Studies on resistance pattern to Beta-lactam antibiotics in *Staphylococcus aureus* and *Escherichia coli* isolated from milk of buffaloes with mastitis

T H E S I S

Submitted

In partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

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(INDIA)

2017

Appendix – B

DECLARATION OF STUDENT

I hereby declare that the experimental Research work and interpretation of the thesis entitled **"Studies on resistance pattern to Beta-lactam antibiotics in *Staphylococcus aureus* and *Escherichia coli* isolated from milk of buffaloes with mastitis"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Appendix – C (i)

DECLARATION OF ADVISORY COMMITTEE

Shri. **KARANDE VIKAS VASANT** has satisfactorily prosecuted his course of research for a period of not less than two semesters and that the thesis entitled, **"Studies on resistance pattern to Beta-lactam antibiotics in *Staphylococcus aureus* and *Escherichia coli* isolated from milk of buffaloes with mastitis"** submitted by him is the result of research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY PHARMACOLOGY AND TOXICOLOGY** for the award of **DOCTOR OF PHILOSOPHY** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

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This is to certify that the thesis entitled, **"Studies on resistance Pattern to Beta-lactam antibiotics in *Staphylococcus aureus* and *Escherichia coli* isolated from milk of buffaloes with mastitis"** submitted by **KARANDE VIKAS VASANT** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **DOCTOR OF PHILOSOPY** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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Dedicated to

My Family and Friends

Acknowledgement

Acknowledgement

I owe this unique opportunity to place on record my deep sense of gratitude & indebtedness to my guide and supervisor Dr. (Mrs.) M. M. Gatne, Professor and Head, Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai for her scholastic guidance, prudent suggestions & persistent endeavor throughout the dissertation. The whole credit of research work done by me goes to her for her valuable guidance, hard work, dedication, patience and encouragement. This thesis would not have been possible without the help, support and understanding by her.

I also gratefully acknowledge my advisory committee members, Dr. P.L. Dhande, Professor, Department of Anatomy, Dr. R. V. Gaikwad, Professor, Department of Veterinary Medicine, Dr. R. J. Zende, Associate Professor, Department of Veterinary Public Health, Dr. A. S. Nagvekar, Associate Professor, Department of Veterinary Physiology and for their suggestions, timely support and encouragement and wholehearted help and cooperation.

My special thanks goes to my advisory committee member Dr. S. H. Dalvi, In charge, CIF for providing every possible facility for this research work.

I wish to express my sincere thanks to friends and colleagues Dr. Swati, Dr. Sushma, Dr. Sangram Chavan, Dr. U B. Kumbhar, Dr. Sandip Gaikwad, Dr. Sonal, Dr. Pharande, Dr. Vitthal, Dr. Pramod, Dr. Metkari Dr. Galdhar and Dr. Prashant Masare for their valuable suggestions and technical support for completion of this study.

I am indebted to Dr. A. M. Paturkar, Associate Dean, Bombay Veterinary College for providing me valuable help and advice during the course this study. I also never forget the motivation and support offered by Dr. Arju Somkuwar from time to time.

I would also wish thanks to my students, Dr. Sandip, Dr. Kirti, Dr. Puja, Dr. Ashvini, Priyanka, Parmeshwar, Devika, Shrikant, Jyoti for their help in carrying out my research.

I also wish to express my sincere thanks to Miss Mina Mane, Mr. Sandip Ghadigaonkar, Uday Birje, Tribhuvan Yadav and D. R. Jadhav for every help did they did in the Department.

The words are insufficient to express my feelings about my loving father and mother, without their hard work and support it is not possible to me to reach this destination.

I express my special thanks to my wife Dr. Ashali and Son, Master Malhar for their dedication, continuous support and motivation; I am able to complete this work due to their devotion only.

I am very much thankful to all Bombay Veterinary college staff for their co-operation and motivation throughout the course of study. I also thanks all those who have helped me directly or indirectly in completion of this study.

In the end, I must record my special appreciation to GOD who has always been a source of my strength, inspiration & my achievements.

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LIST OF SYMBOLS / ABBREVIATIONS

%	:	Per cent
- ve	:	Negative
@	:	At the rate
+ ve	:	Positive
µg	:	Microgram
µl	:	Micro liter
µm	:	Micrometer
^{0}C	:	Degree Celsius
AMR	:	Antimicrobial Resistance
BHI	:	Brain Heart Infusion broth
blaZ	:	β- lactamases encoded gene
CIF	:	Central Instrumentation Facility
CLSI	:	Clinical and Laboratory Standards Institute
CM	:	Clinical Mastitis
CMT	:	California Mastitis Test
CNS	:	Coagulase Negative Staphylococci
CPS	:	Coagulase Positive Staphylococci
DNA	:	Deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosphate
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme-linked Immunosorbent Assay
erm (B)	:	Methylase encocded gene
Eta	:	Exfoliative Toxin A
et al.	:	Et alii/alia
Etb	:	Exfoliative Toxin B
etc	:	Et cetera
Fig	:	Figure
Gm	:	Gram
Lit	:	Liter
M	:	Molar
$MgCl_2$	:	Magnesium chloride
MIC	:	Minimum Inhibitory Concentration
Min	:	Minute

Ml	:	Milli litre
Mm	:	Milli molar
MRSA	:	Methicillin Resistant *S. aureus*
MSSA	:	Methicillin Sensitive *S. aureus*
MTCC	:	Microbial Type Culture Collection
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
NSS	:	Normal saline solution
PCR	:	Polymerase Chain Reaction
S. aureus	:	*Staphylococcus auerus*
SCM	:	Subclinical Mastitis
SE	:	Staphylococcal Enterotoxin
sea	:	Staphylococcal Enterotoxin A gene
seb	:	Staphylococcal Enterotoxin B gene
sec	:	Staphylococcal Enterotoxin C gene
sec	:	Second
sed	:	Staphylococcal Enterotoxin D gene
see	:	Staphylococcal Enterotoxin E gene
TBE	:	Tris-borate Ethylene diamine tetra acetic acid
TE	:	Tris EDTA
tst	:	Toxic Shock Syndrome Toxin
UV	:	Ultra violet
V	:	Volts
v/v	:	Volume by volume
viz.	:	*Videlicet*
w/v	:	Weight by volume

Chapter 1

Introduction

INTRODUCTION

Antibiotics have long been considered the “magic bullets” that would end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have also been losing their effectiveness since the beginning of the antibiotic era. Bacteria have adapted defenses against these antibiotics and continued to develop new resistance patterns. In recent years, much attention has been given to minimize and to prevent, the increasing antibiotic resistance. Inappropriate use of antibiotics in human and animals, as more microbial species and strains become resistant, results in difficulty in treating many infectious diseases. The use of antibiotics in raising food animals has also contributed significantly to the pool of antibiotic resistant organisms globally (Hafiz and Jamil, 2016)

Antibiotics have been employed for the treatment of animals for more than seven decades i.e. from the discovery of Sulphonamides and Penicillin. They are used for treatment, prevention of diseases, for improving feed efficiency and production. At low doses they are also used as growth promoters for extended periods. Prophylactic antibiotics are used at low and sub-therapeutic doses to prevent disease in dairy animals (IOM, 1989).

Among the diseases affecting dairy animals, mastitis is the most common and economically significant disease. A variety of bacteria isolated from bovine mastitis cases are the most common causes of contagious and environmental clinical mastitis (CM). Antimicrobial therapy is commonly used for mastitis prevention and control. Unfortunately, despite the best possible antimicrobial treatments, failures of bacteriological cure are common, for mastitis and antimicrobial resistance (AMR) is considered to be one of the reasons for low cure rates. Additionally, AMR in bacteria is a public health hazard, and extensive use of antimicrobials is considered a potentially important driver of AMR. Several strains of bacteria from mastitis cases have been reported to show resistance against multiple antimicrobials such as Penicillin-G, Gentamicin, Streptomycin, Ampicillin, Ciprofloxacin and Oxytetracycline.

β–lactam antibiotics are frequently used in mastitis therapy and the resistance is due to the production of β-lactamases and low-affinity penicillin-binding protein, PBP2A. The term Methicillin Resistant *Staphylococcus aureus* (MRSA) is used to refer to β-lactam antibiotic resistant (i.e. penicillinase producing) *Staphylococci*. Most of the MRSA strains are resistant to Cloxacillin, Flucloxacillin and all β-lactam antibiotics (Penicillins, Cephalosporins and

Imipenams) and often to other class of antimicrobial agents (Multidrug resistant, MDR) They need to be treated with Vancomycin and patients carrying such infections need special attention in clinics. MRSA is one of the most commonly acquired nosocomial infection (Jacoby, 1996). *Staphylococci* resistant to penicillinase resistant Penicillins chromosomally produce a new penicillin binding protein with decreased affinity for these Penicillins. MRSA have been isolated from mastitis milk samples and their presence in bovine mastitis is a potential risk to other exposed cattle and farm workers including veterinarians. In general, the emergence and transfer of AMR bacteria or genetic determinants from animals to human populations via food chain is a growing concern. Comprehensive information on the prevalence of AMR in bovine mastitis pathogens in milk is lacking in India (Chandrasekaran *et al*., 2014).

Microorganisms producing extended-spectrum β-lactamases (ESBL) were identified in early 1980s. ESBLs are enzymes that compromise the efficacy of all β-lactams, except Cephamycin and Carbapenems, by hydrolysis of the β-lactam ring. (Bourjilat *et al*., 2011). ESBL producing *Escherichia coli (E. coli)* are an increasingly significant cause of community-acquired infection worldwide. The resistant strains of organisms show pathogenic and epidemiological characteristics in various ways such as mutation, clonal evolution (Fitzgerald *et al*., 2001) and horizontal gene transfer (Brody *et al*; 2008). These evolutionary processes enhance the pathogenic and antimicrobial resistant properties of some organisms like *Staphylococcus aureus (S. aureus)* strains. The available information is limited regarding the genetic heterogeneity of resistant organisms in mastitic animals in subtropical conditions like India.

Indiscriminate use of antimicrobials in human and veterinary medicine is considered to be the main driver for emergence of resistance in bacteria (Levy and Marshall, 2004). The increased prevalence and dissemination of AMR is in line with the Darwin's principle of "survival of the fittest" (Boerlin and White, 2006). Antimicrobial use over longer duration changes the microbial ecology in a given environment such that resistant strains become dominant in the bacterial population (Levy, 1998). Intense screening of the factors supporting the emergence of resistance among pathogens in human and animals became essential due to increased prevalence of AMR, and the associated negative health outcomes (Bager *et al*., 1999 and Codex., 2005).

Alongwith the human medical professionals, it is also a duty of a veterinarian to save effectiveness of anti-infective agents by avoiding their misuse and to use these chemical weapons against bacteria strategically.

Alexander Fleming, the discoverer of penicillin, had already warned in his Nobel prize speech in 1945, that bacteria could become resistant to antimicrobial agents. The development of resistance in microorganisms is accelerated by the selection pressure exerted by widespread use of antibacterial drugs. Resistant strains are able to propagate and spread where there is non-compliance with infection prevention and control measures. The development of antimicrobial resistance among bacteria is currently one of the world's most pressing public health problems. The centre for disease control and prevention estimated that the total of Methicillin Resistant *Staphylococcus* Infections (MRSI) in US hospitals and communities has increased from 2 percent in 1974 to almost 63 percent in 2004. Similarly, with *Salmonella* being an important cause of food-borne diarrheal disease in human beings, the reduction in the number of antibiotics available for effective treatment of *Salmonella* related infectious diseases in human and animals has become a serious concern. Globally, *Salmonella* exhibits extensive resistance profiles which have been associated with higher rates of morbidity and mortality and the use of antimicrobials in food producing animals. The causes of AMR are hypothesized to include the abuse and misuse of antibiotics in both human and animals.

Misuse of antimicrobial agents both in human and animals has narrowed the potential use of antibiotics for the treatment of infections in human and animals. The use of antimicrobials combined with improvements in sanitation, nutrition and immunization has led to a dramatic decrease in deaths and a major gain in human life expectancy in later half of 20^{th} century. However, with the increased use of antimicrobials, AMR has emerged as one of the greatest threats to human health security and a most pressing problem of serious concern to public health, animal health and also food safety authorities.

One of the solution to minimize the effects of AMR could be the production of new antimicrobials, but unfortunately, the development of new antimicrobials has not been enough to meet the increased demand for new antibiotics due to AMR. Therefore, region specific antimicrobial resistance data should be obtained in order to use specific antibiotics. Also, as

there is steep decline in the discovery of new antimicrobials one has to go for other options through herbs and homoeopathy as alternatives to current chemotherapeutic.

The antibiotic surveillance programs in various regions are undertaken to decide the strategy of use of particular antibiotic in a particular area. The seriousness of antimicrobial resistance threat has prompted governments of different countries to initiate surveillance programs, which include bacteria of animal origin. These programs provide a tool to globally assess the extent of problem, to follow the evolution over time, and to evaluate the effectiveness of control measures. Such systems include "National Antimicrobial Resistance Monitoring System" (NARMS) in United States, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPRAS) in Canada and the Danish Integrated Antimicrobial Resistance Program (DANMAP) in Denmark. (Boerlin and White, 2013).

The animal husbandry practices in India especially in large animal sector are quite unique. Drying of animals at the end of lactation period is hardly followed. The concept of raising the animals for beef purpose is still in infancy and therefore manner in which particular antibiotic is advocated to be used is modified to suit the local husbandry practices. Some antibiotics developed for use in beef animals are employed for treatment of lactating animals. The monitoring system for residues of antibiotics does not exist. Also the antibiotics are available freely on the chemist's counter to the layman. All these factors are likely to contribute to the development and prevalence of resistant strains. However, the systematic coordinated efforts to find out its extent in various regions are still not launched though in recent years there are reports describing prevalence of resistant bacterial population in different areas.

In the present study it was thought appropriate to find out extent and intensity of AMR in selected pockets of Mumbai suburb where lactating animals are reared. As a step towards it, Department of Pharmacology and Toxicology, Bombay Veterinary College, started screening the samples from SCM cases of buffaloes, and attempts are being made to study the resistance pattern of *S. aureus* and *E. coli*.

This study is sought to identify the species responsible for causing bovine mastitis in Mumbai and Thane regions, and to assess the antibiotic resistance pattern found in these microorganisms. The present study is also carried out with aim to further enrich the knowledge

of dairy scientists and Veterinarians in order to assist in the effective control and treatment of mastitis.

Therefore, the present work is planned with following objectives:

1. To study incidence of SCM in selected farms of Mumbai and Palghar regions
2. To isolate *S. aureus* and *E. coli* from buffalo milk.
3. To study their resistance pattern against β-lactam group of antibiotics.
4. To determine MIC of β-lactam antimicrobials for *S. aureus* and *E. coli.*
5. Molecular detection of resistance of *S. aureus* using PCR technique by targeting *nuc* and *mecA* genes.
6. Molecular detection of resistance of *E. coli* using PCR technique by targeting *TEM*, *SHV* and *CTX-M* genes.
7. To correlate resistance pattern exhibited by these organisms through antibiotic sensitivity testing, MIC and PCR technique.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

2.1 Mastitis:

Mastitis, or inflammation of the mammary gland, is predominantly due to the effects of infection by bacterial pathogens, although mycotic or algal microbes play a role in some cases. Pathologic changes to milk-secreting epithelial cells from the inflammatory process often bring about a decrease in functional capacity. Depending on the pathogen, functional losses may continue into further lactations, which may reduce productivity and potential weight gain for suckling offspring. Although most infections result in relatively mild clinical or subclinical local inflammation, more severe cases can lead to agalactia or even profound systemic involvement, resulting in death. Mastitis has been reported in almost all domestic mammals and has a worldwide geographic distribution. Climatic conditions, seasonal variation, bedding, housing, density of livestock populations and animal husbandry practices may affect the incidence and etiology. However, it is of greatest frequency and economic importance in species that primarily function as producers of milk for dairy products, particularly dairy cattle and buffaloes in developing countries like India. Mastitis is considered as one of the most predominant and most costly infectious disease of the dairy cattle industry worldwide (Seegers *et al*., 2003; Petrovski *et al*., 2006).

2.1.1 Incidence of Mastitis:

In Europe, the problem of mastitis is considered as a highly relevant issue, not only for the economic losses to producers, but also for the hygienic production of milk and the safety of dairy products for human consumption (Moroni *et al*., 2005). The prevalence of mastitis in dairy cattle recorded in 1997 by Wilson *et al*. was high in United Kingdom (UK). SCM was the main form of mastitis in dairy herds, exceeding 20 to 50 percent of dairy cows in given herds among the European countries (Pitkala *et al*., 2004).

Fadlelmoula *et al*. (2007) reported incidence of mastitis in Germany as 27.57 percent of the quarters and 49.59 percent of the whole milk samples. They conducted the field study to investigate the factors that influenced the udder health status of dairy cows in Thuringia-Germany. Out of 64,542 milk samples 56,950 were udder quarter samples and 7592 were whole milk samples, from 10,741 dairy cows in 48 dairy farms which were found positive when subjected to SCM detection by CMT.

Ebrahimi *et al*. (2007) reported 29 percent prevalence of SCM from Iran where 120 out of 620 cow milk samples were positive when detected with CMT.

Plozza *et al*. (2011) conducted a survey to know the occurrence of mastitis in South Wales Australia among 382 dairy farmers to acquire information on the relevant risk factors associated with SCM. They reported the average herd prevalence of SCM among the 189 respondents (response rate 49.5 percent) as 29 percent, whereas Chen *et al*. (2012) reported 34 percent prevalence of SCM from North West China by investigating total 1860 cows.

This clearly indicates the drastic increase in the prevalence of mastitis especially the SCM, which is an alarming situation for the dairy sector in the world. Several authors from India and other countries also reported incidence of SCM between 10 to 60 percent.

Sharma and Sindhu (2007) reviewed the mean incidence of CM in India during 1980 to year 2000, where occurrence ranging from 10 to 50 percent in cows and 5- 20 percent in buffaloes was observed, however after year 2000, higher incidence of SCM ranging from 20 to 83 percent in cows and 45 percent in buffaloes was evident. They analyzed the data from more than 100 studies spread over 21 states of India and inferred that the overall prevalence of mastitis ranged from 25 to 97 percent with a mean of about 50 percent. Another report (Pankaj *et al*., 2012) based on earlier published reports suggested that the average prevalence of

mastitis in 1960s to early 1990s, was not more than 30 percent but increased afterwards to even more than 60 percent in India.

2.1.1. A. World- wide incidence and causative agents:

Several authors have reported prevalence of SCM from different parts of the world and India. The reported incidence of SCM and epidemiology of causative agents in different parts of the world are reviewed and tabulated in **Table 2.1.**

2.1.1. B. Incidence in India and causative agents:

In India the prevalence of SCM and CM shows variable epidemiology. Different geographical regions and managemental practices mainly affect the incidence of mastitis in developing countries like India. The reported incidence and causative agents of CM and SCM are tabulated in **Table 2.2.**

Table 2.1: Incidence of CM, SCM and related causative agents recorded globally:

Reference	Species of animal	Origin and country	No of animals screened	Percent prevalence of SCM / CM	Organisms or species isolated
Soomro *et al*. (1997)	Buffalo	Hyderabad (Pakistan),	200	SCM - 33	--
Qazi *et al*. (1999)	Buffalo	45 different small livestock units/herds in Lahore (Pakistan)	1000 quarters milk samples from 300 buffaloes	SCM - 14.3	--
Khan *et al*. (2004)	Buffalo	Khan District of Pakistan	50	SCM- 26	--
Hashmi *et al*. (1980)	Buffalo and Cow	Lahore, Pakistan	250	45.45 buffaloes and cows 50 SCM	*Streptococci* (36.82%), *Staphylococci* (43.09%), *Coliforms* spp. (12.15%), *Pseudomonas* (2.92%), *Corynebacterium* (4.18%)
Anwar and Chaudhary (1983)	Buffalo	Lahore, Pakistan		47.50 SCM	*Staphylococci* (40%), *Streptococci* (45.06%), *E. coli* (25%) and *Pseudomonas* (5%)
Ghuman (1967)	Buffalo	Faisalabad district of Pakistan	225	44.25 SCM	*S. aureus* 114, *E. coli* (2) and *Pseudomonas aeruginosa (1)* and *Str. agalactiae*, each from one sample of milk
Sargeant, *et al*. (1998)	Cow	Dairy herds of Ontario, Canada	2840	SCM -19.8	*S. aureus* (6.7%), *Streptococcus agalactiae* (0.7%), other *Streptococcus* spp. (14.1%), coliforms (17.2%), and other *Staphylococcus* spp. (28.7%).
Karimuribo *et al*. (2000)	Cow	Tanzania	188 cows	SCM - 80	

Dego and Tareke (2003)	Cow (indigenous, Jersey and HF)	Dairy farms in Ethiopia	307	14.9 CM and 25.4 SCM	
Dhakal *et al*. (2007)	Buffalo (Murrah Cross)	Nepal	355	23 SCM and 332 CM cases, Seasonal incidence was Summer (37.31) , Autumn (31.66) , Winter (23.2) , Spring (7.83)	*Staphylococcus* 33.3%
Bradley, *et al*. (2007)	Cow	97 dairy farms in England and Wales	480	SCM-47(estimated from historic farm records) and 71 % (estimated from the samples collected).	*Coagulase-negative Staphylococci* (CNS) (14·90 %) followed by *S. uberis* (13·80 %) and *Corynebacterium* species (9·90 %), S. *aureus* or *Coagulase-positive Staphylococci* (CPS) accounted for 10 % of the samples whereas *E. coli* was 19·80%
Azmi *et al*. (2008)	Cow (HF)	Thuleil, Jordan	220	SCM - 47.1	In CM samples Coliform - 26.10%, *Streptococcus* spp 8.70%, *S. aureus* 40.6%, *Proteus* spp. 1.4%, *Corynebacterium* spp. 5.8% *Pseudomonas* spp. 4.3% , mixed (*Corynebacterium*, *Streptococci*, *Staphylococci* and Coliforms) 7.3% and others 5.8%. While in SCM Coliforms were 31.9%, *Streptococcus* spp. 7.2%, *S. aureus* 42.7%, *Proteus* spp. 2.2%, *Corynebacterium* spp. 2.9%, mixed and others 13.1%.

Mdegela *et al*. (2009).	Cow	Tanzania	91 cows from 69 farms	SCM - 51.6	
Abera *et al*, (2010)	Cow	Adama town, East Shoa, Ethiopia	300	36.7 (110/300 cases) SCM	*S. aureus* (44.5%)
Mekibib *et al*. (2010)	Cow (cross bred)	Holeta Town, Central Ethiopia	107	48.6 (52/107 cases) SCM	*S. aureus* (47.1%) followed by CNS (30.1%). Other bacterial isolates included *Streptococcus* (7.20%), *E.coli* (4.60%), *Micrococcus* species (3.30%), *Klebsiella* spp., *Pneumoniae* spp. (3.30%), *Enterobacter* (1.30%), *Corynebacterium* species (2.00%) and *Bacillus* (1.30%)
Islam *et al*. (2011)	Cow	Tangail sadar upazila of Bangladesh	200	SCM - 29.	
Almaw, *et al* (2012).	Cow	Small-scale dairy farms (1–5 cows) Ethiopia	90	CM 21.26	
Chavoshi and Husaini (2012)	Buffalo	Northwest Iran.	400	SCM 9.5	CNS 34.50%, *S. aureus* 5.00%
Gebrewahid *et al*. (2012)	Goat and Sheep	Tigray Regional State, North Ethiopia	390 lactating animals comprising 255 Goats and 135 Sheep	Goat 18.03 and Sheep 28.14	CNS (44.70%), *S. aureus* (27.70%), *E. coli* (17.00%) and *Streptococci* (10.63%)
Suleiman *et al*. (2012)	Cow	Plateau State, Nigeria	339	SCM -30.90	*S. aureus* (98%)
Moges *et al*. (2012)	Cows (HF, crossbreds and local zebu)	Ethiopia	183	4.90 CM and 30.60 SCM	--

Anbagi and Kshash (2013)	Cow	Al- Najaf province, Iraq	388	SCM- 44.32	*S. aureus* (25%)
Ikiz *et al*. (2013)	Cow	Marmara Region of Turkey	270	SCM -48.89	*S. aureus* was detected in 4.44%
Siddiquee *et al*. (2013)	Cow (Cross bred)	Mymensingh, Bangladesh	158	SCM - 55.1	--
Alharbi (2014)	Sheep and Goat	Qassim Region, central of Saudi Arabia	562	SCM - 9.43	*S. aureus* (84.91%), CNS (5.66%), *S. agalactiae* (7.55%) and *E. coli* (1.88%).
Barua *et al*. (2014)	Cow	Chittagong district of Bangladesh	444 quarter samples of 111 (56 from commercial dairy farms and 55 from backyards) lactating dairy cows	SCM by CMT, WST and SFMT were 32.43 (n=144), 33.56 (n=149) and 31.53 (n=140), respectively.	--
Idriss *et al*. (2014)	Cow	Nitra, Slovakia	390	SCM- 73.85	*S. aureus (*9.74) and *E. coli* (12.82 %)
Meh *et al*. (2014)	Cow	Barisal district in Bangladesh	200	SCM - 28.50	*Staphylococcus* spp. (73.33%).
Galván *et al*. (2015)	Cow	Guanajuato, Mexico	535	SCM - 52 (n = 278)	*CNS* (42%)
Abdel-Rady and Mohammed Sayed	Cow	Assiut, Egypt	350 dairy cows	SCM- 19.14	*S. aureus*, *Streptococcus agalactiae* and *E. coli* from the positive CMT samples with prevalence 52.5, 31.25 and 16.25%, respectively
Gonalez *et al*. (1980)	Cow	Argentina	129 SCM	--	*S. aureus* (43% of samples) *S. epidermidis* (21%), *Str. uberis* (19%), *Str. agalactiae* (13%), *Str. dysgalactiae* (9%), *Corynebacterium pyogenes* (1.3%), *Corynebacterium bovis* (7%) and coliform (1.7%).

Table 2.2. Incidence of SCM and major causative agents reported from India:

Reference	Species	Area	No of animals screened	Percent Incidence SCM / CM	Organisms found
Prabhakar *et al*. (1995)	Buffalo	District Ludhiana of Punjab state.	421	SCM - 9.5	*Staphylococci* (34.21% S. *aureus* and 13.16% CNS), followed by *Str*. *agalactiae* (14.74%), *E. coli* (10.53%), *Pseudomonas* spp (7.89%), Str. pyogenes (3.95%), *Klebsiella* spp. (3.95%), *Str*. *dysgalactiae* (2.63%), *Proteus* spp. (2.63%), *Str. uberis, Diptheroids*
Tiwari *et al*. (2000)	Cow (crossbred, Gir and Malvi)	Mhow and Indore	400 lactating cows (crossbreds, Gir and Malvi)	SCM in Gir, Malvi and crossbred cows recorded was 66.67, 66.67 and 47.10 respectively	--
Lalrinthuanga *et al*. (2003)	Cow	Aizwal, Mizoram	987 quarters of 248 cows	SCM - 37.5 (animals) and11.66 (quarters)	CNS (55.1%) , *Streptococci* (22.45%), *S. aureus* (7.14%), *Corynebacteria* (6.12%), *E. coli* (3.06%), *Proteus* spp. (20.04%), *Klebsiella* (2.04%) and *Citrobacter* spp (2.04%).
Patil *et al*. (2005)	Buffalo	Bidar Karnataka	2424	SCM- 28.3	--
Sudhan *et al*. (2005)	Cow (Cross Bred)	Organized dairy farms of J and K. India	352	SCM - 43.33	*S. aureus* (56.89%) Micrococcus *spp*. (15.51%) *Bacillus spp.* (12.06%), *Staphylococcus epidermidis* (8.62%), *Klebsiella spp.* (3.44%), *E. coli* (1.72%) and *Corynebacterium spp.* (1.72%)

Sharma *et al.* (2007)	Buffalo	Chhattisgarh India	500	SCM - 72 %	59 (53.64%) isolates belonged to *Staphylococcus* sp. *Streptococcus* sp. were the second largest mastitogen group accounting for 18.18% of isolates followed by *E. coli* **(**14.54%) *Corynebacterium* sp. (8.18%) and *Diplococcus* sp. (5.95%).
Sharma and Sindhu, (2007)	Buffalo	Hisar Haryana	2057	SCM - 64.9	38.81% *Staphylococcus* spp., 32.4% *Streptococcus* spp., 11.80% *E. coli*, 5.2% *Corynebacterium* spp., 1.36% *Bacillus* spp., 2.03% *Klebsiella* spp., 0.78% *Pseudomonas aeruginosa*, 0.14% *Proteus* , 0.14% yeast
Harini and Sumathi, (2011)	Cow	Ramanagara district of Karnataka	250	SCM - 75	*S. aureus* (58%) and *E. coli* (23.5%) followed by *Staphylococcus epidermidis* (8%), *Streptococcus* sp. (5.5%), *Klebsiella* sp. (3%) and *Bacillus* sp. (2%).
Kurjogi and Kaliwal, (2011)	Cow	Dharwad, Karnataka	150	SCM- 83	*S. aureus* 28.10% followed by *E. coli* 21.08%, CNS 18.91%, *Streptococcal* species 15.13%, *Bacillus* species 7.56% and *Bacillus subtillis* 3.24%.
Ranjan *et al*. (2011)	Cow	Jharkhand, India	190 mastitis cases	SCM - 47.37 in rainy season and summer 42.26	*S. aureus* (27.37%), CNS spp. (12.63%), *E. coli*. (08.95%), *Pseudomonas* spp. (07.89%), *Streptococcus* spp. (05.79%)
Mohit *et al*. (2012)	Buffalo	Adhartal, Jabalpur, MP	120	SCM -40% (120/300)	--
Pankaj *et al*. (2012)	Cow (Cross bred)	Hisar, Haryana	95	SCM - 64.21(61/95)	38.66 % CPS and 29.33 % were CNS followed by *Streptococcus dysgalactiae* (22.66%)
Patel *et al*. (2012)	Cow (Cross bred)	Anand, Gujarat	10539	SCM - 46.80	*S. aureus* 30.64%, *E. coli* 10.21%,
Jeykumar *et al*. (2013)	Cow	Namakkal district, Tamil Nadu	74		32 (44.44) for *Staphylococci* species, 4 (5.5%) for *Streptococci* species, 30 (41.66%) for *E. coli* were positive.

Pankaj *et al*. (2013)	Buffalo	Hisar, Haryana	82	SCM- 29.26	15.90 % were CPS and 47.72% were CNS followed by *Streptococcus dysgalactiae* 25% *Streptococcus agalactiae* 9.09%, and *Streptococcus uberis* 2.27% and 13.63%, respectively
Charaya *et al*. (2014)	Buffalo	Hisar, Haryana	564	SCM - 56.73	*S. aureus* 140 (38.04%), *Streptococcus dysgalactiae* 112 (30.43%), *Streptococcus agalactiae* 13 (3.53%), *E. coli* 74 (20.10%) and *Corynebacterium* pyogenes 29 (7.88%).
Chandrasekaran *et al*. (2014)	Cow	Tamilnadu	401	235 Mastitis samples (56.1%.)	184 (45.89%) were positive for *E. coli* , 162 (40.4%) were positive for *S. aureus* , and 12 (2.99 %) were positive for MRSA,
Mir *et al*. (2014)	Cow (cross-bred)	Ludhiana, Patiala, Moga, Bathinda and Ferozpur districts of Punjab	218	SCM - 57.80	*Staphylococci* (41.04%), (30.60%), *Streptococci* (21.27%) and others (7.09%).
Sahu *et al*. (2014)	Cow and Buffalo	Bareilly, India	1022	SCM - 73.77 (754 quarter milk samples)	9.3 % and 14.3 % were *S. aureus* on the basis of culture and Polymerase Chain Reaction (PCR) technique respectively.
Ali *et al*. (2015)	Buffalo	UP, India	48	SCM -31.25	*S. aureus* based on growth characteristics on Mannitol salt agar, 3 (20%) and 2 (13.33%) for *E. coli* .

2.1.2 Economic losses due to mastitis:

Mastitis is the economically most important disease of dairy animals (Halasa *et al*. 2007). It is very difficult to quantify the cost of bovine SCM, however most experts accept that SCM costs the average dairy farmer more than does CM.

Zhao and Lacasse (2008) reviewed the report published by Wilson *et al*. in the year 1997 where presuming a 45 percent prevalence of SCM, the cost had been calculated at an average of $ 180 to $ 320 per case in New York and Pennsylvania. Around 70 percent of this cost was associated with a reduction in milk production.

Average decrease in milk yield due to CM and SCM was estimated to be 50 percent and 17.5 percent, respectively and the prevalence of bovine mastitis continues to affect the dairy herds throughout the world in spite of continued research activity on the problem over the century (Sadana, 2006).

In the United States, the economic loss in the dairy industry due to mastitis was about US $2 billion, and in the United Kingdom, it was £300 million annually, whereas in the Netherlands, the estimated cost varied from €114 to €182/cow per year (Viguier *et al*., 2009; Szweda *et al*., 2013)

NAAS policy paper 2013 also reviewed the reports by Sharma et. al. (2012) on the basis of several studies conducted at the United States, it showed that costs related to mastitis on dairy farms are approximately U$ 200 per cow/year. This gives an annual loss of two billion dollars for dairy industry. It is generally agreed by National Academy of Agricultural Sciences, India, that at least 70 percent of economic loss is due to reduction in milk production and discard of milk from sick animals. Other causes are the elimination of milk containing residues of antibiotics used in treating sick animals, loss of genetic stock by culling cows early and therefore more expensive replacement, veterinary fees, cost of medicines and payment of extra labour hours

Patnaik *et al.* (2013) reviewed that losses in India were about Rs.52.9 crore annually (Dhandha and Sethi., 1962) and these were reported to have increased to Rs. 6053.21 crore annually in the year 2001 (Dua, 2001). Out of this, loss of Rs. 4365.32 crore (70 percent - 80 percent loss) had been attributed to sub clinical version of udder infections According to many researchers, the prevalence of SCM was highly observed in high yielding animals. However, tremendous thrust on cross breeding programme and launching of operation flood in later years resulted in tremendous increase in high yielding bovine population, leading to many fold increase in economic loss.

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 was estimated to be Rs. 7165.51 crores in a year (Bansal and Gupta, 2009).

Apart from its economic importance it is also a matter of public health significance as mastitis reduces milk yield and alters its composition (Motwani, 2011).

In another report from India by Pdadmas (2011), the annual economic loss due to mastitis had been calculated to be Rs. 7165.51 crores; losses being almost same for cows (3649.56 crores) and buffaloes (3515.95 crores).

Motwani (2011) reported mastitis as multi factorial and a costly problem affecting all milk producing ruminants in India. Unfortunately, there is no simple solution to it. As per 2006 estimates referred in ICAR's National Agricultural Innovation Project, the estimated annual loss due to mastitis alone was nearly Rs. 16,702 millions.

Singh *et al*. (2014) studied and reported economic losses due to important diseases of bovines. Overall morbidity rates of mastitis, Hemorrhagic septicemia

and Surra were 15.5, 7.1 and 5.3, percent respectively. The losses due to mastitis per lactation in nondescript (ND), crossbred cows and buffaloes were Rs. 868.34, 1,314 and 1,272/-, respectively which was equivalent to 5,210, 36,795 and 24,175 INR during all the productive life of each animal.

Sinha and Thombare (2013) conducted a cross sectional farm sample survey to assess the consequences of mastitis on farmer's economy. Daily milk records of 187 animals from 28 farms were investigated by personal interview methods. Extra-resources used for the treatment and reduced revenues in terms of production were quantified and aggregated. The overall loss of mastitis from dairy animals was recorded Rs.1390.46 per lactation, in which 48.53 percent was from milk loss followed by veterinary expenses (36.57 percent), and additional cost of labour. Greater was the loss in crossbred due to its high production yield affected during the mastitis period. The cost of treating an animal was Rs. 508.52, including cost of medicines (31.10 percent) and services (5.47 percent).

However still very limited published data are available to quantify production losses and expenditures related to mastitis in developing countries, and thus to assess the economic impact of the disease.

2.1.3 Microorganisms most frequently associated with mastitis:

Bacteria are the most common cause of bovine mastitis. Several reports clarified that more than 137 microbes are considered as etiological agents of mastitis (Watts, 1988). The microbial causes of mastitis include a wide variety of microorganisms (aerobic and anaerobic bacteria, mycoplasma, yeasts and fungi). The most common and important microorganisms of bovine mastitis are *Streptococci, Staphylococci, E. coli* and other coliforms (Giesecke *et al*.,1994; Quinn *et al*., 1994; Radostits *et al*., 2000). The degree of importance of a specific agent, as a cause of mastitis in dairy cows, is mostly dependent on the nature of the organism, the pathogenicity of the agent, the challenge dose required to cause

infection, and is influenced by management practices. Because most pathogens involved in mastitis are ever-present, mastitis can be managed but not eradicated (Petzer, 2009).

According to Kuang *et al*. (2009) the major contagious pathogens in SCM and CM comprise *S. aureus, S. dysgalactiae, S. agalactiae* and the major environmental pathogens comprise *E. coli* and *S. uberis.*

Bovine mammary glands are exposed to different types of bacteria during lactation and in nonlactating periods. Pathogens commonly isolated from mastitic milk can be classified as noncontagiuous (are mainly environmental) and contagious pathogens. The environmental pathogens include *Streptococcus dysgalactiae, Strept. uberis, E.coli,* and CNS species, while the contagious pathogens include *S. aureus* and *Streptococcus agalactiae* (Zhao and Lacasse, 2008).

Most cases of bovine mastitis are caused by various types of bacteria, and bacteria of the genus *Staphylococcus* are one of the most frequent pathogens causing mastitis worldwide. Conventionally, the genus *Staphylococcus* is divided by the coagulase test into CNS and CPS species of which, CNS have often been considered to be minor important pathogens that cause intramammary infections (IMI). In contrast, recent studies on mastitis prevalence have revealed that CNS may be of major importance in some countries (Pyorala and Taponen, 2009).

All dairy herds have cows with SCM; however, the prevalence of infected cows varies from 5 –75 percent, and quarters from 2 –40 percent. Many different pathogens can establish a chronic infection in which clinical signs of mastitis will manifest only occasionally. The primary focus of most SCM programs is to reduce the prevalence of the contagious pathogens *S agalactiae* and *S aureus*, as well as other gram-positive cocci, most notably *Streptococcus dysgalactiae* (which may also be contagious or an environmental pathogen), *Streptococcus uberis*, enterococci, and numerous other CNS, including *Staphylococcus hyicus*, *Staphylococcus*

epidermidis, *Staphylococcus xylosus*, and *Staphylococcus intermedius*. Herds have been identified that have considerable SCM caused by gram-negative rods such as *Klebsiella* sp, *Serratia marcescens*, *Pseudomonas aeruginosa*, and other atypical pathogens such as mycotic and algal microbes (Erskine, 2014).

It appears that in developing countries, where mastitis control is not in place, contagious organism like *S. aureus, Str. agalactiae E. coli* and *Corynebacterium* are the most predominant mastitis pathogens. Before the advent of era of mastitis control (period before 1960's), these organisms were the most prevalent etiologic agents of mastitis in the currently developed countries like North America, Europe, Australia etc. (Singh *et al*., 2014)

2.1.3 A. Bovine *S. aureus* mastitis

2.1.3 A. 1. *S. aureus* identification

S. aureus belongs to the family of *Micrococcaceae* and the group of *Staphylococci*. It is a gram-positive, catalase positive, usually oxidase-negative, facultative anaerobic cocci; *S*. *aureus* can be differentiated from other *Staphylococcal* species on the basis of gold colony pigmentation **(Plate 1),** their productivity to coagulase, fermentation of mannitol, and production of heat stable thermonuclease. Variation may be found in the colony color and may range from orange to grayish or grayish white in color (Carter *et al*., 1994).

Historically, infections caused by *S. aureus* were reported first by Sir Alexander Ogston, a Scottish surgeon, more than hundred years ago. At the end of the eighteenth century *S. aureus* was reported to cause mastitis in cattle (Haveri, 2008).

2.1.3 B. Bovine *E. coli* mastitis:

2.1.3 B. 1. *E. coli* Identification:

The organisms reveal Gram-negative, pink colored with rod shaped appearance and are arranged in single or in pair and form metallic sheen on EMB agar **(Plate 2).** Confirmation can be done by biochemical tests like Sugar fermentation, Catalase positive, Methyl red and indole positive reaction.

Mastitis caused by *E. coli* is common in high-producing cows and buffaloes. The severity and outcome of *E. coli* mastitis vary between animals of the same herd and between different lactation stages in the same animal. Variation in susceptibility of cows to *E. coli* mastitis and disease severity can be caused by differences in infecting bacteria or cows' immune response. Incidence of *S. aureus* and *E. coli* was observed in subclinical and clinical cases of mastitis, by several researchers as tabulated in Table 2.1 and 2.2

2.2 Antimicrobial Resistance:

Antimicrobial resistance (AMR) is when organisms become irresponsive to the commonly used antimicrobials for which previously they were susceptible. Since the discovery of penicillin in the late 1920s, hundreds of antimicrobial agents have been developed for anti-infective therapy. Antimicrobials have become indispensable in decreasing morbidity and mortality associated with a host of infectious diseases. The emergence of AMR was not an unexpected phenomenon and was predicted by Alexander Fleming, who warned in his Nobel prize lecture in 1945 against the misuse of Penicillin. (Boerlin and White., 2013).

As per Witte (1998) a strain is classified as resistant according to microbiological definition, when it grows in the presence of higher concentration of an antimicrobial than other strains of the same species. Clinically, AMR is defined

as the ability of a microorganism to withstand the effect of a normally acquired concentration of an antimicrobial at the site of infection following standard treatment procedures.

Antimicrobial therapy is generally the most common way of treating mastitis in dairy cattle. Unfortunately, despite best possible antimicrobial treatments, bacteriological cure rates (e.g. of *S. aureus* mastitis) seldom exceed 50 percent. AMR is potentially one of the reasons for treatment failures (Barkema *et al*., 1998), hence antimicrobial susceptibility testing of udder pathogens is an important step in defining appropriate farm–level treatment protocols.

The miracle of antibiotic discovery has been threatened by the emergence of superbugs. Superbug is the terminology used to describe resistant organisms. Reygeart (2013) commented that the one of those superbugs, MRSA was causing more deaths in human per year in the United States than HIV. Superbugs also add to the healthcare costs, which is billions of dollars per year. In addition to this, the superbugs also lead to the emergence of resistance to other classes of antibiotics, such as Vancomycin, Aminoglycosides, Erythromycin, Tetracyclines and Fluoroquinolones.

Moon *et al*. (2007) reported resistance among *S. aureus* to Penicillin or Ampicillin after long term use of β-lactam in agriculture and healthcare setting in U.S.

Resistance to commonly used antimicrobials is frequently encountered with *Streptococci* species, *S. aureus* and CNS. Cure rates of *S. aureus* infections are poor after antibiotic treatment (Luthje and Schwarz, 2006).

Kardar (2005) also warned escalation of resistance problem and emergence of MDR strains that cause disease in human and animals.

Report (Anon. 2013) in WHO document**,** emphasized that, monitoring for the development of AMR in humans has been carried out since antimicrobials first became widely available, it was initially usually limited to local programmes designed to guide patient therapy. As resistance to new antimicrobials emerged, and multiple drug resistance developed and spread, the need for comprehensive surveillance systems for AMR was recognized as a public health priority throughout the world.

2.2.1 AMR surveillance programs:

The seriousness of AMR threat has prompted governments of different countries to initiate surveillance programs, which include bacteria of animal origin. These programs provide a tool to globally assess the extent of problem, to follow the evolution over time, and to evaluate the effectiveness of control measures. Such systems include “National Antimicrobial Resistance Monitoring System” (NARMS) in United States, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPRAS) in Canada and the Danish Integrated Antimicrobial Resistance Program (DANMAP) in Denmark (Boerlin and White 2013).

There are several other programs run worldwide for antimicrobial resistance and animal health monitoring as enlisted **(Appendix II).**

In India during the last decade, a large number of new initiatives have been launched by various agencies to address this problem related to human health. These include India CLEN (Indian Clinical Epidemiology Network) which has generated some quality data on AMR in pathogens like Pneumococcus, *H. influenzae* across the country; IIMAR (Indian Initiative for Management of Antibiotic Resistance) was launched in March 2008, with WHO support, by a consortium of NGOs to promote prudent use of antimicrobials, INSAR (Indian Network for Surveillance of Antimicrobial Resistance) a network of 20 laboratories in the private as well as public sector across the country to generate quality data on AMR,

organization by the ICMR of an expert group meeting in December 2009 and an Indo-Swedish workshop held at New Delhi on 2 February 2010 to discuss a joint strategy for containment of AMR. (WHO 2010). There are still gaps in reporting systems and use and availability of data at national level. In animal healthcare however, such a network program does not seem to be operational.

2.2.2 Methods to study AMR:

Very few methods are being used for antimicrobial susceptibility and resistance. Few of the methods described are as below. (Rubin *et al.*, 2011).

a. Disc diffusion (Bauer-Kirby Procedure)
b. Antimicrobial gradient method or E-test: conducted in same way as disc tests. Strips in various concentrations are placed on agar.
c. Agar dilution Method.
d. Broth Microdilution Method.
e. Broth Macrodilution Method.
f. PCR techniques: detection of genes responsible for resistance

2.2.3: Antibiogram studies for *S. aureus* and *E. coli*.

S. aureus is a major pathogen of bovine mastitis worldwide. Despite implementing intensive control measures, it is difficult to eradicate the intramammary infections caused by this pathogen and it remains a substantial economic problem. Antimicrobial therapy is one of the measures for controlling *staphylococcal* mastitis. The cure rates for *S*. *aureus* mastitis are affected by several factors, including AMR of the isolates. The determination of antimicrobial susceptibility of clinical isolates is required not only for therapy but also for monitoring the spread of resistant strains throughout the populations. Although antimicrobial susceptibility testing is a valuable tool in determining the best therapeutic choice against a mastitis pathogen, in practice it is rarely performed and

therapy decisions are usually made empirically. Therefore, susceptibility data for a large number of isolates in an area or region can be useful to veterinarians (Salmon, 2002 and Guler *et al*., 2005).

The high frequency of Penicillin and Ampicillin resistance observed in *S. aureus* is frequently related to β-lactamase producing strains and Oxacillin resistance can be explained by the low *invitro* sensitivity to β-lactam antibiotics (Corrente *et al*., 2003; Kaszanyitzky *et al*., 2004; Bernabe *et al*., 2005).

S. aureus β-lactam resistance represents a worldwide epidemiological alert because of the presence of Methicillin resistant strains (Madigan *et al*., 1999; Bernabe *et al*., 2005) that can produce severe intrahospital infections caused by MRSA strains characterized for the presence of the *mecA* gene (Seguin *et al*., 1999; Bernabe *et al*., 2005). *nuc* gene, is specifically identified in *S. aureus* (Brakstab *et al*., 1992).

Table 2.3 and 2.4 indicates global reports and records of resistance among various microorganisms in various regions.

Table 2.3: Resistance recorded in *S. aureus* from different parts of the world through antibiogram studies.

Author/ year	No of Samples	Region / country	Resistance to	Sensitivity to	Method of study	Source / disease
Martin *et al*. (1987)	1419	Chile	Lincomycin (38.9%), Amoxicillin (38.1%), Penicillin (28.8%), Ampicillin (26.0%), and Cefquinome (24.7%).	--	DDT and MIC with Microdilution method	CM
Gentilini *et al*. (2000)	206	Argentina	83 (40.3%), 24 (11.6%), 16 (7.7%) and 7 (3.4%) *S. aureus* isolates for Penicillin, Erythromycin, Pirlimycin and Gentamicin respectively.	--	DDT and MIC	Bovine Mastitis
Sabour *et al*. (2004)	288	Canada (Ontario, Québec, Prince Edward Island).	25 % or more to Penicillin, Pirlimycin, Tetracycline, Ceftiofur, Tilmicosin, Erythromycin, Penicillin-Novobiocin Combination, Cephalothin, Oxacillin, and Sulfadimethoxine.	--	Culture and DDT	Bovine Mastitis
Bernabe *et al* (2005)	22	Toluca Valley, Mexico	11 for Penicillin (50%), Ampicillin 12 (54.54%), Oxacillin 6 (27.27%) and Cephalothin 3 (13.63%).	--	DDT and PCR (*nuc* and *mecA* detection)	CM
Dhakal *et al*. (2007)	355	Nepal	--	Penicillin 60 to 80%, Ampicillin (53 to 70%)	Culture and DDT	SCM
Adwan (2006)		Palestine	Ampicillin (75.8)	--	DDT	CM cases local goats and Frisian cows

Ebrahimi *et al*. (2007).	620	central Iran	14.28% to Penicillin and 28% to Streptomycin, Oxytetracycline	--	Culture and DDT	SCM
Guler *et al*. (2005)	265	Turkey	63.3% of the strains against β-lactam antibiotics, Penicillin and Ampicillin.	--	Culture and DDT	CM
Nichita *et al* (2007),	503	Punjab, Ludhiana	Ampicillin (53.68%) Penicillin (45.13%) and Erythromycin (35.19%).	--	Culture and DDT	Raw milk samples
Akindele *et al*. (2010)	100	Nigeria	100% and 96% resistant rate obtained for Penicillin and Ampicillin	--	Culture and DDT	Human hosptial samples
Abera *et al*. (2010)	140	Adama town, Ethiopia	Penicillin (94.4%), Trimethoprim Sulfamethoxazole (58.3%) and Amoxicillin (36.1%)	--	Culture and DDT	CM and SCM
Kurjogi and Kaliwal. (2011)	150	Dharwad, Karnataka		Cloxacillin 21.15, Ampicillin 15.38, Penicillin 1.92, Carbenicillin 48.07, Cephalothin 63.46	Culture and DDT	SCM
Kaliwal *et al*. (2011)	76	Dharwad region of Karnataka.	(76.77%) Penicillin followed by Ampicillin (70.59%), Amoxicillin (63.24%), Ciprofloxacin (26.48%), Methicillin (23.53%), Cefotaxime (20.59%)	--	Culture and DDT	CM
Adesola (2012)	177	Nigeria	100% resistance for Tetracycline and Sulphadimidine, while 97.37%, 88.16%, 69.08% and 67.11% showed resistance to Ampicillin, Neomycin, Nalidixic acid and Streptomycin respectively	--	Culture and DDT	CM
Pankaj *et al*. (2012)	326	Haryana	Penicillin 88.8, Ampicillin 88.8, Cloxacillin 100, Amoxicillin 94.4, Ceftriaxone 100 , Cefoperazone 100 and Cephalexin 94.4 percent	--	Culture and DDT	SCM

Pankaj *et al*. (2012)	95 cows	Hisar	-	Penicillin (84.9) Ampicillin 56, Cloxacillin 100, Amoxicillin 96.2, Ceftriaxone 100 Cefoperazone 100, Cephalexin 100	Culture and DDT	SCM
Memon (2013) *et al*.		Eastern China	29 % to Penicillin, Oxacillin, Oxytetracycline and Chloramphenicol	--	DDT and PCR (*mecA* and *blaZ* detection)	Mastitis
Arefi *et al*. (2014)	25	Iran	Penicillin G (92%) followed by Ampicillin (73%) and Cloxacillin (68%).	--	culture and PCR	Cheese Markets of Iran,
Vishnupriya *et al*. (2014)	158	Chennai, South India	81.82% were resistant to Ampicillin, 72.78% to Amoxicillin, 63.64% to Penicillin G, 54.54% to Methicillin and Oxacillin, and 50% to Cloxacillin	CPS, 63.64% were sensitive to Ceftriaxone, 50% were sensitive to Enrofloxacin, Cloxacillin and Ciprofloxacin, and 45.54% were sensitive to Tetracycline, Methicillin and Oxacillin.	Culture and PCR	SCM
Sumathi *et al*. (2008)	75	Bangalore	--	Cephalexin (47%), Penicillin (45%),	culture	SCM
Charaya *et al*. (2014)	564	Hissar	--	Penicillin G 29.28, Ampicillin 67.85, Cloxacillin 63.57, Ceftriaxone 79.28, Amoxicillin 52.14 and Cefoperazone 81.14%	culture	SCM

Mir *et al*. (2014)	218	Ludhiana, Patiala, Moga, Bathinda and Ferozpur districts of Punjab	--	Ampicillin 67.80, Amoxicillin 74.58, Penicillin 76.27 %	culture	SCM
Mohanty *et al*. (2013)	144	Bhubaneswar Orissa	Amoxicillin 55.88 , Penicillin 82.35, Cephalexin 55.88, Cefixime 66.17, Ceftriaxone+ Tazobactam 11%	Amoxicillin 44.11, -G 0 Penicillin 17.64, Cephalexin 44, Cefixime 33.82 and Ceftriaxone + Tazobactam 88 %	Culture	Mastitis cases
Mubarack *et al*. (2012)	152	Coimbatore District, Tamilnadu, South India	Out of 152 isolates of *S. aureus* resistance was detected in 63 (41.44%), 39 (25.65%), 21(13.81%), 18 (11.84%), 6 (3.94%) and 5 (3.28%) isolates for Penicillin, Streptomycin, Erythromycin, Tetracycline, Ampicillin and Cephalothin,			SCM
Patnaik *et al*. (2014)	75	Ranchi Jharkhand	Cephalexin 5%, Ampicillin 95%		Culture and DDT	CM
Patel *et al*. (2012)	4932	Anand district, Gujrat	--	Ampicillin/ Cloxacillin 7.66 Penicillin-G 2.13	culture	CM
Gawande *et al*. (2016)	113	veterinary clinics teaching hospital, Chennai	Methicillin (92%), Penicillin (92%),	--	Culture	Raw Milk Samples

Ikiz *et al*. (2013)	270	Marmara Region of Turkey	--	Cefoperazone 24.95, Ampicillin/Sulbactam 66.66, Ampicillin 8.33, Amoxicillin Clavulanic acid 66.66, Amoxicillin 16.66, Penicillin G 75, Ceftiofur 0	Culture and DDT	SCM
Idriss *et al*., 2014	288	In Nitra, Slovakia	Amoxicillin 18.42, Amoxicillin + clavulanat 5.26, Cephalexin + Kanamycin 5.26, Ceftiofur 5.26, Cloxacillin 7.89, Penicillin 10.53	Amoxicillin 78.95, Amoxicillin + clavulanat 94.74, Cephalexin + Kanamycin 94.74 , Ceftiofur 94.74 , Cloxacillin 92.11, Penicillin 86.84	Culture and DDT	SCM
Abera *et al*., 2010	140	Adama town, Ethiopia	Amoxicillin 26.2, Penicillin 94.4	Amoxicillin 63.8 Penicillin 5.6	Culture and DDT	SCM
Chandrasekaran *et al*., 2014	235	Tamilnadu	Penicillin (63.5%) followed by Amoxicillin (61.5%), and Methicillin (52.9%).	Amoxicillin and Sulbactam (69.2%) and Ceftriaxone (69.2%t).	Culture and DDT	SCM
Szweda *et al*. (2013).	123	Poland	Amoxicillin (n=22, 17.9%), Ampicillin (n=28, 22.8%), Penicillin (n=29, 23.6%)			SCM
Cervinkova *et al*. (2013)	335		27.7% to Ampicillin			SCM

Alekish (2013) *et.al*	205	Jordan	87.00% to Penicillin and 79% to Ampicillin		Culture and DDT	CM
Najeeb, (2013) *et al.*	90	Punjab, Pakistan	(58.69%) was recorded against Penicillin		Culture and DDT	SCM
Sharma *et al*. (2015)	80	Mathura, India.	100% resistance against Penicillins followed by Vancomycin (88.89%), Cefixime, Methicillin, Novobiocin (66.67% each), Amoxyclav, Colistin (55.56% each), Ampicillin/Sulbactam, Cefalexin, Cefazolin, Cefoperazone, Enrofloxacin, Floxidin, Meropenem (33.33% each), Cefuroxime, (22.22% each).		Culture and DDT	SCM

Resistance in *Staphylococci* has been associated with the production of β-lactamases and low-affinity penicillin-binding protein, PBP2a. The presence of β-lactamases in coagulase negative *Staphylococci* (CNS) has been observed both in human and veterinary isolates. There were reported presence of β lactamases in CNS that caused mastitis in lactating dairy cows. (Archer and Scott 1991; Odd and Maeland 1997; Gentilini *et al*., 2000; Taponen *et al*., 2012). High resistance of the *S. aureus* to Ampicillin may have been due to production of β-lactamase by the organism and it has been reported that *S. aureus* are the principal Gram-positive bacteria in which β-lactamase resistance can develop very quickly (Watts and Salmon 1997; Adesola 2012).

Thaker *et al*. (2013) conducted the study in Anand city, Gujarat, with aim of isolation of *S. aureus* from milk and milk products (pedha and curd) and determined antibiogram pattern of *S. aureus* isolates. The samples (n = 160) were collected under aseptic precautions and enriched in Peptone Water (PW) followed by direct plating on selective media viz. Baird-Parker Agar. The presumptive *S. aureus* isolates were identified by biochemical tests. Antibiogram pattern of *S. aureus* was evaluated by disc diffusion method. Analysis of result revealed that 160 samples of milk (100) and milk products i.e. curd (30) and pedha (30) resulted in the isolation of 10 isolates (6.25 %) of *S. aureus* that showed highest sensitivity towards Cephalothin (100.00 %), Co-trimoxazole (100.00 %), Cephalexin (100.00 %) and Methicillin (100.00 %) followed by Gentamicin (90.00 %), Ciprofloxacin (80.00 %), Oxacillin (70.00 %), Streptomycin (60.00 %) and Ampicillin (60.00 %). The pattern clearly indicated that overall high percent of *S. aureus* isolates were resistant to Penicillin-G (100.00 %) followed by Ampicillin (40.00 %), Oxytetracycline and Oxacillin (20.00 %) and Streptomycin and Gentamicin (10.00 %)

Awandkar *et al*. (2013) monitored the antibiotic resistance trends in the clinical cases of bovine mastitis from Nanded, Latur, Osmanabad and Beed districts of Maharashtra. A total of 300 dairy animals including cross bred and indigenous cattle and buffaloes over the period of five years (2008 to 2012) suffering from CM were monitored for antibiotic resistance following standard disc diffusion method.

The gradual decrease in sensitivity was observed for Ciprofloxacin, Enrofloxacin, Streptomycin, Cefotaxime and Chloramphenicol. Higher sensitivity was recorded for combination of Ceftriaxone + Tazobactam and Amoxicillin + Sulbactam as compared to Ceftriaxone and Amoxicillin alone, respectively. The study signified the selection of proper antibiotics in therapy of mastitis. The results of their investigation demonstrated the development of resistance to frequently used antimicrobials in bovine mastitis.

Table 2.4: Resistance recorded in *E. coli* from different parts of the world through antibiogram studies.

Author/ year	No of Samples	Region / country	Resistance to	Sensitivity to	Method of study	Source / disease
Chandrasekaran *et al*. (2014)	235	Tamilnadu	Penicillin (63%) followed by Amoxicillin (52.1%), and Methicillin (45.4%).	Amoxicillin and Sulbactam (74%), and Ceftriaxone (69%).	DDT and culture	Mastitis
Idriss *et al*. (2014)	288	Nitra, Slovakia		Amoxicillin 18.00 (S) 0.00 (I) 82.00 (R) Amoxicillin + Clavulanate 94.00 (S) 4.00 (I) 2.00 (R) Cephalexin + Kanamycin 96.00 (S) 2.00 (I) , 2.00 (R) Ceftiofur 100.00, Cloxacillin 2.00 (S) 0.00 (I) 98.00 (R), Penicillin 4 (S) and 96 (R)	DDT and culture	Mastitis
Brînda *et al*. (2010)	184	Timisoara, Romania		Highest sensitivity was to Cephalothin (92%), followed by the sensitivity to Oxacillin (48%) Ampicillin (20%)	DDT and culture	Mastitis
Bakir *et al*. (2011)	50	Batna and Setif Governorates (East of Algeria)		Penicillin (100) Cefoxitin (100%)	Culture and DDT	mastitis
Ebrahimi *et al*. (2007)	180	Central Iran	Penicillin, Oxytetracycline and Streptomycin were 88.23%, 82.35% and 76.47% respectively.		Culture and DDT	
Jan *et al*. (2009)	135	Nagpur	52% Ampicillin, and Amoxicillin.		Culture and DDT	UTI (human) patients
Alekish *et al*. (2013)	205	Jordan	84.5% Ampicillin		Culture and DDT	CM
Najeeb, *et al*. (2013)	90	Punjab, Pakistan	(58.69%) Penicillin		DDT	CM

2.2.4. Gene expression in *S. aureus:*

PCR is confirmed useful tool for differentiating S. *aureus* from other *Staphylococcus* spp. strains, by the *nuc* gene identification (Brakstad, *et al*., 1992).

Brakstad *et al*. (1992) studied detection of *S. aureus* by PCR amplification of the *nuc* Gene. Synthetic Oligonucleotide primers of 21 and 24 bases, respectively, were used in the PCR to amplify a sequence of the *nuc* gene, which encodes the thermostable nuclease of *S. aureus*. A DNA fragment of approximately 270 bp was amplified from lysed S. *aureus* cells or isolated DNA. The PCR product was detected by agarose gel electrophoresis. The PCR for amplification of the *nuc* gene has potential for the rapid diagnosis of S. *aureus* infections by direct testing of clinical specimens, including specimens from patients with ongoing antimicrobial therapy.

From Thailand, Siripornmongcolchai *et al*. (2002) reported that the detection of the *mecA* gene by PCR is the gold standard for identifying MRSA PCR assays. The purpose of their study was to evaluate the presence of *mec*A gene in 100 clinical isolates of *S. aureus* using PCR with the two pairs of primers. The results were compared to the broth dilution MIC method, Oxacillin salt screening method (OSS) and Oxacillin disc agar diffusion method (ODD). A discrepancy between the *mec*A primer sets 1 and 2 was shown by 15 out of 1000 isolates. Three isolates (3%) without the *mec*A gene showed discrepancies with phenotypic methods. The sensitivity, specificity and positive and negative predictive values for the 154 and 533 bp products of *mec*A were 79, 85, 83, 81 and 94, 100, 100, 94%, respectively.

From Poland, Kuzma *et al*. (2003) examined forty milk samples from mastitic cows bacteriologically and by PCR. *S. aureus* strains were isolated from 29 samples. All the samples were subjected to PCR for the presence of *nuc* gene, which is specific for *S*. *aureus*. Crude DNA from the isolated bacteria and from milk was extracted by rapid boilling and lysis with lysostaphin and proteinase K. The expected PCR product, amplicon of 270 bp was found in all *S. aureus* isolates.

The *mecA* gene, indicates the capacity of expressing genetic resistance to Methicillin, which constitutes a risk to animals and human (Vannuffel, *et al*. 1995; Olsen *et al*. 1998 ,

Corrente *et al*., 2003; Bernabe *et al*., 2005) by establishing an important risk factor in public health from the MRSA animal origin strains increasing the risk of infection amplification to human.

Bernabe *et al*. (2005) studied the *S*. *aureus* resistance pattern in bovine mastitis milk samples using β-lactam antibiotics. All the *S*. *aureus* isolations studied were confirmed by PCR, showing *nuc* gene. In the control strains *mecA* gene was only confirmed in the field S. *aureus* 305 strain, presenting the 310 bp amlpicon. Of the six ORSA strains just one showed a band corresponding to the *mecA* gene, characterized as MRSA.

França *et al*. (2012) studied AMR of *Staphylococcus* spp. collected from small ruminant mastitis in Brazil by disc diffusion test and by detection of the presence of *mecA*, *blaZ*, *ermA*, *ermB*, *ermC* and *msrA* genes by PCR. The isolates were most resistant to Amoxicillin (50.0%), Streptomycin (42.8%), Tetracycline (40.4%), Lincomycin (39.0%) and Erythromycin (33.8%). Although phenotypic resistance to Oxacillin was observed in 12.8% of the isolates, none harbored the *mecA* gene. However, 45.7% of the isolates harbored *blaZ* indicating that β-lactamase production was the main mechanism associated with *Staphylococci* resistance to β-lactams.

Suleiman *et al*. (2012) studied the prevalence and antibiotic resistance profiles of MRSA isolated from bovine mastitic milk in Plateau State, Nigeria. From 339 quarters of 85 cows, 105 (30.9%) were found to be mastitic, where 103 (98%) *S. aureus* were isolated from the mastitic quarters. Seventy three of the isolates tested against 12 antibiotics used in the study area, showed twenty six (35.6%) to be resistant to Oxacillin and ten other antibiotics. All the 73 isolates were susceptible to Vancomycin and resistant to Penicillin. PCR was used to detect the gene *mecA* in 2(7.6%) of the 26 ORSA, and the blaZ gene in all the 26 ORSA. The MIC of Oxacillin 1µg/ml for the *mecA* positive isolates was 2.4µg/ml to ≥ 10µg/ml higher than the non–*mecA* isolates 1.2µg/ml-2.5µg/ml.

Methicillin resistance is much more commonly reported in CNS species than in S. *aureus*. Resistant CNS species have been found to carry the *mecA* gene, which is responsible for conferring Methicillin resistance (Pyorala and Taponen., 2009).

CNS species which carry the *mecA* gene could possibly be a source of Methicillin resistance through a mechanism known as horizontal gene transfer. Co-infections with CNS and *S. aureus* are common in bovine mastitis infections. If this mechanism were to occur during a co-infection with S. *aureus* and a CNS species containing the *mecA* gene, it is possible that the *S. aureus* strain could pick up this gene, resulting in the acquisition of Methicillin resistance. Horizontal gene transfer has also been implicated as the possible method by which *S*. *aureus* originally obtained the *mecA* gene when it was first described in humans (Brody *et al*., 2008).

Vanderhaeghen *et al*. (2010) commented that the MRSA has emerged as a major nosocomial pathogen in human hospitals. This problem had remained limited to hospital settings, but MRSA has been emerging rapidly in animals in recent years and represents an important example of both the spread of resistance and the links between resistance in human and animal medicine. The remarkable ability of *S. aureus* to acquire drug resistance has lead to the emergence of MRSA. This resistance is caused by an alternative penicillin-binding protein, called PBP2a. PBP2a is encoded by the *mecA* gene located in the mobile genetic element called *Staphylococcal* cassette chromosome (SCCmec) Methicillin resistance is caused by the acquisition of the *mecA* gene. This gene encodes an alternative penicillin-binding protein, called PBP2a, which has a low affinity for β-lactam antibiotics.

Vyletelova *et al*. (2011) studied occurrence and characteristics of Methicillin resistant *S. aureus* and Methicillin Resistant *Staphylococci* in raw milk manufacturing from research institute for cattle breeding ltd., Vikýřovice, Czech Republic. Total 1729 samples were examined and 634 strains were isolated. Generic identification of the *Staphylococci* isolates was done by biochemical tests and all *S*. *aureus* and CNS isolates were checked by the PCR method for the presence of *mecA* gene, which is responsible for Methicillin resistance.

MRSA includes *S*. *aureus* that have acquired a gene *mecA*, giving resistance to Methicillin and essentially to all other β-lactam antibiotics. MRSA was first reported as a nosocomial pathogen in 1961, soon after Methicillin was introduced into human medicine to treat penicillin resistant *Staphylococci* (Fizgerald *et al*., 2001).

Gandra *et al*. (2005) standardized the multiplex PCR for the identification of CPS from the standard strains of *S. aureus* (ATCC 10832, ATCC 29213 and FRI-100), *Staphylococcus*

epidermidis (ATCC 14990). *nuc1* and *nuc2* primers demonstrated specificity for *S. aureus* because only in the reactions in which DNA from this species was present the estimated 458 amplicon fragment was amplified.

Bennimath *et al*. (2011) reported amplification and sequencing of *mecA* gene from Methicillin resistant *S. aureus*. The clinical samples were collected selectively form the patients who were infected from *S. aureus* in around Bagalkot region of Karnataka state India. The amplified product of *S. aureus mecA* gene showed partial homology with *S. aureus* strain M600 penicillin binding protein 2a (*mecA*) gene, partial cds, which is available in public databases. Amplification of specific gene and sequencing of *mecA* gene gives insight into pharmaceutical aspects to design new effective drugs for treatment of Methicillin resistance S. *aureus*.

Duran *et al*. (2012) carried out study to evaluate the association between the antibiotic susceptibility patterns and the antibiotic resistance genes in *Staphylococcal* isolates obtained from various clinical samples of patients attending a teaching hospital in Hatay, Turkey. Total of 298 *Staphylococci* clinical isolates were subjected to antimicrobial susceptibility testing. The genes implicated in resistance to Oxacillin (*mecA*), Gentamicin (*aac*(*6*') /*aph*(*2*"), *aph*(*3*'-*IIIa*, *ant*(*4*')-*Ia*), Erythromycin (*ermA, ermB, ermC*, and *msrA*), Tetracycline (*tetK, tetM*), and Penicillin (*blaZ*) were amplified using multiplex PCR method. The majority of *Staphylococci* tested possessed the *blaZ* gene (89.9%).Methicillin resistance rate among 139 *S. aureus* isolates was 16.5 and 25.9 percent of *S. aureus* carried *mecA* gene. Of the 159 CoNS isolates, Methicillin resistance rate was 18.9 and 29.6 percent carried *mecA* gene

Memon *et al*. (2013) studied genotypes, virulence factors and AMR genes of *S. aureus* isolated in bovine SCM from Eastern China. MIC results showed resistance to erythromycin in all isolates. A high frequency of MRSA (29%) was observed and these isolates were also highly resistant to Penicillin, Oxacillin, Oxytetracycline and Chloramphenicol than Methicillin sensitive *S. aureus* (MSSA) isolates. Thirteen pathogenic factors and seven resistance genes including *mecA* and *blaZ* gene were checked through PCR. High resistance rate against Methicillin was found but no isolate was positive for *mecA* gene, whereas *blaZ* and *tetK* were detected in 82 and 56% isolates, respectively.

Buyukcangaz *et al*. (2013) studied the *mecA* gene prevalence in *S. aureus* Isolates from dairy cows in Turkey from milk samples of mastitic cattle by PCR. Milk samples (n:1600) from 50 different Holstein Freisian herds were evaluated by CMT and 480 CM cases were diagnosed and taken for further investigation. Out of 480 samples, 151 had been identified to harbor *S. aureus* by APIStaph® (Biomereux) identification panel and the results were evaluated by API-web system. Kirby Bauer Disc Diffusion Test was used for determination of Cephoxitin® (Oxoid) susceptibility. By PCR, 24 isolates (15.89 %) were found to carry *mecA* gene and yielded an amplification product of 154 bp. This report represents that a significant number of MRSA was found among *S. aureus* isolates in mastitis cases.

Although MRSA is mostly associated with the acquiring *mecA* gene, the role of inappropriate antibiotics use should also not be underestimated in formation of bacterial resistance and multidrug resistant strains (Chambers, 1997, Buyukcangaz *et al*., 2013).

S. *aureus* β-lactamic resistance represents a worldwide epidemiological alert because of the presence of Methicillin resistant strains (Madigan *et al*., 1999, Bernabe *et al*., 2005) that can produce severe intrahospital infections caused by MRSA strains characterized for the presence of the *mecA* gene (Seguin *et al*. 1999, Bernabe *et al*., 2005).

Frey *et al*. (2013) studied the genetic characterization of AMR in CNS from bovine mastitis milk. CNS (n = 417) were isolated from bovine mastitis cases and identified by matrix-assisted laser desorption/ionization time-offlight mass spectrometry. Resistance to Oxacillin (47.0% of the isolates), fusidic acid (33.8%), Tiamulin (31.9%), Penicillin (23.3%), Tetracycline (15.8%), Streptomycin (9.6%), Erythromycin (7.0%), Sulphonamides (5%), Trimethoprim (4.3%), Clindamycin (3.4%), Kanamycin (2.4%), and Gentamicin (2.4%) was detected. Resistance to Oxacillin was attributed to the *mecA* gene in 9.7% of the Oxacillin-resistant isolates. The remaining Oxacillin-resistant CNS did not contain the mecC gene or *mecA1* promoter mutations. Methicillin-resistant CNS isolates were diverse, as determined by *mecA* gene sequence analysis, *Staphylococcal* cassette chromosome *mec* typing, and pulsed-field gel electrophoresis. Oxacillin resistance, which is the indicator of *mecA* gene-mediated Methicillin resistance, was the most frequent resistance phenotype (47.0% of isolates).

Enany *et al*. (2013) studied the prevalence of *coagulase (coa)* gene and *mecA* gene of *S. aureus* isolated from bovine CM. A total of 78 quarter milk samples were collected from clinical cases of mastitis after the investigation of 100 lactating Friesian cows at Ismailia Governorate. By bacteriological examination the percentage of S. *aureus* in bovine CM was (28.2%). Ten isolates were subjected to PCR for detection of coagulase gene (*coa*) gene, (100%) were positive. PCR protocol used for amplification and detection of (*mecA*) gene of MRSA as a confirmatory diagnosis of their resistance to Methicillin antibiotic. All MRSA isolates were 100% positive for *mecA*.

Benhamed and Kihal (2013) reported occurrence of *mecA* genes in *S. aureus* organisms isolated from bovine mastitis. Study was conducted to investigate the phenotypic and genotypic characterization of *S. aureus* involved in dairy cow mastitis in West Algeria. A total of 141 isolates of *S. aureus* isolated from quarter milk samples were collected from dairy cows. All retained *S. aureus* species contained *gyr* gene and were identified by molecular typing. The presence of resistance was evaluated in *S. aureus*. The strains of *S. aureus* showed high level of resistance to Erythromycin, Ciprofloxacin, Penicillin and a susceptibility to Kanamycin. All strains showed also a high resistance to Tetracycline, Gentamicin and Bacitracin. *Staphylococci* AMR was assessed by detection of *mecA* gene.

Ashraf *et al*. (2014) studied the Detection of species-specific gene *nuc* in *S. aureus* from chickens by real- time PCR assay. A total of 318 samples were collected as follows: 108 sample from different farms of apparently healthy broiler & layer (108) and 210 samples from diseased layer & broiler chickens. The samples were examined bacteriologically, 164 (51.6%) *Staphylococcal* isolates were recovered from the 318 sample. 37/164 (22.6%) coagulase positive *S. aureus* were isolated. Confirmation was made by Real Time PCR for *nuc* gene as *S. aureus*. The isolated *S. aureus* was highly sensitive to Vancomycin, Amoxicillin + Clavulanic acid and Cephalothin by 84.5%, 83.8% and 78.4 respectively and highly resistant to Ampicillin, Oxacillin and Penicillin by 75.7%, 73% and 70.2% respectively.

Ahmed *et*. *al*. (2014) studied disc diffusion method versus PCR for *mecA* gene in detection of ORSA in university children's hospital in Damascus, Syria. In their study, 61 isolates of *S. aureus* were isolated from the Children's Hospital from ill children aged from one day to 13

years. Antimicrobial susceptibility testing to Oxacillin (OXA) was conducted by disc-diffusion method. Then, *mecA* genes were assayed by PCR. They showed that 21% of isolates were resistant to OXA by ODD method, while 29.5% (18% ORSA and 11.5% OSSA) of isolates possessed *mecA* gene by PCR-based *mecA* detection. Two isolates were resistant to OXA based on ODD method, although they lacked *mecA* gene.

Vishnupriya *et al*. (2014) studied Methicillin resistant *Staphylococci* associated with bovine mastitis in Tamilnadu, South India. A total of 158 milk samples from bovine mastitis cases and 126 nasal swabs from the animal handlers were sampled in and around Pondicherry. The presence of *Staphylococcal* organism was confirmed by PCR amplification using the genus specific primers and among the isolated *Staphylococci*; Methicillin resistance was identified by genetic amplification of Methicillin resistant gene *mecA*. Out of 158 mastitis milk samples; 96 and 19 bovine isolates were found to be positive for *Staphylococcal* genus specific *nuc* and Methicillin resistant (*mecA*) gene PCR, respectively.

Velasco *et al*. (2014) used the Multiplex Real-Time PCR for detection of *S. aureus* , *mecA* genes from selective enrichments from animals and retail meat. A total of 234 samples were examined (77 animal nasal swabs, 112 retail raw meat, and 45 deli meat). The multiplex real-time PCR targeted the genes: *nuc* (identification of *S. aureus*), *mecA* (associated with Methicillin resistance). Among *S. aureus* isolated *nuc* gene was observed in all the isolates whereas 32 were showing presence of *mecA*.

Medeiros *et al*. (2015) studied AMR of *Staphylococcus* spp. isolates from cases of mastitis in buffalo in Brazil. The aim of their study was to determine resistance patterns and the presence of *mecA*, *blaZ*, and efflux pump in *Staphylococcus* spp. isolated from cases of mastitis in Brazilian buffalo herds. Susceptibility to antimicrobials was determined by the disc diffusion test and detection of the *mecA* and *blaZ* genes by PCR. The percentages for resistance to the drugs tested were: 71.8% to Penicillin, 49.2% to Amoxicillin, 65.8% to Oxacillin, 62.3% to Cefquinome, 44.7% to Cephalonium, 43.2% to Cephalothin, 45.2% to Ceftriaxone and 53.7% to Cephalexin. Simultaneous resistance to four or more antimicrobial drug groups was observed in 112 isolates, using the *mecA* (11) and blaZ (79) genes.

2.2.5 ESBL and expression of resistant genes:

ESBL producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. ESBLs are enzymes capable of hydrolyzing penicillin, broad-spectrum cephalosporins and monobactams, and are generally derived from *TEM* and *SHV*-type enzymes. ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species. Although the prevalence of ESBLs is not known, it is clearly increasing, and in many parts of the world 10–40% of strains of *E. coli* and *Klebsiella pneumoniae* expresses ESBLs. ESBL-producing *Enterobacteriacae* have been responsible for numerous outbreaks of infection throughout the world and pose challenging infection control issues. Clinical outcome data indicate that ESBLs are clinically significant and, when detected, indicate the need for the use of appropriate antibacterial agents. Unfortunately, the laboratory detection of ESBLs can be complex and, at times, misleading. Antibacterial choice is often complicated by multi-resistance. Many ESBL producing organisms also express Amp β-lactamases and may be co-transferred with plasmids mediating Aminoglycosides resistance. In addition, there is an increasing association between ESBL production and Fluoroquinolones resistance. (Rupp and Fey, 2003)

Production of ESBLs, specifically by *E. coli*, has caused a major concern in several countries, being frequently implicated in human infections. In the last few years, ESBL-containing *E. coli* strains have also been found and reported in healthy animals**.** (Brinas *et al*., 2005; Costa *et al*., 2006).

Sikarwar and Gopal (2015) took a review of *E. coli* resistance to β-lactam group of antimicrobials. As per their review, the most commonly prescribed drugs for *E.coli* infection are β-lactam antibiotics. There are some *E.coli* strains that become resistant to antibiotic drugs. Bacteria resist by producing β-lactamase to avoid antibiotic mechanisms of action. Extended-spectrum ß-lactamases (ESBLs) are a group of these ß-lactamase enzymes that have the ability to inactivate ß-lactam antibiotics such as Cephalosporin, Monobactams, Penicillins and Carbapenems. Most of them also have the ability to hydrolyze fourth-generation Cephalosporins such as Cefepime and Cefpirome. As a result, bacteria that produce ESBLs become resistant to most of the ß-lactam antibiotics.

ESBLs do not active against Cephamycin and Carbapenems. There are two most common types of ESBLs found in *E.coli*; *TEM* and *SHV* enzyme types. ESBL-producing *E.coli* creates a lot of challenges to doctors, microbiologists, clinicians, scientists and other healthcare providers because of their dynamic evolution and epidemiology, therapeutic limitation, challenges in diagnostic and their prevention and infection control measures.

E. coli is commonly found in the environment and also observed in many diseased or healthy human and animals. Many a times resistant *E. coli* strains showed presence of β-lactamase enzymes and the gold standard to identify this is the PCR technique.

Amaya *et al*. (2011) studied the AMR of intestinal *E. coli* isolates from children. Their study was aimed to determine the AMR pattern in a collection of 727 intestinal *E. coli* isolates from the faeces of children in Leon, Nicaragua, from central America, between March 2005 and September 2006. All samples had been screened previously for the presence of diarrhoeagenic *E. coli* (DEC) by multiplex PCR. In general, AMR among the 727 intestinal *E. coli* isolates was high for Ampicillin (60%), Trimethoprim–Sulfamethoxazole (64%) and Chloramphenicol (11%). Resistance to Ceftazidime and/or Ceftriaxone and a pattern of multiresistance was related to *CTX-M-5*- or *CTX-M-15*-producing *E. coli* isolates when subjected to PCR studies.

Brinas *et al*. (2005) studied β-lactamases in Ampicillin-resistant *E. coli* isolates which were recovered from foods of animal origin (*n*= 20), feces of humans (*n* = 49) and healthy animals (*n* = 55). All the isolates were subjected for antimicrobial sensitivity tests against β-lactam antimicrobials and showed resistance for Ampicillin, Cefazolin, Cefoxitin, Cefotaxime, and Ceftriaxone. All (124) were Isolates were showing resistance for Ampicillin. PCR showed that 103 isolates were positive for *TEM* and negative for SHV and OXA. Three *E. coli* isolates showed a positive reaction for OXA, and one showed a positive reaction for SHV.

Kaur *et al*. (2013) reported occurrence of the *CTX-M, SHV* and the *TEM* genes among the ESBL producing isolates of *Enterobacteriacae* in the Hospital of North India (Ludhiana, Punjab). Clinical isolates from urine (344), pus (109), blood (15), IV/ central line tip (10), sputum (12) and body fluid (10) specimens were processed. The organisms which were identified, included *E. coli* (351), *K. pneumoniae* (74), *K. Oxytoca* (21), *Proteus mirabilis* (15), Proteus *vulgaris* (9), Enterobacter *spp* (15) and *Citrobacter spp* (15). Antimicrobial susceptibility testing

was done and the ESBL detection was carried out for all the isolates by the CLSI confirmatory method. A majority of *E.coli* in the study possessed the *CTX-M* genes (59.32%).

Sharma *et al*. (2010) reported detection of *TEM* & *SHV* genes in *E. coli* & *Klebsiella pneumoniae* isolates in a tertiary care hospital from India. PCR carried out on plasmid DNA alone detected 30 percent ESBL positive isolates using *TEM* primer and 38 percent using SHV primer, whereas PCR for both plasmid and chromosomal DNA showed 56 percent positivity for *TEM* and 60 percent positivity for SHV.

Zaniani *et al*. (2012) reported the prevalence of *TEM* and *SHV* genes among extended-spectrum β-lactamases producing *E. coli* (82) and *Klebsiella pneumoniae (78)* isolated from out-patients and hospitalized patients and they were examined by Oxoid combination disc test and PCR methods. They found that 43.9% of *E. coli* and 56.1% of *K. pneumoniae* produced ESBLs. The frequency of SHV and *TEM* among the ESBLs producing isolates were 14.4% and 20.6%, respectively.

Geser *et al*. (2012) reported occurrence and characteristics of ESBL producing *Enterobacteriaceae* in food producing animals, minced meat and raw mastitis milk. *E. coli* isolates (67) from cattle *E. coli* mastitis were analyzed. As many as 8.6% samples yielded ESBL producers after an enrichment step. Of the total of 91 isolates, 89 were *E. coli*. PCR analysis revealed that 78 isolates (85.7%) produced *CTX-M* ESBLs while six isolates (6.6%) produced *CTX-M.* Five detected ESBLs (5.5%) belonged to the SHV group and two isolates (2.2%) contained a *TEM*-type. A total of 27 *CTX-M* producers were additionally PCR-positive for *TEM*-β-lactamase.

Yaochi *et al*. (2014) studied ESBL producing *E. coli* from the milk of cows with CM in Southern Taiwan. A total of 57 *E. coli* isolates were characterized genetically and biochemically. ESBL genes and variations in virulence genes and genomes of these *E. coli* isolates were investigated by the antimicrobial susceptibility test, simplex and multiplex PCR. All *E. coli* isolates were resistant to Cloxacillin (100%) and to a lesser extent (50%) to Tetracycline, Neomycin, Gentamicin, Ampicillin, Ceftriaxone, Cefotaxime and Ceftazidime. The predominant bla gene was *blaTEM*, followed by *blaCMY*, *blaCTX*, *blaSHV*, and *blaDHA* among the six (10.5%) ESBL-producing *E. coli* carrying *blaCTX-M15, blaCTX-M55,* or *blaCTX-M14*.

Bourjilat *et al*. (2011) studied the prevalence of ESBL-producing *E. coli* in a community, to analyze the relationship between strains studied, and to characterize the ESBL genes involved in resistance in Casablanca, Morocco. ESBL production was detected by the double disc synergy test. Genes encoding ESBLs *(blaTEM, blaCTM, blaSHV*) were identified by PCR and DNA sequencing. Seven ESBL-producing *E. coli* were identified among the 535 *E. coli* isolates. Most of them expressed a *CTX-M* enzyme (6/7) with a predominance of *CTX-M-15* (6/6). Two strains possessed *TEM* in combination with *CTX-M-15* or *SHV-5.*

Wu *et al*. (1994) studied effect of hyperproduction of *TEM-1* 1-Lactamase on in-vitro Susceptibility of *E. coli* to three lactam antibiotics. The susceptibility of 173 *TEM-1* producing isolates of *E. coli* was assessed by determination of MICs by the agar dilution method. MICs of Amoxicillin, Mezlocillin, Cephaloridine, and, to a smaller extent, Amoxicillin-Clavulanic acid were higher for isolates that produced large amounts of β-lactamase than for isolates that produced smaller amounts. Higher concentrations of Clavulanic acid had progressively greater effects on Amoxicillin MICs, but even at 8 µg/ml some of the isolates with high β-lactamase activities remained resistant or only moderately susceptible to Amoxicillin. All the isolates were inhibited by Clavulanic acid (in the absence of Amoxicillin) at concentrations of 16 to 32 µg/ml. *TEM-1* β-lactamase activity was inhibited *in-vitro* by Clavulanic acid, but not totally, with approximately 2% of the initial activity remaining at 2 µg/ml and 0.4% remaining at 8 µg/ml. These findings suggested that the amount of β-lactamase activity is a major determinant of the degree of resistance to several β-lactam antibiotics and can make the difference between susceptibility and resistance to some compounds, notably the combination of Amoxicillin with Clavulanic acid.

Hassan *et al*. (2010) conducted the study to determine the antimicrobial resistant genes and some virulence factors produced by clinical isolates of *E. coli* and *S. pyogenes* isolated from different sources. A total of 60 *E. coli* and 42 *S. pyogenes* were studied for antibiotic susceptibility pattern, resistance genes and different resistance enzymes production. Nearly 79.4 % of the isolates were multiple resistant as they were resistant to all antimicrobial classes used in the study. All *E. coli* isolates and only two *S. pyogenes* were β-lactamase producers. Detection of *bla-TEM* gene revealed that it was amplified on plasmid DNA of 23 isolates out of 25 *E. coli* isolates. *bla-TEM* gene was amplified on plasmid DNA of 23 out of 25 β-lactam resistant *E. coli i*solates.

Alipourfard and Nili (2010) studied the susceptibility of different antimicrobials to ESBL producing *E. coli* and *K. pneumoniae* isolated from wound swabs, blood, urine, fluid, tracheal aspirates and sputum in Shahid Bahonar Hospital of Tehran from July, 2007 to June, 2008. A total of 115 ESBL producing isolates were obtained from outdoor and indoor patients. Out of 115 isolates, 60% were *E. coli* and 40% were *K. pneumoniae*. All ESBL-producing isolates were confirmed using the CLSI approved double-disc diffusion method. Urine (70.4%) was the main source of ESBL-producing isolates from all patients, followed by blood (16.5%). All isolates were susceptible to both Imipenam and 100% resistant to Meropenem. Aztreonam, Ampicillin, Co-amoxyclav and Ampicillin/Sulbactam. This study showed that the frequency of ESBL producing strains of *E*. *coli* and *K. pneumoniae* was high in both hospital and community levels.

Hamdoon (2011) studied ESBL in *E. coli* from clinical samples for the detection of β - lactamase production, ESBL activity was tested. The double disc synergy test was performed using Ceftriaxone and a combination disc of Amoxicillin 20 µg and Clavulanic acid 10 µg. Out of the total 136 *E. coli* isolates, 58.82% were found to be ESBL producers. The most effective antimicrobial agent against the isolates was amikacin (85%), followed by Ciprofloxacin (67.6%), while all the isolates were fully resistant to Penicillin, Cepharadine, Cephalothin and Carbenicillin. Multi-drug resistance (MDR) was found to be more among the ESBL producers.

Gundogan and Avci (2013) studied the prevalence and antibiotic resistance of extended ESBL producing *E. coli* (45) and *Klebsiella* species isolated from foods (chicken drumsticks and minced meat, raw milk, white cheese and ice cream samples) of animal origin in Turkey. The double-disc synergy test was used to determine ESBL production. ESBL production was detected in 20 of 45 *E. coli* (44.4%). Resistance of all isolates to 14 antibiotics was determined by the disc diffusion method. All isolates showed resistance to Ampicillin but none exhibited resistance to Imipenam, Cefepime and Piperacillin/Tazobactam. *E. coli* spp. isolates had also been found resistant to Cefotaxime, Ceftazidime, Ceftriaxone, Aztreonam, Tetracycline and Ciprofloxacin. All isolates were resistant to two or more antimicrobial agents.

Timofte *et al*. (2014) did molecular characterization of *E. coli CTX-M-15* β-lactamases from bovine mastitis isolates in the United Kingdom. Total six isolates from the cases of CM, where response to β- lactam drugs was poor, were subjected to disc diffusion tests with Penicillin G, Amoxicillin-Clavulanic acid, Ceftiofur, and Cefquinome. After phenotypic

confirmation of ESBL, MIC was studied which showed reduced MICs to Cefotaxime and Ceftazidime by more than threefold concentrations when used alongwith Clavulanic acid. PCR studies evaluated the presence of *TEM* and *SHV* genes in all the six isolates.

Ilyas *et al*. (2014) carried out study in Peshawar, Pakistan, in order to isolate and identify the ESBLs producing *E. coli* in the urine samples. A total of 195 urine samples were collected from the UTI suspected patients admitted at Lady Reading hospital, a tertiary care unit in Peshawar, Khyber Pakhtoonkhawa (KPK), Pakistan. The isolated *E. coli* were screened for ESBLs production by double disc synergy method. Out of 68 positive samples 58 (85.2%) were *E. coli.* Thirty eight out of 58 *E. coli* samples were identified as ESBLs producers and MDR strains.

Costa *et al*. (2006) reported the detection of *E. coli* harboring extended-spectrum β-lactamases of the *CTX-M*, *TEM* and *SHV* classes in faecal samples of wild animals in Portugal. After microbiological confirmation of the 72 *E.coli* isolates from the faecal samples those were subjected to sensitivity tests and detection of ESBLs by using antimicrobial discs (Ampicillin, Amoxicillin + Clavulanic acid, Cefoxitin, Ceftazidime, Cefotaxime, Aztreonam, Imipenam, Gentamicin, Amikacin, Tobramycin, Streptomycin, Nalidixic acid, Ciprofloxacin, Sulfamethoxazole/Trimethoprim, Tetracycline and Chloramphenicol). More than 75% isolates showing β-lactam resistance confirmed the presence of *TEM, SHV* and *CTX* genes which expressed the ESBLs

Chapter 3

Materials & Methods

MATERIAL AND METHODS:

3.1 Materials:

3.1.1 Study area and population:

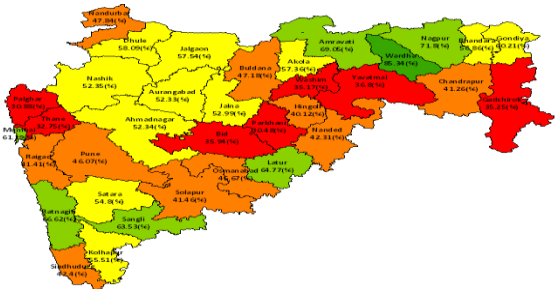
Study population included in the present work was lactating buffaloes from various farms located in and around Mumbai city **(Fig -1).** The present research work was carried out on 750 lactating buffaloes from different stables of Mumbai and Palghar Districts of Maharashtra during July, 2014 to May, 2016. The laboratory works were performed at the Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai 400012. The buffaloes were reared under intensive husbandry system with raised floor. They were often provided with some green and dry grass in addition to concentrate diet and kept together in common shed with clean drinking water supply.

3.1.2 Sampling from the study population

The milk samples were collected after screening for SCM using California mastitis test (CMT). Composite milk samples stripped from all the lactating quarters, considered as single sample from each animal was tested and quarter wise prevalence was not considered.

3.1.3 Glasswares:

All the glasswares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plates, slides, vials and agglutination test tubes were soaked in a household dishwashing detergent solution overnight, contaminated glassware was disinfected in 2% sodium hypochlorite solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven overnight at 50°C.



Safale, Dahisar, Palghar

Unit 19, Maharaja Dairy Goregaon

Unit 21, Maharaja Dairy Goregaon

3.1.4 Dehydrated microbiological Media

All the dehydrated microbiological media such as Mannitol Salt agar base, Eosin methylene blue (EMB) agar media, Mueller-Hinton agar media, Nutrient broth, Dextrose broth, Lactose broth, Mannitol broth, Sucrose broth used under study were procured from Himedia, India.

3.1.5 Reagents

The chemicals and reagents used during the study were: Di-sodium hydrogen phosphate (0.2M $Na_2HPO_4.12H_2O$) , Potassium di-hydrogen phosphate (0.2M KH_2PO_4 . $2H_2O$), Dehydrated sodium citrate , Phosphate Buffered Saline (PBS), Physiological Saline Solution (PSS), Methyl Red (MR), Voges-Proskauer (VP), Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents were used as required during the experiment.

3.1.6 Antimicrobial discs:

Discs of different concentrations (as per CLSI guidelines) of β-lactam group of antimicrobials were procured from Hi Media, Mumbai.

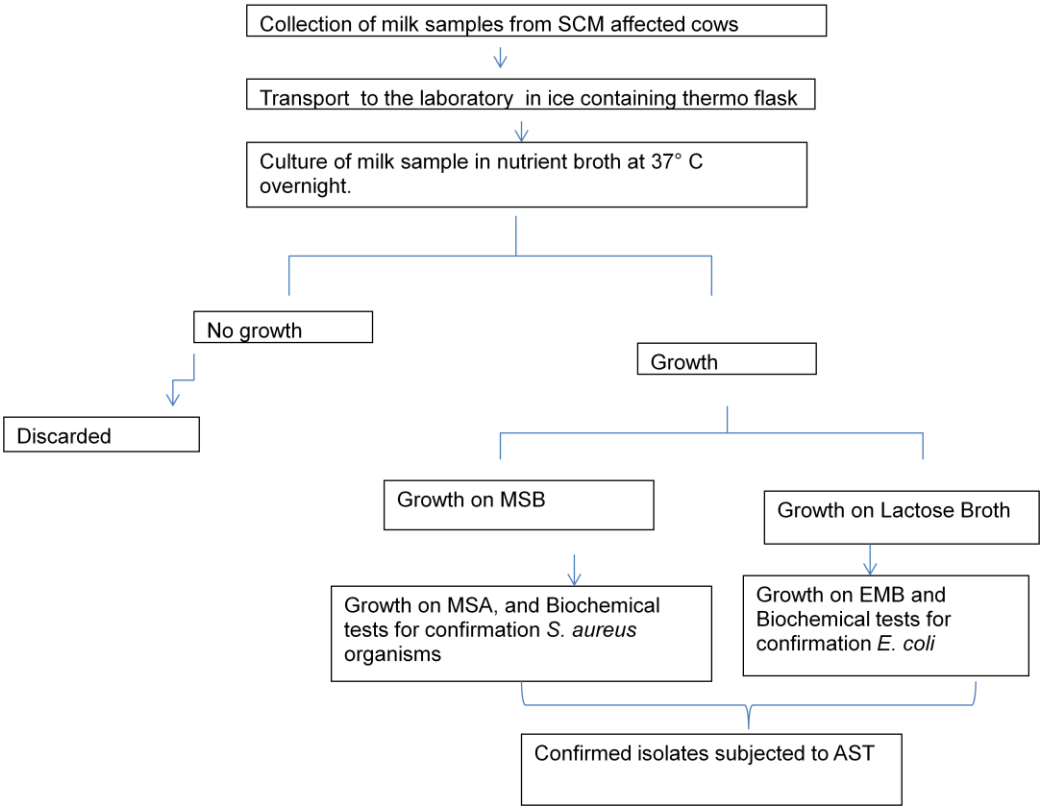
3.2 Methods

3.2.1 Experimental design (Fig. 3.2)

The experimental work was divided into following steps.

1. Screening of buffaloes for SCM
2. Collection of CMT positive milk samples.
3. Isolation and identification of the *S. aureus* and *E. coli*.
4. Determination of antibacterial sensitivity of *S. aureus* and *E. coli* against β-lactam antibiotics.
5. Determination of MIC of β-lactam antibiotics against *S. aureus* and *E. coli*.
6. Amplification of *nuc*, *mecA*, *SHV*, *TEM* and *CTX-M* gene.
7. Result analysis.

Fig 3.2: Experimental Design



3.2.2. Biowaste Disposal:

All the used and unused media and cultures were wrapped in autoclavable yellow bags and autoclaved at 121^{o}C for 15 minutes which were then submitted to the biowaste disposal agency of BMC Mumbai.

3.2.3 Collection of milk samples:

The milk samples were collected from the 750 lactating buffaloes from various farms located in Mumbai and Palghar region. They were examined for SCM using CMT. After CMT screening total 310 positive milk samples out of 750 milking animals were collected and transported on ice to the Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai, for further analysis.

3.2.4 Principle of CMT:

CMT reagent when mixed with milk reacts with leucocytes (white blood cells) that are usually present in large numbers when an infection occurs. When this reaction occurs, the reagent-milk mixture thickens or gels in proportion to the number of leucocytes present and indicates the severity of the inflammation. The greater the reaction, the higher the CMT scores.

3.2.5. Preparation of CMT reagent:

CMT reagent was prepared using sodium hydroxide 15g, teepol 5 ml, bromo thymol blue 0.1 g and finally added distilled water up to 1000 ml. Every time fresh CMT reagent was used for testing SCM.

3.2.6 CMT Procedure:

Milk samples from each animal were collected in a clean CMT paddle. The CMT paddle has four shallow cups marked A, B, C, and D to help identify the individual quarter from which the milk is obtained. From each animal quarter 5ml milk was poured in respective cup. An equal

amount of CMT solution was added to each cup. CMT paddle was then gently rotated in a circular motion to thoroughly mix the contents. Mixing was done for not more than 10 seconds. Test results were read quickly as visible reaction disintegrates after about 20 seconds. The reaction was visually scored. The more the gel formation, the higher is the score.

Table 3.1: Score data for CMT

Sr. No.	Mastitis score	Reaction type
1	T – Trace	Slight thickening
2	1 – Weak positive	Distinct thickening but no gel formation
3	2– Distinct positive	Immediate thickening with slight gel formation
4	3 – Strong positive	Gel formation with elevated surface

Samples from distinct positive and strong positive reactions were considered for further analysis.

3.2.7. **Microbiological analysis**:

3.2.4.1 Isolation and identification of *S. aureus*

3.2.7.1 A. Enrichment:

All the milk samples (1.5ml) each were inoculated in 13.5 ml of enrichment broth (Mannitol salt broth) separately and incubated at 37^{o}C for 48 hours.

3.2.7.1 B. Inoculation on selective media:

3.2.7.1 B.1. Mannitol Salt agar preparation:

One hundred and eleven grams of dehydrated Mannitol Salt agar base (HI- MEDIA, India) powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the

medium completely. The medium was then sterilized by autoclaving at 121°C and 15lbs pressure for 15 minutes. After autoclaving the medium was put into water bath at 45-50°C to reduce the temperature.

3.2.7.1 B.2.Inoculation of *S. aureus* :

About 10-15ml of sterilized Mannitol salt agar was poured in sterile glass petridishes to form thick uniform layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed and kept in sterile area. Suspension culture from enrichment broth (Mannitol salt broth) was streaked on these plates and incubated at 37°C overnight. The sterility of the medium was checked by incubating at 37°C overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.7.1 B.3.Steps followed for isolation of *S. aureus:*

1. Inoculums were picked up with a sterile inoculating loop and were spread on an area of the medium in the petridishes.
2. The loop was sterilized by heating red hot in a flame.
3. The inoculums were spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single direction in each parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.2.7.1. C Identification of *S. aureus* on Mannitol Salt Agar (MSA):

MSA is a selective media used for isolation of pathogenic *Staphylococci.* It contains Proteose peptone as a nitrogen source, Beef extract as a source of growth factors, nutrients and trace elements and Mannitol as a source of carbon. The differential nature of the media is due to

Phenol red indicator, which detects a change in pH due to the fermentation of Mannitol, by some strains of *Staphylococci. S. aureus* ferments Mannitol to produce yellow colonies surrounded by a yellow zone **(Plate 1).** Coagulase-negative strains of *S. aureus* and *Staphylococcus epidermidis* which are usually non-fermenters produce pink-red colonies. The selectivity of the media towards *Staphylococci* is due to the high concentration of sodium chloride.

3.2.7.1. D. Morphological characters:

Incubation of materials from nutrient broth into mannitol salt agar plates which after inoculation, if positive for *Staphylococcus*, were indicated by the growth of circular, small, smooth, convex and gray white or yellowish colonies. The red or pinkish red color of the mannitol salt agar media was discolored into yellow color which indicated the growth and presence of *S. aureus*.

3.2.7.1 E. Staining methods

Gram's staining method was used to study the morphological and staining characteristics of bacteria and to provide information about the presumptive bacterial identification as per recommendation of Merchant and Packer (1969). The procedure was as follows:

A small colony was picked up from MSA plates with a bacteriological loop, smeared on separate glass slide and fixed by gentle heating. Crystal violet was then applied on each smear to stain for two minutes and then washed with running water. Few drops of Gram's iodine was then added to act as a mordant for one minute and then again washed with running water. Acetone alcohol was then added (acts as decolorizer) for few seconds. After washing with water, safranin was added as counter stain and allowed to stain for two minutes. The slides were then washed with running water, blotted and dried in air and then examined under microscope with high power objects (100x) using oil immersion lens.

3.2.7.1. F. Interpretation:

Gram's staining was performed to determine the size, shape, and arrangement of bacteria according to the methods described by Cowan and Steel (1970). If the organism found as Gram-positive, cocci, arranged in cluster, it indicated *Staphylococcus* spp.

3.2.7.1 G. Biochemical test:

The presumptive *S. aureus* colonies were subjected to Gram's staining. The organisms with grape like clusters of Gram-positive cocci were inoculated into Brain Heart infusion (BHI) broth and overnight incubated at 37˚C. After purification, the isolates were subjected for biochemical and molecular characterization. The biochemical characterization was carried out as per the method described by Agarwal *et al*. (2003) **(Table 3.2).** Details of chemicals and reagents used for biochemical tests are given in Appendix – I. Biochemical test were performed as described below.

Table 3. 2. Biochemical characteristics of *S. aureus*

Sr. No.	Biochemical characteristics	Reaction
1	Grams Reaction	+
2	Catalase	+
3	Oxidase	-
4	Indole	+
5	Methyl Red	+
6	Voges Proskauer	+
7	Nitrate Reduction	+
8	Glucose	+
9	Lactose	+
10	Sucrose	+

3.2.7.1 G. i. Catalase test

The presumptive *S. aureus* isolates were tested for their catalase activity as per the method of Feldsine *et al.* (2002). A drop of 3% hydrogen peroxide (H_2O_2) was taken on a clear glass slide and the single *S. aureus* colony picked up with sterile tooth pick was placed into it. It was critically observed for formation of gas bubbles, colonies showing bubble formation were considered positive for catalase.

3.2.7.1 G. ii. Oxidase test

The test was performed as per the methods of Cruickshank *et al.* (1975). The strip of filter paper was soaked in freshly prepared solution of one percent tetramethyl-p-phenylene-diamine dye (HiMedia). The presumptive colony was taken with the help of sterile glass rod and rubbed on filter paper. The development of deep purple colour within 5-10 seconds was considered as a strong oxidase positive reaction, while no coloration even after 60 seconds was considered as oxidase negative test.

3.2.7.1 G. iii. Methyl Red test

The test was performed as per the methods of Cruickshank *et al.* (1975). Briefly, each of the presumptive *S. aureus* isolates was inoculated with 0.5 ml sterile glucose phosphate broth and overnight incubated at 37˚C. Then, one drop of methyl red (MR) solution was added and observed for development of color. Bright red color formation was considered as MR positive.

3.2.7.1 G. iv. Voges-Proskauer test

This test was also performed as per the methods of Cruickshank *et al.* (1975). Single presumptive colony of *S. aureus* was inoculated in 2 ml glucose phosphate peptone water and incubated at 37^0C for 24 h. Thereafter, 0.2 ml of 40% Potassium hydroxide (KOH) solution followed by 0.6 ml 5% α -naphthol solution was added and shaken vigorously with aeration. The cotton plug was removed and the tube left at room temperature for 1 h. The development of pink red color in the solution indicates VP- positive test.

3.2.7.1 G. v. Indole test

This test was performed as per method described earlier (Agrawal *et al*., 2003).Indole production is detected by Kovac's reagent. Loopful culture of *S. aureus* inoculated in 3 ml sterile tryptone water and incubated at 35-37°C for 48 hr. After incubation 0.5 ml of Kovac's reagent added in that and examined for a red color in the surface layer within 10 min. Kovac's reagent contains p- dimethylaminobenzaldehyde. This react with the indole to produce a red coloured compound. The development of red color at the surface layer indicates Indole positive test.

3.2.7.1 G. vi. Sugar fermentation test:

Preparation of Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto-peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 minutes and pH adjusted to 7.0, cooled and then filtered through filter paper. Phenol red indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation tube and placed inverted. These were then sterilized by autoclaving at 1.2 kg/cm^2 and 121°C for 15 minutes. The sugars used for fermentation were prepared separately by 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in by autoclaving at 100°C for 30 minutes. The volume of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at 37°C for 24 hours.

Sugar fermentation test was performed to identify *Staphylococcus* spp. For sugar fermentation tests, the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose, and mannitol were inoculated with a loopful of broth culture of the isolated organisms and incubated at 37°C for 18 hours. The isolates if positive, ferment five sugars viz. dextrose, maltose, lactose, sucrose, and mannitol, the organism produces acid in all cases and changes color of media from reddish to yellowish. Acid production was indicated by the change of the color reddish to yellowish in the medium.

3.2.7.2. **Isolation and Identification of *E. coli:***

3.2.7.2. A. Enrichment:

Milk sample 1.5ml of each was inoculated separately in 13.5 ml of enrichment (lactose) broth and incubated for 48 hours at 37^{o}C.

3.2.7.2. B. inoculation on Selective Media:

Preparation of Eosin Methylene Blue agar:

Thirty six grams of EMB agar base (HI-MEDIA, India) was added to 1000 ml of water in a flask and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 121°C and 15 lbs pressure for 15 minutes and cooled down to 50°C. The medium was shaken in order to oxidize the methylene blue (i.e. to restore its blue color). Then 10 ml of medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37°C overnight to check their sterility. The petridishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use.

EMB as a Selective Media:

EMB agar is a selective medium used for isolation of *Escherichia coli, Enterobacter aerogenes* etc. It contains peptic digest of animal tissue which serves as source of carbon, nitrogen, and other essential growth nutrients. Phosphate buffers the medium. Lactose and sucrose are the sources of energy by being fermentable carbohydrates. Sucrose is added to the medium as an alternative carbohydrate source for typically lactose-fermenting, gram-negative bacilli, which on occasion do not ferment lactose or do so slowly. Methylene blue and Eosin-Y inhibit gram-positive bacteria to a limited degree. These dyes serve as differential indicators in response to the fermentation of carbohydrates. The ratio of eosin and methylene blue was adjusted approximately to 6:1.

Inoculation:

A loopful of culture from lactose broth was streaked on Eosin Methylene Blue (EMB) Agar. The plates were incubated for 24 hours at 37^{o}C. The coliforms produce purplish black colonies due to taking up of methylene blue-eosin dye complex, when the pH drops. The dye complex is absorbed into the colony. Non-fermenters probably raise the pH of surrounding medium by oxidative deamination of protein, which solubilizes the methylene blue-eosin complex resulting in colourless colonies. **(Plate 2)**

3.2.7.2. C. Identification of *E. coli.* of on EMB agar

Inoculums from lactose broth were inoculated into EMB agar plates which after incubation, will show smooth, circular colonies with dark centers and metallic sheen if *E. coli.*

3.2.7.2. D Gram's staining method:

Gram Staining was performed as per the method described in the **section 3.2.7.1 D** *E. coli* will reveal Gram negative, pink color, large rod shape appearance, arranged in single or paired.

3.2.7.2. E. Biochemical test

For confirmation of *E. coli* isolates biochemical tests such as Catalase test, Methyl red test, VP test, Indole test and sugar fermentation tests were performed as described in the preceding sections of *S. aureus* (3.2.7.1.G.i. to G.vi.).

3.2.7.3. Maintenance of stock culture

Sterile buffered glycerine (20%) method was used for the maintenance stock culture for each of the bacterial isolates. Twenty percent of pure glycerine and 80 percent of PBS were

mixed to make 20% sterile buffered glycerine. A loopful of thick bacterial culture was mixed with 20% sterile buffered glycerine in eppendorf tubes and preserved at -20° C. By this method bacteria can be preserved with no deviation of their original character for one year (Buxton and Fraser, 1977).

3.2.7.4. Antibiotic sensitivity test

3.2.7.4. A. Preparation of Mueller-Hinton agar

Thirty-eight grams of dehydrated Mueller-Hinton agar medium was suspended in 1000 ml distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121°C and 15 lbs pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45°C in a water bath and distributed to sterile petridishes. After solidification, petridishes were placed in an incubator for 24 hours at 37°C to check sterility and then placed in a refrigerator at 4°C until use.

3.2.7.4. B. Antimicrobial discs

To determine the drug sensitivity of different bacterial isolates with different types of antimicrobials; commercially available antimicrobial discs (Himedia Mumbai.) were used. The antimicrobial discs were applied to the plates within 15 minutes after inoculation. The discs were placed individually with sterile forceps (12 discs on a 150-mm plate) and then gently pressed down onto the agar. This prevented overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disc began immediately; therefore, once a disc contacted the agar surface, the disc was not moved. The following antibiotics were tested against the selected organisms with their disc concentration. The concentration of antibiotic contained in the disc is also indicated in following table **(Table 3.3).**

Table 3.3. Antimicrobial agents with their disc concentration

Sr. No.	Antimicrobial Disc Used	Concentration(µg/disc)	Abbreviation
1	Amoxicillin + Clavulanic acid	20/10	AMC
2	Cefepime	30	CPM
3	Ceftazidime	30	CAZ
4	Oxacillin	05	OX
5	Ampicillin + Sulbactam	10/10	A/S
6	Methicillin	5	MET
7	Ceftazidime + Clavulanic acid	30/10	CAC
8	Aztreonam	50	AT
9	Cefotaxime	30	CTX
10	Cefotaxime + Clavulanic acid	30/10	CEC
11	Cefepime + Clavulanic acid	30/10	CFC
12	Ampicillin	10	AMP
13	Amoxicillin	30	AMX

3.2.7.4. C. Antimicrobial susceptibility testing

Susceptibility or resistance of different antibiotics was measured *in-vitro* by employing the Kirby-Bauer method (Bauer *et al*., 1966). This method allowed for rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc.

A suspension of test organism was prepared in lactose broth by overnight culture for 24 hours at 37°C. Sterile glass spreader was used to spread the culture homogenously on the Mueller-Hinton agar media. Antibiotic discs were applied aseptically to the surface of the inoculated plates at an appropriate place. The plates were then inverted and incubated at 37°C

for 24 hours. After incubation, they were examined and the diameters of the zone of complete inhibition were observed.

The disc diffusion method was used to test antimicrobial susceptibility assay according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009). The steps of disc diffusion method are mentioned below.

1. The top of the colony was touched with a loop and the growth was transferred into nutrient broth at 37°C for overnight.

2. The solution was inoculated into Mueller-Hinton agar.

3. The antibiotic discs were placed onto Mueller-Hinton agar and incubated at 37°C for 24 hours and the plates were examined.

4. The diameter of zones was measured in mm including the diameter of the disc using meter ruler according to the guidelines of CLSI (2009).

3.2.7.4. D. Recording and interpreting results

After the discs were placed on the plates, the plates were inverted and incubated at 37°C for 16 to 18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The value was compared with the zone-size table. The zones of growth inhibition were provided by Clinical and Laboratory Standards Institute (CLSI, 2007). Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation tables (Table 3.4 and 3.5) updated according to the Clinical and Laboratory Standards Institution (CLSI, 2009 and 2014).

Table 3.4: Standard zone of inhibition for *S. aureus* (CLSI 2009 and 2014):

Antimicrobial discs	Diameter of the zone observed (mm)		
	Sensitive	Intermediate	Resistance
Ampicillin 10 µg	≥14	12-13	≤11
Ampicillin + Sulbactam 10/10	≥14	12-13	≤11
Cefepime 30µg	≥18	15-17	≤14
Cefepime 30µg + Clavulanic acid10 µg	≥18	15-17	≤14
Cefotaxime30µg	≥23	15-22	≤14
Cefotaxime30µg + Clavulanic acid10 µg	≥23	15-22	≤14
Amoxicillin	≥20	-	≤19
Amoxicillin 20µg + Clavulanic acid 10µg	≥20	-	≤19
Methicillin	≥14	10- 13	≤9
Oxacillin 30µg	≥13	11-12	≤10

Table 3.5: Standard zone of inhibition for *E. coli* (CLSI guidelines 2009 and 2014):

Antimicrobial discs	Diameter of the zone observed (mm)		
	Sensitive	**Intermediate**	**Resistance**
Ampicillin 10 µg	≥17	14-16	≤13
Ampicillin + Sulbactam 10/10	≥15	12-14	≤11
Cefepime 30µg	≥18	15-17	≤14
Cefepime 30µg + Clavulanic acid10 µg	≥18	15-17	≤14
Cefotaxime30µg	≥26	23-25	≤22
Cefotaxime30µg + Clavulanic acid10 µg	≥26	23-25	≤22
Amoxicillin 30µg	≥18	14-17	≤13
Amoxicillin 20µg + Clavulanic acid 10µg	≥18	14-17	≤13
Ceftazidime 30µg	≥21	18 -20	≤17
Ceftazidime 30µg + Clavulanic acid10 µg	≥21	18 -20	≤17
Aztreonam	≥21	18 - 20	≤17

3.2.7.5. Detection of ESBL production by *E.coli* organisms:

3.2.7.5.A. ESBL detection method.

E. coli were first screened for ESBL production by phenotypic method and then phenotypic confirmatory test was done as per CLSI guidelines, 2012. For phenotypic screening of ESBL, CLSI (2012) has recommended the use of any of the Ceftazidime, Aztreonam, and Cefotaxime discs for screening of ESBL producers. More than one of these agents was used for screening to improve the sensitivity of ESBL detection, as CLSI has recommended the method only in 2012 guidelines.

3.2.7.5. B. Procedure:

Loopful colonies were grown in nutrient broth overnight at 37^{o}C. Inoculum with turbidity from nutrient broth was spread on agar plates. MHA plates were inoculated by lawn culture method using a sterile cotton swab. With a sterile forceps Ceftazidime, Cefotaxime, and Aztreonam discs were placed on the MHA plate and the plate was incubated at 35°C for 18–24 hours.

3.2.7.5. C. Phenotypic confirmatory methods:

Muller Hinton agar plates were inoculated with the test organisms. Then Ceftazidime, Amoxicillin and Cefotaxime discs were used alone and in combination with Clavulanic acid. Increase in zone diameter of ≥5mm for either antimicrobial agent tested in combination with Clavulanic acid against its zone when tested alone were confirmed as ESBL produced by *E.coli*

3.2.7.5.D. Cephalosporin / Clavulanate combination discs:

Cefotaxime (30 µg) or Ceftazidime discs (30 µg) with or without Clavulanate (10 µg) was used for phenotypic confirmation of the presence of ESBL as recommended by CLSI (2012) guidelines. A lawn culture of *E. coli* was made on the MHA plate and discs were placed at an appropriate distance from each other and incubated aerobically overnight at 37°C. A difference in zone of inhibition of 5 mm of either of cephalosporin discs and their Clavulanate containing discs indicates production of ESBL.

3.2.8. Detection of resistant genes of *S. aureus* and *E. coli:*

3.2.8. A. Isolation of genomic DNA from *S. aureus and E. coli.*

3.2.8. A.1. *S. aureus* DNA isolation:

Genomic DNA from bacterial strains was extracted as per the standard protocol provided by manufacturer of kit (Promega). Organisms from plates were inoculated and grown overnight in Nutrient broth in incubator at 37ºC. One ml culture was centrifuged for 2 minutes at 13,000–16,000 × *g**. Supernatant was discarded and pellet obtained at bottom was used in further steps. Pelleted cells in the 2 ml capacity tubes were suspended in 480 µl 50 mM EDTA and Lytic enzyme(s) (120 µl) [lysozyme and/or lysostaphin] were added to the suspension. All the tubes were incubated at 37°C for 30–60 minutes. Tubes containing suspension were centrifuged for 2 minutes at 13,000–16,000 × *g** and supernatant was discarded. With the help of pipette, 600 µl Nuclei Lysis Solution was gently added and mixed. Tubes were Incubated for 5 minutes at 80°C, then kept for cooling at room temperature. Then 3 µl of RNase Solution was added and mixed gently and incubated at 37°C for 15–60 minutes, and kept at room temperature for cooling. After cooling 200 µl of protein precipitation solution was added and vortexed for 5 min and all the samples then Incubated on ice for 5 minutes and centrifuged at 13,000–16,000 × *g** for 3 minutes. Supernatant was transferred to a clean tube containing 600 µl of isopropanol kept at room temperature and mixed well. Pellet was obtained after centrifugation and supernatant was decanted. To pellet cells, 600 µl of 70% ethanol kept at room temperature was added and again centrifuged. Ethanol was aspirated and pellet was allowed to dry for 15 minutes. Pellet was then rehydrated with 100 µl of Rehydration Solution for 1 hour at 65°C or overnight at 4°C. Isolated DNA was stored at -20°C till further use.

3.2.8. A.2. *E. coli* DNA isolation:

Fresh colonies from broth were used for isolation of DNA. 1 ml of culture was centrifuged for 2 minutes at 13,000–16,000 RPM. Supernatant was discarded. With the help of pipette 600 µl Nuclei Lysis Solution was gently added and mixed. Tubes were Incubated for 5 minutes at 80°C, then kept for cooling at room temperature. Then 3 µl of RNase solution was added and mixed gently and incubated at 37°C for 15–60 minutes, and kept at room temperature for cooling. After cooling 200 µl of protein precipitation solution was added and vortexed for 5 min

and all the samples then incubated on ice for 5 minutes followed by centrifugation at 13,000–16,000 × *g** for 3 minutes. Supernatant was transferred to a clean tube containing 600 µl of isopropanol kept at room temperature and mixed well. Pellet was obtained after centrifugation and supernatant was decanted. To pellet cells 600 µl of 70% ethanol maintained at room temperature was added and again centrifuged. Ethanol was aspirated and pellet was allowed to dry for 15 minutes. Pellet was then rehydrated with 100 µl of rehydration solution for 1 hour at 65°C or overnight at 4°C. Isolated DNA was stored at -20°C till further use.

3.2.8.. B. Selection of primers and PCR conditions:

Designing of primers for cloning and expression of *nuc* and *mecA* gene were procured from Invitrogen ltd. which were designed accordining to the Bali *et al*. (2010)

Table 3.6 .Oligonucleotide sequences and PCR conditions used for detection of antibiotic resistant genes in *S. aureus* and *E. coli*

Genes	Oligonucleotide sequences (5′-3′)	Product size (bp)	PCR conditions	Annealing temperature (A)
mecA	F-GGGATCATAGCGTCATTATTC' R-AACGATTGTGACACGATAGCC	527	ID – 95˚C/5 min. Followed by 38 cycles at D – 95˚C/30 sec. A – E – 65˚C/1.5 min. FE– 65˚C/10 min.	60˚C
nuc	**F-**TCAGCAAATGCATCACAAACAG **R-**CGTAAATGCACTTGCTTCAGG	255		60˚C
SHV	**F**- CGCCTGTGTATTATCTCCCT **R**- CGAGTAGTCCACCAGATCCT	293		59˚C
TEM	**F**- TTTCGTGTCGCCCTTATTCC **R-** ATCGTTGTCAGAAGTAAGTTGG	403		59˚C
CTX-M	**F-** CGCTGTTGTTAGGAAGTGTG **R-** GGCTGGGTGAAGTAAGTGAC	569		58˚C

F= Forward Sequence, R = Reverse Squence, ID – Initial denaturation, D = Denaturation, A= Annealing, E = Extension, FE = Final Extension.

3.2.8. C. PCR amplification of genes.

3.2.8. C. i. Preparation of the mix

Amount of PCR master mix was calculated for the 100 samples. Readymade PCR master mix was obtained from Sigma Aldrich, India. Aliquot of master mix were prepared for required number of PCR tubes (24 µl per tube)

The genomic DNA of *S. aureus* and *E. coli* were isolated as explained in the section 3.2.8. A. and DNA templates were subjected to PCR with specific primers (Table 5) for the detection of antibiotic resistance genes *viz. nuc, mecA,* for *S. aureus* and *TEM, SHV* and *CTX-M* genes for *E. coli.* PCR reaction was performed in 25 µl volume containing; 1.5 µl of each of the *nuc, mecA,* for *S. aureus* and *TEM, SHV* and *CTX-M* genes for *E. coli* primer pairs. The volume of this mix was adjusted to 25 µl with Nuclease free water (NFW). PCR reactions were performed with a final reaction volume of 25 µl using 0.25 ml thin wall PCR tube, with forward and reverse primers 7 µl + 12.5 µl PCR master mix (Promega) + 2.5 µl DNA template + 3 µl NFW. All above components were mixed by gentle shaking and DNA amplification was carried out in thermal cycler (Verity, Applied Biosystems) with the following thermal cycling conditions; A pre-PCR step at 95°C for 5 min was applied. A total of 38 cycles were run at the following conditions: denaturation at 95°C for 30 sec, annealing for 35 sec, at respective temperatures mentioned in table and extension at 65°C for 1.5 min. After the final cycle, the preparation was kept at 65°C for 10 min to complete the reaction and holding at 4^0C until further use. Amplified PCR product in 5 µl volume was further separated by electrophoresis in 3 percent agarose gel stained by ethidium bromide and visualized under UV.

3.2.8. C. ii. Agarose gel Electrophoresis:

Gel was prepared by boiling agarose in 0.5x Tris Borate Electrophoresis (TBE) buffer. After cooling at 50^0C, 0.5 mg/mL ethidium bromide was added to the agarose solution. Electrophoresis was carried out at 100 volts for 45 min. After electrophoresis gel was visualized under a UV transilluminator (G-BOX F3 Syngene, India). Mol Bio™ 100bp DNA Ladder (Sigma pvt ltd) was used as a molecular size marker.

3.2.8.D Statistical Analysis:

Microsoft excel was used for statistical analysis. The variables were compared using a Completely Randomised design for correlating results of PCR, MIC and AST, as appropriate. Differences were considered significant when the $p<0.05$, using the WASP2 software.

Chapter 4

Results & Discussion

RESULTS AND DISCUSSION

While reviewing present status of mastitis in India, reports express that most of the investigations were made pertaining to epidemiological data, clinical manifestations, diagnosis, bacterial isolation, *in-vitro* drug sensitivity, and treatment of SCM. Mastitis, on account of causing serious wastage and undesirable milk quality, is emerging as a major challenge to dairy development of tropics. SCM is reportedly more important in India (varying from 10–50% in cows and 5–20% in buffaloes) than CM (1–10%). The factors like herd size, agro climatic conditions of the region, variations in socio-cultural practices, milk marketing, literacy level of the animal owner, system of feeding, and management were found important affecting the incidence of SCM. In many countries including India, almost all approved intramammary antibiotics are labeled for treatment of *Streptococci* and *Staphylococci,* and there are no approved products for treatment of mastitis caused by number of different other pathogens. In the U.S. only two antimicrobial classes are represented among commercially available products that are approved by the U.S. Food and Drug Administration (FDA). Those classes are β-lactams (Amoxicillin, Ceftiofur, Cephapirin, Cloxacillin, Hetacillin, and Penicillin) and a Lincosamide (Pirlimycin). While several products have been withdrawn from the U.S. market, no new intramammary antibiotics for lactating cows have been approved since 2006 (Anon, 2015). There is no clear-cut group wise demarcation between intramammary formulations and systemic use of antibiotics and many classes of antibiotics are employed for intramammary use. β-lactams are most commonly used for treatment of mastitis and other animal diseases.

As most of drugs in mastitis treatments are administered simply based on observation of inflammation (without knowing the bacteria), most systemic treatments are difficult to justify both medically and from consumers point of view.

Several reports are published regarding one or other aspects of mastitis from different parts of country. However, no systematic studies regarding specific class of antimicrobials were reported from Mumbai or Maharashtra. As Mumbai is

the largest milk consumer city of Maharashtra, milk from several parts of Maharashtra and Gujarat is being supplied to Mumbai. There are many buffalo stables in western parts of Mumbai and Palghar districts. These buffalo farms, with considerable number of animals, are run by farmers in this area. As all lactating animals are reared, it is essential to find prevalence of mastitis in this area. However, reports citing mastitis prevalence or resistance of organisms arising through this area are lacking. Many veterinarians during discussion, accepted the fact of reduced efficacy of important antimicrobials. In addition many drugs and antibiotics are available as over the counter medicines, and are rampantly used by farm owners without consulting Veterinarians. Hence, it could be hypothesized that problem of bacterial resistance to routine important antimicrobials might be prevailing. The present study was planned to study prevalence of SCM, and monitor AMR of mastitis organisms from this region.

4.1 Prevalence of SCM

The present study was carried out during July, 2014 to May, 2016. In order to study prevalence of SCM, bacterial resistance against β-lactam antibiotics in *S. aureus* and *E. coli* isolated from milk of buffaloes with SCM samples were collected from Mumbai and Palghar Districts.

Prevalence is defined as the number of cases of disease or infection existing at any given time in relation to the unit of population in which they occur. It is a static measure as compared with the dynamic measure (Anon, 2013).

Incidence describes the number of new cases in a particular area in a specific time period (Anon, 2013). However, prevalence and incidence are often used synonymously.

For screening of SCM, six different buffalo farms were identified from Mumbai and Palghar districts. All farms were having similar husbandry practices.

In the present study total of 750 buffalo milk samples were collected and screened with the CMT. Out of theses 310 milk samples were found positive for SCM. Formation of gel after mixing of CMT reagent with milk was considered as a positive for SCM. Even if a single quarter was involved, it was considered as a case of SCM. When milk from more than one quarter revealed gel formation, the composite sample from affected quarters was taken. These samples were transported to the Department of Pharmacology and Toxicology, Bombay Veterinary College for further microbiological analysis. In the present investigation the mean prevalence of SCM was found to be 41.33%. Farmwise prevalence of SCM is expressed in *Table 4.1 and fig 4.1*

The literature search regarding prevalence studies revealed variable sample sizes from minimum 50 to maximum 1000 or more samples. The various samples sizes observed through previous literature search are, 2057 by Sharma and Sindhu (2007), 10539 (Patel *et al*., 2012), 1022 **(**Sahu *et al*., 2014), 218 (Mir *et al.,* 2014), 564 (Charaya *et al*., 2014), and 48 (Ali *et al*., 2015).

Table 4.1. Farmwise prevalence of SCM in collected samples

Place /Farm	No of samples	No of samples found Positive for SCM	Percent Prevalence
Maharaja Dairy unit 21 Goregaon	205	90	43.90
Maharaja dairy unit 19 Goregaon	155	72	46.45
Palghar	90	46	51.11
Safale	97	32	32.98
Dahisar Palghar	88	38	43.18
Virar	115	32	27.82
Total	**750**	**310**	**41.33**

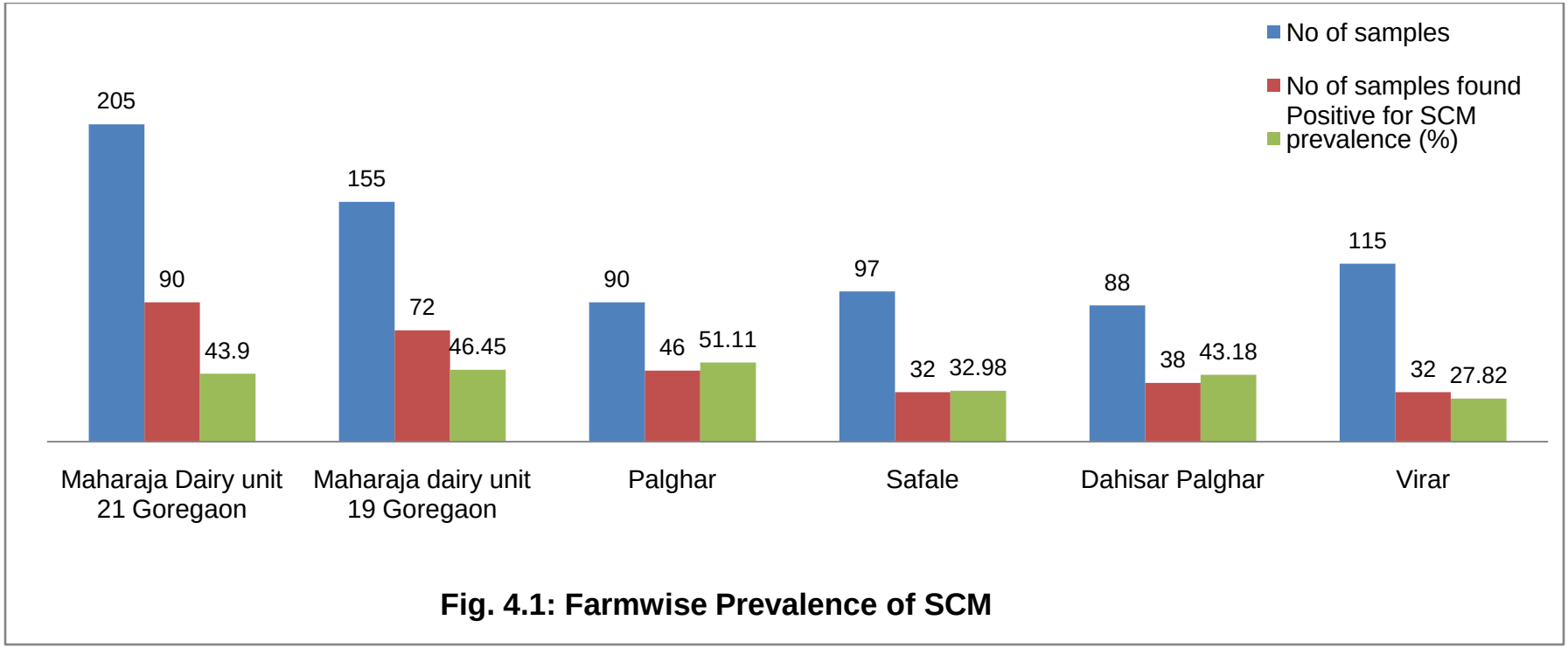


Fig. 4.1: Farmwise Prevalence of SCM

Out of six dairy animal farms, relatively higher prevalence of SCM was recorded from dairy farm located at Palghar (51.11%) followed by Unit 19 Goregaon (46.45%), unit 21 Goregaon (43.90%), Dahisar (43.18%), Safale (32.98%) and Virar (27.82%) farms. All together, the occurrence of SCM was observed in the range of 27 – 51 percent at all farms sampled during the study.

Estimates of prevalence of SCM and CM in dairy cows have been reported earlier world over (Gianneechini *et al.,* 2002; Mekibib *et al.,* 2010; Rahman *et al.,* 2010; Bachaya *et al.,* 2011; Ayano *et al.,* 2013; Ondiek *et al.,* 2013; Saidi *et al.,* 2013) as well as from India (Tiwari *et al.,* 2000; Kalorey *et al.,* 2007; De and Mukherjee, 2009; Supriya *et al.,* 2010; Kurjogi and Kaliwal, 2011; Srinivasan *et al.,* 2013 and Ali *et al.,* 2015). Estimated prevalence of SCM and CM in bovines recorded by these researchers revealed wide variations in the range of 10-70% or more.

A cross sectional study carried out by Mekibib *et al*. (2010) in dairy farms of central Ethiopia also revealed prevalence of mastitis in cows, as high as 71 percent, out of which 22.4 and 48.6 percent cases suffered from CM and SCM, respectively. The quarter level prevalence recorded by them was 44.9 percent, out of which 34.8 percent was SCM. The overall quarter wise prevalence of SCM as 35.25 percent was recorded in the study of Bachaya *et al*. (2011) conducted in Pakistan and 28.77 percent in Algeria by Saidi *et al*. (2013).

Study of Rahman *et al*. (2010) in dairy cows reared at Government dairy farm in Bangladesh, revealed the overall SCM as 51.3 percent. Relatively high (82.9%) incidence of SCM in cows was observed by Ondiek *et al*. (2013) in Kenya. Findings of Ayano *et al.* (2013) from Ethiopia studied on commercial dairy farms recorded prevalence of SCM as 41.02 percent which is almost similar to the observation of present study.

Reported prevalence of SCM in dairy animals in India ranged from 10-70 percent. Comparatively less prevalence of SCM in cow (14.17%) was recorded by Supriya *et al*. (2010). Kurjogi and Kaliwal (2011) also recorded prevalence of mastitis in dairy cattle from Karnataka, where prevalence of CM and SCM was recorded as 8 and 72 percent, respectively. Tiwari *et al.* (2000) also observed relatively high (47.10% - 66.67%) incidence of SCM in cross bred and indigenous cows of Madhya Pradesh. Similarly, very high prevalence (90.76%) of SCM in cows from Vidarbha was also reported in the study of Kalorey *et al.* (2007). Present results are in agreement with the findings of study carried out by De and Mukherjee (2009) at organized dairy farm in which nearby similar estimates of SCM (42.93%) were revealed. Srinivasan *et al*. (2013) and Ali *et al.* (2015) had observed 26.21 and 31.25 percent prevalence of SCM from Namakkal and IVRI, respectively.

Systematic review and meta-analysis of prevalence of SCM for a period of 1995 – 2014 in dairy cows in India has been recently published by Banger *et al.* (2015). The pooled estimates of prevalence of SCM on cow basis were found to be 46.35 percent. Their findings revealed very high prevalence of SCM in dairy cows in India. However, prevalence of CM noted was very low as compared to earlier studies carried out in India by Tiwari *et al*., (2000); De and Mukherjee, (2009); Kurjogi and Kaliwal, (2011).

From the literature reviewed it is evident that occurrence of farms with ‘nil’ prevalence of SCM is impossible as the prevalence depends upon many factors like milk yield, host species, number of lactation and mainly standard hygienic practices adopted at any farm. The incidence can be lowered through adoption of standard hygienic practices of udder and milkers.

4.2 Biochemical characterization of the isolates

4.2. a. Biochemical characterization of the presumptive *S. aureus* isolates:

The presumptive *S. aureus* colonies were subjected to Gram's staining. The organisms with grape like clusters of Gram-positive cocci were inoculated into peptone water and incubated overnight at 37˚C. After purification, the isolates were subjected for biochemical and molecular characterization. The biochemical characterization was carried out as per the method described by Agarwal *et al*. (2003) Biochemical tests were performed as described below. Isolates showing positive reactions for Gram, catalase, Indole, methyl red, Voges Proskauer etc and negative for oxidase were confirmed as *S. aureus* and such 168 isolates were used for further screening. Detailed results of Biochemical characterization of *S. aureus* are given in **Annexure III**

4.2 b. Biochemical characterization of the presumptive isolates of *E.coli*

Although selective plating is specific, presumptive identification of *E. coli* was based on Gram's straining, morphology, catalase, oxidase and IMVIC test. The presumptive *E. coli (102)* colonies were subjected to Gram's staining. The Gram negative rod shaped organisms were inoculated into lactose broth and overnight incubated at 37°C. After purification, the isolates were subjected for biochemical and molecular characterization. The biochemical characterization was carried out as per the method described by Agarwal *et al*. (2003) The isolates showing positive results for catalase methyl red and indole tests and negative reactions for Gram, oxidase and citrate utilization were considered as *E. coli* and such 94 isolates were used for further screening. Detailed results of Biochemical characterization of *E. coli* are given in **Annexure IV**

4.3 Prevalence of *S. aureus* and *E. coli:*

Prevalence of *S. aureus* and *E. coli* was studied by isolating them from 310 SCM positive samples obtained through six different buffalo farms

a. Prevalence of *S. aureus*:

For isolation of *S. aureus,* milk samples from animals with SCM were processed according to the method described by Baur *et al.* (1966) and CLSI (2014) guidelines.

Out of 310 SCM milk samples, *S. aureus* was isolated from 168 samples. The overall prevalence of *S. aureus* in relation to SCM cases was 54.19%

There are plenty of literatures available on detection of bacterial isolates including *S. aureus* from CM and SCM milk collected from bovines world over (Gianneechini *et al.,* 2002; Mekibib *et al.,* 2010; Rahman *et al*., 2010; Saidi *et al*., 2013 and Ayano *et al.,* 2014) as well as from India (De and Mukherjee, 2009; Harini and Sumathi, 2011; Kurjogi and Kaliwal, 2011; Ranjan *et al*., 2011; Sentitula *et al.,* 2012; Srinivasan *et al*., 2013 and Ali *et al*., 2015). Although, above mentioned researchers revealed *S. aureus* as a predominant bacterial pathogen causing CM and SCM, isolation rate of *S. aureus* in these studies was found in the range of 20 – 58 percent.

Nationwide survey of microbial etiology of cases of SCM in dairy cows in Sweden conducted by Persson *et al.* (2011) revealed *S. aureus* and coagulase negative *Staphylococci* as the most frequently isolated pathogens. Farm hygienic practices observed in majority of the farms under referred studies were not up to the mark in case of *S. aureus*, which is reported to be the cause of high isolation rates.

Santos *et al*. (2014) showed the association of prevalence of *S. aureus* with cross contamination, poor hygienic conditions during milking, contaminated water and infected milker's hand.

b. Prevalence of *E. coli.*

Out of 310 SCM milk samples, *E. coli* was isolated from 94 samples. The overall prevalence of *E. coli* in relation to SCM cases was 30.32%

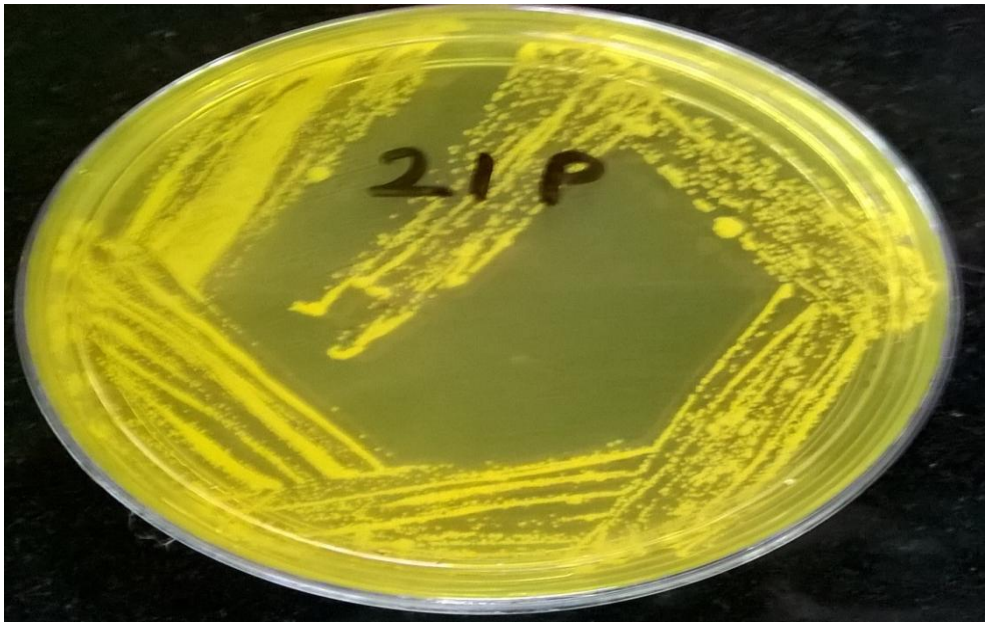


Plate 1: *S. aureus* showing golden yellow colonies on mannitol salt agar

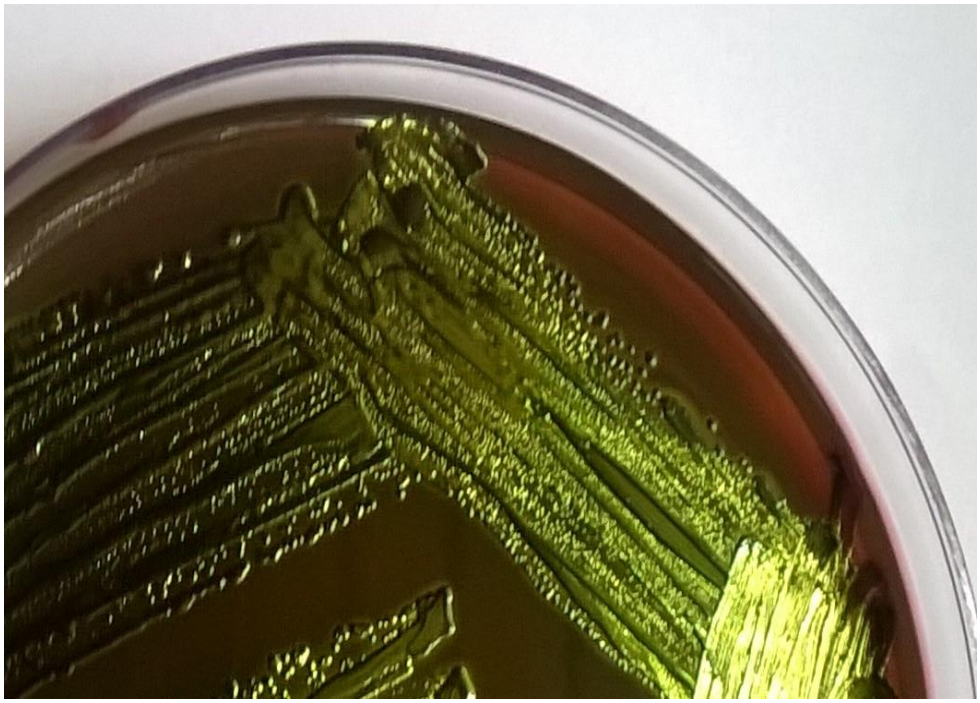


Plate 2: *E. coli* showing colonies with metallic sheen on EMB agar

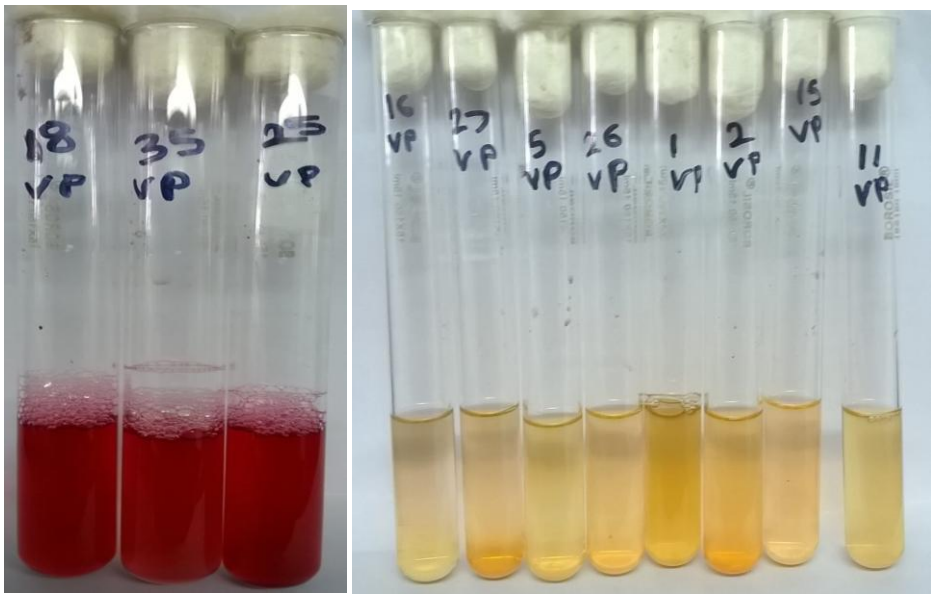


Plate 3: Voges Prausker test – left side positive (*S. aureus*) and right side negative (*E.coli*)

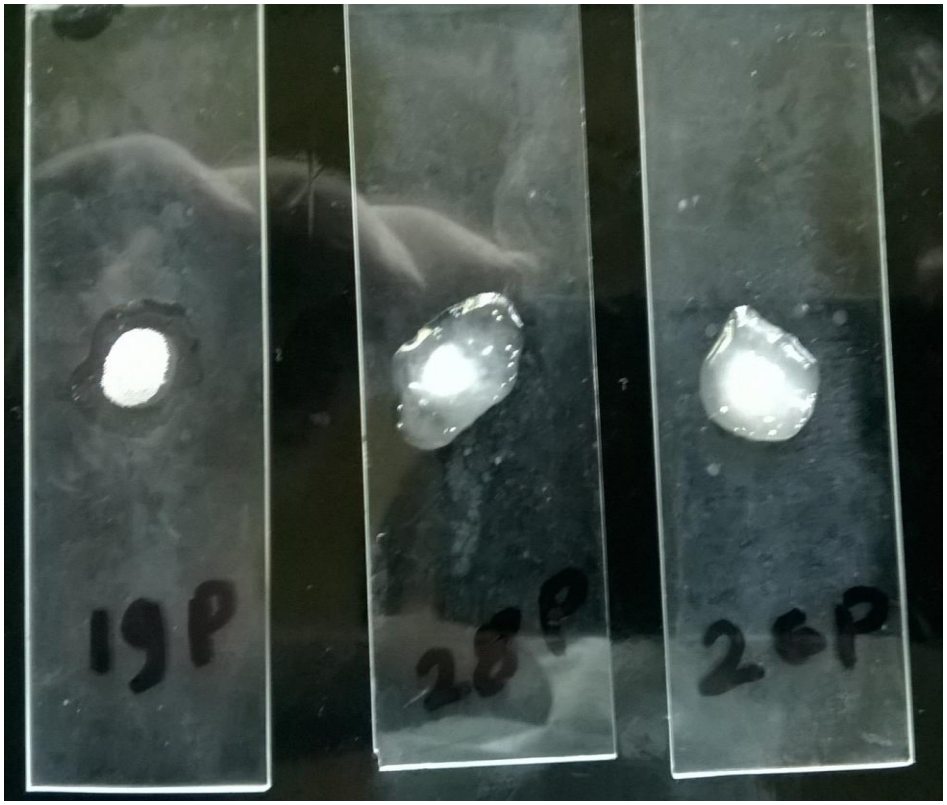


Plate 4: *S. aureus* showing positive catalase test

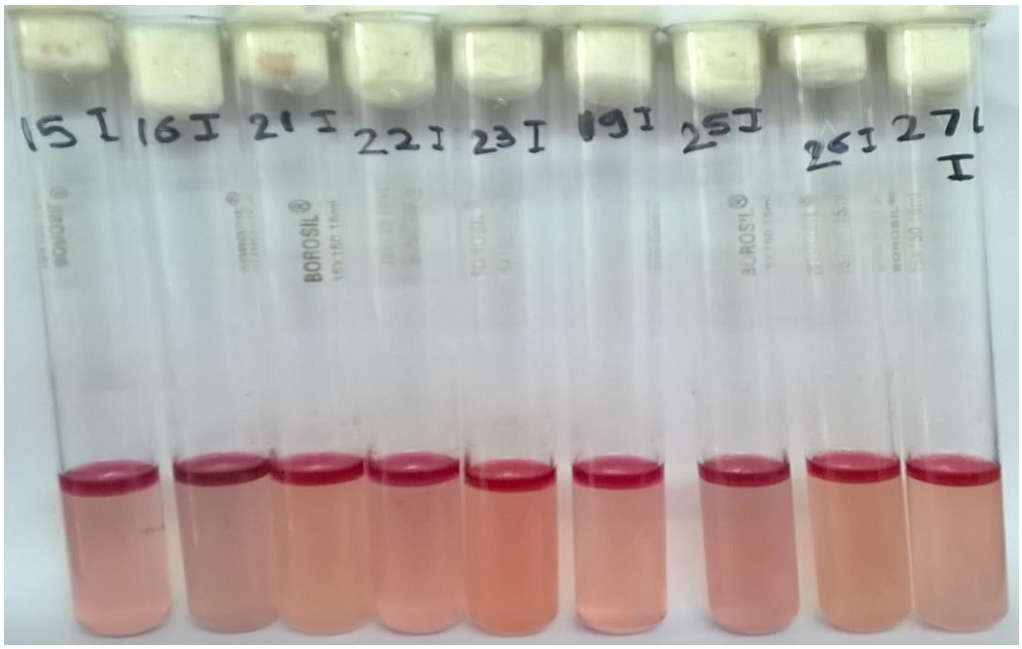


Plate 5: *E.coli* showing positive Indole test

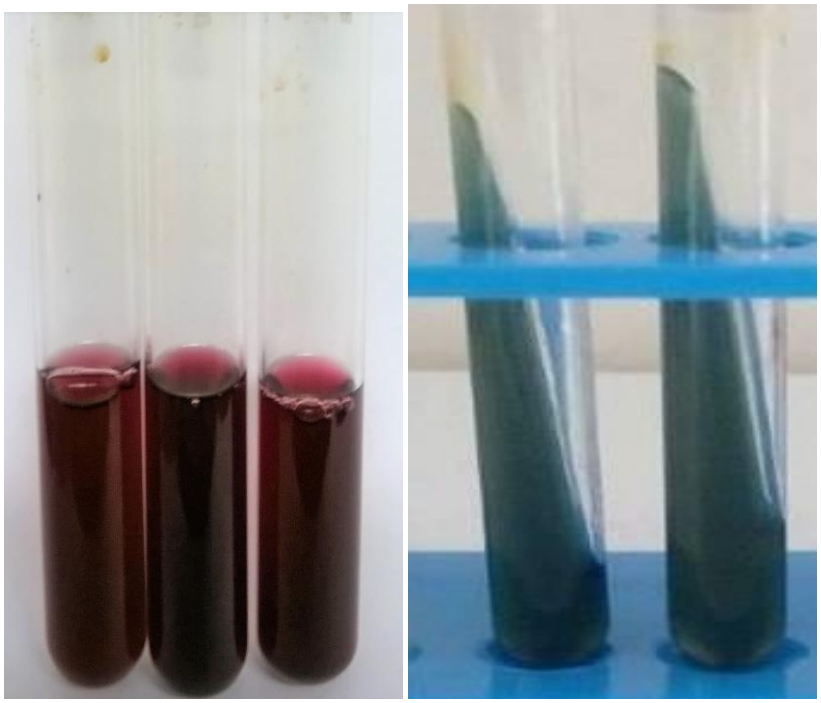


Plate 6: *E. coli* showing positive methyl red test (left) and negative citrate test (right)

E. coli due to its ubiquitous distribution has been isolated from cases of bovine mastitis by number of investigators from India (Ranjan *et al*., 2011; Kurjogi and Kaliwal, 2011; Palaha *et al.,* 2012 and Hegade *et al.,* 2013) as well as from other parts of the world, (Dopfer *et al*., 1999; Bradley and Green 2001; Lira *et al*., 2004; Momtaz *et al*., 2012; Abera *et al*., 2010; Alekish *et al*., 2013; Tesfaye *et al.*, 2013 and Mahamoud *et al*., 2015). All these investigators have recorded prevalence of *E. coli* isolated from bovine mastitis in the range of 6 to 35 percent.

E. coli by far is the most common species isolated from more than 80 percent of the cases of coliforms mastitis (Bradley *et al*., 2007).

Anwar and Chaudary (1983) from Lahore, Pakistan, reported 25% incidence of *E. coli* among the tested subclinical cases of bovine mastitis, whereas in 1967 lowest incidence (4%) was reported by Ghuman (1967) from Pakistan.

From England Bradley, *et al*. (2007) reported 19.8% occurrence of *E. coli* among the tested 480 milk samples, where the incidence of SCM was 47%.

From Ethiopia, Mekibib *et al*. (2010) reported only 4.6% incidence of *E. coli* among the tested 52 SCM cases. Whereas in case of sheep and goats SCM cases, the reported incidence noted by Gebrewahid *et al*. (2012) was 17%. Similarly, Alharbi (2014) reported 1.88% incidence of SCM cases in sheep and goat from Saudi Arabia.

In India Kurjogi and Kaliwal investigated the status of CM and SCM among dairy cattle in Dharwad district; Karnataka in 2011. A total of 185 isolates were recovered from 120 milk samples. The major pathogens isolated from the milk samples were *S. aureus* and *E. coli* (21.08%). They reported incidence of *E. coli as* 21.0**8**%.

Ranjan *et al.* (2011) studied bovine mastitis in different climatic conditions in Jharkhand India. Among 190 milk samples confirmed positive for bovine mastitis by

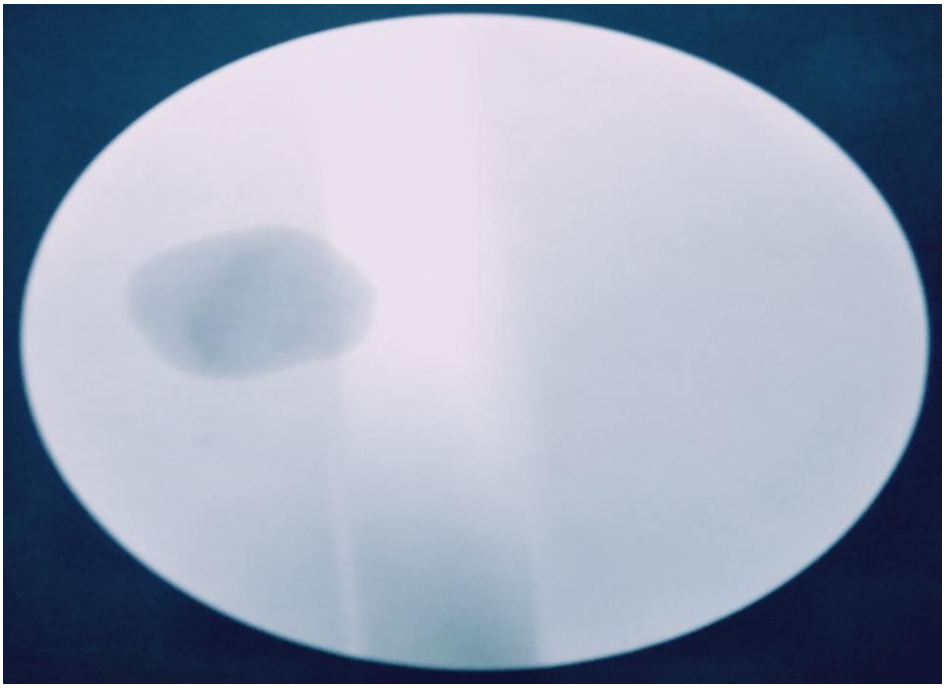


Plate 7: *S. aureus* showing negative oxidase test

CMT, somatic cell count and White side test, 138 (72.63 %) samples were positive for bovine mastitis and 8.95% were *E. coli*.

Palaha *et al.* (2012) detected *E. coli* from the udder of the dairy farm buffaloes in Phagwara region, Punjab, India. A total of 135 swabbed samples were collected randomly from the udder of buffaloes in ten dairy farms. Out of 135 samples examined, 23 (17.03%) were positive for *E. coli*.

In most countries major mastitis pathogens reported are *Streptococci* and *S. aureus.* However, *E. coli* may also reflect considerable impact on cow health, milk quality and animal productivity. Along with *E. coli* other fecal origin Gram-negative organisms may cause bovine mastitis to lesser extent, therefore the term coliform mastitis is frequently used to identify intramammary infections caused by Gram-negative bacteria. They are basically considered as environmental pathogens (Hogan and Smith, 2003). Coliform bacteria occupy many habitats in cow's environment *viz.* bedding material, feed and fodder, water, farm refuse, sewage, effluent and most importantly intestinal tract and dung and include *Enterobacter*, *Shigella*, and *Klebsiella* organisms.

The portal of entry of Gram-negative bacteria into the mammary gland is the teat canal. The manner in which coliform bacteria traverse the teat canal is unknown but probably involves an opportunistic entry into the gland. Endotoxin is the primary virulence factor of Gram-negative bacteria responsible for the damage to the cow. Coliform mastitis can result in bacteraemia and septicaemia as blood milk barrier is destroyed. A primary source of bacterial contamination is bedding, therefore use of germicidal sanitizers on teat immediately preceding milking (pre dipping) can significantly reduce the incidence of new coliform during lactation (Hogan and Smith, 2003).

As an environmental pathogen, the incidence may depend on hygienic conditions and managemental practices. This could be the reason behind the variable incidences of *E. coli* at different geographical locations.

4.4 Detection of Antimicrobial Resistance (AMR):

β-lactams play major role in the treatment of human and animals. Many of the β-lactams like Amoxicillin, Cloxacillin, Ampicillin and their combinations with β-lactamase inhibitors are being used in human and animal treatments (Table 4.2). Therefore antimicrobial discs were selected depending on their use in animal treatment.

Table 4.2: β-lactams used commonly in bovines:

Sr. No.	Antimicrobials	Common indications
1	Amoxicillin + Clavulanic acid	Respiratory tract infections, urinary tract infections, soft tissue infections
2	Cefepime	Used in horses and extra label use in cattle and buffaloes
3	Amoxicillin	Respiratory tract infections, urinary tract infections, soft tissue infections
4	Cefotaxime	RTI, UTI, *Staphylococcal* and *E. coli* infections.
5	Ampicillin + Sulbactam	Mastitis, RTI, UTI, METRITIS, PHARYNGITIS
6	Ampicillin	Mastitis, RTI, UTI, METRITIS, PHARYNGITIS

4.4.1 AMR of *S. aureus* through disc diffusion method.

All *S. aureus* isolates obtained under study were further subjected for antimicrobial sensitivity/resistance pattern by disc diffusion method as described by Bauer *et al*. (1966) and as per the recommendations of CLSI, (2014). This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc.

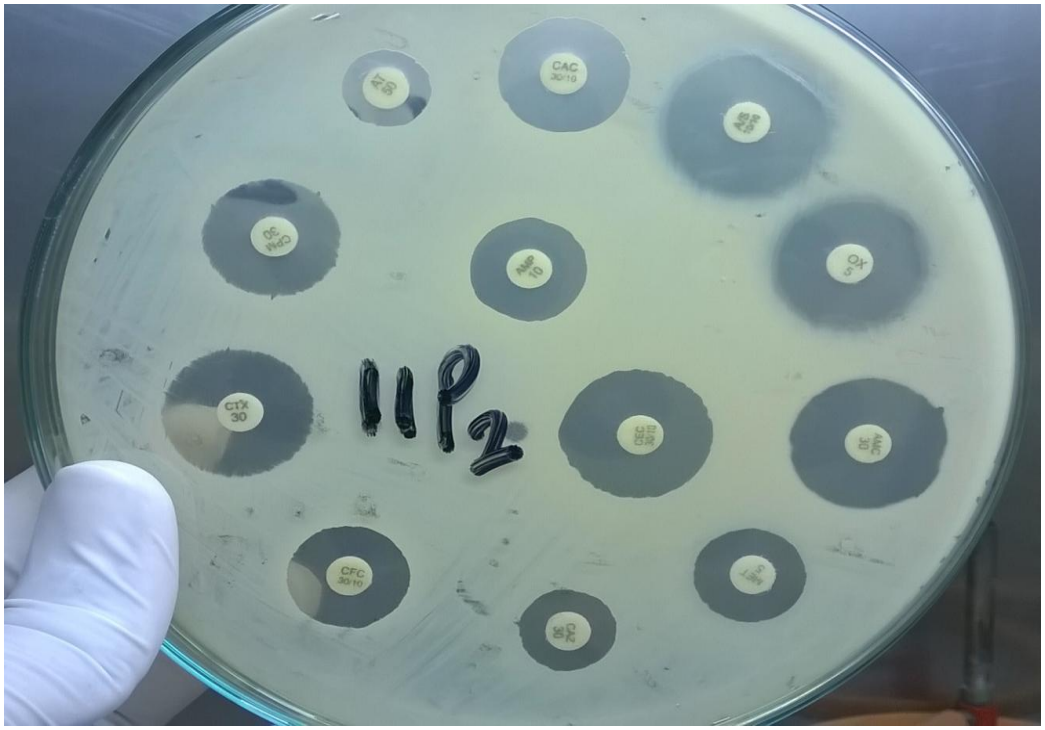


Plate8: *S. aureus* showing susceptibility different β-lactam antimicrobials on Muller Hinton agar

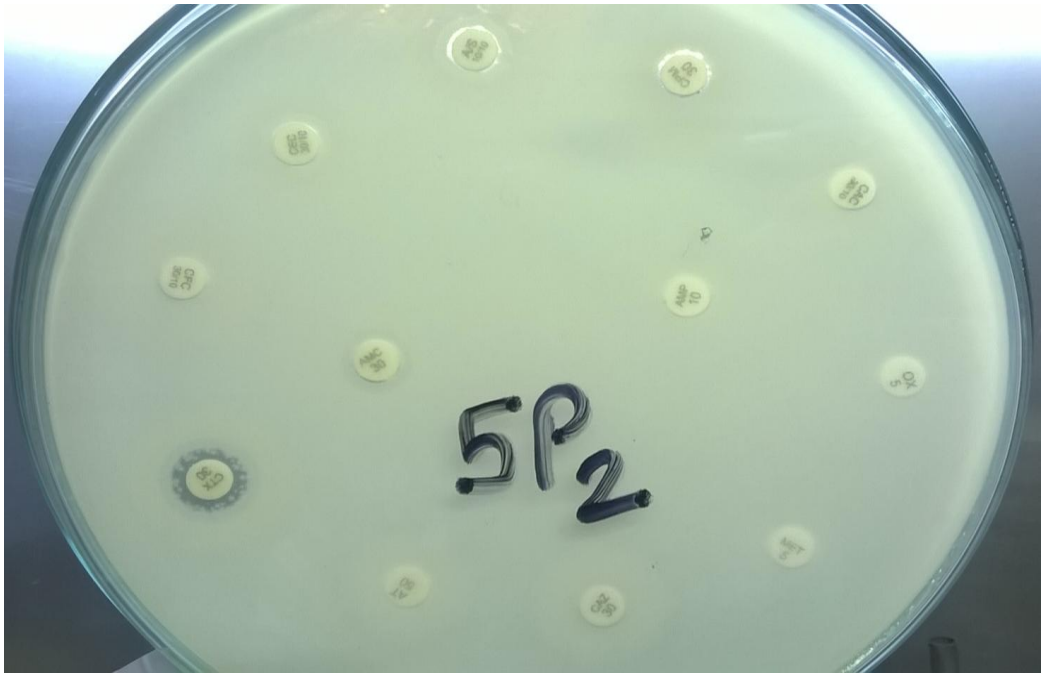


Plate 9: *S. aureus* showing resistance to different β-lactam antimicrobials on Muller Hinton agar.

The readings for the selected β-lactam antimicrobials were interpreted as described in CLSI guidelines (2009 and 2014). The results of antimicrobial resistance pattern of *S. aureus* are presented in Table 4.3.

Table 4.3, shows that, maximum sensitivity of the *S. aureus* was observed to Cefepime + Clavulanic acid (77.38%), followed by Cefotaxime + Clavulanic acid (73.80%), Amoxicillin + Clavulanic acid combination (73.21%), Methicillin (70.23%) , Oxacillin (60.04), Ampicillin+ Sulbactam combination (76.78%), Cefotaxime (61.30%), Cefepime (56.54%), Amoxicillin (43.45%) and Ampicillin (42.85%) **(Plate 8 and 9).**

Table 4.3: Antimicrobial resistance pattern of *S. aureus*.

Antimicrobials	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	42.85 (72)	-	57.14 (96)
Ampicillin + Sulbactam	65.47 (110)	3.57(6)	30.95 (52)
Cefepime	64.28 (108)	17.85 (30)	17.85 (30)
Cefepime + Clavulanic acid	77.38 (130)	7.14 (12)	15.47 (26)
Cefotaxime	61.30 (103)	15.47 (26)	22.02 (37)
Cefotaxime + Clavulanic Acid	73.80 (124)	10.11 (17)	16.07 (27)
Amoxicillin	43.45 (73)	-	56.54 (95)
Amoxicillin + Clavulanic Acid	73.21 (123)	-	26.18 (45)
Methicillin	70.23 (118)	5.95 (10)	23.80 (40)
Oxacillin	69.04 (116)	0	30.95 (52)

Details regarding sensitivity of the individual organisms to each of the antimicrobial are tabulated in **Annexure I**

Disc diffusion technique is most widely used method for determination of the susceptibility of animal pathogens in set up to determine the correct treatment

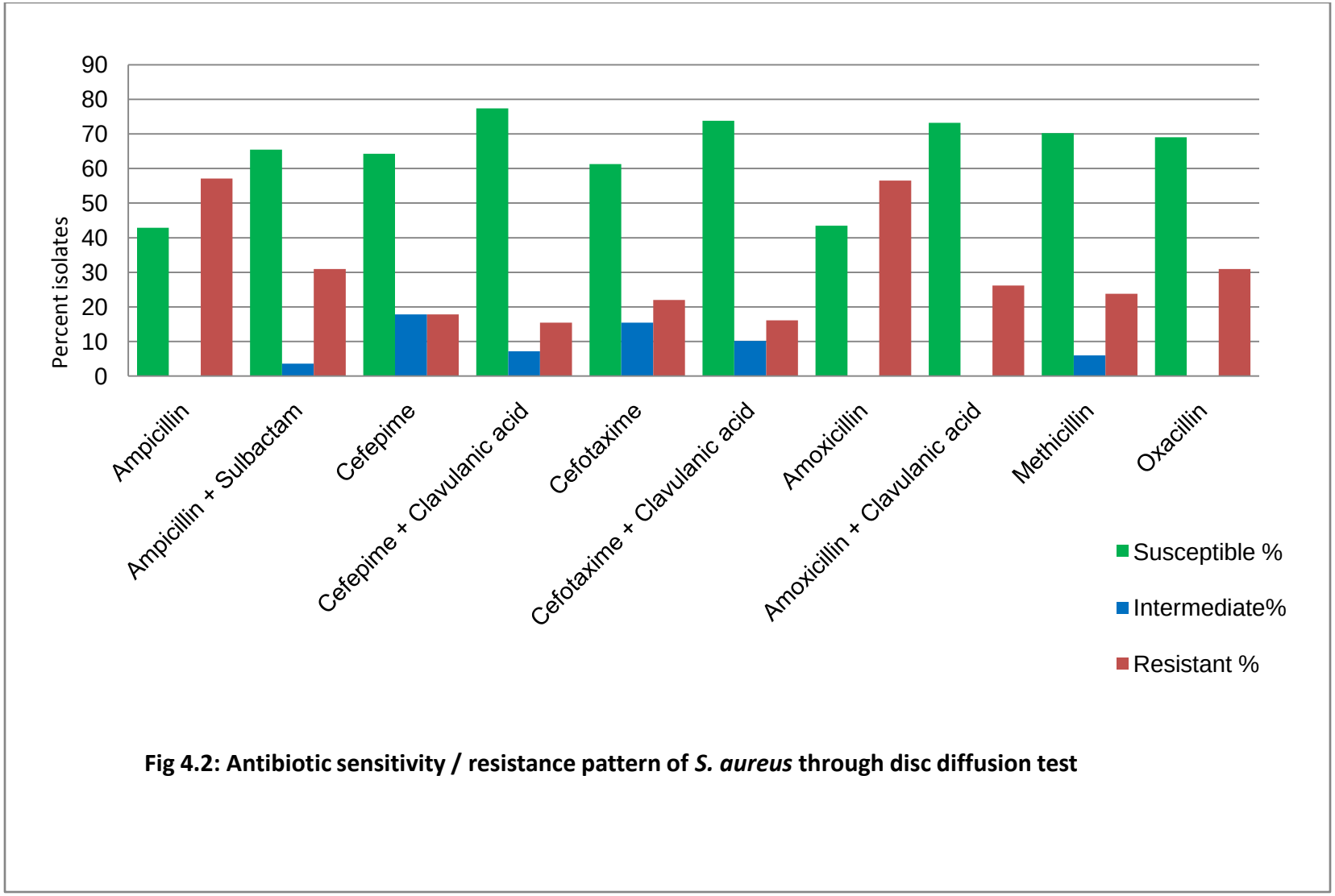


Fig 4.2: Antibiotic sensitivity / resistance pattern of *S. aureus* through disc diffusion test

choice. The only disadvantage is that outcomes are reported on qualitative basis. The *in-vitro* AMR of the *S. aureus* isolates examined from samples of bovine mastitis was high. Isolates of *S. aureus* had an alarming level of resistance to Ampicillin and Amoxicillin.

The reported percentage of β- lactam resistant *S. aureus* in cases of bovine mastitis world over detected by disc diffusion method are variable (Gentillini *et al.,* 2000; Gianneechini *et al.,* 2002; Kalmus *et al.,* 2011; Persson *et al.,* 2011 and Saini *et al*., 2012) and in India Penicillin resistant *S. aureus* strains procured from bovine mastitis were isolated by maximum researchers v*iz.,* 28.9 percent (Kumar *et al*., 2010), 45 percent (Sumathi *et al.,* 2008), 100 percent (Thaker *et al*., 2013), 76.77 percent (Kaliwal *et al.,* 2011), 83.3 percent (Chandrasekaran *et al*., 2014), 63.5 percent (Chandrasekaran *et al.,* 2014b) and 82.35 percent (Mohanty *et al*., 2013).

Reported resistance of *S. aureus* for Ampicillin by various researchers was 54.54 percent (Bernabe *et al*., 2005), 70.59 percent (Kaliwal *et al*., 2011), 75.8 percent (Adwan 2006), 63.3 percent (Guler *et al*., 2005), 53.68 percent (Nichita *et al*., 2007), 96 percent (Akindele *et al*., 2010) 3.94 percent (Mubarak *et al*., 2012).

In the present study the resistance to Ampicillin and Ampicillin + Sulbactam was 57.14 and 30.95 percent, respectively. It was also observed from the previous reports that high variation in resistance percentage to Ampicillin could be due to different geographical areas and previous exposure to antimicrobials.

Ampicillin is active against many Gram-positive and Gram-negative bacteria. It was the first 'broad spectrum' Penicillin with activity against Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes* and some isolates of *S. aureus* (but not Penicillin-resistant or Methicillin-resistant strains). Its spectrum of activity is enhanced by co-administration of Sulbactam, a drug that inhibits β-lactamase, an enzyme produced by bacteria to inactivate Ampicillin and related antibiotics (Hauser, 2013).

As the introduction and use of Ampicillin alone started in 1961 (Acred, 1962) many organisms became resistant due to production of β-lactamase enzyme. The introduction of Sulbactam since 1987, combined with Ampicillin made β-lactamase producing bacteria susceptible. This is observed in the present study where more percent of *S. aureus* (65.47 percent) was sensitive to the combination as compared to Ampicillin alone (42.85 %).

The results in the present study suggest that inclusion of Sulbactam enhanced the sensitivity and lowered the resistance percentage. However, 100 percent sensitivity could not be achieved indicating that other mechanisms of resistance development might be involved like alterations in PBP and decreased permeability of antimicrobials.

The degree of sensitivity of *S. aureus* towards Cefotaxime and its combination with Clavulanic acid in present study was 61.30 and 73.80 percent, respectively whereas intermediate sensitivity of 15.47 and 10.11 percent showed effectiveness of Cefotaxime than other antimicrobials. Therefore overall sensitivity to Cefotaxime and Cefotaxime + Clavulanic acid could be considered as 76.77 and 83.91 percent, respectively.

In contrast Rajaduraipandi *et al*. (2006) from Tamilnadu interpreted 63.2 percent resistance to Cefotaxime in human isolates of *S. aureus*. In 2011 Chakraborty and associates from Bangalore, India interpreted Cefotaxime resistance in human pus samples as 26.67 percent.

On similar grounds Sonth *et al* (2015) from Bagalkot, Karnataka reported 52.6 percent resistance to Cefotaxime from the *S. aureus* collected from human samples. As a β-lactam antibiotic in the third-generation class of cephalosporins, Cefotaxime is active against numerous Gram-positive and Gram-negative bacteria, including several with resistance to classic β-lactams such as penicillin. It is active against *S. aureus* but not MRSA, S*. epidermidis, Str. pneumoniae, S. pyogenes* and *E. coli* etc

In the present study resistance of *S. aureus* to Amoxicillin and Amoxicillin + Clavulanic acid was 56.54 and 26.18 percent among the tested samples. Amoxicillin is a moderate-spectrum, bacteriolytic, β-lactam antibiotic in the aminopenicillin family used to treat susceptible Gram-positive and Gram-negative bacteria. In general, *Streptococcus, Bacillus subtilis, Enterococcus, Haemophilus* are susceptible to Amoxicillin, whereas *Citrobacter, Klebsiella* and *Pseudomonas aeruginosa* are resistant to it. However, cited literature showed some *E. coli* and most clinical strains of *S. aureus* have developed resistance to Amoxicillin. França *et al*. (2012) from Brazil, reported that resistance of *S. aureus* samples obtained from bovine mastitis to Amoxicillin alone was 50 percent.

Elizabeth *et al*. (2015) studied AMR of *Staphylococcus* spp. isolates from cases of mastitis in buffalo in Brazil and reported 49.2 percent resistance to Amoxicillin. However, very low resistance was reported by Ashraf *et al*. (2014) in *S. aureus* isolated from chickens.

Another study from Maharashtra, India conducted by Awandkar *et al*. (2013) reported the antibiotic resistance trends in the cases of bovine mastitis from Nanded, Latur, Osmanabad and Beed districts of Maharashtra State, India. The rate of acquiring resistance to these antibiotics was high. Higher sensitivity was recorded for combination of Ceftriaxone + Tazobactam and Amoxicillin + Sulbactam as compared to Ceftriaxone and Amoxicillin alone, respectively. The sensitivity to Amoxicillin, Ampicillin and Cloxacillin reported was below 20 percent.

As Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as Penicillin, it is combined with Clavulanic acid, a β-lactamase inhibitor. This drug combination is commonly called Co-Amoxiclav.

The sensitivity of *S. aureus* isolates towards Cefepime and Cefepime + Clavulanic acid in present study was 64.28 and 77.38 percent, respectively. Cefepime is a fourth-generation Cephalosporin antibiotic having extended spectrum of activity against Gram-positive and Gram-negative bacteria, with greater activity against both types of organisms than third-generation agents.

From Brazil, Santos et al. (2014) reported *in-vitro* antibiotic resistance and susceptibility of 34 *S. aureus* isolates obtained from bovine mastitis which were 32.4 and 67.60 percent, respectively.

There are several reports published about the increased sensitivity to cephalosporins if used in combination with β-lactamase inhibitors. Moreover several authors mentioned that, β- lactam resistance can be due to the expression of inducible β- lactamases encoded by the *blaZ* gene, which causes hydrolysis of β - lactam ring of Penicillin (Asfour and Darwish, 2011; Kumar *et al*., 2011; Bagcigil *et al.,* 2012 and Behiry *et al.*, 2012).

In the present study resistance observed to Methicillin and Oxacillin was 23.80 and 30.95 percent, respectively.

The first report on the ability of *S. aureus* to metabolize Penicillin was published in 1940, a year before the antimicrobial was introduced for therapeutic use. However the discovery of Penicillin dramatically reduced the incidence of bacterial infections around the world. This single antibiotic was effective against a large number of bacteria for many years, until *S. aureus* developed the ability to produce β-lactamase, an enzyme that destroys Penicillin. *S. aureus* develops resistance to antibiotics through plasmid-mediated genetic mutations (Chambers, 1997). These mutations confirmed that, S*. aureus* has the remarkable ability to adapt to changing antibiotic environments. The resiliency of *S. aureus* motivated pharmacologists to create a class of semi-synthetic Penicillins that could withstand β-lactamase. These antibiotics became known as β-lactam Penicillins, with Methicillin as the prototype. For years, infections with *S. aureus* were reliably eradicated with Methicillin and its analogs, Nafcillin and Cloxacillin. However, the resourceful bacterium soon became able to resist these β-lactam antibiotics, and the first strain of MRSA was identified in 1961. Since the mid-1980s, antibiotic resistance among nosocomial *S. aureus* isolates has been increasing appreciably.

MRSA is any strain of *S. aureus* that has developed, through horizontal gene transfer and natural selection, multi- resistance to β-lactam antibiotics, which include the Penicillins (Methicillin, Dicloxacillin, Nafcillin, Oxacillin, etc.) and the Cephalosporins (Fitzgerald *et al*., 2001).

S. aureus usually shows limited host specificity, and transfer between different host species may occur (Van *et al*., 2005). The transmission of milk-associated *S. aureus* strains between cows and humans was suggested by Lee (2003). The risk for spread of MRSA from bovine sources into the human population is low. Generally, persons are not at risk as long as raw milk is not consumed. However, persons in close contact with MRSA infected cattle, including Veterinarians, farmers, milkers, and persons working at slaughterhouses, may become colonized from the bovine source.

In addition to Methicillin, strains of *S. aureus* have developed resistance to other antibiotics. MRSA is resistant to Cephalosporins, Erythromycin, Clindamycin, Gentamicin, Trimethoprim-Sulfamethoxazole, and Ciprofloxacin. Vancomycin, a glycopeptide antibiotic, was relied upon until recently to eradicate MRSA infection. As expected, strains of Vancomycin-resistant *S. aureus* (VRSA) have been isolated and are fast becoming a new treatment challenge (Hiramatsu, 2001).

Impaired treatment response is associated with Penicillin resistance of *S. aureus* strains. Importance of prolonged β- lactam associated resistance in *S. aureus* and higher MIC values demonstrated that Ampicillin and Penicillin are consistently the antimicrobial agents to which the *S. aureus* are most commonly resistant (Asfour and Darwish, 2011; Bagcigil *et al.,* 2012; Behiry *et al*., 2012). Highest number of Penicillin resistant *S. aureus* and sensitive to cephalosporins were also observed in the study of Onyenwe *et al*. (2012) and also revealed that 80 percent of bovine isolates were producing β- lactamases.

The high rate of β-lactam resistance amongst *S. aureus* from milk of bovine mastitis is likely due to the wide use of intramammary preparations containing combinations of different antibiotics and broad-spectrum antimicrobials including Penicillin. Numerous factors can influence the overall susceptibility pattern of mastitis pathogens.

Penicillin predicts resistance to other β- lactam group of antimicrobials like Ampicillin. *S. aureus* β- lactamase will be resistant to Penicillin and Cephalosporins. In the present study Methicillin and Oxacillin was included for detection of MRSA where 23.80 and 23.21 percent strains were resistant to Methicillin and Oxacillin. Oxacillin resistance is reported to show resistance to all β- lactam antimicrobial agents. Thus, such high degree of resistance to Oxacillin in the present study is alarming. Oxacillin resistance was not detected by Gianneechini *et al.* (2002). In contrast to the present study no MRSA strains were detected from bovine mastitis originated *S. aureus* isolated in Argentina by Gentillini *et al*. (2000).

High level of β- lactam resistance of *S. aureus* strains was also observed by Moroni *et al.* (2006) from Argentina. They observed very poor *in-vitro* activity of Ampicillin and Amoxicillin where 94 percent of *S. aureus* isolates were found resistant to three or more antimicrobial agents. This level of resistance may probably relate to the presence of strong β-lactamase producers among the tested *S. aureus.*

There are several factors other than antimicrobial usage which influence the susceptibility pattern of mastitis pathogens and the general recommendation is to cull all animals with chronic *S. aureus* intra mammary infections (IMI) (Moroni *et al*., 2006). The control of IMI sustained by *S. aureus* should involve the best management practices and selective antimicrobial usage. Unfortunately most antimicrobial agents used in veterinary medicine still rely on interpretive criteria developed for humans and the validity of these criteria for categorizing veterinary pathogen as susceptible or resistant has not been established. Thus, the usefulness

of susceptibility data is limited to monitoring the percentage of *S. aureus* with MIC above threshold value and to predict efficacy (Moroni *et al*., 2006).

Phenotypic AMR by disc diffusion method was studied in India by several workers and most of the *S. aureus* strains isolated from bovine mastitis were found to be resistant to Penicillin, Amoxicillin, Cephalexin, Oxytetracycline, Methicillin and sensitive to Gentamicin, Chloramphenicol and Enrofloxacin (Sumathi *et al.,* 2008; Kaliwal *et al*., 2011; Mohanty *et al.,* 2013; Thaker *et al.*, 2013 and Chandrasekaran *et al*., 2014).

4.4.1.1. Comparison of activity against *S. aureus* among Ampicillin, Ampicillin + Sulbactam and Methicillin (Table 4.4) .

In the present study the activity of Ampicillin, Ampicillin + Sulbactam and Methicillin was compared in order to get information of *S. aureus* sensitivity towards penicillinase susceptible, penicillinase inhibitor combination and penicillinase resistant antimicrobials, respectively.

Table 4.4 .Comparison of activity among Ampicillin, Ampicillin + Sulbactam and Methicillin

Antimimicrobial	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	42.85	0	57.14
Ampicillin + Sulbactam	65.47	3.57	30.95
Methicillin	70.23	5.95	23.80

From the above comparison it was obvious that Methicillin resistant isolates are also showing resistance to other β-lactams and MRSA responds poorly to the β-lactamase inhibitors. In nutshell present study findings revealed that AMR with special reference to few β- lactams is common in *S. aureus* strains except combinations with Clavulanic acid or other β-lactamase inhibitors which could be better alternative for treating *S. aureus* associated SCM, if used judiciously.

4.4.2 AMR of *E. coli* recorded through disc diffusion:

All the *E. coli* isolates were studied for antimicrobial sensitivity/resistance pattern by disc diffusion method as described by Bauer *et al*. (1966) and as per the recommendations of CLSI, (2014). The results are depicted in Table 4.5

Highest sensitivity of *E. coli* among the tested β-lactam antimicrobials was observed in Cefepime + Clavulanic acid (86.17 percent) followed by Cefepime (80.85 percent), Amoxicillin+ Clavulanic acid (77.65 percent), Ampicillin + Sulbactam (71.27 percent) and Cefotaxime + Clavulanic acid (71.27 percent). Whereas relatively higher resistance was observed to Cefotaxime, Aztreonam and Ceftazidime which was 53.19 percent, 53.19 percent and 42.55 percent, respectively **(Plate 10 and 11).** The Individual sensitivity of *E. coli* isolates is given in **Annexure II**

Table 4.5 : Antimicrobial resistance pattern of *E. coli*.

Antimicrobials	Percent Sensitivity/ resistance		
	Susceptible	Intermediate	Resistant
Ampicillin	38.29 (36)	28.72 (27)	32.97 (31)
Ampicillin + Sulbactam	71.27 (67)	19.14 (18)	9.57 (9)
Cefepime	80.85 (76)	(0)	19.14 (18)
Cefepime + Clavulanic acid	86.17 (81)	8.51 (8)	4.25 (4)
Cefotaxime	37.23 (35)	9.57 (9)	53.19 (50)
Cefotaxime + Clavulanic acid	71.27 (67)	19.14 (18)	9.57 (9)
Amoxicillin	38.29 (36)	28.72 (27)	32.97 (31)
Amoxicillin + Clavulanic acid	77.65 (73)	10.63 (10)	11.70 (11)
Ceftazidime	9.57 (9)	47.87 (45)	42.55 (40)
Ceftazidime + Clavulanic acid	28.72 (27)	52.12 (49)	19.14 (18)
Aztreonam	4.25 (4)	42.55 (40)	53.19 (50)

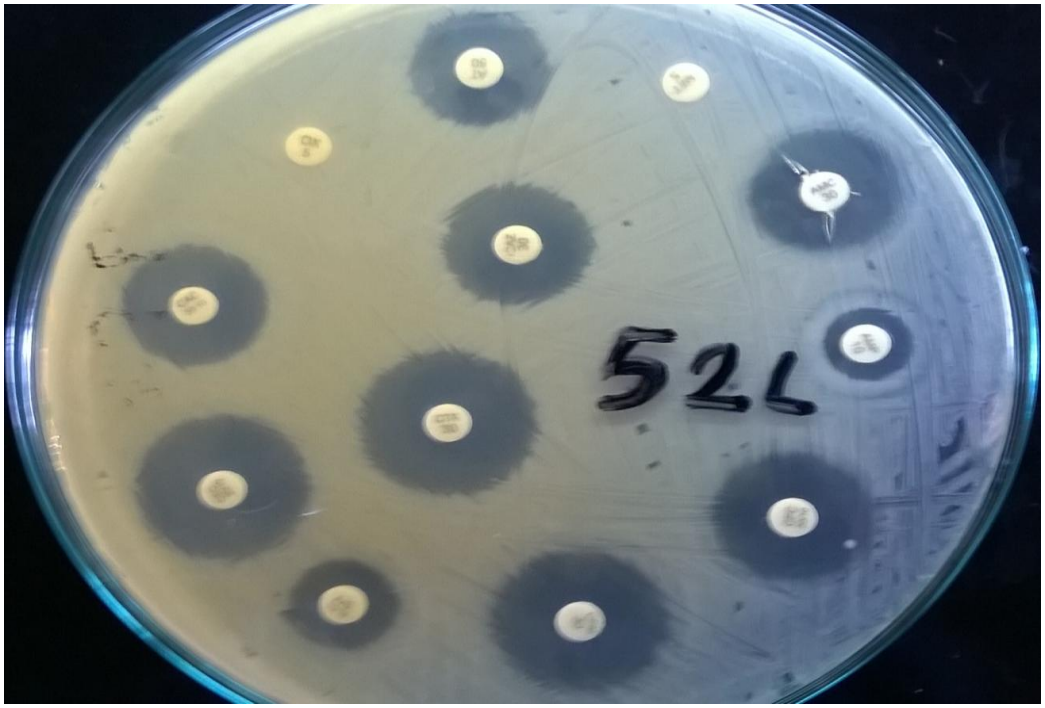


Plate 10: *E.coli* showing susceptibility to different β-lactam antimicrobials on Muller Hinton agar.

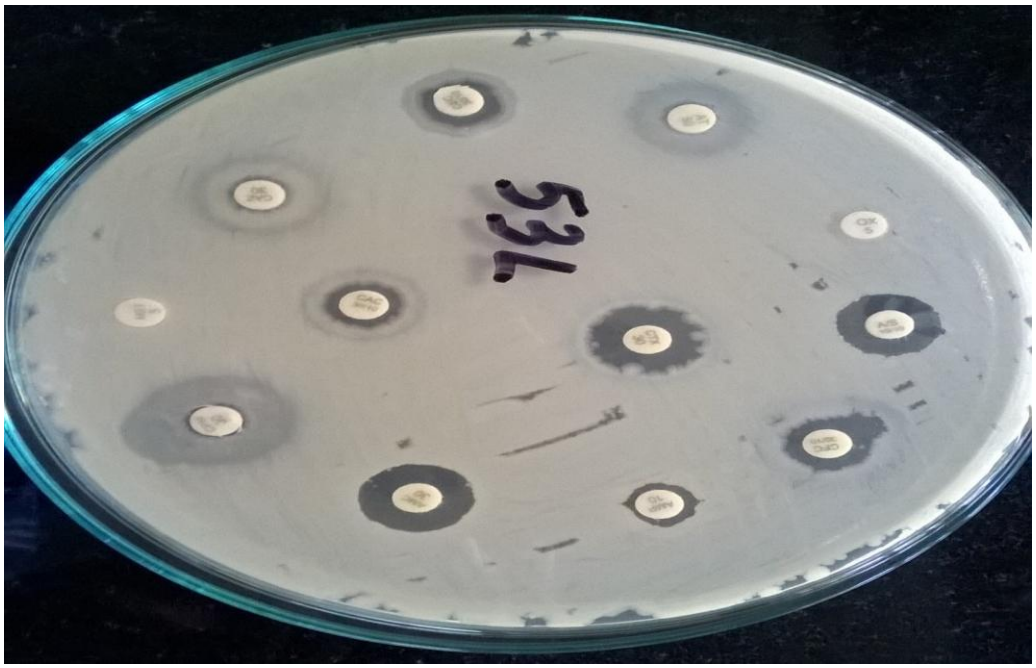


Plate 11: *E.coli* showing resistance to different β-lactam antimicrobials on Muller Hinton agar.

From Brazil, Fernandes *et al.* (2011) studied *E. coli* from CM for AMR by disc diffusion method where, twenty-seven *E. coli* isolates were analyzed, and 14.81 percent (n = 4) resistance to Ampicillin was reported.

In Iran, Momtaz *et al.* (2012) carried out study to detect the virulence factors, serogroups, and antibiotic resistance properties of Shiga toxin-producing *E. coli* (STEC), using 268 bovine mastitic milk samples which were diagnosed through CMT. The disc diffusion method showed that the STEC strains had the highest resistance to Penicillin (100 percent), followed by Tetracycline (57.44 percent), while resistance to Cephalothin (6.38 percent) was the lowest.

Memon *et al.* (2013) investigated prevalence of SCM and coliform mastitis with antimicrobial sensitivity profile of various mastitis-causing organisms in Pakistan. Minimum inhibitory concentration (MIC) results revealed that *E. coli* isolates were resistant to Penicillin group (93-99 percent), Fluoroquinolones (40-74 percent), Cephalosporins (54-66 percent), Oxytetracycline (91 percent), Gentamicin (82 percent) and 100 percent were sensitive to Florfenicol.

Mahmoud *et al.* (2015) carried out studies on *E. coli* mastitis in cattle and buffaloes from Behira Governorate Egypt. *E. coli* isolates (39 i.e. 20.4 percent) were detected in cases of mastitis, 90 cases were suffering from SCM and six (6.8 percent) *E. coli* isolates were detected from these samples. The antimicrobial sensitivity indicated that the most effective antibiotics were Lincospectin (56.6 percent), Danofloxacin (56.6 percent), Enrofloxacin (40 percent) and Ceftiofur (40 percent), while the lowest effective antibiotics were Oxytetracycline and Ampicillin.

Dubal *et al. (*2010) investigated AMR of *S. aureus* and *E. coli* isolates from SCM milk samples of cattle from Sikkim, India and reported Nitrofurantoin and Tetracycline (97.77 percent each) to be the most effective antimicrobials followed by Chloramphenicol (95.55 percent) and Cefotaxime (91.11 percent) against the *S. aureus* and *E. coli* based on results of disc diffusion test.

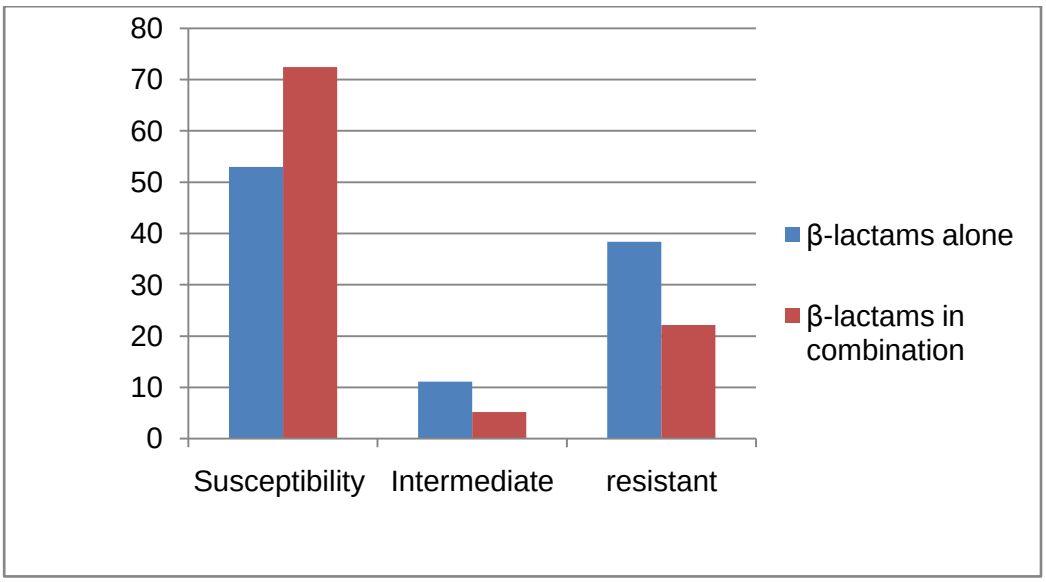


Fig 4.4: comparison of β-lactams when used alone and in combination with β-lactamase inhibitors against *S. aureus*

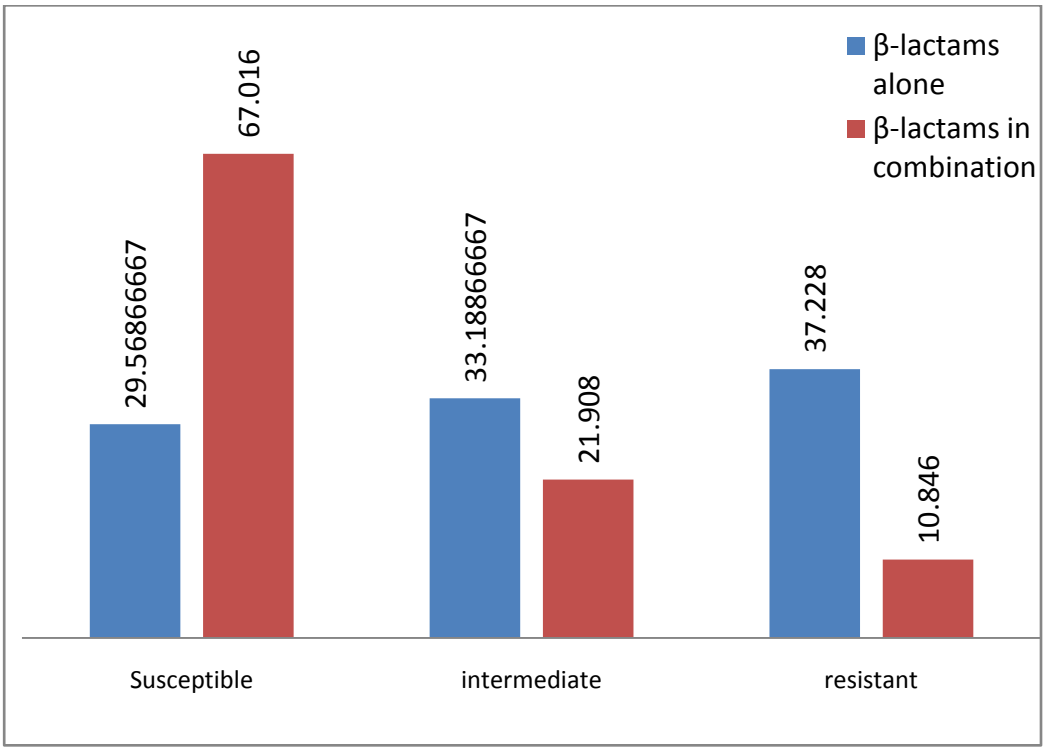


Fig 4.5: comparison of β-lactams when used alone and in combination with β-lactamase inhibitors against *E.coli*

Kurjogi and Kaliwal (2011) from Dharwad district, Karnataka investigated antibiogram pattern for the isolates obtained from bovine SCM, using fourteen antibiotics and reported sensitivity for Kanamycin (76.20 percent), Cloxacillin (75.30 percent), Rifampicin (48.26 percent), Ampicillin (60.86 percent), Penicillin-G (25.61 percent), Carbenicillin (25.41 percent), Chloramphenicol (67.07 percent), Cephalothin (43.57 percent), Tetracycline (78.35 percent), Trimethoprim (38.73 percent), Polymyxin-B (20.08 percent), Streptomycin (57.13 percent), Gentamicin (49.77 percent) and Amikacin (39.02 percent) which were used frequently for the treatment of mastitis and Tetracycline was found to be more effective antibiotic among all the tested antibiotic.

Umar *et al.* (2013) in Pakistan identified bacteria related to mastitis in cattle biochemically and assessed their sensitivity to different antibiotics. Gentamicin, Enrofloxacin, Amoxicillin and Cepharadine showed best efficacy against the mastitis causing bacteria amongst the antibiotics tested. Sensitivity was moderate for Ampicillin, Streptomycin and Kanamycin, whereas least sensitivity was observed to Cloxacillin and Penicillin.

In the present study *E. coli* resistance to Ampicillin and Ampicillin + Sulbactam was 32.97 percent and 9.57 percent which shows that Ampicillin in combination with Clavulanic acid is more effective due to inhibition of β- lactamase enzyme by Clavulanic acid. Also it showed presence of β-lactamase enzyme producing *E. coli*.

Rasmussen *et al*. (2015) from Ghana, reported *E. coli* resistance to Ampicillin as 61 to 69.4 percent in tested local and imported chicken meat samples respectively. From Brazil Koga *et al*. (2015) reported 69 percent resistance to Ampicillin alone for *E. coli*. Kurjogi and Kaliwal (2011) from Karnataka also reported resistance as 60.86 percent. for *E. coli* isolates obtained from bovine SCM. However, the reports on combination of Ampicillin and Sulbactam were scanty.

In the present study, resistance shown by *E. coli* isolates to Cefepime and its combination with Clavulanic acid was 19.14 percent and 4.25 percent. This finding assertively indicated presence of β-lactamase enzyme production by the *E.coli* isolates tested.

Chong *et al*. (2010) from Japan investigated *E. coli* resistance to Cefepime from human patients and reported 46 to 70 percent during three different years from 2006 to 2008.

From India, Kumar *et al*. (2014) also reported 35.55 percent resistant *E.coli* to Cefepime alone. All the samples obtained in their study were sourced from human hospital at Noida.

In contrast, low resistance of *E. coli* by Iqbal *et al*. (2002) against Cefepime (19 percent) when human urinary tract samples tested was reported.

However, reports regarding Cefepime combination with Clavulanic acid were unavailable. The enhanced sensitivity of combination is due to the inhibition of β-lactamase enzyme by the Clavulanic acid.

In the present investigation *E. coli* resistance observed towards Cefotaxime and Cefotaxime + Clavulanic acid was 53.19 and 9.57 percent, respectively. Similar resistance pattern like earlier antimicrobials, was observed here when it was used with and without β-lactamase inhibitors, which shows presence of β-lactamase enzyme among the tested *E. coli* isolates.

Similar kinds of reports are published by Chong *et. al.* (2010) from Japan, where 91 percent *E. coli* resistance to Cefotaxime alone was reported. Iqbal *et al*. (2002) from Pakistan, reported overall 36 percent resistance from human urinary tract *E. coli* infections.

From UP, India Dinesh Kumar *et al*. (2014) also reported similar kind of reports where *E. coli* isolates were obtained from urine, pus and blood samples showing that resistance to Cefotaxime was 72 percent, 72 percent and 60 percent, respectively.

In the present study resistance shown by *E. coli* against Ceftazidime and Ceftazidime + Clavulanic acid was 42.55 percent and 19.14 percent, respectively. Low resistance was observed where Ceftazidime was used in combination with Clavulanic acid. Dinesh Kumar *et al*. (2014) reported *E. coli* resistance from 60 to 70 percent. Resistance was 66.77, 68 percent and 66.77 percent, respectively when *E. coli* were isolated from human urine, pus and blood, respectively.

Very high resistance was reported by Adeyankinnu *et al*. (2014) from Nigeria. They investigated *E. coli* resistance to various antimicrobials including β-lactams and reported 83 percent resistance to Ceftazidime. Oteo *et al*. (2006) also reported 90 percent resistance for Ceftazidime shown by *E. coli* isolates obtained from human hospitals in Spain.

The resistance to Ceftazidime observed in present study was lower than the earlier reports. The isolates were obtained from human subjects in earlier reports which could be the reason behind the sensitivity difference.

In the present study *E. coli* resistance to Amoxicillin + Clavulanic acid was 11.70 percent. Rangel and Marin (2009) from Brazil reported Amoxicillin resistance from the *E. coli* mastitis cases as 31 percent.

In the present investigation *E. coli* resistance to Aztreonam was 53.19 percent. Though the Aztreonam is active against Gram-negative organisms, its use in human and animals is limited due to its narrow spectrum activity. However, the mastitis like diseases are multietiological which limits the use of such antimicrobial, but it is used in human for urinary tract infections and for meningitis treatment in neonatal animals (Prescott, 2013)

β-lactamases are enzymes produced by bacteria (also known as penicillinase) that provide multi-resistance to β-lactam antibiotics such as Penicillins, Cephamycins,and Carbapenems (Ertapenem),although Carbapenems are relatively resistant to β-lactamase. These antibiotics all have a common element in their molecular structure: a four-atom ring known as a β-lactam. Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties. β-lactam antibiotics are typically used to treat a broad spectrum of Gram-positive and Gram-negative bacteria.

β-lactamases produced by Gram-negative organisms are usually secreted, especially when antibiotics are present in the environment. (Neu, 1969). Thus it can be said that these organisms are previously exposed to few β-lactam antimicrobials.

ESBLs are β-lactamases that hydrolyze extended-spectrum cephalosporins with an Oxyamino side chain. These cephalosporins include Cefotaxime, Ceftriaxone, and Ceftazidime, as well as the Oxyamino-Monobactams Aztreonam. Thus ESBLs confer multi-resistance to these antibiotics and related Oxyamino-β lactams. In typical circumstances, they derive from genes for *TEM-1, TEM-2*, or *SHV-1* by mutations that alter the amino acid configuration around the active site of these β-lactamases. A broader set of β-lactam antibiotics are susceptible to hydrolysis by these enzymes. (Emery, 1997)

4.4.2.1. Comparison of β-lactams when used alone and in combination of β-lactamase inhibitors by disc diffusion method.

β-lactamases are a family of enzymes involved in bacterial resistance to β-lactam antibiotics. They act by breaking the β-lactam ring that allows penicillin-like antibiotics to work. Strategies for combating this form of resistance have included the development of new β-lactam antibiotics that are more resistant to cleavage and the development of the class of enzyme inhibitors called β-lactamase inhibitors (Essack 2001). Although β-lactamase inhibitors have little antibiotic activity of their

own, they prevent bacterial degradation of β-lactam antibiotics and thus extend the range of bacterial susceptibility to the drugs.

Table 4.6: a. Comparison of β-lactams when used alone and in combination with β-lactamase inhibitors by disc diffusion method against *S. aureus*

Antimicrobials used	Susceptibility %	Intermediate %	Resistant %
β lactams alone	52.97	11.10667	38.3875
β lactams in combination	72.465	5.205	22.1675

Most β-lactam resistance in Gram-positive bacteria is due to variations in penicillin-binding proteins that lead to reduced binding to the β-lactam. The Gram-positive pathogen *Staphylococcus aureus* produces β-lactamases, but β- lactamase inhibitors play a lesser role in treatment of these infections because the most resistant strains (Methicillin-Resistant *Staphylococcus aureus*) also use variant penicillin-binding proteins. (Wolter and Listor 2013)

Table 4.6: b. Comparison of β-lactams when used alone and in combination with β-lactamase inhibitors by disc diffusion method *E. coli*

	Susceptibility %	Intermediate %	Resistant %
β lactams alone	29.56867	33.18867	37.228
β lactams in combination	67.016	21.908	10.846

In the present investigation *E. coli* organisms showed enhanced susceptibility towards β-lactams when they were used in combination with β-lactamase inhibitors.

The most important use of β-lactamase inhibitors is in the treatment of infections known or believed to be caused by Gram-negative bacteria, as β-lactamase production is an important contributor to β-lactam resistance in these pathogens (Zapun *et al.,* 2008).

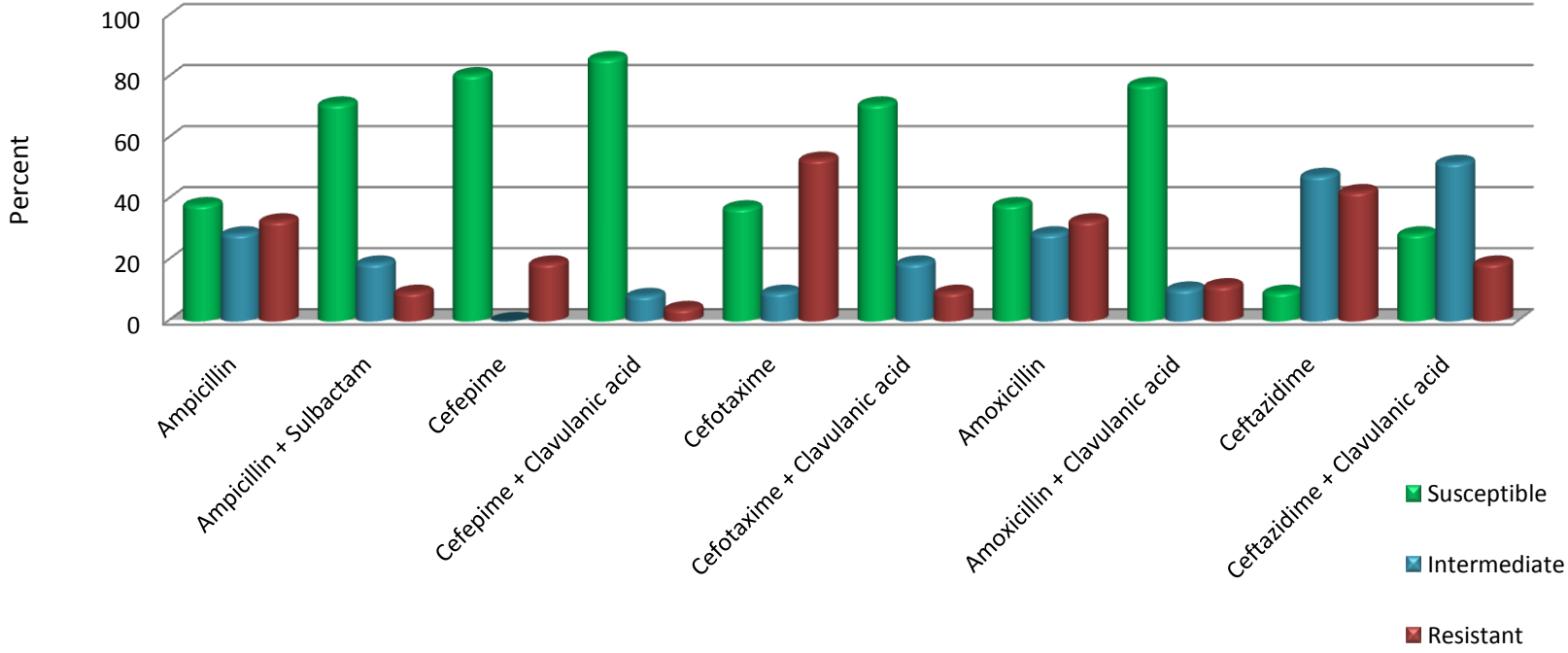


Fig 4.3: Antibiotic sensitivity / resistance pattern of *E.coli*

It was observed that when β-lactams used alone and in combination of β-lactamase inhibitors, susceptibility percentage was enhanced and resistance decreased for combinations for *S. aureus* and *E. coli*.

4.4.3. Detection of MIC:

A. MIC for the *S. aureus*

MIC values were obtained as per the E-test method described by Sader and Pignatari (1994). This is a quantitative method that applies to both, the dilution of antibiotic and diffusion of antibiotic into the medium. The device consists of a predefined, continuous, and exponential gradient of antibiotic concentrations immobilized along a rectangular plastic test strip. After 48 hours incubation a drop-shaped inhibition zone intersects the graded test strip at the inhibitory concentration (IC) of the antibiotic.

How it works?

- A predefined stable antimicrobial gradient is present on a thin inert non-porous plastic carrier strip 5mm wide, 60 mm long known as E-test strip.
- When this E test strip is applied onto an inoculated agar plate, there is an immediate release of the drug.
- Following incubation, a symmetrical inhibition ellipse is produced.
- The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value. The results obtained are summarized in **table 4.7.**

All the isolates of *S. aureus* (30) were selected on the basis of their resistance to Methicillin. The MICs obtained in the present investigation were variable and more than 80% isolates were showing resistance to other β lactams including cephalosporins and their combinations with β-lactamase inhibitors.

Gentilini *et al*. (2000) reported MICs (µg/ml) for the *S. aureus* as 0.5, and 0.125 for Oxacillin, and Ampicillin-Sulbactam, respectively.

In study conducted by Gill *et al*. (1981) from Maryland, USA, MIC of the Penicillin was reported as 0.05 - 1 and more than one in 35 and 64% isolates of *S. aureus* tested, respectively.

Rubin *et al*. (2011) from Saskatchewan, Canada, reported MIC_{50} Amoxicillin/Clavulanate and Ampicillin/Sulbactam for *S. aureus* organisms obtained from bovines as 2 and 4 µg/ml respectively.

The MICs of Ampicillin-Sulbactam, Aztreonam, and other β-lactams tested were not available in CLSI guidelines. As per CLSI guideline (2014), MIC of Oxacillin, if more than 4µg/ml, is considered as resistant. The interpretive criteria can be applied to other β-lactams.

S. aureus showing resistance to Methicillin are called as MRSA and those were included in the present MIC findings. These MRSA in present study showed considerable resistance to other β-lactams also.

MRSA is resistant to the β-lactamase stable antibiotics. The term Methicillin resistance is historically used to describe resistance to any of this class of antimicrobials even though Methicillin is no longer the drug of choice. The acronym MRSA persists and is used interchangeably with ORSA – Oxacillin-resistant *Staphylococcus aureus*. Oxacillin/Methicillin resistance implies resistance to all Penicillins, Cephalosporins, Monobactams, Carbapenems and β-lactam/β-lactamase inhibitor combinations.

The percentage of the resistance in descending order, obtained in the present study through MIC testing was as Oxacillin, Ampicillin, Cefotaxime,

Ampicillin + Sulbactam, Amoxicillin+ Clavulanic acid, Cefepime, Amoxicillin and Cefepime + Clavulanic acid.

Vahdani, (2004) reported resistance pattern of 90 MRSA collected from human patients from hospital of Iran and tested for MIC which showed 100 percent resistance to Penicillin, 92 percent to Ampicillin, and 93 percent to Cefotaxime.

Chin *et al*. (2007) tested Cefepime and Cefepime + Clavulanic acid for its efficacy against MRSA (50) obtained from laboratories in Detroit and reported MIC of more than 64 µg/ml for 90 percent isolates for both.

Namikawa *et al* (2012) tested MRSA (2) obtained from dogs from veterinary hospitals from Japan for their sensitivity against Amoxicillin, Ampicillin, Cefotaxime, Amoxicillin + Clavulanic acid. All the isolates showed presence of *mecA* gene by PCR and resistance to Amoxicillin and Ampicillin.

Vidhani *et al*. (2001) studied MRSA (97) isolates for their sensitivity against β- lactams and reported that none of the MRSA isolates was sensitive to Penicillin and Amoxicillin and only 17(21.5 percent) MRSA were sensitive to Cefotaxime. All the MRSA isolates showed MIC above 4 µg/ml.

The inclusion of β-lactamase inhibitors could not affect the sensitivity of MRSA much as resistance produced by MRSA is through Penicillin binding proteins and not via production of β-lactamase. *S. aureus* intrinsically produces β-lactamase enzymes that breakdown β-lactam antibiotics (eg, Penicillin); these are designated PBP 1 - 4. The β-lactam resistance of MRSA is determined by the production of a novel penicillin-binding protein called PBP 2' (PBP2a), that has a reduced binding affinity for β-lactam antibiotics. This allows MRSA strains to continue cell wall synthesis due to the uninhibited activity of PBP2' even in the presence of otherwise inhibitory concentrations of β-lactam antibiotics.

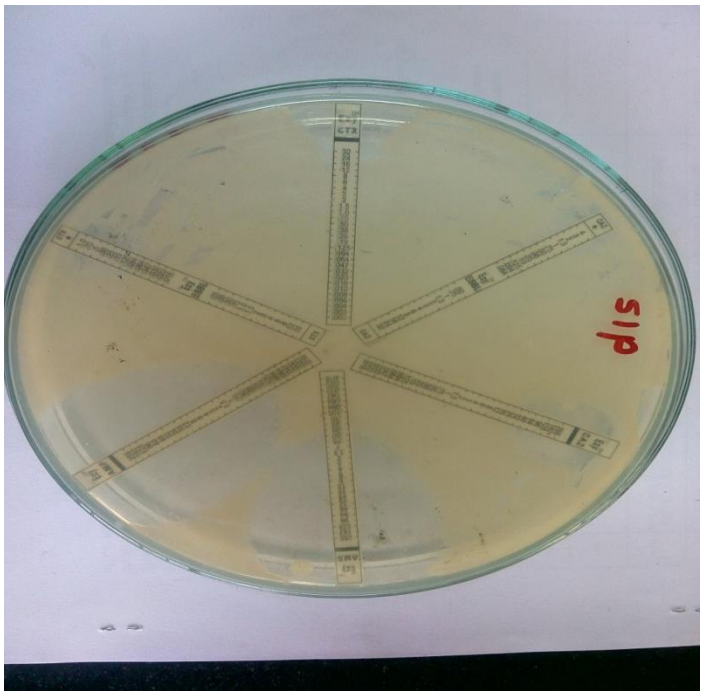


Plate 18: MIC for *S. aureus* isolates

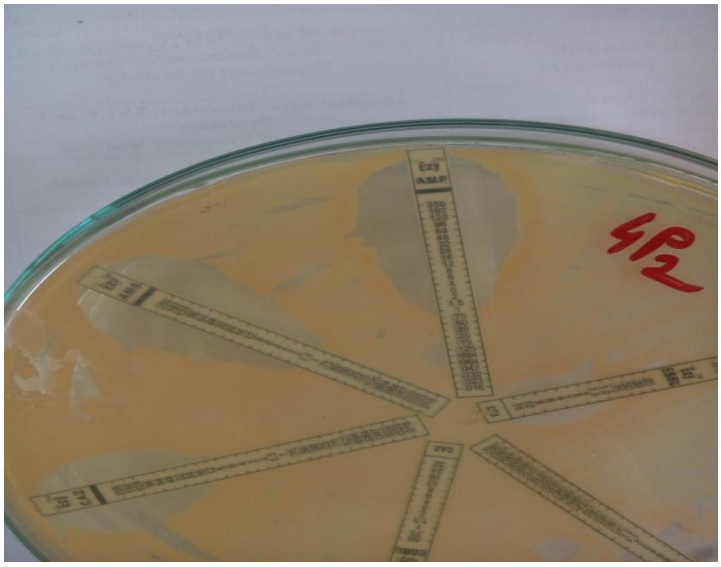


Plate 19: MIC for *S. aureus* isolates

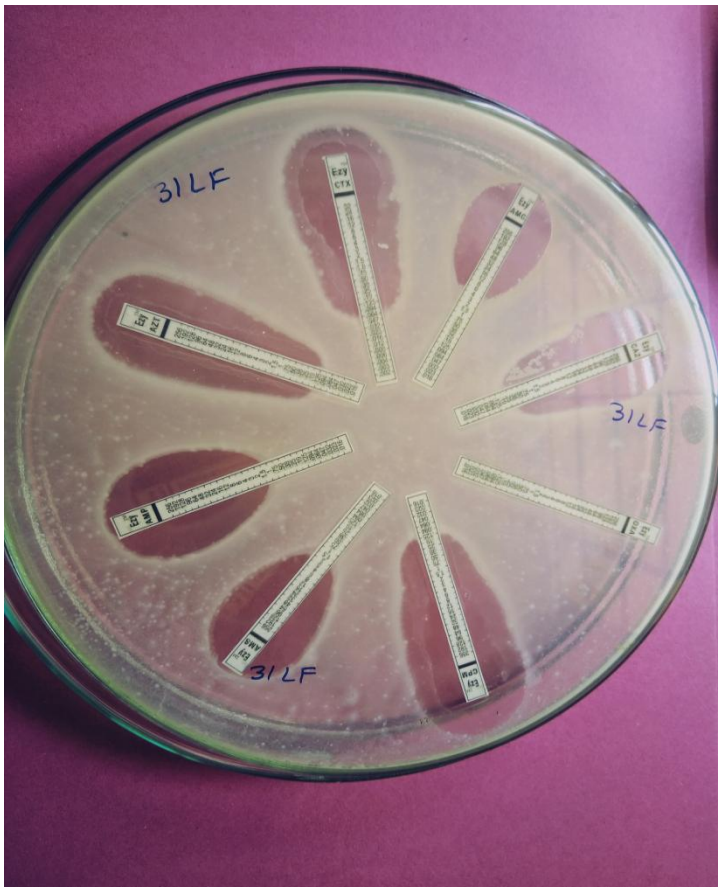


Plate 20: MIC for *E.coli* isolates

Table 4.7: MIC of different antimicrobials in *S. aureus* isolates (n=30) by E-test

Antimicrobials MIC strips	MIC in µg/ml for *S. aureus*	*1* µg/ml or less	*1* µg/ml to *2* µg/ml	*2*µg/ml to *4* µg/ml	*4*µg/ml to *8*µg/ml	*8*µg/ml to *16*µg/ml
Ampicillin	1 - 16		6.66 (2)	6.66 (2)	76.66 (23)	10 (3)
Ampicillin + Sulbactam	1 - 16		10 (3)	16.66 (5)	66.66 (20)	6.66 (2)
Cefepime	1 - 16		16.66 (5)	16.66 (5)	63.33 (19)	3.33 (1)
Cefepime + Clavulanic acid	1 - 16		23.33 (7)	16.66 (5)	56.66 (17)	3.33 (1)
Cefotaxime	1 - 16		6.66 (2)	10 (3)	76.66 (23)	6.66 (2)
Cefotaxime + Clavulanic Acid	1 - 16		13.33 (4)	16.66 (5)	60 (18)	6.66 (2)
Amoxicillin	1 - 16		10 (3)	16.66 (5)	56.66 (17)	16.66 (4)
Amoxicillin+ Clavulanic Acid	1 - 16		10 (3)	16.66 (5)	60 (18)	13.33 (4)
Oxacillin	1 - 16		6.66 (2)	13.33 (4)	80 (24)	

Table 4.8 : Resistance of *S. aureus* obtained by disc diffusion, MIC and PCR (Genotypic) method

Antimicrobials MIC strips	*Resistant isolates by disc diffusion test*	*Resistant Isolates by MIC*	*Detection of mecA*
Ampicillin	93.33 (28)	86.66 (26)	70 % (21)
Ampicillin + Sulbactam	80 (24)	73.33 (22)	
Cefepime	90 (27)	66.66 (20)	
Cefepime + Clavulanic acid	83.33 (25)	66 (18)	
Cefotaxime	80 (24)	83.33 (25)	
Cefotaxime + Clavulanic acid	63.33 (19)	66.66 (20)	
Amoxicillin	86.66 (26)	36.66 (11)	
Amoxicillin+ Clavulanic acid	66.66 (20)	73.33 (22)	
Oxacillin	100 (30)	80 (24)	

Statistical Analysis

Table 4.8 a. Anova Table					
Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean sum of squares**	**F cal**	**F prob**
Treatments	2	924.236	462.118	3.892	0.034
Error	24	2849.847	118.744	-	-
Total	26	-	-	-	-

Coefficient of Variation = 14.663

Table 4.8 b: Showing correlation of resistance obtained by Disc Diffusion Method, Minimum Inhibitory Concentration and PCR method (mecA amplification)

Sr. No	Resistance Method	Mean
1	Disc Diffusion Test	82.590[a]
2	MIC	70.366[b]
3	PCR (mecA detection)	70.00[b]

*Mean should read column wise. Mean showing dissimilar superscript differ significantly at 5% level of significance.

B. **MIC for the *E. coli***

Minimum inhibitory concentration was detected by the MIC strips for the 30 selected *E. coli* isolates which were phenotypically positive for ESBL production and showing resistance to third generation Cephalosporins and Aztreonam **(Plate 12 and 13).** The strips were having increasingly different concentrations of selected antimicrobials (Plate 20) and those were prepared according to CLSI guidelines 2014. The results obtained are summarized in table 4.10

The references citing MICs against *E. coli* for all the tested β-lactams are depicted in table 4.9.

Table 4.9: MIC interpretation criteria for *E. coli* as per CLSI (2014) M100 S-24 (Table 2A)

Antimicrobials	**Sensitive**	**Intermediate**	**Resistant**
Ampicillin	≤8	16	≥32
Ampicillin + Sulbactam	≤8/4	16/8	≥32/16
Amoxicillin + Clavulanic acid	≤8/4	16/8	≥32/16
Cefepime	≤2	4-8	≥16
Cefotaxime	≤1	2	≥4
Ceftazidime	≤4	8	≥16
Aztreonam	≤4	8	≥16

Table 4.10: MIC of different antimicrobials in *E. coli* isolates (n=30)

Antimicrobials MIC strips	MIC in µg/ml	*2*µg/ml to *4* µg/ml	*4*µg/ml to *8*µg/ml	*8*µg/ml to *16*µg/ml	*16*µg/ml to *32*µg/ml	*32*µg/ml to *64*µg/ml	*64*µg/ml to *128*µg/ml
Ampicillin	8 -64	--		23.33 (7)	13.33 (4)	63.33 (19)	--
Ampicillin + Sulbactam	4 - 32	--	50 (15)	40 (12)	10 (3)	--	--
Cefepime	2 - 32	33.33 (10)	13.33 (4)	--	53.33 (16)	--	--
Cefepime + Clavulanic acid	2 - 32	20 (6)	73.33 (22)	--	6.66 (2)	--	--
Cefotaxime	2 - 16	20 (6	50 (15)	30 (9)	--	--	--
Cefotaxime + Clavulanic acid	2 - 16	50 (15)	40 (12)	10 (3)	--	--	--
Amoxicillin	32 - 128	--			--	73.33 (22)	13.33 (4)
Amoxicillin+ Clavulanic acid	4 - 64	--	16.66 (5)	60 (18)	--	23.33 (7)	--
Ceftazidime	4 -64	--	3.33 (1)	20 (6)	73.33 (22)	3.33 (1)	--
Ceftazidime + Clavulanic acid	4 - 32	--	50 (15)	--	50 (15)	--	--
Aztreonam	4 - 64	--	6.66 (2)	--	73.33 (22)	3.33 (1)	--

Table 4.11 : Correlation among resistance of *E.coli* obtained by disc diffusion, MIC and PCR (Genotypic) method.

Antimicrobials	DDT % resistance (No. of isolates)	MIC % resistance (No. of isolates)	*PCR (TEM)*	*PCR (SHV)*
Ampicillin	86.66 (26)	63.33 (19)	76.66 (23)	70.00 (21)
Ampicillin + Sulbactam	10 (3)	10 (3)		
Cefepime	63.33 (19)	53.33 (16)		
Cefepime + Clavulanic acid	13.33 (4)	6.66 (2)		
Cefotaxime	93.33 (28)	80 (24)		
Cefotaxime + Clavulanic acid	26.66 (8)	10 (3)		
Amoxicillin	90 (27)	86.66 (26)		
Amoxicillin+ Clavulanic acid	33.33 (10)	23.33 (7)		
Ceftazidime	93.33 (28)	76.66 (23)		
Ceftazidime + Clavulanic acid	60 (18)	50 (15)		
Aztreonam	93.33 (28)	76.66 (23)		

Table 4.11 a: Analysis of Variance

Source of variation	**Degrees of freedom**	**Sum of squares**	**Mean sum of squares**	**F cal**	**F prob**
Treatments	3	956.120	318.707	4.449	0.015
Error	20	1432.808	71.640	-	-
Total	23	-	-	-	-

Coefficient of Variation = 11.061

Treatments found Significant at 5% level of Significance CD(0.05)= 10.194

Table 4.11 b: Showing correlation among resistance of *E. coli* obtained by Disc Diffusion Method, Minimum Inhibitory Concentration and PCR method (TEM and SHV amplification)

Sr. No	Resistance Method	Mean
1	Disc Diffusion Test	86.658^{a}
2	MIC	72.773^{b}
3	PCR (TEM detection)	76.660^{ab}
	PCR (SHV detection)	70.00^{b}

*Mean should read column wise. Mean showing dissimilar superscript differ significantly at 5% level of significance.

Oteo *et al*. (2006) studied *E. coli* resistance to few β-lactam antimicrobials and reported > 16 µg/ml MIC for all Ampicillin, Amoxicillin-Clavulanic acid, Piperacillin-Tazobactam, Ceftazidime, Cefepime and Cefoxitin, whereas for Cefotaxime, it was > 32 µg/ml.

Wang *et al.* (2015) also investigated MICs against *E. coli* isolates collected from human hospitals in China and reported as Cefotaxime, Ceftriaxone and Aztreonam with MIC value more than 128 µg/ml. whereas Ceftazidime and Cefepime were showing MIC value of 64 µg/ml.

From Missouri, Yang *et al*. (1998) also studied *E. coli*. Isolated and reported MICs for the Ampicillin, Amoxicillin + Clavulanic acid, Ceftazidime and Cefotaxime, as > 128 µg/ml., > 16 µg/ml., > 128 µg/ml. and 1µg/ml, respectively.

Doi *et al*. (2009) reported MIC of Ampicillin, Ceftazidime, Cefotaxime, Cefepime and Aztreonam against *E. coli,* as 128, 256, 256 and 128 µg/ml respectively.

MIC obtained in the present investigation is very low compared to the results of aforementioned authors however resistance to Ampicillin, Ceftazidime, Cefotaxime, Cefepime and Aztreonam is observed as 63.33, 76.66, 80, 53.33 and 76.66 percent respectively.

During the past century the excitement of discovering antibiotics as a treatment of infectious diseases has given way to a sense of pleasure and acceptance that when faced with AMR there will always be new and better antimicrobial agents to use. Now, with clear indications of a decline in the interest of pharmaceutical companies in anti-infective research, at the same time when multi-drug resistant micro-organisms continue to be reported, it is very important to review the prudent use of the available agents to fight these micro-organisms. Injudicious use of antibiotics is a global problem with some countries more affected than others. There is no dearth of interest in this subject with scores of scholarly articles written about it. While over the counter access to antibiotics is mentioned as an important contributor towards injudicious antibiotic use in developing nations, as shown in a number of studies, there are many provider, practice and patient characteristics which drive antibiotic overuse in developed nations such as the United States. As new antibiotic development dropped since year 1980, and the overall interest of pharmaceuticals in production of nonantimicrobials, the judicious use of these available antimicrobials is the need of hour.

4.4.4 Detection of ESBL Producing *E. coli*

Detection of extended spectrum B-lactamase (ESBL):

Phenotypic confirmatory test for detection of ESBL by double disc diffusion test: A double disc diffusion test was performed with Amoxicillin Clavulanic acid surrounded by Aztreonam and third generation Cephalosporin discs Cefotaxime and Ceftazidime (Freitas *et al*., 2003). The difference of 5mm or more among the antimicrobials when used with and without β-lactamase inhibitors was considered as indicators of ESBL production **(Plate 12 and 13).**

ESBL production in *E. coli* isolates were studied as per recommendations of CLSI (2014). Out of Ninty four (94) *E. coli* isolated, 28 were observed to be ESBL producers.

Multidrug-resistant (MDR) *E. coli* strains are often isolated from animals in veterinary hospitals and they are frequently associated with opportunistic infections, however, currently, detailed knowledge of the resistance mechanisms with reference to the ESBL production in this bacterium cultured from mastitic animals is limited. Overall prevalence of ESBL producing *E. coli* recorded in the present study was 29.78 percent which is according to the previous findings of Bandopadhyay *et al.* (2015). There are adequate numbers of references available in the data base on phenotypic AMR in the *E. coli* isolated from mastitis and majority of the strains revealed to be resistant to commonly used antimicrobials in the mastitis treatment *viz.* Trimethoprim sulphate, Ampicillin, Penicillin, Cephalosporins, Streptomycin, and Kanamycin (Fernandes *et al.,* 2011; Saini *et al.,* 2012; Memon *et al.,* 2013; Momtaz *et al.,* 2012; Metzger and Hogan, 2013; Umar *et al.,* 2013; Kumar *et al.,* 2012 and Liu *et al.,* 2014). Although few antimicrobial belonging to β- lactam group like Penicillin and Cephalosporins were included in the study of these earlier investigators, no systematic study with reference to the ESBL producing *E. coli* strains was undertaken. Therefore, this study was also conducted to know the extent of *E. coli* in terms of virulent ESBL production phenotypically and genotypically.

4.4.5 : Detection of genes and ESBL by genotypic method:

In the present study detection of *TEM, SHV* and *CTX-M* genes was performed. All these genes express ESBLs showing resistance to β-lactam class of antimicrobials. In the present study 28 isolates were showing presence of ESBLs phenotypically. For detection of above

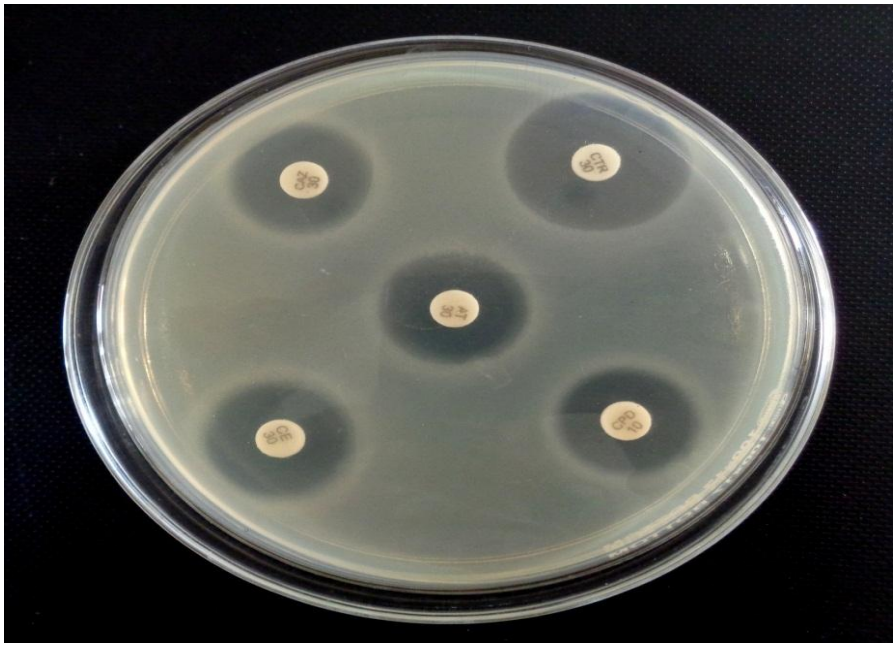


Plate 12: ESBL screening from *E.coli* organisms

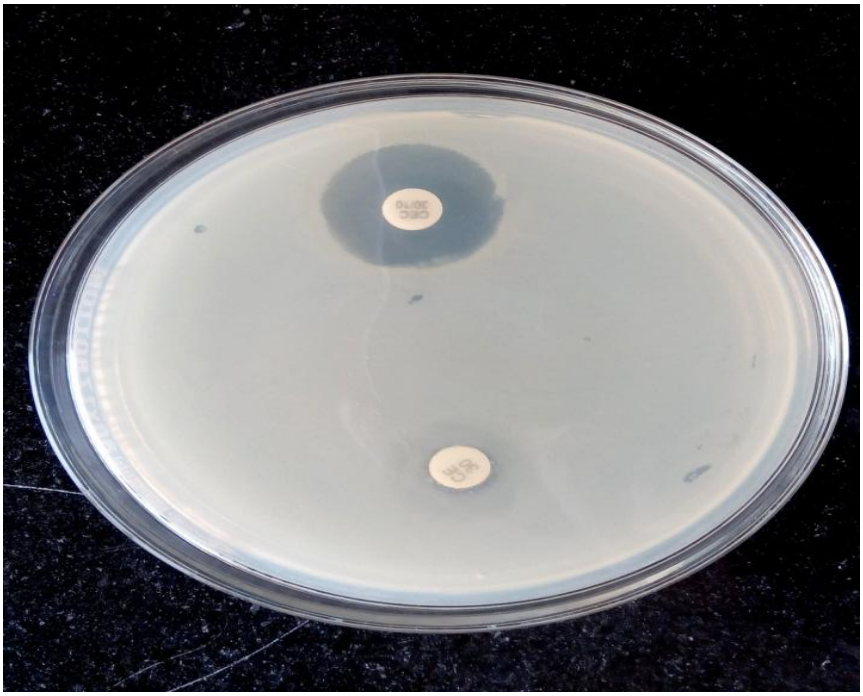


Plate 13: Confirmation of ESBL by using Ceftazidime and Ceftazidime + Clavulanic acid

mentioned genes total 30 isolates were selected including ESBL positive isolates. Genotypically *CTX-M, TEM* and *SHV* in *E.coli* were observed in 0, 23 and 21 isolates respectively. Gene detection in *E. coli* and their results are discussed in section 4.4.3.

4.4.6 Detection of genes in *S. aureus*:

In the present study randomly 30 isolates out of 40 showing resistance to Methicillin and other antimicrobials of β-lactam group, were recruited for detection of *nuc* and *mecA* genes.

A. Detection of *nuc* gene

Amplification of Thermonuclease (*nuc*) gene is used to detect and quantify *S. aureus* which is specifically known as *S. aureus* species specific gene (Studer *et al*., 2008). Therefore in the present study selected 30 isolates were subjected to detection of *nuc* gene, which is a specific for thermonuclease gene present in *S. aureus*. Many authors reported identification of *S. aureus* by detectining *nuc* gene using PCR.

In the present study all the isolates were showing presence of *nuc* gene, of amplicon size of 255bp, which confirmed the species as *S. aureus.* Results of the present investigations are in agreement with several authors, who reported presence of *nuc* gene specifically in *S. aureus* **(Plate 15).**

S. aureus strains produce an extracellular thermostable nuclease (thermonuclease or TNase) with a frequency similar to that at which they produce coagulase (Madison *et al*., 1983). The TNase is a protein with a molecular mass of 17,000 Da (Kovacevic *et al*., 1985). It is an endonuclease, degrading both DNA and RNA, and the enzymatic activity can resist 100^{o}C for at least one hour. (Lachica *et al*., 1971). An enzymatic test for TNase production is used in many laboratories for the identification of *S. aureus* isolates (Lachica *et al*., 1972).

Gao *et al*. (2011) reported that 100 percent of *S. aureus* isolates were showing presence of *nuc* gene, where all the isolates were obtained from bovine SCM and identification was done by growing them on selective media and biochemical characters

According to Brakstab *et al*. (1992) *nuc* gene, is specifically identified in *S. aureus* isolates collected from bovine SCM and it can considered as gold standard in identification of coagulase positive Staphylococci or *S. aureus.*

B. Detection of *mecA*.

In the present study 40 isolates were showed resistance to Methicillin and 70 to Oxacillin, when tested with disc diffusion tests. Only 30 isolates were recruited for the detection of *mecA* genes in the present investigation which were showing resistance to both Methicillin and Oxacillin. Detection of *mecA* was done as per the method described by Poulsen *et al*. (2003). Primers were selected which were having 21 base pairs. Out of 30 isolates 70 percent (21) isolates were showing presence of *mecA* gene **(Plate 14).**

The Methicillin resistance is heterogeneous in nature and level of resistance varies according to the culture conditions and β-lactam antibiotics used. Most of the isolates show heterogeneous resistance in routine culture conditions and therefore detection of presence of *mec A* gene by PCR is accepted as gold standard. It is the most reliable and fundamental method for identifying MRSA (Ciftci *et al*., 2009). Findings of Ciftci *et al*. (2009) revealed that among *S. aureus* strains found as Oxacillin resistant phenotypically, only 30.7 percent were positive for *mecA* gene. As per Asfour and Darwish (2011) isolated 25 (52 percent) and 118 (67 percent) *Staphylococcus* strains from 223 milk samples collected from cows with CM and SCM, respectively in Egypt. Phenotypic prediction of *mecA* gene presence was conducted by both Methicillin and Oxacillin disc diffusion tests.

In the present study also resistance to Oxacillin (30.95 percent) and Methicillin (23.80) was observed and *mecA* gene was detected in only 70 percent *S. aureus* isolates showing resistance to Methicillin phenotypically. Present results are in consistence with the observations of Chandrasekaran *et al*. (2014) who recorded 10.34 percent incidence of *blaZ* and *mecA* genes in *S. aureus* isolated from mastitic dairy cows.

In contrast, all Oxacillin resistant isolates showing positivity to *mecA* was also revealed in the study of Coelho *et al*. (2009) conducted in the Brazil. Extensive study from India on Sahiwal mastitis by Kumar *et al*. (2011) revealed 13.08 percent *S. aureus* strains resistant to

~ 527 bp

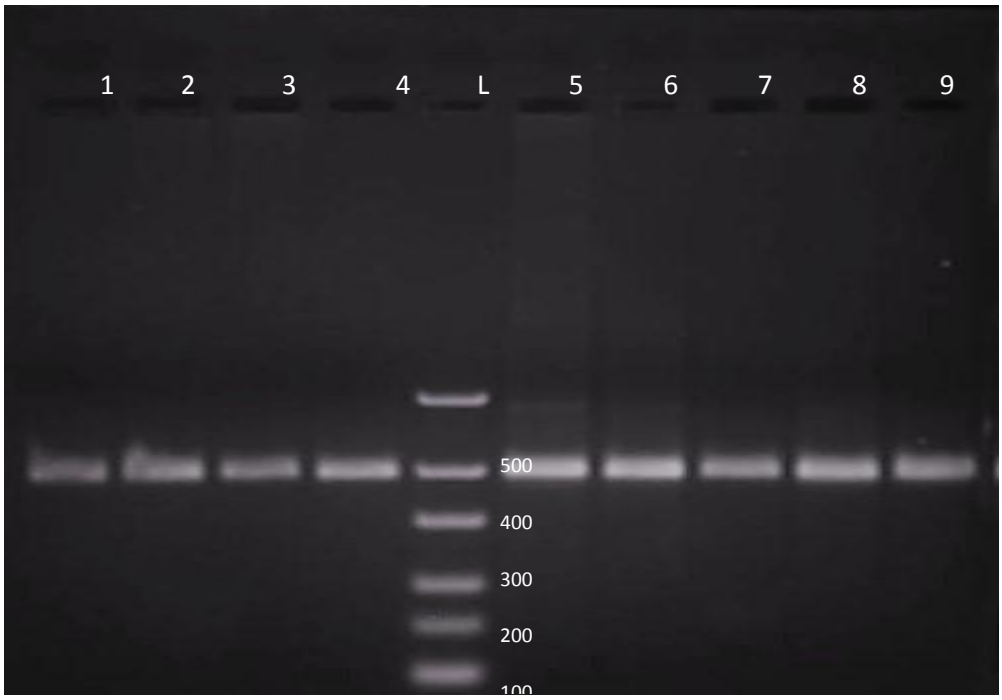


**Plate 14:Detection of antimicrobial resistance genes (mecA) in*S. aureus isolates*
Lane L: 100 bp DNA Ladder , Lane 1 – 4 and 5 - 9 : Amplified*mecA*gene**

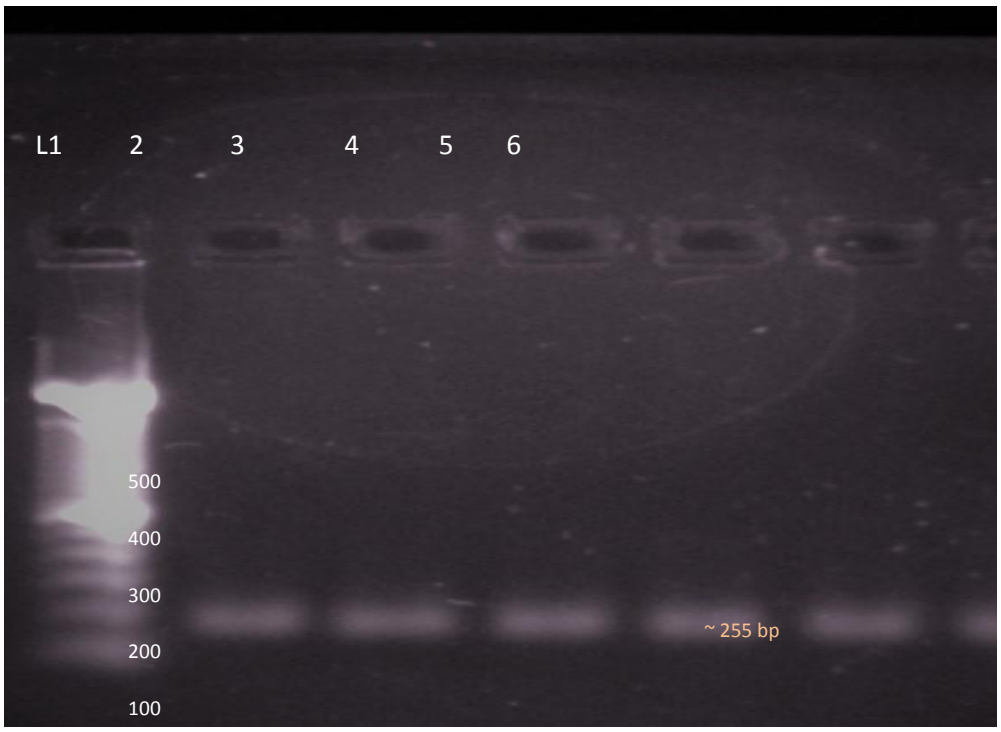


Plate 15:Detection of genes (*nuc*) in*S. aureus isolates.* Lane L 100bp ladder Lane 1-6 amplified *nuc* gene

Methicillin, out of which 71.43 percent MRSA amplified *mecA* gene. Aslantas *et al*. (2011) revealed single or various combinations of resistance genes v*iz., MRSA ermB and ermC* in erythromycin resistant *S. aureus* isolates from bovine SCM in Turkey. Their findings warned prudent use of these antimicrobials and establish continuous surveillance to prevent the spread of antimicrobial resistant *Staphylococci*.

The present findings are in agreement with Kumar *et al*. (2011), where 71.43 percent strains of *S. aureus,* tested were *mecA* positive.

Similarly, close correspondence between phenotypic and genotypic test was observed for majority of *S. aureus* isolates from bovine CM and SCM by Asfour and Darwish (2011). Bagicigil *et al*. (2012) also revealed all the 78 β-lactam positive *S. aureus* isolates of bovine mastitis expressed the *blaZ* gene.

Epidemiologically and genetically related Methicillin resistant *Staphylocooci* could be revealed in the study of Vishnupriya *et al*. (2014) which indicates the transmission of these strains between human and animals. Link between genotype and AMR in bovine mastitis related *S. aureus* was studied in detail by Sakwinska *et al.* (2011) and their observations revealed strong association of Penicillin resistant clusters highlighting the fact that the knowledge of local epidemiology is essential for accurate treatment by veterinarians. They further emphasized the need of more research on molecular types for better knowledge of both local and global epidemiologies of *S. aureus* clones.

Variation in phenotypic and genotypic AMR of Staphylococci from bovine mastitis milk was also observed by Kot *et al.* (2012). They have also revealed 47.4 percent coagulase negative *Staphylococci* (CNS) carrying *tetK* gene and 11.1 percent of ML resistant CNS strains harboring *ermB* gene. Resistance to Methicillin, Tetracycline and macrolides was detected more frequently in strains from CM mastitis as compared to strains from SCM. They have concluded that use of PCR method alone for detection of resistance to these antimicrobials in CNS from cattle is not reliable. Present findings also support this statement and phenotypic resistance should be correlated with genotypic pattern.

4.4.7 Detection of *CTX-M, TEM* and *SHV* genes:

AMR in bacteria has emerged as a problem in both human and veterinary medicine. One of the currently most important resistance mechanisms in *Enterobacteriacae*, which reduces the efficacy even of modern expanded-spectrum Cephalosporins (except Cephamycins, and Carbapenems) and Monobactams is based on plasmid-mediated production of enzymes that inactivate these compounds by hydrolyzing their β-lactam ring. Such resistance is encoded by an increasing number of different point-mutational variants of classical broad-spectrum β-lactamases (BSBL). These variants are called ESBL and most are derivates of *TEM* and *SHV* β -lactamase families, whereas other groups, such as *CTX-M*, *PER* and *KPC* b-lactamases have been described more recently (Coque *et al*., 2008)

A. Detection of *CTX-M*:

Farm animals are well recognized as a potential reservoir of ESBL-producing *E. coli*, and therefore, it has been considered that the spread of such resistant bacteria or determinants may occur via the food chain. The aim of this study was also to determine occurrence of *E. coli* strains producing *CTX-M* type ESBL production, however, none of the isolates showed positivity for the *CTX-M* type by PCR in the present investigation.

Genomic resistance of *CTX-M* type ESBL in *E. coli* isolated from cases of bovine mastitis has been reported earlier (Geser *et al.,* 2012; Watson *et al.,* 2012; Dahmen *et al.,* 2013; Ohnishi *et al.,* 2013; Bandopadhyay *et al.,* 2014; Timofte *et al.,* 2014 and Davis *et al.,* 2015).

Genes encoding *CTX-M2/15* have been detected in very low frequency (0.22 percent) from Japan in the *Enterobacteriacae* isolates causing bovine mastitis by Ohnishi *et al.* (2013). Extensive investigation of Timofte *et al.* (2014) on molecular characterization of *CTX-M-15* producing *E. coli* from bovine mastitis emphasized the importance of detecting ESBL producing bacteria in food production animals and the need for routine screening of isolates for ESBL production in veterinary diagnostic laboratories. They have further concluded that, to preserve the efficacy of extended-spectrum Cephalosporins for the treatment of problematic cases of bovine mastitis or other conditions, it is essential that culture and sensitivity testing always be performed, especially for recurrent infections, and therefore, empirical therapy should be

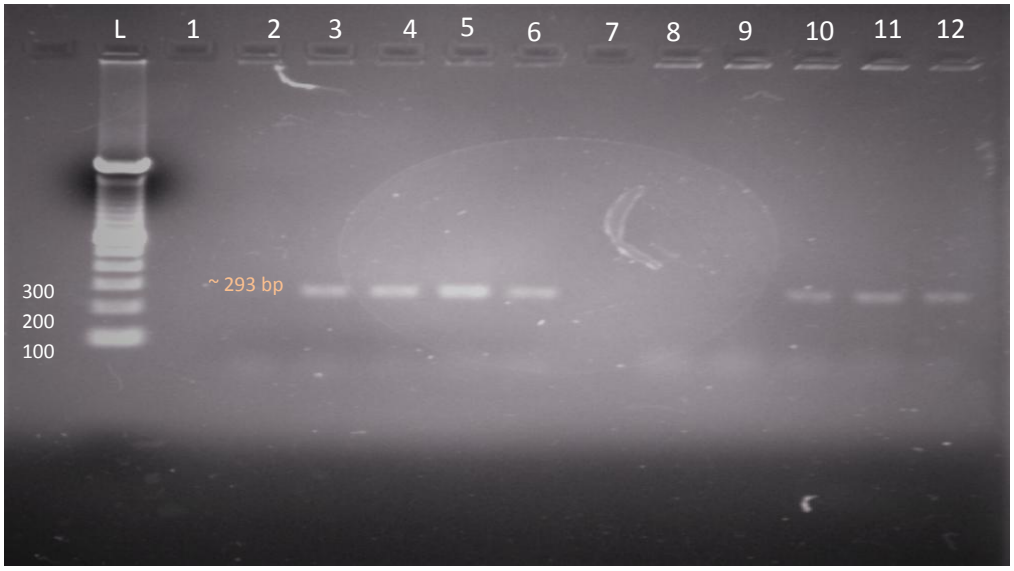


Plate 16: Detection of antimicrobial resistance genes *(SHV)* in *E. coli* isolates .Lane L: 100 bp DNA Ladder , Lane 3 -6 and 10-12 : Amplified *SHV* gene in *E.coli* isolates. Lane 1,2, 7, 8 and 9 - negative

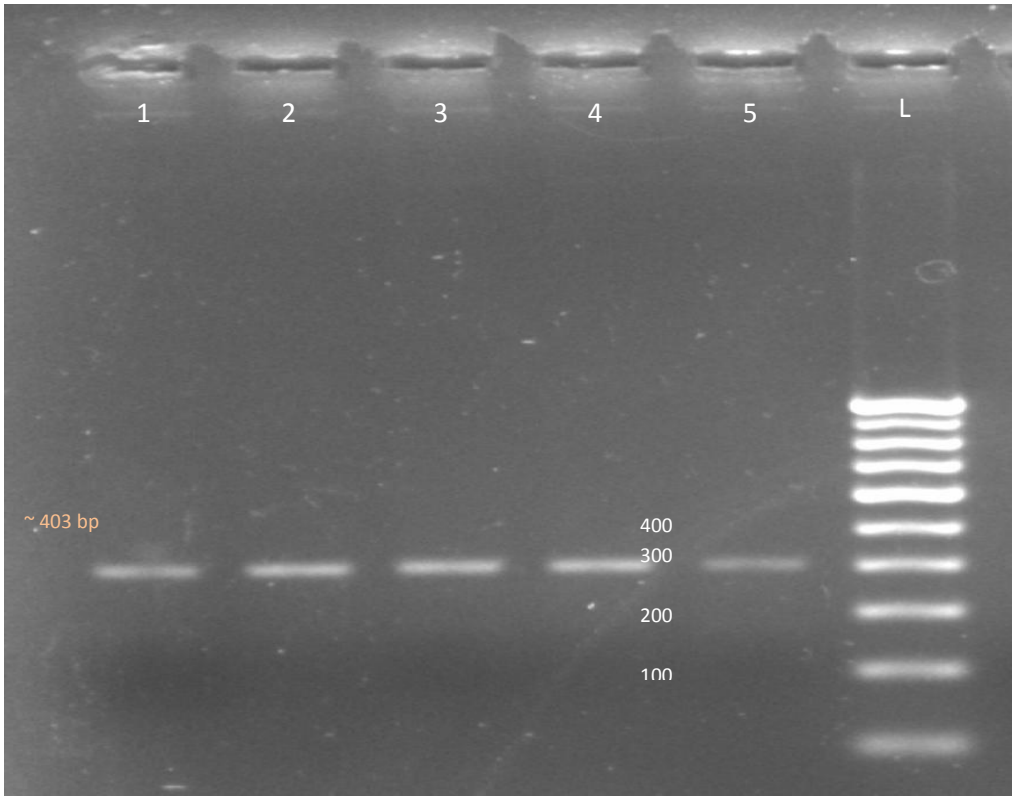


Plate 17: Detection of antimicrobial resistance genes *(TEM)* in *E. coli* isolates .Lane L: 100 bp DNA Ladder , Lane 1-5 Amplified *TEM* gene in *E.coli* isolates

avoided in such cases. Antimicrobial susceptibility testing before prescribing antibiotic in human too was suggested by Kumar *et al.* (2014) who have detected ESBL producing *E. coli* in very high percent (55.55 percent) from human samples. Present findings also support these recommendations and further suggest that isolation of pathogen and culture sensitivity to antimicrobials including ESBL inhibitors should make compulsory to avoid problem of AMR in mastitis pathogen in particular and other food borne zoonotic pathogens in general.

B. Detection of *TEM*:

In the present study, 23 isolates out of 30 were showing presence of *TEM* gene with amplicon size of 403 bp. Genotypic expression of ESBL was shown by 23 Isolates out of 28, which were ESBL positive phenotypically. In the present study, there was presence of *TEM* gene by PCR and overall it was 82.14 percent **(Plate 17).**

C.Detection of SHV:

In the present study 21 isolates out of 28 were showing presence of *SHV* gene with amplicon size of 293 bp. Out of 28 phenotypically ESBL positive 21 had confirmed presence of *SHV* gene by PCR and overall it was 75 percent **(Plate 16).**

In the last decade a variety of plasmid mediated ß-lactamases have emerged in Gram-negative bacteria. These included both ESBLs and AmpC. ESBLs confer resistance to a variety of ß-lactams, including Penicillins, first – second – third and fourth generation Cephalosporins, and Monobactams, but usually not to the Carbapenems or the Cephamycins. The most frequent ESBLs in *Enterobacteriacae* belong to the *TEM, SHV*, and *CTX-M* families. This resistance is conferred to the plasmids which can replicate within the species of many genera and even families, and such plasmids are defined as broad-host-range plasmids. Risk of ESBL producing bacterial pathogens to human origination from animal source is an established fact (Tenover, 2006; Liebana *et al.,* 2013; Timofte *et al.,* 2014). A variety of farm management factors may facilitate the introduction and spread of ESBL producing bacteria. The establishment of risk factors for occurrence of ESBL/AmpC producing bacteria is complicated by scarcity or lack of accuracy of reliable data.

Therefore, further research is needed to understand more about the driving forces that have led to the rapid spread of these resistant bacteria in many countries worldwide. Most ESBL producing strains may carry additional resistances such as to sulfonamide and other commonly used veterinary drugs. Therefore, persistence and dissemination of ESBL producing bacteria can be selected for by the use in food-producing animals, not only of cephalosporins, but also of other compounds such as Amoxicillin, Sulphonamides, Trimethoprim, Fluoroquinolones, and Aminoglycosides (Liebana *et al.,* 2013).

In the present study occurrence of *TEM* and *SHV* was observed from 23 and 21 isolates out of 30 selected *E.coli* strains. *TEM* (76.66 percent) and *SHV* (70 percent) of isolates showing resistance to two or more antimicrobials.

Timofte *et al.* (2014) concluded, that to preserve the efficacy of extended-spectrum cephalosporins for the treatment of problematic cases of bovine mastitis or other conditions, it is essential that culture and sensitivity testing always be performed, especially for recurrent infections, and therefore, empirical therapy should be avoided in such cases. Antimicrobial susceptibility testing before prescribing antibiotic in human too was suggested by Kumar *et al.* (2014) who have detected ESBL producing *E. coli* in very high percent (55.55 percent) from human samples. Present findings also support these recommendations and further suggest that isolation of pathogen and culture sensitivity to antimicrobials including ESBL inhibitors should be made compulsory to avoid problem of AMR in mastitis pathogen in particular and other food borne zoonotic pathogens in general.

For several decades, antibiotic-resistant bacteria have created a constant problem in hospital settings. The emergence of ESBL producing bacteria, particularly *E. coli* and *K. pneumoniae*, is now a critical concern for the development of therapies against bacterial infection. ESBLs consist of three major genetic groups: *TEM*, *SHV*, and *CTX-M* types. Nosocomial infections due to *TEM* and *SHV*-producing *K. pneumoniae* strains were frequently documented until the late 1990s. The number of reports on community-acquired infections caused by *CTX-M* producing *E. coli* strains have dramatically increased over the last decade; however, *K. pneumoniae* strains, of either the *TEM* or *SHV* types, are persistent and important ESBL producers. The spread of ESBL genes is associated with various mobile genetic elements, such as transposons, insertion sequences, and integrons. The rapid dissemination of

ESBL genes of the *CTX-M* type may be related to highly complicated genetic structures. These structures harboring ESBL genes and mobile elements are found in a variety of plasmids, which often carry many other antibiotic resistance genes. Multidrug-resistant *CTX-M-15*-producing *E. coli* strains disseminate worldwide. Efficient mobile elements and plasmids may have accelerated the genetic diversity and the rapid spread of ESBL genes, and their genetic evolution has caused an emerging threat to the bacteria for which few effective drugs have been identified. (Chong, 2011).

4.4.8: Correlation among Disc diffusion, MIC and PCR results:

In the present investigation disc diffusion test and MIC methods show good agreement with PCR in detection of resistant gene *mecA* in case of *S. aureus*. Presence of *mecA* gene was observed in 70 percent (21) isolates out of the 30 tested isolates of *S. aureus.* Resistance shown by disc diffusion method was in the range of 63-100 percent whereas; MIC method showed resistance in the range of 36 to 86 percent. Phenotypically all the 30 isolates were resistant to Methicillin. However, few isolates were showing resistance to other β-lactam antimicrobials also. After statistical analysis was done by student T test to find out correlation among these three methods, where no significant difference was observed among the results obtained by these methods.

When all the methods were compared statistically with completely randomized design **(Table 4.8 c),** no significant difference could be observed among MIC and PCR method of resistance detection. However, resistance obtained by disc diffusion method differ significantly from MIC and PCR method regarding *S. aureus* resistance.

In the current investigation MIC was established for *E.coli* for 30 selected isolates on the basis of results obtained through disc diffusion test. Phenotypically 28 isolates were ESBL positive by disc diffusion method. However, ESBL positive isolates by phenotypic method were also positive for presence of *TEM* (23) and SHV (21) genes except *CTX-M*. None of the isolates was positive for *CTX-M* gene. Therefore, presence of ESBL can be interpreted on the basis of *TEM* and *SHV* genes, which usually express ESBLs and also responsible for resistance to β-lactams.

When all the methods were compared statistically with completely randomized design **(Table 4.11 b),** PCR method of TEM detection showed no significant difference for MIC and Disc diffusion test. However, MIC method showed correlation with SHV detection. It indicates, that PCR methods of resistant gene detection are useful for resistance identification.

Chapter 5

Summary & Conclusions

CHAPTER V

SUMMARY AND CONCLUSIONS

SCM is a major a problem in the bovines especially dairy cows and it is mostly associated with managemental practices. *S. aureus and E. coli* are important bacterial pathogens responsible for contagious bovine mastitis globally. Since it is ubiquitous in the affected dairy cows and associated environment, practical solutions for its control totally rely on implementation of hygienic measures at farm level. Both organisms *are* virulent and thus knowledge of strains harboring multiple enterotoxin types is very important in management of mastitis due to this pathogen. Data on distribution of *S. aureus and E. coli* enterotoxin types and their resistance to β-lactam group of antimicrobials in India is largely lacking, this study was undertaken to assess the prevalence of *S. aureus* and *E. coli* antimicrobial resistance to β-lactam as per the guidelines of CLSI.

During present investigation a total of 750 buffaloes were sampled from six farms namely Maharaja dairy farm (Unit 21 Goregaon), Maharaja dairy farm (Unit 19 Goregaon), Palghar, Safale, Dahisar and Virar. The samples were screened for SCM by CMT. Out of 750 milk samples, 310 were positive for CMT, thus the overall prevalence of SCM recorded was 41.33 percent. Relatively higher prevalence was recorded at Palghar farm (51.11 percent) followed by unit 19 Goregaon (46.45 percent), unit 21 Goregaon (43.90 percent), Dahisar (43.18 percent), Safale (32.98 percent) and Virar (27.82 percent). All together occurrence of SCM was observed in the range of 27-51 percent from all farms sampled during the study.

The occurrence of *S. aureus* and *E. coli* in the present investigation was 54.19 (n= 168) and 30.32 (n= 94) percent, respectively. The aim of present study was also to assess the resistance pattern of *S. aureus* and *E.coli* against selected β -lactam antimicrobials. Therefore sensitivity of *S. aureus* was assessed against Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid, Methicillin and Oxacillin through antimicrobial disc diffusion, which was found to be 42.85, 65.47, 64.28, 77.38, 61.30, 73.80, 43.45, 73.21, 70.23 and 69.04 percent, respectively. The most effective β-lactam antimicrobials found in present investigation in descending order were Cefepime + Clavulanic acid > Cefotaxime + Clavulanic acid >

Amoxicillin + Clavulanic acid> Oxacillin > Ampicillin + Sulbactam > Cefotaxime> Ampicillin. Whereas great resistance was observed to Ampicillin and Amoxicillin when used alone.

Similarly sensitivity of *E.coli* was assessed against Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid, Ceftazidime, Ceftazidime + Clavulanic acid and Aztreonam antimicrobial discs which was found to be 38.29, 71.27, 80.85, 86.17, 37.23, 71.27, 38.29, 77.65, 9.57, 28.72 and 4.25 percent, respectively. The most effective β-lactam antimicrobials to treat *E.coli* infections, found in present investigation in descending order were Cefepime + Clavulanic acid > Cefepime> Amoxicillin + Clavulanic acid > Ampicillin + Sulbactam > Cefotaxime + Clavulanic acid. Whereas great resistance was observed to Cefotaxime, Ceftazidime, Aztreonam and Ampicillin.

On the basis of results of sensitivity tests randomly 30 isolates selected, which were showing resistance to two or more β-lactam antimicrobials. The MIC was tested with the E-test method by keeping strips on the media plate containing *Staphylococcus* organisms.

The MIC was tested with the E-test method by keeping strips on the media plate containing *Staphylococcus* organisms. The MIC in µg/ml against *S. aureus* investigated for Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid and Oxacillin. Among all the Methicillin Resistant *S. aureus showing resistance to* Ampicillin (86.66 percent), Ampicillin + Sulbactam(73.33 percent), Cefepime (66.66 percent), Cefepime + Clavulanic acid (60), Cefotaxime (83.33 percent), Cefotaxime + Clavulanic acid (66.66 percent), Amoxicillin (70 percent), Amoxicillin + Clavulanic acid (73.33 percent) and Oxacillin (80 percent) when tested for MIC. Resistance to Methicillin and other antimicrobials was observed during disc diffusion test and same isolates when subjected for *mecA* expression by PCR, 70 percent isolates found positive. From the results of MIC it was obvious that lowered MIC was found where the antimicrobials are used in combination with β-lactamase inhibitors.

Similarly 30 isolates of *E. coli* on the basis of phenotypic ESBL detection were studied for their MIC and PCR analysis to detect *TEM*, *SHV* and *CTX-M* genes. Resistant *E.coli* isolates observed by MIC method for Ampicillin (63.33 percent), Ampicillin + Sulbactam (10

percent), Cefepime (53.33 percent), Cefepime + Clavulanic acid (6.66 percent), Cefotaxime (80 percent), Cefotaxime + Clavulanic acid (10 percent), Amoxicillin (86.66 percent), Amoxicillin + Clavulanic acid (23.33 percent), Ceftazidime (76.66) and Ceftazidime + Clavulanic acid (50).

In the present study ESBL producing *E.coli* was also investigated through results obtained in the disc diffusion tests. Confirmatory test for detection of ESBL was performed with Amoxicillin + Clavulanic acid, Amoxicillin alone, Aztreonam and third generation Cephalosporin discs of Cefotaxime and Ceftazidime. From the total 94 *E.coli* isolates total 28 isolates were found to be ESBL producers. ESBL producers generally do not respond to β–lactam antimicrobials if used alone. However the present study isolates showed enhanced susceptibility to β-lactam antimicrobials when used in combination with β-lactamase inhibitors like Clavulanic acid and Sulbactam.

During present study same 30 isolates which were selected for MIC studies were subjected to PCR for detection of *nuc* and *mecA* genes. All the 30 isolates exhibited presence of *nuc* gene, which confirmed that all were *S. aureus* isolates. Resistance to Methicillin and other antimicrobials was observed during disc diffusion test and same isolates when subjected for *mecA* expression by PCR, 70 percent isolates found positive.

Similarly 30 isolates of *E. coli* after studying their MIC were subjected to PCR analysis to detect *TEM*, *SHV* and *CTX-M* genes. All these genes express ESBLs showing resistance to β-lactam class of antimicrobials.

MIC obtained in the current investigation was done on 30 selected isolates on the basis of results obtained through disc diffusion test. Isolates showing positive results for *TEM* and *SHV* by PCR were resistant to other β-lactam antimicrobials when used alone. None of the isolates was found positive for *CTX-M* gene. However *TEM* and *SHV* genes were observed in 23 and 21 isolates respectively.

Therefore presence of ESBL can be interpreted on the basis of *TEM* and SHV genes, which are usually expresses ESBLs and also responsible for resistance to β-lactams.

Present study revealed that AMR with special reference to β- lactams is common in *S. aureus* and *E. coli* strains. Among all the tested antimicrobials, Ampicillin + Sulbactam, Cefepime + Clavulanic acid and Amoxicillin + Clavulanic acid are the efficient β-lactams against *S. aureus* causing SCM. Similarly Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime + Clavulanic acid and Amoxicillin + Clavulanic acid could be better alternative for treating *E. coli* associated SCM, if used judiciously.

Present findings suggest that phenotypic resistance should be correlated with genotypic pattern using PCR targeting resistant genes to decide the antimicrobial therapy for mastitis control.

Following conclusions could be drawn from the observations of the present investigation,

1. Overall prevalence of SCM in the present study was 41.33 percent.
2. According to CLSI recommendations, *S. aureus* and *E. coli* strains isolated in the present study are resistant to many β-lactam antimicrobials when used alone. Therefore, β-lactams should be used in combination with β-lactamase inhibitors after screening with antimicrobial sensitivity testing.
3. Resistance to β-lactam group of antibiotics tested phenotypically and genotypically is alarming and highlights the need of prudent use of these antimicrobials by veterinarians in treatment of mastitis.
4. For determining the antimicrobial resistance in *S. aureus* isolates from bovine mastitis, both phenotypic and genotypic assays should be used simultaneously.
5. Continuous monitoring of antimicrobial resistance pattern at herd level is recommended.
6. Detection of MRSA and avoiding use of β-lactams is recommended.
7. Detection of ESBL and use of β-lactamase inhibitor combinations to treat ESBL positive cases is recommended.
8. Co-ordinated efforts at national level are required to monitor antibiotic resistance to take policy decision regarding use of antimicrobials in dairy herds.

Proposed Area of Future Research

PROPOSED AREA OF FUTURE RESEARCH:

- Generation of resistance pattern data from different parts of Maharashtra for important animal pathogens and useful antimicrobials.

- To describe and predict the dynamics of antibiotic resistance development at the level of the drug target, the microbe and the host..

- Extensive collection of resistant mutant strains of *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium tuberculosis* and other bacterial species that are well-characterized with regard to genetics and resistance mechanisms.

- To use a defined set of strains to perform the in vitro and in vivo experiments.

- Examination of fluoroquinolones, beta-lactams, aminoglycosides, macrolides, and other important antibiotics.

- To develop and implement methods to rapidly diagnose resistance patterns in bacteria. Means Development of a rapid test for antibiotic resistance usable in any clinical setting.

- Generation of antibiotic dosing protocols that reduce resistance development.

- Expectations: Results obtained should provide knowledge and insight of general nature applicable virtually to any combination of bacterium and resistance.

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Appendix

APPENDIX- I

1) EMB Agar

Ingredient	Gms/liter
Tryptone	:10.00
Dipotassium phosphate	:2.00
Lactose	:5.00
Sucrose	:5.00
Eosin Y	:0.4
Methylene Blue	:0.065
Agar	:13.5
pH	:7.1 ± 0.2

Suspend 36.0 gms of powder in 1000 ml distilled water.Heat with freqente agitation to dissolve ingredients completely. Avoid overheating and sterilize by autoclaving at 15lbs pressure (121˚C) for 20 min.

2) Mannitol Salt Agar Base

Mannitol Salt Agar Base	: 111.00gm
Distilled water	: 1000ml
pH	: 7.4±0.2
Autoclaved at 121^0C for 20 min	

3) Brain heart infusion (BHI) Broth

Ingredient	Gms/liter
Peptic digest of animal tissue	:10.00
Calf brain, infusion (solids)	:12.50
Beef heart infusion(solids)	:5.00
Dextrose	:2.00
Sodium chloride	5
Disodium phosphate	:2.5
pH	:7.4 ± 0.2

Suspend 37.0 gms of powder in 1000 ml distilled water. Heat with frequent agitation to dissolve ingredients completely. Avoid overheating and sterilize by autoclaving at 15 lbs pressure (121˚C) for 15 min.

4) Methyl Red test

A. Medium (Glucose phosphate peptone water)

Peptone	: 5.0gm
Dipotassium hydrogen phosphate	: 5.0gm
Distilled water	: 1000ml
Glucose(10% solution)	: 50 ml
pH	: 7.6 ± 0.2

Autoclaved at 121^0C with 15 lbs for 15 minutes.

B. Methyl Red Indicator Solution

Methyl red	: 0.1gm
Ethanol (95%)	: 300 ml
Distilled water	: 200ml

5) Voges- Proskauer Test - Same as per Methyl Red Test

Voges- Proskauer indicator solution

a. 40% potassium hydroxide solution

Potassium hydroxide	: 40.0 gm
Distilled water	: 100 ml

b. 5% α-Napthol solution

α-Napthol	: 5.0gm
Absolute ethanol	: 100 ml

6) Indole Test

A.Kovac's reagent

p- dimethylaminobenzaldehyde	: 5.0gm
Amyl alcohol	: 75 ml
Conc. HCL	: 25 ml

7) Oxidase reagent

Tetra methyl-p- phenylnediaminedihydro- chloride	: 0.1gm
Distilled water	: 10 ml

8) Media for antimicrobial test

a) Mueller Hinton (MH) Agar No. 2 (Dehydrated, HiMedia)

Ingredients	Grams/liter
Casein acid hydrolysate	:17.50
Beef heart Infusion	:2.00
Starch, soluble	:1.5
Agar	:17.00
pH	:7.3 +0.2

Suspended 38 gm in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure, 121^{0}C for 20 min. The molten medium was cooled to about 50^{0}C temperature and poured into sterile petri plates.

9) Reagents for Polymerase Chain Reaction (PCR)

1) Agarose

Agarose	:1.2 g
TBE (0.5X)	:100 ml
Ethidium bromide (1%)	:7.5 µl

2) Reagents for agarose gel electrophoresis:

A) Tris-borate EDTA (TBE) buffer (5X stock solution):

Tris base/Tris buffer	:54.0 gm
Boric acid	:27.5 gm
0.5M EDTA (pH 8.0)	:20 ml
Distilled water	:upto 1000 ml

Stored at room temperature.For use, dilute 1:10 (0.5X) with distilled water for agarose gel electrophoresis.

B) Gel loading dye:

Bromophemol blue	:0.25%
Sucrose in water	:40.0%

C) Ethidium bromide solution (10mg/ml):

Ethidium bromide	:0.1 gm
Distilled water	:10 ml

Appendix II

Integrated Antimicrobial Resistance Surveillance in Other Countries

- Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) - This program monitors trends in antimicrobial use and antimicrobial resistance in selected bacterial organisms from human, animal, and food sources across Canada.
- Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS) (In Spanish) - This program maintains information on resistant bacteria from humans, animals, and animal products and monitors trends in antimicrobial resistance.
- Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) - This program conducts surveillance of antimicrobial consumption and resistance in bacteria from animals, food, and humans.
- Norwegian Veterinary Institute, Report of Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway - This national health registry collects data about antibiotic resistance of bacterial isolates from animals and humans to determine the incidence and prevalence of antibiotic resistance and monitor changes over time.
- Swedish Veterinary Antimicrobial Resistance Monitoring Programme (SVARM) - This program monitors and analyzes the development of antimicrobial resistance in bacteria from animals and in bacteria from food of animal origin. Results are published in a yearly report.

International & Consensus Organizations

- Codex ad hoc Intergovernmental Task Force on Antimicrobial Resistance - This task force developed science-based guidelines to assess the risk to human health associated with non-human use of antimicrobials and to provide advice on risk management activities to reduce such risk.

- Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing CLSI is an international, voluntary, nonprofit, interdisciplinary standards-setting and education organization. The CLSI Subcommittee on Antimicrobial Susceptibility Testing reviews a variety of data to develop antimicrobial susceptibility test methods, interpretive criteria and quality control parameters for use in clinical laboratories.
- World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR) AGISAR is an international group of experts that advises WHO on integrated surveillance of antimicrobial resistance and the containment of food-related antimicrobial resistance.
- Global Foodborne Infections Network (GFN) The Global Foodborne Infections Network (GFN) equips countries to better detect and control foodborne and other enteric infections through increased capacity for integrated laboratory-based surveillance, and collaboration among human, veterinary, and food sectors.
- Transatlantic Taskforce on Antimicrobial Resistance (TATFAR) The objectives of this task force are to increase the mutual understanding of US and EU activities and programs relevant to the antimicrobial resistance issues. The main outcome of the taskforce is a report, including a set of recommendations for future cooperation in the global fight to keep antimicrobials effective.
- World Organisation for Animal Health (OIE) This is an intergovernmental organization responsible for improving animal health worldwide.
- OIE List of Antimicrobial Agents of Veterinary Importance Find recent recommendations from OIE on the use of fluroquinolones and the third and fourth generation cephalosporins in animals on page 4.
- The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) This project collects information on how antimicrobial medicines are used in animals across the European Union (EU). This type of information is essential to identify possible risk factors that could lead to the development and spread of antimicrobial resistance in animals.

- European Committee on Antimicrobial Susceptibility Testing (EUCAST) EUCAST is a standing committee that deals with breakpoints and technical aspects of phenotypic in vitro antimicrobial susceptibility testing.
- European Food Safety Agency (EFSA) EFSA is a Biological Monitoring Unit that monitors and analyzes the situation on antimicrobial resistance in food and animals across Europe.
- European Antimicrobial Resistance Surveillance Network (EARS-Net) This is a European wide network of national surveillance systems, providing European reference data on antimicrobial resistance for public health purposes.

Source Ref:
http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm418884.htm accessed online on 16.02.2017

Annexure

Annexure I

Zone of Inhibition of the Antimicrobials. *S.aureus*

Sample No	Code	Ampicillin	Ampicillin + Sulbactam	Cefepime	Cefepime + Clavulanic acid	Cefotaxime	Cefotaxime + Clavulanic acid	Amoxicillin	Amoxicillin + Clavulanic acid	Methicillin	Oxacillin
1	1	0	0	0	0	12	0	8	10	0	0
2	36	32	32	20	30	26	30	19	33	22	30
3	12	34	35	18	22	18	21	16	22	15	14
4	26	13	19	19	26	21	30	16	16	15	24
5	16	31	31	20	28	27	23	25	31	24	32
6	13	13	15	16	21	24	29	15	15	0	0
7	30	30	29	23	27	27	18	28	32	22	29
8	32	10	14	17	14	19	28	12	13	15	22
9	27	28	27	18	27	25	18	28	29	19	25
10	18	13	16	17	14	17	23	16	16	13	20
11	5	24	22	18	22	20	21	22	25	14	20
12	11	0	14	16	13	19	16	11	11	16	22
13	33	35	36	18	27	10	16	9	16	8	11
14	7	34	36	17	25	10	17	13	17	8	12
15	31	34	34	24	32	10	32	15	34	24	31
16	3	16	15	19	17	29	20	22	18	0	0
17	23	35	36	19	22	19	23	12	25	15	16
18	17	25	24	17	20	19	23	17	26	15	22
19	35	12	24	22	16	20	19	22	19	18	22
20	24	0	12	16	11	23	16	18	11	10	16
21	34	19	16	12	16	17	20	12	20	12	16
22	6	0	11	13	10	16	15	14	11	11	16
23	29	0	14	18	16	19	19	11	15	15	18
24	4	24	20	16	21	19	23	13	22	14	21
25	21	28	28	19	24	23	27	16	30	17	24
26	28	12	17	19	14	22	21	22	16	19	27
27	8	26	24	18	24	24	27	16	26	16	22
28	14	34	33	23	31	30	31	16	33	25	30
29	2	18	25	16	18	19	20	17	20	14	21
30	25	19	23	16	18	18	20	18	21	12	21
31	6P	7	8	13	21	14	24	21	21	0	0
32	52P	19	25	20	18	22	23	22	27	15	22
33	14P	0	10	20	16	12	24	0	5	0	0
34	53P	19	27	20	15	20	21	22	27	16	22
35	18P	26	27	17	24	24	27	21	27	20	22
36	2P	15	23	27	23	30	21	21	23	23	32
37	1P	10	17	20	17	27	27	14	20	17	24
38	51P	0	11	0	5	14	11	13	13	0	0
39	42P2	24	24	11	15	22	22	10	12	15	24
40	54P	20	25	20	19	22	24	15	27	14	23
41	15P2	16	24	16	15	18	20	15	23	13	20
42	55P	7	14	10	16	16	18	12	20	12	23
43	105P2	0	5	0	0	10	05	5	5	0	0
44	4P	0	10	20	16	12	24	0	5	0	0
45	20P2	20	27	18	19	22	23	14	24	19	16
46	8P	0	0	0	0	0	14	10	0	0	0
47	58P	4	8	6	12	12	16	12	16	15	15
48	21P2	15	23	25	15	28	29	16	18	21	31
49	15P	4	8	6	12	12	16	12	16	15	15
50	11P2	17	24	19	17	20	21	14	22	16	20
51	7P2	27	27	22	25	25	30	16	28	22	26
52	21P	14	22	27	26	27	28	22	25	24	25
53	**9P**	**0**	**15**	**13**	**14**	**19**	**19**	**0**	**0**	**0**	**0**
54	12P2	5	0	0	0	0	14	10	13	0	0
55	16P	14	17	15	14	18	19	12	18	0	0
56	1P2	0	5	0	0	13	10	5	5	0	0
57	22P2	0	10	0	5	14	5	8	10	0	0
58	17P2	19	27	18	17	21	22	22	24	20	24
59	13P	24	28	18	22	23	26	22	28	17	24
60	3P	4	9	10	14	14	18	12	18	17	16

Zone of Inhibition of the Antimicrobials. *S.aureus*

Sample No	CODE	Ampicillin	Ampicillin + Sulbactam	Cefepime	Cefepime + Clavulanic acid	Cefotaxime	Cefotaxime + Clavulanic acid	Amoxicillin	Amoxicillin + Clavulanic acid	Methicillin	Oxacillin
61	57P	4	8	6	12	12	16	12	16	15	15
62	10P	0	0	0	12	17	13	0	12	0	0
63	12P2	24	18	21	23	23	27	13	25	14	26
64	13P2	21	19	19	21	23	23	14	27	13	23
65	25P	35	22	25	29	29	33	15	34	14	25
66	19P2	20	19	17	19	19	21	12	26	12	21
67	10P2	0	11	18	15	23	23	12	14	13	20
68	125P	0	0	0	0	13	0	17	0	0	0
69	17P	25	18	21	26	25	27	24	30	15	29
70	7P	0	0	0	0	14	0	0	0	14	10
71	5P	0	0	0	0	14	10	0	10	0	0
72	59P	22	22	20	19	19	24	19	26	20	27
73	2P	6	8	9	12	12	16	10	16	12	12
74	15P2	0	00	0	10	16	11	0	11	0	0
75	23P2	0	0	0	0	14	10	0	10	0	0
76	9P2	6	8	8	12	12	16	14	16	15	15
77	19P	4	8	6	12	12	16	12	16	15	15
78	4P2	0	0	10	8	14	10	10	10	0	0
79	56P	0	0	0	0	14	10	0	10	0	0
80	24P	0	21	18	18	20	21	20	27	20	19
81	25P2	17	18	16	17	18	23	20	26	19	13
82	16P2	0	0	0	0	0	14	0	0	0	0
83	14P2	26	23	22	21	24	24	17	29	18	28
84	23P	34	22	23	29	28	34	21	32	20	30
85	20P	21	19	22	19	22	23	23	21	22	26
86	22P	16	22	20	23	22	21	18	18	19	26
87	18P2	19	15	17	18	20	21	10	23	11	22
88	11P	15	12	17	14	19	20	12	16	13	23
89	8P2	40	30	13	38	35	42	21	42	20	34
90	24P2	12	13	17	16	21	21	19	15	18	25
91	101P	16	17	24	20	25	23	0	20	0	0
92	110P	18	23	19	19	21	22	22	26	16	23
93	151P	0	11	19	15	19	23	14	7	0	0
94	158P	20	26	19	16	21	22	28	28	15	20
95	131P	24	26	18	22	22	26	0	28	20	21
96	140P	16	24	26	24	31	30	23	22	22	32
97	102P	9	18	20	16	24	26	15	18	17	23
98	143P	0	8	0	3	12	10	20	12	0	0
99	139P	23	24	10	14	21	23	12	10	14	23
100	111P	19	24	19	20	21	25	15	26	13	22
101	142P	17	23	15	16	19	21	15	24	13	20
102	159P	16	23	16	15	20	19	25		13	23
103	132P	0	4	0	0	9	4	0	5	0	0
104	103P	18	23	25	20	29	30	14	22	23	34
105	152P	21	26	17	18	20	24	19	24	18	16
106	133P	0	16	17	19	20	24	9	16	0	0
107	112P	19	23	16	17	20	22	13	24	14	20
108	160P	14	22	24	14	29	18	12	17	20	30
109	171P	16	23	20	18	20	20	10	21	15	19
110	104P	26	26	22	24	24	30	14	29	21	25
111	134P	16	24	28	23	28	18	16	28	18	19
112	114P	0	14	14	13	20	18	14	19	16	0
113	148P	4	15	17	15	15	19	10	18	0	0
114	175P	15	16	14	14	18	18	14	18	0	0
115	145P	0	4	0	0	11	10	20	4	0	0
116	153P	0	9	0	4	12	5	20	9	0	0
117	135P	18	26	19	18	20	23	20	25	12	22
118	105P	25	27	17	20	22	25	17	29	18	25
119	172P	25	29	20	24	20	25	21	28	18	23

Zone of Inhibition of the Antimicrobials. *S.aureus*

Sample No	CODE	Ampicillin	Ampicillin + Sulbactam	Cefepime	Cefepime + Clavulanic acid	Cefotaxime	Cefotaxime + Clavulanic acid	Amoxicillin	Amoxicillin + Clavulanic acid	Methicillin	Oxacillin
120	149P	24	23	19	20	20	23	21	28	20	25
121	173P	0	0	0	10	16	14	0	13	0	0
122	115P	25	17	20	23	24	26	14	24	13	26
123	106P	22	18	18	20	24	23	13	26	14	24
124	154P	34	20	26	18	28	35	14	33	15	25
125	117P	20	18	17	20	20	20	12	25	12	20
126	136P	0	10	17	14	22	22	13	13	12	21
127	144P	27	27	20	23	24	28	0	31	17	23
128	126P	23	19	19	20	25	24	15	26	24	30
129	150P	22	21	22	23	24	28	16	25	15	25
130	174P	35	20	26	29	30	32	19	30	18	29
131	130P	20	20	22	18	19	23	20	25	19	26
132	155P	23	21	22	20	23	25	23	25	24	26
133	107P	0	0	00	9	15	10	0	12	0	0
134	177P	0	0	0	0	13	9	0	10	0	0
135	157P	15	13	23	14	23	22	22	18	16	25
136	137P	13	12	20	19	21	29	14	17	14	24
137	146P	0	0	11	9	25	9	0	11	10	0
138	182P	25	16	21	20	20	26	15	26	14	24
139	187P	0	20	19	19	21	20	20	26	20	10
140	190P	18	19	15	16	17	24	19	25	20	12
141	108P	30	25	25	20	24	25	21	26	20	25
142	156P	25	22	22	20	23	23	18	30	17	28
143	138P	34	21	22	30	27	35	20	30	21	31
144	147P	20	20	21	20	20	23	22	20	23	25
145	180P	18	21	20	22	22	20	19	19	18	26
146	184P	20	14	15	19	20	20	11	24	10	22
147	109P	14	13	16	15	20	21	13	15	12	22
148	192P	38	29	12	37	34	41	20	40	21	35
149	195P	14	14	18	15	20	22	18	16	19	26
150	240P	0	0	0	0	10	0	0	11	0	0
151	201P	31	30	19	29	25	29	16	34	22	30
152	212P	12	18	19	15	21	21	0	15	14	25
153	210P	30	30	29	29	26	29	15	16	28	30
154	204P	14	16	15	20	25	23	20	16	0	0
155	215P	29	29	24	26	26	30	22	31	23	30
156	230P	9	16	16	15	20	19	17	14	15	23
157	222P	27	26	19	26	14	29	0	30	20	16
158	202P	12	15	16	13	18	19	14	15	12	21
159	228P	25	21	19	21	19	24	13	25	15	20
160	216P	0	13	15	12	20	30	13	12	15	21
161	221P	35	34	23	31	30	31	13	33	25	30
162	224P	15	16	20	18	20	21	0	19	0	0
163	214P	25	18	19	20	21	24	23	25	14	23
164	203P	12	25	21	17	22	20	18	21	19	23
165	219P	0	13	15	12	17	17	0	10	9	15
166	208P	18	15	13	15	18	21	14	21	13	15
167	212P	0	12	14	11	15	16	20	12	12	15
168	223P	0	15	17	15	19	20	15	16	10	17

Annexure II

Zone of Inhibition of the Antimicrobials. *E. coli*

Sample No	Code	Ampicillin	Ampicillin + Sulbactam	Cefepime	Cefepime + Clavulanic acid	Cefotaxime	Cefotaxime + Clavulanic acid	Ceftazidime	Ceftazidime + Clavulanic acid	Amoxicillin	Amoxicillin + Clavulanic acid	Aztreonam
1	1	0	11	13	17	10	17	11	16	13	13	10
2	2	10	13	16	17	18	20	12	17	20	17	12
3	5	0	13	14	19	17	17	14	18	22	18	14
4	7	10	18	19	23	21	22	18	22	20	19	17
5	9	12	12	13	16	15	15	10	15	0	14	10
6	11	36	34	33	36	40	40	31	35	16	38	27
7	12	0	13	16	18	15	15	14	19	18	15	12
8	13	11	10	11	14	13	14	11	13	19	13	12
9	14	35	32	34	12	30	35	0	36	19	37	0
10	15	12	11	12	15	14	16	11	14	17	15	10
11	16	0	16	11	20	17	16	13	16	0	14	12
12	19	0	13	11	12	12	13	12	16	20	15	12
13	21	0	12	15	19	16	16	13	17	19	16	12
14	22	10	15	14	18	19	19	17	20	17	19	15
15	23	10	13	12	18	19	16	14	18	16	16	13
16	26	0	11	10	16	11	13	10	15	0	12	11
17	27	10	11	12	14	12	17	10	15	22	13	10
18	32	0	12	13	20	16	17	13	16	18	18	13
19	33	10	16	17	21	21	21	17	21	20	18	18
20	34	0	11	13	15	14	15	10	13	18	14	10
21	36	0	13	17	18	18	15	13	17	16	15	14
22	18L	15	18	22	24	20	24	20	20	17	21	19
23	25L	18	17	23	21	21	24	22	22	10	22	21
24	55L	17	15	20	22	19	22	20	21	11	21	18
25	58L2	0	10	0	15	0	10	0	8	15	10	0
26	24L	16	15	19	19	19	20	16	17	19	21	16
27	5L	17	18	20	20	21	20	18	18	10	22	17
28	16L2	17	16	20	22	20	23	19	19	30	22	17
29	7L	18	18	23	22	20	22	19	19	14	22	19
30	10L	16	14	20	20	17	21	17	18	12	20	17
31	53L2	10	14	0	15	12	11	0	10	0	15	0
32	52L	12	15	22	24	20	24	20	20	10	21	19
33	16L2	10	16	10	18	19	22	19	20	11	20	18
34	57L	16	15	22	23	20	23	17	20	12	20	18
35	10L	0	10	20	22	18	22	16	16	13	12	15
36	8L	0	12	0	14	10	10	0	10	12	15	0
37	3L	20	16	26	26	23	26	22	22	11	21	20
38	9L	10	14	19	21	18	21	18	19	17	20	18
39	14L	16	16	23	24	20	24	20	21	10	21	19
40	15L2	16	16	20	22	20	22	18	18	10	19	17
41	2L2	35	31	23	30	31	34	16	33	12	38	14
42	5L	35	32	23	31	32	34	17	33	17	38	15
43	61L	0	12	16	14	9	18	10	15	10	12	9
44	91L	9	12	15	17	17	16	11	16	11	17	11
45	84L	0	12	20	13	18	17	15	17	15	18	13
46	90L	9	17	22	20	20	22	19	20	19	20	16
47	71L	11	11	15	12	16	16	10	14	10	15	9
48	62L	35	35	36	34	40	40	30	34	30	39	26
49	93L	0	12	16	17	15	14	14	20	14	14	11
50	73L	10	11	13	10	14	14	12	13	12	13	12
51	64L	36	31	11	35	29	36	0	35	0	36	0
52	92L	11	11	14	13	15	15	10	14	10	15	10
53	88L	0	15	19	10	16	16	11	15	11	15	10
54	94L	0	12	11	10	13	13	12	15	12	15	12
55	83L	34	34	35	34	39	39	13	35	13	40	25
56	77L	0	12	19	14	15	16	12	16	12	15	12
57	81L	16	11	15	12	15	15	11	15	11	16	10
58	79L	10	14	18	15	15	15	17	20	17	15	14
59	63L	0	10	15	10	10	12	10	14	10	11	10
60	96L	9	10	15	12	12	16	10	14	10	12	10
61	84L	0	11	20	12	15	16	12	15	12	17	12

Zone of Inhibition of the Antimicrobials. *E. coli*

Sample No		Ampicillin	Ampicillin + Sulbactam	Cefepime	Cefepime + Clavulanic acid	Cefotaxime	Cefotaxime + Clavulanic acid	Ceftazidime	Ceftazidime + Clavulanic acid	Amoxicillin	Amoxicillin + Clavulanic acid	Aztreonam
62	31LF	9	15	20	16	20	25	15	20	17	19	9
63	15LF	0	10	16	14	15	22	11	14	12	15	10
64	13LF	0	12	17	16	19	26	14	19	12	15	14
65	10LF	9	14	17	15	18	24	18	19	12	18	14
66	33LF	0	11	17	15	17	24	12	17	12	18	11
67	52LF	0	12	17	15	18	25	13	19	12	18	13
68	5L2	10	15	0	11	16	21	0	9	8	16	0
69	10L2	10	15	0	11	16	21	0	9	8	16	0
70	5LF	17	17	22	20	16	23	22	22	22	23	20
71	58LF	10	15	0	11	16	21	0	9	8	16	0
72	12LF	0	10	0	11	16	22	0	9	8	9	0
73	58L	0	11	9	15	10	0	11	9	15	10	9
74	53L	0	11	9	15	0	11	9	15	10	0	11
75	10L	0	10	0	11	16	22	0	9	8	9	0
76	18L	0	11	9	15	10	0	11	9	15	10	9
77	16L2	0	11	9	15	10	9	0	11	9	15	10
78	8L	16	16	20	20	16	22	20	20	13	22	15
79	9L	0	11	9	15	10	20	0	11	9	15	10
80	77L	0	11	9	15	10	0	11	9	15	10	9
81	79L	0	11	9	15	10	9	12	12	0	11	9
82	63L	10	15	0	11	16	21	0	9	8	16	0
83	96L	16	16	0	11	9	15	10	9	12	12	0
84	84L	0	11	9	15	10	9	12	12	12	13	14
85	95L	9	15	10	0	11	9	15	10	9	19	19
86	80L	15	15	20	20	22	22	18	19	12	19	19
87	67L	0	11	9	15	10	9	12	12	12	13	14
88	99L	0	11	0	10	15	17	0	10	11	14	0
89	100L	9	15	10	9	12	12	9	19	18	19	19
90	97L	9	15	10	9	12	12	9	19	18	20	20
91	61L	16	15	22	20	25	25	20	20	18	21	20
92	91L	16	15	20	20	21	21	18	19	18	20	19
93	90L	34	30	24	30	30	35	17	32	22	37	10
94	31LF	33	31	24	31	30	35	19	34	23	37	9

Annexure III

Biochemical characteristics of *S. aureus*

Sample No	Code	Grams Reaction	Catalase	Oxidase	Indole	Methyl Red	Voges Proskauer	Nitrate Reduction	Glucose, Lactose, Sucrose
		+	+	-	+	+	+	+	+
1	1	+	+	-	+	+	+	+	+
2	36	+	+	-	+	+	+	+	+
3	12	+	+	-	+	+	+	+	+
4	26	+	+	-	+	+	+	+	+
5	16	+	+	-	+	+	+	+	+
6	13	+	+	-	+	+	+	+	+
7	30	+	+	-	+	+	+	+	+
8	32	+	+	-	+	+	+	+	+
9	27	+	+	-	+	+	+	+	+
10	18	+	+	-	+	+	+	+	+
11	5	+	+	-	+	+	+	+	+
12	11	+	+	-	+	+	+	+	+
13	33	+	+	-	+	+	+	+	+
14	7	+	+	-	+	+	+	+	+
15	31	+	+	-	+	+	+	+	+
16	3	+	+	-	+	+	+	+	+
17	23	+	+	-	+	+	+	+	+
18	17	+	+	-	+	+	+	+	+
19	35	+	+	-	+	+	+	+	+
20	24	+	+	-	+	+	+	+	+
21	34	+	+	-	+	+	+	+	+
22	6	+	+	-	+	+	+	+	+
23	29	+	+	-	+	+	+	+	+
24	4	+	+	-	+	+	+	+	+
25	21	+	+	-	+	+	+	+	+
26	28	+	+	-	+	+	+	+	+
27	8	+	+	-	+	+	+	+	+
28	14	+	+	-	+	+	+	+	+
29	2	+	+	-	+	+	+	+	+
30	25	+	+	-	+	+	+	+	+
31	6P	+	+	-	+	+	+	+	+
32	52P	+	+	-	+	+	+	+	+
33	14P	+	+	-	+	+	+	+	+
34	53P	+	+	-	+	+	+	+	+
35	18P	+	+	-	+	+	+	+	+
36	2P	+	+	-	+	+	+	+	+
37	1P	+	+	-	+	+	+	+	+
38	51P	+	+	-	+	+	+	+	+
39	42P2	+	+	-	+	+	+	+	+
40	54P	+	+	-	+	+	+	+	+
41	15P2	+	+	-	+	+	+	+	+
42	55P	+	+	-	+	+	+	+	+
43	105P2	+	+	-	+	+	+	+	+
44	4P	+	+	-	+	+	+	+	+
45	20P2	+	+	-	+	+	+	+	+
46	8P	+	+	-	+	+	+	+	+
47	58P	+	+	-	+	+	+	+	+
48	21P2	+	+	-	+	+	+	+	+
49	15P	+	+	-	+	+	+	+	+
50	11P2	+	+	-	+	+	+	+	+
51	7P2	+	+	-	+	+	+	+	+
52	21P	+	+	-	+	+	+	+	+
53	**9P**	+	+	-	+	+	+	+	+
54	12P2	+	+	-	+	+	+	+	+
55	16P	+	+	-	+	+	+	+	+
56	1P2	+	+	-	+	+	+	+	+
57	22P2	+	+	-	+	+	+	+	+
58	17P2	+	+	-	+	+	+	+	+
59	13P	+	+	-	+	+	+	+	+
60	3P	+	+	-	+	+	+	+	+

Zone of Inhibition of the Antimicrobials. *S.aureus*

Sample No	CODE	Grams Reaction	Catalase	Oxidase	Indole	Methyl Red	Voges Proskauer	Nitrate Reduction	Glucose, Lactose, Sucrose
61	57P	+	+	-	+	+	+	+	+
62	10P	+	+	-	+	+	+	+	+
63	12P2	+	+	-	+	+	+	+	+
64	13P2	+	+	-	+	+	+	+	+
65	25P	+	+	-	+	+	+	+	+
66	19P2	+	+	-	+	+	+	+	+
67	10P2	+	+	-	+	+	+	+	+
68	125P	+	+	-	+	+	+	+	+
69	17P	+	+	-	+	+	+	+	+
70	7P	+	+	-	+	+	+	+	+
71	5P	+	+	-	+	+	+	+	+
72	59P	+	+	-	+	+	+	+	+
73	2P	+	+	-	+	+	+	+	+
74	15P2	+	+	-	+	+	+	+	+
75	23P2	+	+	-	+	+	+	+	+
76	9P2	+	+	-	+	+	+	+	+
77	19P	+	+	-	+	+	+	+	+
78	4P2	+	+	-	+	+	+	+	+
79	56P	+	+	-	+	+	+	+	+
80	24P	+	+	-	+	+	+	+	+
81	25P2	+	+	-	+	+	+	+	+
82	16P2	+	+	-	+	+	+	+	+
83	14P2	+	+	-	+	+	+	+	+
84	23P	+	+	-	+	+	+	+	+
85	20P	+	+	-	+	+	+	+	+
86	22P	+	+	-	+	+	+	+	+
87	18P2	+	+	-	+	+	+	+	+
88	11P	+	+	-	+	+	+	+	+
89	8P2	+	+	-	+	+	+	+	+
90	24P2	+	+	-	+	+	+	+	+
91	101P	+	+	-	+	+	+	+	+
92	110P	+	+	-	+	+	+	+	+
93	151P	+	+	-	+	+	+	+	+
94	158P	+	+	-	+	+	+	+	+
95	131P	+	+	-	+	+	+	+	+
96	140P	+	+	-	+	+	+	+	+
97	102P	+	+	-	+	+	+	+	+
98	143P	+	+	-	+	+	+	+	+
99	139P	+	+	-	+	+	+	+	+
100	111P	+	+	-	+	+	+	+	+
101	142P	+	+	-	+	+	+	+	+
102	159P	+	+	-	+	+	+	+	+
103	132P	+	+	-	+	+	+	+	+
104	103P	+	+	-	+	+	+	+	+
105	152P	+	+	-	+	+	+	+	+
106	133P	+	+	-	+	+	+	+	+
107	112P	+	+	-	+	+	+	+	+
108	160P	+	+	-	+	+	+	+	+
109	171P	+	+	-	+	+	+	+	+
110	104P	+	+	-	+	+	+	+	+
111	134P	+	+	-	+	+	+	+	+
112	114P	+	+	-	+	+	+	+	+
113	148P	+	+	-	+	+	+	+	+
114	175P	+	+	-	+	+	+	+	+
115	145P	+	+	-	+	+	+	+	+
116	153P	+	+	-	+	+	+	+	+
117	135P	+	+	-	+	+	+	+	+
118	105P	+	+	-	+	+	+	+	+
119	172P	+	+	-	+	+	+	+	+

Zone of Inhibition of the Antimicrobials. *S.aureus*

Sample No	CODE	Grams Reaction	Catalase	Oxidase	Indole	Methyl Red	Voges Proskauer	Nitrate Reduction	Glucose, Lactose, Sucrose
120	149P	+	+	-	+	+	+	+	+
121	173P	+	+	-	+	+	+	+	+
122	115P	+	+	-	+	+	+	+	+
123	106P	+	+	-	+	+	+	+	+
124	154P	+	+	-	+	+	+	+	+
125	117P	+	+	-	+	+	+	+	+
126	136P	+	+	-	+	+	+	+	+
127	144P	+	+	-	+	+	+	+	+
128	126P	+	+	-	+	+	+	+	+
129	150P	+	+	-	+	+	+	+	+
130	174P	+	+	-	+	+	+	+	+
131	130P	+	+	-	+	+	+	+	+
132	155P	+	+	-	+	+	+	+	+
133	107P	+	+	-	+	+	+	+	+
134	177P	+	+	-	+	+	+	+	+
135	157P	+	+	-	+	+	+	+	+
136	137P	+	+	-	+	+	+	+	+
137	146P	+	+	-	+	+	+	+	+
138	182P	+	+	-	+	+	+	+	+
139	187P	+	+	-	+	+	+	+	+
140	190P	+	+	-	+	+	+	+	+
141	108P	+	+	-	+	+	+	+	+
142	156P	+	+	-	+	+	+	+	+
143	138P	+	+	-	+	+	+	+	+
144	147P	+	+	-	+	+	+	+	+
145	180P	+	+	-	+	+	+	+	+
146	184P	+	+	-	+	+	+	+	+
147	109P	+	+	-	+	+	+	+	+
148	192P	+	+	-	+	+	+	+	+
149	195P	+	+	-	+	+	+	+	+
150	240P	+	+	-	+	+	+	+	+
151	201P	+	+	-	+	+	+	+	+
152	212P	+	+	-	+	+	+	+	+
153	210P	+	+	-	+	+	+	+	+
154	204P	+	+	-	+	+	+	+	+
155	215P	+	+	-	+	+	+	+	+
156	230P	+	+	-	+	+	+	+	+
157	222P	+	+	-	+	+	+	+	+
158	202P	+	+	-	+	+	+	+	+
159	228P	+	+	-	+	+	+	+	+
160	216P	+	+	-	+	+	+	+	+
161	221P	+	+	-	+	+	+	+	+
162	224P	+	+	-	+	+	+	+	+
163	214P	+	+	-	+	+	+	+	+
164	203P	+	+	-	+	+	+	+	+
165	219P	+	+	-	+	+	+	+	+
166	208P	+	+	-	+	+	+	+	+
167	212P	+	+	-	+	+	+	+	+
168	223P	+	+	-	+	+	+	+	+

Annexure IV

Biochemical properties of *E. coli*

Sample No	Code	Grams Reaction	Catalase	Oxidase	Indole	Methyl Red	Voges Proskauer	Citrate Utilization	Oxidase
1	1	-	+	-	+	+	-	-	-
2	2	-	+	-	+	+	-	-	-
3	5	-	+	-	+	+	-	-	-
4	7	-	+	-	+	+	-	-	-
5	9	-	+	-	+	+	-	-	-
6	11	-	+	-	+	+	-	-	-
7	12	-	+	-	+	+	-	-	-
8	13	-	+	-	+	+	-	-	-
9	14	-	+	-	+	+	-	-	-
10	15	-	+	-	+	+	-	-	-
11	16	-	+	-	+	+	-	-	-
12	19	-	+	-	+	+	-	-	-
13	21	-	+	-	+	+	-	-	-
14	22	-	+	-	+	+	-	-	-
15	23	-	+	-	+	+	-	-	-
16	26	-	+	-	+	+	-	-	-
17	27	-	+	-	+	+	-	-	-
18	32	-	+	-	+	+	-	-	-
19	33	-	+	-	+	+	-	-	-
20	34	-	+	-	+	+	-	-	-
21	36	-	+	-	+	+	-	-	-
22	18L	-	+	-	+	+	-	-	-
23	25L	-	+	-	+	+	-	-	-
24	55L	-	+	-	+	+	-	-	-
25	58L2	-	+	-	+	+	-	-	-
26	24L	-	+	-	+	+	-	-	-
27	5L	-	+	-	+	+	-	-	-
28	16L2	-	+	-	+	+	-	-	-
29	7L	-	+	-	+	+	-	-	-
30	10L	-	+	-	+	+	-	-	-
31	53L2	-	+	-	+	+	-	-	-
32	52L	-	+	-	+	+	-	-	-
33	16L2	-	+	-	+	+	-	-	-
34	57L	-	+	-	+	+	-	-	-
35	10L	-	+	-	+	+	-	-	-
36	8L	-	+	-	+	+	-	-	-
37	3L	-	+	-	+	+	-	-	-
38	9L	-	+	-	+	+	-	-	-
39	14L	-	+	-	+	+	-	-	-
40	15L2	-	+	-	+	+	-	-	-
41	2L2	-	+	-	+	+	-	-	-
42	5L	-	+	-	+	+	-	-	-
43	61L	-	+	-	+	+	-	-	-
44	91L	-	+	-	+	+	-	-	-
45	84L	-	+	-	+	+	-	-	-
46	90L	-	+	-	+	+	-	-	-
47	71L	-	+	-	+	+	-	-	-
48	62L	-	+	-	+	+	-	-	-
49	93L	-	+	-	+	+	-	-	-
50	73L	-	+	-	+	+	-	-	-
51	64L	-	+	-	+	+	-	-	-
52	92L	-	+	-	+	+	-	-	-
53	88L	-	+	-	+	+	-	-	-
54	94L	-	+	-	+	+	-	-	-
55	83L	-	+	-	+	+	-	-	-
56	77L	-	+	-	+	+	-	-	-
57	81L	-	+	-	+	+	-	-	-
58	79L	-	+	-	+	+	-	-	-
59	63L	-	+	-	+	+	-	-	-
60	96L	-	+	-	+	+	-	-	-
61	84L	-	+	-	+	+	-	-	-

Biochemical properties of *E. coli*

Sample No		Grams Reaction	Catalase	Oxidase	Indole	Methyl Red	Voges Proskauer	Citrate Utilization	Oxidase	Oxidase
62	31LF	-	+	-	+	+	-	-	-	-
63	15LF	-	+	-	+	+	-	-	-	-
64	13LF	-	+	-	+	+	-	-	-	-
65	10LF	-	+	-	+	+	-	-	-	-
66	33LF	-	+	-	+	+	-	-	-	-
67	52LF	-	+	-	+	+	-	-	-	-
68	5L2	-	+	-	+	+	-	-	-	-
69	10L2	-	+	-	+	+	-	-	-	-
70	5LF	-	+	-	+	+	-	-	-	-
71	58LF	-	+	-	+	+	-	-	-	-
72	12LF	-	+	-	+	+	-	-	-	-
73	58L	-	+	-	+	+	-	-	-	-
74	53L	-	+	-	+	+	-	-	-	-
75	10L	-	+	-	+	+	-	-	-	-
76	18L	-	+	-	+	+	-	-	-	-
77	16L2	-	+	-	+	+	-	-	-	-
78	8L	-	+	-	+	+	-	-	-	-
79	9L	-	+	-	+	+	-	-	-	-
80	77L	-	+	-	+	+	-	-	-	-
81	79L	-	+	-	+	+	-	-	-	-
82	63L	-	+	-	+	+	-	-	-	-
83	96L	-	+	-	+	+	-	-	-	-
84	84L	-	+	-	+	+	-	-	-	-
85	95L	-	+	-	+	+	-	-	-	-
86	80L	-	+	-	+	+	-	-	-	-
87	67L	-	+	-	+	+	-	-	-	-
88	99L	-	+	-	+	+	-	-	-	-
89	100L	-	+	-	+	+	-	-	-	-
90	97L	-	+	-	+	+	-	-	-	-
91	61L	-	+	-	+	+	-	-	-	-
92	91L	-	+	-	+	+	-	-	-	-
93	90L	-	+	-	+	+	-	-	-	-
94	31LF	-	+	-	+	+	-	-	-	-

Abstract

THESIS ABSTRACT

a)	Title of the thesis (in Capital letters)	**:**	STUDIES ON RESISTANCE PATTERN TO BETA-LACTAM ANTIBIOTICS IN *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* ISOLATED FROM MILK OF BUFFALOES WITH MASTITIS
b)	Full name of student	**:**	Karande Vikas Vasant
c)	Name and address of Major Advisor	**:**	Dr. (Mrs). M. M. Gatne, Professor, Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai-400012
d)	Degree to be awarded	**:**	Ph.D.
e)	Year of award of degree	**:**	2017
f)	Major subject	**:**	Veterinary Pharmacology and Toxicology
g)	Total number of pages in the thesis	**:**	
h)	Number of words in the abstract	**:**	
i)	Signature of Student	**:**	
j)	Signature, Name and address of forwarding authority (HOD / SH) Date:	**:**	Dr. (Mrs). M. M. Gatne, Professor, Department of Pharmacology and Toxicology, Bombay Veterinary College, Parel, Mumbai-400012
	Signature of the Associate Dean		

ABSTRACT

The aim of this study was to determine the predominant species responsible for bovine mastitis in a subset of Mumbai and Palghar region's dairy herds, and to assess the presence of antibiotic resistance in these pathogens. In this study, 750 milk samples were obtained from buffaloes selected from six different stables of Mumbai and Palghar districts. Samples positive for SCM were identified using the California mastitis test. After screening with CMT 310 samples were found positive from these stable giving overall incidences of 41.33 percent. Relatively higher prevalence was recorded at Palghar farm (51.11 percent) followed by unit 19 Goregaon (46.45 percent), unit 21 Goregaon (43.90 percent), Dahisar (43.18 percent), Safale (32.98 percent) and Virar (27.82 percent). All together occurrence of SCM was observed in the range of 27-51 percent from all farms sampled during the study. The occurrence of *S. aureus* and *E. coli* in the present investigation was 54.19 (n= 168) and 30.32 (n= 94) percent respectively. The aim of present study was also to assess the resistance pattern of *S. aureus* and *E.coli* against selected β-lactam antimicrobials. Therefore sensitivity of *S. aureus* was assessed against Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid, Methicillin and Oxacillin antimicrobial discs which was found to be 42.85, 65.47, 64.28, 77.38, 61.30, 73.80, 43.45, 73.21, 70.23 and 69.04 percent respectively. The most effective β-lactam antimicrobials found in present investigation in descending order were Cefepime + Clavulanic acid > Cefotaxime + Clavulanic acid > Amoxicillin + Clavulanic acid> Oxacillin > Ampicillin + Sulbactam > Cefotaxime> Ampicillin. Whereas great resistance was observed to Ampicillin and Amoxicillin when used alone. Similarly sensitivity of *E.coli* was assessed against Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid, Ceftazidime, Ceftazidime + Clavulanic acid and Aztreonam antimicrobial discs which was found to be 38.29, 71.27, 80.85, 86.17, 37.23, 71.27, 38.29, 77.65, 9.57, 28.72 and 4.25 percent respectively. The most effective β-lactam antimicrobials to treat *E.coli* infections, found in present investigation in descending order were Cefepime + Clavulanic acid > Cefepime> Amoxicillin + Clavulanic acid > Ampicillin + Sulbactam > Cefotaxime + Clavulanic acid. Whereas great resistance was observed to Cefotaxime, Ceftazidime, Aztreonam and Ampicillin. On the basis of results of sensitivity tests randomly 30 isolates of *S. aureus* were selected showing resistance to two or more β-lactam antimicrobials including Methicillin. The MIC was tested with the E-test method by keeping strips on the media plate containing *Staphylococcus* organisms. The MIC in µg/ml against *S.*

aureus investigated for Ampicillin, Ampicillin + Sulbactam, Cefepime, Cefepime + Clavulanic acid, Cefotaxime, Cefotaxime + Clavulanic acid, Amoxicillin, Amoxicillin + Clavulanic acid and Oxacillin. Among all the Methicillin Resistant *S. aureus showing resistance to* Ampicillin (86.66 percent), Ampicillin + Sulbactam(73.33 percent), Cefepime (66.66 percent), Cefepime + Clavulanic acid (60), Cefotaxime (83.33 percent), Cefotaxime + Clavulanic acid (66.66 percent), Amoxicillin (70 percent), Amoxicillin + Clavulanic acid (73.33 percent) and Oxacillin (80 percent) when tested for MIC. Resistance to Methicillin and other antimicrobials was observed during disc diffusion test and same isolates when subjected for *mecA* expression by PCR, 70 percent isolates found positive. Lowered MIC was found where the antimicrobials are used in combination with β-lactamase inhibitors. Similarly 30 isolates of *E. coli* on the basis of phenotypic ESBL detection were studied for their MIC and PCR analysis to detect *TEM*, *SHV* and *CTX-M* genes. Resistant *E.coli* isolates observed by MIC method for Ampicillin (63.33 percent), Ampicillin + Sulbactam (10 percent), Cefepime (53.33 percent), Cefepime + Clavulanic acid (6.66 percent), Cefotaxime (80 percent), Cefotaxime + Clavulanic acid (10 percent), Amoxicillin (86.66 percent), Amoxicillin + Clavulanic acid (23.33 percent), Ceftazidime (76.66) and Ceftazidime + Clavulanic acid (50). By PCR None of the isolate was found to be positive for *CTX-M* gene. However *TEM* and *SHV* genes were observed in 23 and 21 isolates respectively. *E. coli* isolates showing ESBL positive phenotypically showed presence of *TEM* (76.66 percent) and *SHV* (70 percent) genes. Lowered MIC was observed against ESBL positive isolates when subjected to β-lactamase inhibitor combinations. Findings suggested the use of genotypic methods for characterization of *S. aureus* strains in terms of virulent types and antimicrobial resistance pattern to decide the antimicrobial therapy for mastitis control.

Appendix G

प्रबंध सारांश

१. प्रबंधाचे नाव	:	सबक्लीनीकल स्तनदाह प्रभावित म्हशींच्या दुधातून मिळालेल्या स्टाफायलोकॉकस ऑरियस आणि इश्चर्शिया कोलाय या जीवाणूंची बीटा लॅक्टम प्रतिजैवकांसाठीच्या रोधनक्षमतेचा अभ्यास करणे
२. विद्यार्थ्याचे नाव	:	कारंडे विकास वसंत
३. मार्गदर्शकाचे नाव व पत्ता	:	डॉ. सौ. मधुमंजिरी मु. गटणे औषधशास्त्र व विषशास्त्र विभाग मुंबई पशुवैद्यकीय महाविद्यालय परेल, मुंबई-४०० ०१२.
४. पदवी	:	पदव्युत्तर/आचार्य पदवी
५. पदवी प्रदान करण्याचे वर्ष	:	२०१७
६. मुख्य विषय	:	औषधशास्त्र व विषशास्त्र
७. प्रबंधाची एकुण पाने	:	
८. सारांशचे एकुण शब्द	:	
९. विद्यार्थ्याची सही	:	
१०. विभाग प्रमुखाचे नाव, सही, आणि पत्ता दिनांक	:	डॉ. सौ. मधुमंजिरी मु. गटणे औषधशास्त्र व विषशास्त्र विभाग मुंबई पशुवैद्यकीय महाविद्यालय परेल, मुंबई-४०० ०१२.
११. सहयोगी अधिष्ठाता मुंबई पशुवैद्यकीय महाविद्यालय परेल, मुंबई-४०० ०१२.	:	

प्रबंध सारांश

सदरचा प्रबंध हा मुबंई आणि पालघर विभागातल्या जनावरांच्या स्तनदाह या रोगास कारणीभूत ठरणाऱ्या जंतूचा शोध घेणे आणि त्यामधील प्रतिजैविक विरोधी गुणाचे अस्तित्व शोधणे या उद्देशाने केला गेला.

सदर अभ्यासासाठी जवळपास ७५० म्हशींच्या दुधाचे नमुने पालघर आणि मुबंई जिल्ह्यातून गोळा करण्यात आले. कॅलिफोर्निया मस्टायटीस टेस्ट (CMT) च्या सहाय्याने सबक्लिनिकल स्तनदाहाचे (ज्याची चिन्हे व लक्षणे इतकी सौम्य असतात की, त्यावरुन रोगनिदान करणे अशक्य होते) सकारात्मक नमुने ओळखण्यात आले. सी. एम. टी. च्या सहाय्याने तपासणी केली असता एकूण ७५० नमून्यांपैकी ३१० नमूने हे सकारात्मक आढळले याचा अर्थ सबक्लिनीकल मस्टायटीस चे एकूण सरासरी प्रमाण ४१.३३% इतके आढळले. एकूण नमुन्यांपैकी सर्वात जास्त टक्केवारी ही पालघर तबेल्यात (५१.११%) त्यापाठोपाठ गोरेगाव युनिट १९ मध्ये ४६.४५% , युनिट २१ गोरेगावमध्ये ४३.९०%, दहीसर तबेल्यात ४३.१८%, सफाळे येथील तबेल्यात ३२.९८% आणि विरार येथे २७.८२% इतके आढळले. सर्व संबंधीत तबेल्यामध्ये सबक्लिनीकल मस्टायटीसचे प्रमाण २७ ते ५१ % या दरम्यान मिळाले. सदरच्या अभ्यासात स्टेफायलोकोकस ऑरीयस आणि इश्चर्शिया कोलाय यांचे प्रमाण अनुक्रमे ५४.१९% आणि ३०.३२% आढळले. सदरच्या प्रयोगामध्ये एस. ऑरीयस आणि ई. कोलाय या जीवाणूंचा बीटा लॅक्टम या प्रतिजैविकाच्या विरोधातील रोधन अभ्यासला गेला. त्या अनुषंगाने एस. ऑरियस या जीवाणूची संवेदनशीलता ही ॲम्पिसीलीन, ॲम्पिसीलीन + सलबॅक्टम, सेफेपाईम, सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड, सिफोटॅक्सीम, सिफोटॅक्सीम + क्लॅव्हुलॅनिक ॲसिड, ॲमॉक्सिसिलीन, ॲमॉक्सिसिलीन + क्लॅव्हुलॅनिक ॲसिड, मेथिसीलीन आणि ऑक्सॅसिलीन या प्रतिजैविकांच्या चकत्या वापरुन अभ्यासण्यात आली ती अनुक्रमे ४२.८५, ६५.४७, ६४.२८, ७७.३८, ६१.३०, ७३.८०, ४३.४५, ७३.२१, ७०.२३ आणि ६९.०४% एवढी आढळली. सदर प्रतिजैविकांचा त्यांच्या उपयोगितेनुसार उतरता क्रम लावला असता तो सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड, सिफोटॅक्सिम + क्लॅव्हुलॅनिक ॲसिड, ॲमॉक्सिसीलीन + क्लॅव्हुलॅनिक ॲसिड, ऑक्सॅसिलीन, ॲम्पिसीलीन + सलबॅक्टम, सिफोटॅक्सिम आणि सर्वात शेवटी ॲम्पिसीलीन असा आढळून आला. त्याचप्रकारे ई. कोलाय या जीवाणूची संवेदनशीलता ही ॲम्पिसीलीन, ॲम्पिसीलीन + सलबॅक्टम, सेफेपाईम, सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड, सिफोटॅक्सीम, सिफोटॅक्सीम + क्लॅव्हुलॅनिक ॲसिड, ॲमॉक्सिसिलीन, ॲमॉक्सिसिलीन + क्लॅव्हुलॅनिक ॲसिड, सेफटॅझिडींम, सेफटॅझिडींम + क्लॅव्हुलॅनिक ॲसिड आणि ॲझट्रीव्होनाम या प्रतिजैवीकांच्या विरोधात अभ्यासली गेली कि जी अनुक्रमे ३८.२९, ७१.२७, ८०.८५, ८६.१७, ३७.२३, ७१.२७, ३८.२९, ७७.६५, ९.५७, २८.७२ आणि ४.२५%

इतकी होती. सदरच्या अभ्यासात ई. कोलाय च्या विरोधात सर्वात उपयोगी प्रतिजैविक हे उतरत्या क्रमाने सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड, त्यापाठोपाठ सेफेपाईम, ॲमॉकसीसीलीन + क्लॅव्हुलॅनिक ॲसिड, ॲम्पिसीलीन + सलबॅक्टम आणि सेफोटॅक्सीम + क्लॅव्हुलॅनिक ॲसिड हे आढळले, तसेच सर्वात जास्त प्रतिजैविक विरोध हा सीफोटॅक्सीम, सेफटॅझिडींम, ॲझट्रीव्होनाम आणि ॲम्पिसीलीन यांना जाणवला. एस. ऑरियस च्या रोधन क्षमतेच्या अभ्यासावरुन एकूण तीस जीवांणू की, जे मेथिसीलीन सह दोन किंवा अधिक प्रतिजैविकांचा विरोध दर्शवत होते असे निवडले व त्यांचा मिनीमम इनहिबीटरी कॉसंट्रेशन व त्यासाठी कारणीभूत जनुके अभ्यासण्यासाठी वापर केला गेला.

स्टेफायलोकॉकस जीवाणूंच्या आगारावर ई -टेस्ट पट्टी ठेवून मिनीमम इनहिबीटरी कॉसंट्रेशन अभ्यासले. एम आय सी. ही मायक्रोग्रॅम प्रति, मिलीलिटर या एककात ॲम्पिसीलीन, ॲम्पिसीलीन + सलबॅक्टम, सेफेपाईम, सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड, सेफोटॅक्सीम, सेफोटॅक्सीम + क्लॅव्हुलॅनिक ॲसिड, ॲमॉक्सिसिलीन, ॲमॉक्सिसिलीन + क्लॅव्हुलॅनिक ॲसिड, ऑक्सॅसिलीन या प्रतिजैविकासांठी अभ्यासण्यात आली. एस. ऑरीयस जीवाणू, की जे मेथिसीलीन ला विरोध दर्शवत होते ते इतर प्रतिजैविकांना सुद्धा विरोध दर्शवत होते जसे की, ॲम्पिसीलीन (८६.६६%) ॲम्पिसीलीन + सलबॅक्टम (७३.३३%), सेफेपाईम (६६. ६६%), सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड (६०%), सीफोटॅक्सीम (८३.३३%), सीफोटॅक्सीन + क्लॅव्हुलॅनिक ॲसिड (६६.६६%), ॲमॉक्सिसिलीन(७०%), ॲमॉक्सिसिलीन + क्लॅव्हुलॅनिक ॲसिड (७३.३३%), ऑक्सॅसीलीन (८०%), इतक्या प्रमाणात विरोध दर्शविला. डिस्क डीफ्युजन आणि पीसीआर या दोन पद्धतीमधील नातेसंबंध तपासला असता डिस्क डीफ्युजन या पद्धतीमधील मेथिसीलीन विरोधी ७०% एस. ऑरीयस जीवाणुंनी mecA या जनुकाचे अस्तित्व दर्शविले. जेव्हा प्रतिजैवके बीटा लॅक्टेमज इनहीबीटर घटकांसोबत वापरली तेव्हा त्यांनी कमी एम आय सी.दर्शविली. त्याच धर्तीवर ई.कोलाय जीवाणूंमधील ईएसबीएल हा घटक डिस्क डीफ्युजन पद्धतीने शोधून असे तीस जीवाणू एम आय सी. आणि पीसीआर (कि ज्यामध्ये TEM, SHV, आणि CTX ही जनुके असण्याचा संभव आहे) या पद्धतीने अभ्यासले. ई.कोलाय या जीवाणूंनी ई-टेस्ट पद्धतीने दर्शविलेली रोधन क्षमता ही ॲम्पिसीलीन (६३.३३%), ॲम्पिसीलीन + सलबॅक्टम (१०%), सेफेपाईम (५३. ३३%), सेफेपाईम + क्लॅव्हुलॅनिक ॲसिड (६.६६%), सीफोटॅक्सीन (८० %), सेफोटॅक्सीन + क्लॅव्हुलॅनिक ॲसिड (१०%), ॲमॉक्सिसिलीन (८६.६६%), ॲमॉकसीसीलीन + क्लॅव्हुलॅनिक ॲसिड (२३.३३%), सेफ्टॅझिडींम (७६.६६%), सेफ्टॅझिडींम + क्लॅव्हुलॅनिक ॲसिड (५०%) इतकी आढळली. पीसीआर तंत्राच्या सहाय्याने ज्यावेळी गुणसूत्रांचा अभ्यास केला त्यावेळी CTX–m गुणसूत्र कुठल्याही जीवाणूंमध्ये सापडला नाही. तरीही TEM आणि SHV ही जनुके अनुक्रमे २३ आणि

२१ ई कोलाय जीवांणूमध्ये सापडली. ईएसबीएल सकारात्मक ई. कोलाय मध्ये TEM (८२.१४%) आणि SHV (७५%) ही गुणसूत्रे आढळली. बीटा लॅक्टेमेज इनहीबीटर सोबत वापरल्यानंतर ईएसबीएल सकारात्मक नमून्यांमध्ये कमी एमआयसी आढळली. सदरचे निष्कर्ष असे सांगतात अनुंवाशिक तंत्रज्ञान हे प्रतिजैविक विरोध तपासण्यासाठी आणि त्या अनुषंगाने स्तनदाह रोगाच्या नियंत्रणासाठी औषध निवडीसाठी अतिशय उपयुक्त आहे.

Vita

VITA

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