9 per cent), ceftriaxone (53.8 per cent) and resistant to amoxicillin (100 per cent), oxytetracycline (55.8 per cent), penicillin G (100 per cent) and oxacillin (80.8 per

cent) and this concur with the findings of San Martin *et al.,* (2012) and Saini *et al.,* (2012).

The MIC break point for pencillin G, oxytetracycline, amoxicillin and oxacillin in *S.aureus* mastitis in the present study was 0.25, 16, 0.5 and 4 μg /mL. Hence, they were considered as resistance. The high resistance of pencillin G, oxytetracycline, amoxicillin and oxacillin in *S.aureus* mastitis in the present study could be attributed to the indiscriminate use of these drugs and intramammary preparations used by the owner without the prescription of the veterinarian.

In the present study, MIC_{50} of the MRSA strains tested were: 15.62, 31.25, 31.25, 62.5, 62.5, 62.5, 62.5 and 125 for enrofloxacin, gentamicin, amoxicillin+sulbactam, oxytetracycline, ceftriaxone, amoxicillin, pencillin G and oxacillin respectively.

The breakpoints for gentamicin, oxytetracycline, ceftriaxone, enrofloxcain, amoxicillin, penicillin G, amoxicillin+sulbactam and oxacillin were 8, 8, 4, $\geq$ 0.5, 0.25, 0.12, 4, and 2 µg/ ml for MRSA respectively (Febler *et al.,* 2010).

Based on the breakpoints the result indicate that MRSA is sensitive to gentamicin (24.9 per cent), enrofloxcain (8.3 per cent), amoxicillin+sulbactam (50 per cent), ceftriaxone (8.3 per cent) and resistant to amoxicillin (100 per cent), oxytetracycline (73.4 per cent), penicillin G (100 per cent) and oxacillin (100 per cent) and this concur with findings of Febler *et al.,* (2010).

Though resistance was shown against enrofloxacin *in vitro* it had a good efficacy *in vivo*. This may be due to reduction in bacterial count, action of NSAID that will reduce the leukocyte count.

The MIC break point for pencillin G, oxytetracycline, amoxicillin and oxacillin in MRSA mastitis in the present study was 0.12, 8, 0.25 and 2 μg /mL. Hence, they were considered as resistance. The high resistance of pencillin G, oxytetracycline, amoxicillin and oxacillin in MRSA mastitis in the present study could be attributed to the indiscriminate use of these drugs and intramammary preparations used by the owner without the prescription of the veterinarian.

Only a few studies in India have been carried out to assess MRSA status among mastitis infections. The presence of MRSA signifies alarming levels of Resistance. Further it highlights the need for preventing the indiscriminate use of antibiotics.

5.11 POLYMERASE CHAIN REACTION

5.11.1 Confirmation of the mastitis bacteria by targeting specific genes for different strains

The incidence of *E.coli* mastitis and *S.aureus* mastitis were 50.64 and 89.66 per cent respectively by PCR targeting specific genes namely 16s-23s rRNA.

Whereas, Kumar *et al.* (2013) reported 100 per cent positivity of *E.coli* in mastitis milk samples by simplex PCR using published primers of 23s rRNA.

Kwon *et al.,* (2005) opined that 16S rRNA sequence similarity has been shown to be very high, 90 to 99 per cent, in 29 *Staphylococcus* species studied.

Sindhu *et al.* (2010) recorded that out of 367 milk samples from crossbred cows, 83 (22.61 per cent) milk samples revealed presence of *Staphylococcus* Spp. by genus specific PCR, while conventional microbiological methods could detect 78 (21.25 per cent) samples as positive for *Staphylococcal* mastitis.

The incidence of MRSA mastitis by targeting specific gene *mec*A and *bla*Z in the study was 10.34 per cent (12/116). This study was in agreement with Haveri, (2008), Asfour and Darwish, (2011) and Soares *et al*., (2012).

In the present study, all the 12 tested isolates were positive for *blaZ* and *mec*A gene by PCR and also resistant to penicillin and methicillin by *in-vitro* disk diffusion test (100 per cent) and showing a good relationship between the two techniques which also agreed with, a similar study (Asfour and Darwish, 2011) who reported a 100 per cent and 50 per cent resistance to penicillin and Oxacillin.

Penicillin has been used overtime and it is antibiotic of choice for drying – off. This could be the reason for the 100 per cent resistance by the *S.aureus* isolates against this antibiotic. The MIC result of this study showed a higher value for the

*mec*A isolates. It can be due to sub-optimal doses of antibiotic used against infection with MRSA and also high resistance to these antibiotics due to selective pressure.

MRSA isolates also need to be screened for the PVL (Panton-Valentine leukocidin). Screening for this would enable to determine the virulence of these isolates and also indicating the possibility of human transmission. Avoiding routine antimicrobial use in food animals, to decrease selection pressures, might decrease the prevalence of MRSA among cow. However, in this study, we have not looked into the presence of PVL gene.

5.11.2 Screening of milk samples by Multiplex PCR

Phuektes *et al.* (2001 and 2003), Gillespie and Oliver (2005) and Pradhan *et al.* (2011) used the multiplex PCR for detection of different mastitis pathogens in milk sampls. In our study, out of the 235 positive milk samples, single specific bands of *S. aureus*, *E.coli* and MRSA in 104, 119 and 12 samples were observed; Two bands of *S. aureus* + *E.coli* , *S.aureus* + MRSA were observed in 2 and 12 samples and three bands of *E.coli* + *S.aureus* + MRSA were observed in 2 samples by multiplex PCR (Plate 28).

5.12 Comparison of different tests in the identification of methicillin resistance in *S.aureus* mastitis cows

Higher incidence of methicillin resisatnce was recorded by ABST and nitrocefin test. Even though low positive incidence was recorded by MRSA alert kit test and PCR target (*mec*A and *bla*ZA) gene, they were equally found to be useful in the confirmation of methicillin resistance when compared to ABST and nitrrocefin test.

Van duijikeren *et al.,*(2004) and Loeffler and Lloyd, (2010) opined that detection of *mec*A and *bla*Z gene by PCR was gold standard test for confirmation of methicillin resistance. Selepak and Witebsky, (1985) reported that MRSA alert kit test was found useful in the detection of methicillin resistance in β lactamase resistance *S.aureus*. The results of the present study concurred with the above said authors.

5.13 THERAPEUTIC EVALUATION

Therapeutic evaluation of different groups were done based on clinical signs, pH, EC, SCC and CMT.

5.13.1 Clinical Signs

Complete remission of clinical signs were observed in 93.28 per cent in *E.coli*, 94.43 per cent in *S.aureus* and 94.43 per cent in MRSA.

In *E.coli* group, 74.1 per cent, 67.75 per cent, 76.67 per cent and 64.52 per cent of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively showed normal milk colour.

In *S.aureus* group, 65.25 per cent, 65.25 per cent, 72.43 per cent and 68.98 per cent of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively showed normal milk colour.

In MRSA group, 50 per cent, and 50 per cent of cases treated with amoxicillin+sulbactam and enrofloxacin respectively showed normal milk colour.

Local clinical signs, such as swelling, pain and firmness of the inflamed mammary quarters, were less severe in the treated cows (Hoeben *et al.,* 2000).

5.13.2 Milk profile

5.13.2.1 *E.coli*, *S.aureus*

In the present study, in all the treatment groups of *E.coli* and *S.aureus* mastitis, the post treatment pH, SCC was significantly decreased when compared to pre treatment pH, SCC values indicated that the treatment was effective in controlling the inflammation. The post treatment electrical conductivity was significantly increased when compared to pre treatment electrical conductivity value. Marked reduction in CMT scores were also recorded indicating that the treatment was effective in controlling the inflammation.

5.13.2.2 Therapeutic evaluation of antibiotic in treatment of mastitis

In the present study, cows affected with *E.coli* and *S.aureus* mastitis treated with amoxycillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed uniform improvement in clinical mastitis.

Enrofloxacin is a broad spectrum antibiotic used for treatment of localized and systemic infections (Owens *et al.* 1997 and Sol *et al.* 2000).

Dosogne *et al.* (2002) reported that systemically enrofloxacin had very good distribution in mammary gland with a higher concentration in milk and a significant decrease in milk loss in *E.coli* mastitis.

Constable *et al.*(2008) and Evira *et al.*(2009) recommended fluroquinolone as the first antibiotic of choice for *E.coli* mastitis because of pharmacokinetic and pharmacodynamic properties.

When, enrofloxacin was administered, its active metabolite ciprofloxacin had reached higher concentration and maintained in blood and milk (Kaartinen *et al.,*1995 and Rantala *et al.,* 2002). Milk does not significantly interfere with the antimicrobial activity of enrofloxacin *in vitro* (Fang and Pyorala,1996). Fluoroquinolones were shown to have positive immunomodulatory effects by increasing the killing ability of neutrophils, which may contribute to therapeutic effect (Hoeben *et al.,*1997).

Olson *et al.*(2002) opined that gentamicin as one of the most efficient antibiotics for bovine mastitis which was also proved in the present trial. Gentamicin is a bactericidal, concentration dependent, drug primarily used for treatment of Gram negative infections.

Tufani *et al.* (2012) conducted therapeutic trial with gentamicin at a dose rate of 2.5 mg/kg intramuscular twice daily for 3.32 days (average) and found 84.21 per cent clinical recovery.

The combination of β lactam antibiotics with β lacatmase inhibitors, such as clavulanic acid and sulbactam, increases the activity of antibiotics against bacteria (Francis, 1989). β lactamase activity at a higher rate in *Staphylococci* and this enzyme

might be the main factor in the development of resistance to the β lactam antibiotics like pencillin, ampicillin and amoxicillin. Amoxicillin+ clavulanic acid, danofloxacin, enrofloxacin and cloxacillin are the most effective antibiotics against *Staphylococci* (Turutoglu *et al.,* 2002).

Wilson *et al.*(1999) and De Oliveira *et al.(*2000) opined that Amoxicillin in combination with β lactamase inhibitors as a potentially useful antibiotic for the treatment of mastitis. Later Sharma *et al.,*(2010) and Tufani *et al.,*(2010) reported a very effective clinical recovery of bovine mastitis after intramammary infusion of amoxicillin+sulbactam.

Amoxicillin alone or in combination with β–lactamase inhibitors is potentially useful for the treatment of mastitis caused by pathogenic organisms (Roberson *et al.,* 2004 and Sharma *et al.,* 2010).

In human medicine, efficacy of AMX/SUL combination in the treatment of bacterial infections, including *E. coli* and *Acinetobacter baumannii* were studied by Bantar *et al.,* (1999) and Bantar *et al.,* (2009). Hui *et al.* (2013) also reported that a good bactericidal activity *in vitro* was achieved for AMX/SUL (4:1) combination against these common mastitis pathogens in cows.

Sachin kumar (2007) suggested a decrease in pH and electrical conductivity of milk in Ceftriaxone treated cows.

Ceftriaxone is a bactericidal drug, belonging to third generation cephalosporin, which act by inhibiting bacterial cell wall synthesis. It has remarkable activity against Enterobacteriaceae (Prescott and Baggot, 1994) and *Staphylococcus* Spp. (Trailovic *et al.,* 1992; Prescott and Baggot, 1994; Mathur, 2004 and Sumati *et al.,* 2008).

5.13.2.3 MRSA

In the present study the post treatment pH and SCC significantly decreased when compared to pre treatment pH and SCC indicating that the treatment was effecting in controlling the inflammation. The post treatment electrical conductivity significantly increased when compared to pre treatment electrical conductivity indicating that the treatment was effective in controlling the inflammation. Reduction

in CMT scores were also recorded indicating that the treatment was effective in controlling the inflammation.

Based on the pH, EC and SCC changes, enrofloxacin was found to be superior in the management of MRSA mastitis in cows.

Based on the MIC_{50} breakpoint results, enrofloxcain (8.3 per cent) and amoxicillin+sulbactam (50 per cent) were sensitive but active against MRSA and agree with Febler *et al*., (2010).

Though Enrofloxacin was resistant *in vitro* (5 out of 6 cases) but it had a good efficacy *in vivo* (3 out of 6 cases). This might be due to immunomodulatory, concentration dependent and post antibiotic effect.

The efficiency of a β –lactamase inhibitor associated with the detection of blaZ gene in 55 per cent of oxacillin –resistant strains and positive results obtained from nitrocefin test gives us a clue that β –lactamases must be greatly implicated in the detected resistance.

Enrofloaxacin was found most effective antibiotic against MRSA (Ganiere *et al.,*2001; Kenar *et al.,* 2012 and Azeezz *et al.,* 2012) followed by norfloxacin and chloramphenicol (Imran *et al.,* 2010).

The lower efficacy of amoxicillin + sulbactam noticed in the current study might be due to development of resistance by bacterial strains which was confirmed by PCR (*mec*A and *bla*Z gene).

Further investigations are needed to study the PVL genes in *mec*A positive MRSA positive animals for zoonotic transmission from cows to animals.

5.14 Economic Impact of mastitis

The average milk yield loss/ day was more in *E.coli* mastitis followed by MRSA and *S.aureus.* But the average discarded milk remained almost same in all the three groups. The treatment cost of all three groups were same. The total milk yield loss in *E.coli* was higher than *S.aureus* and MRSA. However, post treatment milk

yield loss was higher in MRSA mastitis compared to *E.coli* and *S.aureus* which contributes to the more total economic loss in this group of clinical mastitis.

These findings were in partial agreement with Sharma *et al.* (2012) who reported almost the same pattern of economic impact in a study on Karan Fries cattle. Although average milk yield loss was more in *E.coli* mastitis and post treatment milk yield loss was higher in MRSA mastitis. The treatment cost remains comparable in all the groups.

The above findings indicated that the loss during only one episode of outbreak during lactation. However, if there are multiplies of such outbreakes the economic impact will be much higher than what is calculated in the present study.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The study entitled "Evaluation of antibiotic resistance mastitis in dairy cows" was undertaken at Large Animal Clinic Medicine Unit of Madras Veterinary College Teaching Hospital and dairy farms in Coimbatore district. The study was conducted with an attempt to study the prevalence of drug resistant mastitis, clinico-pathological changes and their pattern of antibiotic resistance in dairy cows. Comparative evaluation of different therapeutic protocols for clinical management of resistant mastitis and assessment of the economic impact of drug resistant mastitis were undertaken for the study.

Based on culture, isolation and sensitivity tests, cows with resistant mastitis were grouped as follows,

Group I *E.coli* (n=119)

Group II *Staphylococcus aureus* (n=104)

Group III Methicillin resistant *staphylococcal aureus (MRSA)* (n=12)

Group IV Apparently healthy cows (n=20)

Based on Antibiotic sensitivity tests, treatment trials were conducted. Group I and Group II were treated with gentamicin, ceftriaxone, enrofloxacin and amoxycillin + sulbactam and Group III were treated with amoxicillin+sulbactam and enrofloxacin. The post treatment evaluation was performed after 7 days of treatment.

The incidence of clinical mastitis at Madras Veterinary College Teaching Hospital was 9.57 per cent, out of which 1.90 per cent was acute mastitis and incidence in organized farm was 16.56 per cent, out of which 11.08 per cent was acute mastitis.

The incidence of resistant mastitis was 56.1 per cent. The predominant resistant causative pathogen was *E.coli* (50.64 per cent) followed by *S. aureus* (44.25 per cent) and

MRSA (5.11 per cent). However, no drug resistance was recorded in *Streptococcus* Spp. and *Bacillus* Spp.

The incidence of resistant mastitis in both organized (42.13 per cent) and unorganized (52.87 per cent) dairy farms are similar and hence environmental contamination need not be the prime factor in causing clinical mastitis. Highest incidence was observed in early stage of third lactation. The incidence of mastitis was highest in hind quartets than fore quarters.

Haematobiochemical changes were reduced Hb, PCV and TEC, leukocytosis with neutrophilia and lymphopenia, hypoalbuminemia, and hyperglobulinemia. A significant increase in ALP and AST were observed in early lactation which might reflect the negative energy balance leading on to mobilization of fat and there on to fatty liver.

In vitro antibiotic sensitivity test, *E.coli, S.aureus* and MRSA organisms showed more sensitivity to enrofloxacin, amoxicillin+sulbactam, gentamicin and ceftriaxone and had highest resistant to penicillin followed by amoxicillin, oxytetracycline and methicillin. Most of the *E.coli* and *S.aureus* isolates were found to be resistant.Whereas, most of the MRSA isolates were found to be multi-drug resistant.

Nitrocefin and MRSA alert kit was found to be useful in preliminary screening of β lactamase production and methicillin resistance in clinical mastitis.

Based on the MIC breakpoints, gentamicin, enrofloxacin, amoxicillin+sulbactam, ceftriaxone were sensitive against *E.coli*, *S.aureus* and MRSA and amoxicillin, oxytetracycline, penicillin G and oxacillin were resistant against these organisms.

The incidence of *E.coli* and *S.aureus* mastitis by targeting specific gene 16s-23s r RNA was 50.64 and 44.26 per cent respectively. The incidence of MRSA mastitis by targeting specific gene *mec*A for β lactamse and *bla*Z for alternate penicillin binding protein PBP2 (methicillin resistance) was 10.34 per cent.

Out of the 235 positive milk samples, multiplex PCR showed single specific bands of *S. aureus*, *E.coli* and MRSA in 104, 119 and 12 samples respectively and two bands of *S. aureus* and *E.coli*, *S.aureus* and *MRSA* in 2 and 12 samples respectively. Positive for three bands of *E.coli*, *S.aureus* and MRSA in 2 samples (Plate 28).The PCR products were subjected to BLAST analysis and confirm the identity.

A highly significant increase in pH and SCC and significant decrease in electrical conductivity were noticed in all clinical mastitis prior to treatment and showed slow return after treatment. In *E.coli* and *S.aureus* mastitis treated with amoxycillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin were showed uniform clinical recovery. However, in MRSA mastitis, enrofloxacin was found to be highly effective in comparison to amoxicillin+sulbcactam.

The milk yield loss was more in *E.coli* mastitis compared to *S.aureus* and MRSA mastitis. However, similar economic impact was observed in *E.coli, S.aureus* and MRSA mastitis.

The following conclusions were derived from the study.

1. Incidence of drug resistant mastitis was 54.6 per cent. The predominant resistant causative pathogen was *E.coli* (50.64 per cent) followed by *S. aureus* (44.25 per cent) and MRSA (5.11 per cent). However, no drug resistance was recorded in *Streptococcus* Spp. and *Bacillus* Spp.

2. The incidence of resistant mastitis in both organized and unorganized dairy farms are similar and hence environmental contamination need not be the prime factor in causing clinical mastitis.

3. Higher incidence of mastitis was observed in early stage of third lactation. A significant increase in ALP and AST were observed in early lactation which might reflect negative energy balance and fatty liver

4. pH, electrical conductivity and SCC of milk were reliable indicators for post treatment evaluation of clinical mastitis.

5. Higher incidence of resistant mastitis was observed in *E.coli* and *S.aureus* isolates. Whereas, MRSA isolates were found to be multi-drug resistant.

6. ABST and Nitrocefin test were found to be useful in the preliminary screening of β lactamase resistance. MRSA alert kit and *mec*A and *bla*Z target gene PCR were equally found to useful in the confirmation of MRSA.

7. Amoxicillin+sulbactam, enrofloxacin, ceftriaxone and gentamicin were equally effective in the management of resistant mastitis caused by *E.coli* and *S.aureus*. In MRSA mastitis enrofloxacin was found to be superior in the management even though *in vitro* it was resistant.

8. The milk yield loss was more in *E.coli* mastitis compared to *S.aureus* and MRSA mastitis. However, similar economic impact was observed with regard to single episode of mastitis.

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