Molecular detection of antibiotic resistance genes against gram positive bacteria isolated from mastitis milk of cows in Malkangiri district of Odisha

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BHUBANESWAR-751003
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Molecular detection of antibiotic resistance genes against gram positive bacteria isolated from mastitis milk of cows in Malkangiri district of Odisha

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By

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CERTIFICATE-I

This is to certify that the thesis entitled **"Molecular detection of antibiotic resistance genes against gram positive bacteria isolated from mastitis milk of cows in Malkangiri district of Odisha"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** to the Odisha University of Agriculture and Technology is a faithful record of bona fide and original research work carried out by **Sthitiprajna Panigrahi, Adm. No. 191922004** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received by her from various sources during the course of investigation has been duly acknowledged.

CHAIRMAN
ADVISORY COMMITTEE

CERTIFICATE-II

This is to certify that the thesis entitled **"Molecular detection of antibiotic resistance genes against gram positive bacteria isolated from mastitis milk of cows in Malkangiri district of Odisha"** submitted by **Sthitiprajna Panigrahi, Adm. No. 191922004** to the Odisha University of Agriculture and Technology, Bhubaneswar in partial fulfilment of the requirements for the degree of **Master of Veterinary Science (Veterinary Epidemiology and Preventive Medicine)** has been approved/disapproved by the students' advisory committee and the external examiner.

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Place: Bhubaneswar

Date: 23-2-2022

Sthitiprajna Panigrahi

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ABBREVIATIONS

%	:	Percent
µl	:	Microliter
^{0}C	:	Degree Centigrade
AMR	:	Antimicrobial Resistance
ARG	:	Antibiotic Resistance Genes
CDC	:	Centers for Disease Control and Prevention
CoNS	:	Coagulase negative staphylococci
DNA	:	Deoxyribonucleic acid
et al.	:	and associates
Fig	:	Figure
MDR	:	Multi-drug resistant bacteria
ml	:	Mililiter
mm	:	Milimeter
PCR	:	polymerase chain reaction
SCM	:	Subclinical mastitis
WHO	:	World Health Organization

ABSTRACT

Antibiotic resistance genes against bacterial pathogens isolated from mastitis milk of cows in the Malkangiri area of Odisha were investigated in this study.

A total of 520 cows were screened from various herds in Malkangiri district of Odisha during March 2021 to August 2021, milk samples were taken from 15 lactating cows who had signs of clinical mastitis. Microbiological and/or 16S rDNA sequencing of 15 milk samples revealed seven isolates as *Mammalicoccus sciuri* and the other isolate was *Enterococcus faecium*. 85.7% isolates were resistant to penicillin, 14.2% to tetracycline and cefoperazone, 28% to streptomycin, as revealed by disk diffusion assay. Further antibiotic resistance *genes viz blaZ, aacA-aphD, tetK, tetM* were detected by conventional PCR using specific primer. One *Mammalicoccus sciuri* isolate had amplified *tetK* gene whereas one isolate of *Enterococcus faecium* had amplified both genes (*tetK* and *tetM*) of tetracycline. Phylogenetic analysis of all the *Mammalicococcus sciuri* isolates showed high degree of similarity between our isolates and *Mammalicoccus sciuri* isolates obtained from GenBank.

Efficacy of tetracycline is likely to deteriorate in the future. Overuse of tetracycline in the treatment of mastitis may cause the dissemination of genetic resistance mechanisms among *Staphylococcus* spp. and as a result, tetracycline is no longer effective. It is the first ever antimicrobial resistance study in backyard region of Odisha, provide baseline data for the global threat.

CHAPTER-I

INTRODUCTION

Livestock sector plays a significant role in Indian economy. India has surpassed China as the world's greatest milk producer, accounting for 20.17 percent of global milk output. Milk is considered as a complete food containing all six essential nutrients which makes it a perfect complement to cereal based diet. If anything happens to the udder tissue or mammary gland it will directly affect the production of milk. Mastitis is one such prevalent, complex and economically significant disease in dairy industry, with losses owing to gradual reduction in milk yield, cost of treatment, cost of discarded milk, cost of veterinary service, early culling and labor cost. Antibiotics have been used as a preventive and curative treatment of mastitis since long, hence microbes evolved and became resistant to commonly used antibiotics. Hence it urges extensive research on the phenotypic and genotypic study of resistivity against antibiotics for different microbes in different geographical area. Understanding genetics and evolutionary biology of antibiotic resistance may help to develop therapeutic options which in turn may reduce the risk of resistance evolving in the future.

Mastitis is a condition in which the mammary gland becomes inflamed, resulting in change in physical and chemical characteristic of the milk and alteration in macroscopic and microscopic tissue of udder. Popularly it is classified as clinical and sub-clinical mastitis (SCM). A broad spectrum of microorganisms including bacteria, fungi, mycoplasmas and viruses are the causative agents of mastitis. The most prevalent infections linked with SCM are *Staphylococcus* spp., notably and coagulase-negative staphylococci (CoNS) as reported by Harmon (1994) and Djabri *et al*. (2002). Infection increases with age due to loosening of sphincter, poor quality management of housing and bedding which is conducive for the proliferation of pathogens and their virulence. Collectively these factors facilitate increased resistance to antibiotics, now a serious concern.

Antibiotics have long been used since so many decades and considered as a "magic bullet" that would terminate infectious diseases. However, there is rise in

resistance profiles. Antibiotic resistance was seen since the beginning of its use and is increasing to peak levels in all areas of the globe. Antibiotic resistance may arise as a result of mutation and horizontal resistance gene transfer in mobile genetic cassettes (Adam and Roy, 2013). It depends on the class of antibiotics and on the genetic flexibility of bacterial species. To fight antimicrobials, *Stahylococci* have evolved four main resistance mechanisms: drug trapping, alteration of the drug binding site, deactivation of the drug by enzymes and enhanced efflux of the drug (Pantosti *et al.,* 2007). Since the discovery of sulfonamide, penicillin, and streptomycin in 20th century there is subsequent rise of antibiotic resistance. Penicillin started showing resistance after 1year of its discovery i.e. 1942 followed by methicillin (1960), extended spectrum cephalosporins (1983), vancomycin (1988), fluconazole (1988), imipenam (1996), ciprofloxacin (2007), azithromycin (2011), ceftazidime-avibactem (2015), amphotericin-B (2016) (CDC Report, 2020). Antimicrobial Resistance (AMR) is a complicated issue which poses threat to animal and human well-being, food safety, national and world economy.

There are multiple antibiotic resistance genes for a particular class of antibiotic. The resistance genes against beta lactam antibiotic are *blaZ, mecA*, *femA* (Yang *et al.,* 2018) and for broad spectrum beta lactam antibiotics are bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$. (Gow *et al.,* 2008, Harini *et al.,* 2013, Hedmann *et al.,* 2019). Tetracycline antibiotic resistance determinants includes *tetK, teM, tetC, tetL, tetM, tetO* etc., Aminoglycoside resistance genes are *aacA, aphD* (Yang *et al.*, 2018). Rifampicin resistance gene is *rpoB*, Quinolone resistance gene is *qnrB, qnrA* etc., erythromycin resistance genes are *ermA, ermB, ermC* and streptomycin resistance genes so on. 20,000 resistance genes have been discovered so far for a number of antibiotics (Liu *et al.,* 2009). Mode of actions and resistance mechanisms varies with antibiotic classes. Organisms show resistance to β-lactam antibiotics by targeting peptidoglycan synthesis and removes the antibiotics from the organisms through efflux, hydrolysis, altered target, similarly resistance to aminoglycoside by targeting translation and tetracycline resistance is mainly through phosphorylation, acetylation, nucleotidylation, altered target, efflux and monoxygenation (Wright, 2007). Most commonly used/ commercially available antibiotics to treat mastitis are β-lactam group of antibiotics *viz* penicillin (Pendistrin-SH), cefoperazone sodium (Mastiwok), cefuroxime sodium (Uddercef) and tetracycline, neomycin (Mastijet forte).

Antibiotic use in animals may increase the chance of drug-resistant microorganisms being transmitted to people, either directly (Juhász-Kaszanyitzky *et al.,* 2007) or by transferring resistance genes from farming to people or from environment to human. Antibiotic resistance is responsible for 25000 yearly fatalities in the European Union and 23,000 annual deaths in the United States, as reported by Centers for Disease Control and Prevention (CDC). Every year, in the United States up to 2 million people get a drug-resistant illness. Researchers anticipate that by the year 2050, antibiotic resistance would kill 10 million people globally, surpassing cancer as the main cause of death. There is a reported incidence of MRSA transmission between cows and farm workers. The United States and China are the world's top consumers of antibiotics in food production. Agriculture uses 80 percent of all antibiotics in the United States, according to the Food and Drug Administration (FDA), with pigs and poultry receiving ten times more antibiotics than cows and sheep. Hence antibiotic resistance would be a serious concern.

Declined rate of novel antibiotic discovery along with frequent emergence of antibiotic resistance against existing antibiotics dims the prospect by narrow down benefits of antibiotic use. The pace of evolution of resistance is determined by the genetic background and therapy. In some cases nucleotide substitution is the main cause of rifampicin resistance in *M. tuberculosis* and resistance cassette causes methicillin resistance in *Staphylococcus aureus*. Detection of antibiotic resistance gene is faster and cost effective. Information acquired from genome sequencing of infectious pathogens will increasingly been used in therapeutic and infection control techniques (Paterson, 2006). Genotypes can provide information on a pathogen's current drug sensitivity as well as its future potential for resistance and dissemination. Further sequencing thereof could reveal if a drug-resistant strain has precursors of resistance genes which could be altered to boost expression or activity.

Solutions to reduce the AMR incidence could be attempted through development of new antimicrobials and its judicious use with increased sanitation, herd hygiene, nutrition. But, unfortunately the development of novel antibiotics is remarkably slow to meet the increased demand. Only three new antibiotics have been licensed in the previous 30 years, indicating that the number of approved medications is decreasing. Therefore, knowledge on region specific antimicrobial resistance data is the need of the day to address AMR.

Realizing gradual decline of antimicrobial effect against bovine mastitis and increased drug resistance, an attempt was made to evaluate the genotypic and phenotypic resistivity pattern of antimicrobials in mastitis milk in the Malkangiri district of Odisha. The present study was also conducted with the aim to add or upgrade the knowledge of dairy scientists and veterinarians in the field of prevention and treatment of mastitis.

Current study was planned with the following objectives:-

1. Isolation and identification of bacterial pathogens from bovine mastitis milk samples in Malkangiri district of Odisha.
2. Molecular detection of resistance genes against β-lactam antibiotics (*blaZ*), tetracycline (*tetK, tetM*) and aminoglycoside (*aacA-aphD*) antibiotics.

CHAPTER-II

REVIEW OF LITERATURE

The relative importance of the subject in question necessitates a critical survey of the research conducted in India and abroad pertaining to detection of antimicrobial resistance encoding genes against gram positive bacteria isolated from mastitis milk of cows so as to give a wider perspective that will enlighten the salient characteristics which will be an invaluable asset in interpreting the results. An attempt was made in this chapter to review a range of the information available on the following heads;

2.1. Antimicrobial resistance
2.2. Detection of β-lactam resistance genes
2.3. Detection of tetracycline resistance genes
2.4. Detection of aminoglycoside resistance genes

2.1. Antimicrobial resistance

When microorganisms acquire resistance or become irresponsive to antimicrobials that they were previously sensitive to, the term "antimicrobial resistance (AMR)" is employed. There is pre-existence of resistance mechanisms one year before the administration of first antibiotic i.e. penicillin in 1940. Since then the exponential use of antimicrobials causes the dawn of AMR among microbes efficiently. ARGs (Antibiotic resistance genes) are thought to play a role in resistance. Resistance genes found both in chromosome and plasmid. Transfer of resistance genes occurs between organisms mainly through conjugation, transposons, transformation or transduction.

The antibiotic resistome is a compendium of all the resistance genes present in pathogens, antibiotic producers and non-pathogenic bacteria. Resistome was first coined in 2006 by Wright (2007). Resistance mechanisms were classified as acquired (vertically or horizontally transferred, taxonomic nonspecific), intrinsic (only vertically transmitted, taxonomic specific), silent/cryptic resistance (phenotypically sensitive, functional but not expressed), and protoresistance (phenotypically sensitive, little/no activity). Miraculous discovery of antibiotics has been accompanied by the evolving of resistance among microbes. Overuse and indiscriminate use of

antimicrobials is the leading cause of development of resistivity in microbes. There are more than 20,000 potential resistance genes of nearly 400 different types discovered so far (Liu *et al.,* 2009).

β -lactamase resistance gene is globally distributed and mutations of the genes that code for the enzymes might wake up the extended spectra of resistance. CTX-M, which was discovered in the 1990s and obtained from environmental *Kluyvera* strains, was the first enzyme to hydrolyze extended spectrum β– lactamase. There are different modes of action for different antibiotics. Mode of actions and resistance mechanisms of commonly used antibiotics as reported by Wright (2007) for β – lactam antibiotics is by targeting peptidoglyacan synthesis that removes the antibiotics through efflux, hydrolysis, altered target; targets translation for aminoglycoside and for tetracycline the resistance mechanism is mainly through phosphorylation, acetylation, nucleotidylation, altered target, efflux and monoxygenation.

In the United Kingdom, the first methicillin-resistant *Staphylococcus aureus* was found in 1961. All β -lactam antibiotics, including cephalosporins and carbapenems, were shown to be resistant to it. Two major mechanism of resistance in gram positive bacteria are; enzymatic degradation by the synthesis of β-lactamses or by altering the target site i.e. penicillin binding protein PBP by either acquiring from external DNA or by altering the existing PBP genes as studied by Munita *et al*. (2015) and Berger and Bachi (2002).

2.2. Detection of resistance genes against β –lactam antibiotics in bacteria isolated from bovine mastitis milk -

2.2.1. World

Turutoglu *et al*. (2019) investigated the presence of the *mecA* gene producing methicillin resistance in *S. aureus*, was studied in 18 phenotypically sensitive oxcillin antibiotics in Turkey. Out of which only three had *mecA* genes. Only one of the three *mecA*-positive isolates had genes encoding aminoglycoside resistance gene i.e. *aac(6')/aph(2'')* gene in conjunction with the *aph(3')-IIIa* gene.

Tojo *et al*. (2014) in USA, evaluated an automated fast multiplex assay for fast detection of gram negative bacteria isolated from blood cultures along with its nine β-

lactam antibiotic resistance genes within five minutes hands-on and two hours of run duration per sample. There was cent percent similarity in identification of bacteria between the standard laboratory method and BC-GN assay. All β-lactam antibiotic resistance genes were found by the BC-GN test namely 8 bla_{KPC}, 119 bla_{IMP}, 16 bla_{NDM}, 54 $bla_{CTX\text{-}M}$, 24 $bla_{OXA\text{-}23}$, one $bla_{OXA\text{-}24/40}$, one $bla_{OXA\text{-}48}$,four $bla_{OXA\text{-}58}$ and six bla_{VIM}. Study showed rapid detection of bacteria with earlier detection of its resistance genes which would be useful for optimal patient management.

Yang *et al.* (2016) in Northwest China established the genetic features of antibiotic resistance characteristics in *S. aureus* cultured from cow mastitis milk. Antimicrobial resistance testing was performed on 44 *S. aureus* isolates. Resistance characteristics were determined by *in- vitro* sensitivity test and genes linked to resistance were determined by conventional polymerase chain reaction. 84.09% of *S. aureus* isolates tested positive for penicillin, 20.45% for methicillin, 15.91% for tetracycline, 9.09% for gentamicin, 6.82% for tobramycin, 6.82% for kanamycin and 15.91% for tetracycline. Furthermore, genotyping revealed 100% of *S. aureus* isolates had *rpoB* (rifampicin resistance gene), 95.45% had *blaZ* (penicillin resistance gene) and 22.73 % had *tetK, tetM,* alone or in combination (tetracycline resistance genes) and *ermB* or *ermC* (erythromycin resistance genes), 2.27% of isolates had *aacA-aphD* (aminoglycoside resistance genes) and *mecA* (methicillin resistance gene).

Ahmed *et al.* (2018) reported frequency of subclinical mastitis caused by *Staphylococci* and examined their antibiogram and utilising polymerase chain reaction to assess the prevalence of the *blaZ* and *mecA* resistance genes against β-lactam antibiotics in Egypt. There were 39.8% coagulase-negative staphylococcus (CoNS) isolates collected, representing eight species, of which one was *S. aureus*. *S. xylosus* was the most common (36.7%). Antimicrobial susceptibility tests revealed a high level of sensitivity. Ampicillin and cefoxitin resistance were found, with amoxicillin resistance being moderate. PCR was used to determine above two resistance genes against 20 *S. aureus* and 20 CoNS β -lactam resistant isolates. *blaZ* and *mecA* resistance genes were found in 75 percent and 65 percent of *S. aureus* isolates, respectively, and 70 percent and 55 percent of CoNS isolates.

Zhang *et al*. (2021) studied the presence of *S. dysgalactiae* in bovine clinical mastitis, as well as their antimicrobial resistance profiles and phenotypic and

genotypic profiling of resistant isolates. Milk samples were obtained between January 2014 and May 2016 from clinical mastitis affected dairy cows. The antimicrobial susceptibility of these isolates was assessed using minimum inhibitory concentrations against eight antimicrobial drugs. Most common resistance genes were bla_{TEM}, *ermB*, and *tetM*. At least one of the resistance genes examined was present in all isolates. In addition, 1.1, 12.5, 18.2, 36.4 and 31.8% of isolates tested positive for at least one resistance gene. Between the phenotypic and genotypic resistance profiles, no further statistically significant relationships were found. This study found a high incidence of *S. dysgalactiae* linked with bovine mastitis in Chinese dairy herds.

Girmy *et al*. (2020) conducted a Cross-sectional investigation in 220 dairy cows in Ethiopia. *S. aureus* were isolated and identified from mastitic cows' milk and evaluated the incidence of MRSA. All isolates were resistant to penicillin G and ampicillin phenotypically. *mecA* and *femA* resistance genes were also detected using multiplex PCR.

Zhang *et al*. (2021) in Northern Thailand, documented 227 *S. uberis* strains linked to bovine mastitis. Antimicrobial sensitivity was detected by microdilution method and AMR genes were detected by employing polymerase chain reaction, respectively. Tetracycline resistance was found in the majority of *S. uberis* strains (187/228, 82.02%), followed by ceftiofur resistance (44/228, 19.30%), and erythromycin resistance (19/228, 8.33%). Ceftiofur's MIC50 and MIC90 were 2–4 times higher in 2017 than in 2010 (P 0.01). Between 2010 and 2017, tetracycline and ceftiofur resistance grew considerably (P 0.05). *tetM* (199/228, 87.28 percent) was the most frequent gene found in *S. uberis*, followed by *ermB* (66.23 percent) and *blaZ* (6.58%). There was a significant link between resistance of tetracycline detected phenotypically and *tetM* resistance gene detection by polymerase chain reaction. During the investigation, *tetM* detection rates increased drastically, but *tetO* and *ermB* detection rates decreased significantly.

Mbindyo *et al*. (2021) tested antibiotic resistance profiles both phenotypically and genotypically in 183 *Staphylococci* isolates from 142 dairy cows in Kenya. Phenotypic traits, amplification by PCR assay, nucleotide sequencing and sensitivity testing for antimicrobials were used to classify Staphylococci isolates. Seven resistance genes were detected and the most commonly found genes in *S. aureus* and

CoNS were *blaZ* and *strB*. *S. aureus* and CoNS were found in 29.67 percent and 16.3 percent of cases, respectively.

2.2.2. India

Chandrasekaran *et al*. (2014) studied the prevalence of drug resistivity in clinical mastitis in Tamil Nadu, India. They had isolated and identified the resistant pathogens and categorized into groups *E. coli* (n=119), *S. aureus* (104) and MRSA (12) and the organisms are confirmed by PCR. They had detected resistance genes *mecA* and *blaZ* and also confirmed by sequencing. The particular target genes could be amplified from 119,104, 12 isolates out of 235 milk samples respectively with 50.64%, 89.64% and 10.34% respectively.

Kar *et al*. (2015) in Odisha, determined the prevalence of *E. coli* resistant to extended spectrum beta-lactam antibiotics from both milk sample of cows and faecal sample from poultry. Those isolates were resistant to cephalosporins and monobactam phenotypically were examine for the presence of Beta lactamase resistance genes. Isolates had bla_{SHV}, bla_{CTXM}, bla_{TEM}, bla_{ampC} in 17, 13, 9, 2 isolates by PCR. The sulfonamide resistance determinant *(sul1)* was found in nine isolate, whereas quinolone resistance gene was found in one *(qurB*).

Das *et al*. (2017) reported status of antimicrobial resistance in gram-negative bacteria found in SCM affected cows in West Bengal. *Escherichia coli*, *Proteus, Pseudomonas, Klebsiella, and Enterobacter* were identified. Extended spectrum beta lactamase producing and tetracycline-resistant isolates were detected in 48 percent (24/50) of the gram-negative isolates. 18 (36%) of the 50 gram-negative isolates tested positive for $bla_{CTX\text{-}M}$, while 6 (12%) tested positive for bla_{TEM} genes in PCR. bla_{SHV} genes were not found in any of the isolates. In addition, 5 (10%) of the isolates in this investigation had the *tetA* gene, whereas 8 (16%) carried the *tetB* gene and none of the isolates had *tetC* resistance gene. Study suggested the incidence of antibiotic resistance in gram negative is now on rise.

Kulangara *et al*. (2017) observed 76 Gram positive cocci isolates from 157 milk samples of cows were found to be CoNS, whereas the rest were *Staphylococcus aureus* in Kerala. CoNS were showed largest sensitivity to azithromycin and samllest

to tetracyclines, whereas *S. aureus* were more sensitive to cefoperazone (95%) and least to azithromycin (72.2%). The dilution technique with a commercial strip was used to detect antimicrobial resistance. 65 isolates (33 with the *mecA* gene and 32 with the *blaZ* gene) had β-lactam antibiotic resistance genes. Both genes were detected in 18 isolates. Nine isolates positive for *mecA* gene were showed oxacillin resistant *in-vitro* while seven isolates without the *mecA* or *blaZ* genes showed phenotypic resistance to oxacillin.

Bag *et al*. (2021) investigation restricted to *E. coli* isolated from milk of clinical mastitis affected cows in Bangladesh and detected its antibiotic sensitivity and antibiotic resistance genes. Phenotypically susceptible organisms were further examined for the presence of resistance genes. 94.5% isolates were resistant to ampicillin and tetracycline. 39% of *E. coli* isolates had bla_{TEM-1}.

2.3. Detection of tetracycline resistance gene from mastitis milk

2.3.1. World

Yang *et al.* (2016) in China isolated 44no of *S.aureus* from mastitis milk of cows and detected resistance genes in *S. aureus* by conventional PCR, tetracycline (*tetK, tetM*)-22.73%, against erythromycin (*ermA, ermB, ermC*)-22.73%.

Ali *et al*. (2016) in Egypt studied the number of genes on the bacterial chromosome that is responsible for tetracycline resistance. This study was focused on *tetK* and *tet*(*l*)*(38).* Large number of *S. aureus* isolates had *tet38* gene than the *tetK* gene, and the intriguing findings in this study was that the most MRSA (*mecA* gene positive) isolates had *tetK* gene, but the *tet(38)* gene was found in all *S. aureus* isolates irrespective of methicillin resistance means in both MRSA and methicillin susceptible isolates.

Kaczorek *et al*. (2017) reported the antibiotic resistance pattern of 135 strains of *Streptococcus* spp. on a phenotypic and genotypic level in Poland's southwestern area. Milk samples were taken from clinical mastitis affected dairy cows. Targeted resistance genes (n=14) were detected by PCR. They have also looked at the relationship between the drug resistivity genotypically and phenotypically. *Streptococcus* spp. showed the maximum resistance to kanamycin, gentamicin and

tetracycline, whereas they have showed susceptibility to penicillin, enrofloxacin, and marbofloxacin. There was no constant a linkage between the existence of a particular resistance gene and antimicrobial sensitivity.

El-Razik *et al.* (2017) unveiled resistance characteristics of tetracycline in coagulase-negative staphylococci (CoNS). Both normal and subclinical mastitic (SCM) affected buffaloes' milk samples were collected from various dairy herds in Egypt. Phenotypic and genetic approaches were used to identify CoNS. Different CoNS species isolated *S. intermedius S. xylosus S. epidermidis*, *S. hominis*, *S. hyicus, S. sciuri, S. simulans and S. lugdunensis*. All 28 CoNS isolates tested positive for the 16S rRNA gene specific for the Staphylococci genus and confirmed by seeing absence of thermonuclease (*nuc*) gene which is specific for *Staphylococcus aureus* using nested PCR. Detection *tetK, tetL, tetM, and tetO* tetracycline encoding resistance genes were determined by multiplex PCR. None of the isolates had amplified *tetL, tetM* and *tetO* genes. 50% of the CoNS isolates had amplified *tetK* gene, including all *S. lugdunensis*, *S. hominis*, 66.6% *S. epidermidis*, 45.4% of both *S. intermedius* and *S. xylosus*. Sequencing followed by phylogenetic analysis of the *tetK* gene, uncovered a high degree of similarity between tetracycline encoding resistance genes in CoNS isolates and those in *S. aureus* isolates from Egypt. The frequent transfer of *tetK* resistance genes between CNS and Coagulase positive *Staphylococcus* spp. gained evidence.

Zhang *et al.* (2021) had detected *tetM* (199/228, 87.28%) gene in *S. uberis* isolated from cow’s milk using conventional PCR.

2.3.2. India

Bag *et al*. (2021) investigation restricted to *E. coli* isolated from milk of clinical mastitis affected cows in Bangladesh, and detected its antibiotic sensitivity and antibiotic resistance genes. Phenotypically susceptible organisms were further examined for the presence of resistance genes. All *E. coli* isolates were resistant to tetracycline resistance gene i.e. *tetA*.

Das *et al*. (2017) detected *tetA, tetB, tetC,* and *tetM* in 50 gram negative isolates from subclinical mastitis in cows using conventional PCR in West Bengal.

2.4. Detection of aminoglycoside resistance gene from mastitis milk

Turutoglu *et al*. (2019) in Turkey investigated 18 phenotypically susceptible MRSA isolates obtained from mastitis milk sample. They have recorded the isolates positive for *mecA* gene as well as the *aph(3')-IIIa, aac (6')/aph(2'') and ant(4')-Ia* genes encoding aminoglycoside resistance by PCR. Only one isolate had aminoglycoside-modifying enzymes out of the three *mecA*-positive isolates had genes encoding and this isolates had the *aph(3')-IIIa* gene in conjunction with the *aac(6')/aph(2'')* gene. In three isolates, *aph(3')-IIIa* gene was found. Gentamicin, kanamycin and neomycin resistance was found in these 3 isolates containing the aminoglycoside resistance genes. All three *mecA* resistance genes were similar to those reported in MRSA strains found in human. Findings of this investigation say MRSA obtained from bovine mastitis might have originated in humans.

Yang *et al.* (2016) in China, isolated 44 *S. aureus* isolates from bovine mastitis milk. *S. aureus* isolates tested positive for penicillin (84.09%), erythromycin and vancomycin. Multidrug-resistant *S. aureus* isolates made up 9.09%of the total and detected resistance genes in *S. aureus* by conventional PCR, gentamicin *(aacA-aphD)*-2.27%.

CHAPTER-III

MATERIALS AND METHODS

The current study entitled 'Molecular detection of antibiotic resistance genes against gram positive bacteria isolated from mastitis milk of cows in Malkangiri district of Odisha' was conducted in the Department of Veterinary Epidemiology and Preventive Medicine, CVSc and AH, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar during the period from March 2020 to October 2021.

Fig 1. Map showing Malkangiri district of Odisha

3.1. Study area and population

A total of 520 lactating cows were screened from various herds located in Malkangiri district of Odisha, of which 15 had clinical mastitis with one or more signs such as fever, inappetance and inflammatory signs in udder. Milk samples were collected from the diseased animals. The laboratory tests were performed.

3.2. Collection of milk sample

About 5.0 milliliter of milk sample was collected aseptically in sterile vial from each lactating cows having clinical mastitis. Pooled milk sample was taken from all the secretory quarters and subjected to microbiological investigation.

Fig 2. Collected milk sample in sterile vial

3. 3. Microbiological growth characteristics

3.3.1. Inoculation in nutrient broth

13grams of nutrient broth powder (Hi- Media) was put in 1000ml distilled water. Mixed properly and sterilized in autoclave at 121^{0}C with 15lb pressure for 15 minutes.

Procedure of inoculation

Loopful of milk sample was taken from sterile milk vial with the help of inoculation loop which was allowed to red hot for sterilization by placing it over the flame of bunsen burner. Test tubes were incubated overnight at 37^{0}C.

Fig 3. Inoculation into nutrient broth

3.3.2. Gram staining

Morphological and staining characteristics of bacteria were evaluated by gram's staining.

3.3.3. Culture on mannitol salt agar (MSA)

Mannitol salt agar media was prepared by putting 111grams of mannitol salt agar base (Hi- MEDIA, India) in 1000ml of distilled water in a flask which then mixed and covered tightly with aluminium foil. Then the media was sterilized in autoclave with temperature maintained at 121 ^{0}C with 15 lb pressure for 15 minutes.

Selective media for isolation of *Staphylococci* is mannitol salt agar which is comprised of peptone as one protein source, beef extract and mannitol as a source of carbohydrate. The phenol red indicator, detects alteration in pH due to fermentation of alcohol by some Staphylococci, is responsible for the medium's differential character. *S. aureus* produce yellow colonies by fermenting mannitol. CoNS produce pink-red colonies as they are non- fermenters. Apart from *S. aureus* some other *Staphylococcus* spp produce yellow color colonies is one of the limitation of MSA.

Procedure

About 15-20ml of above sterilized media was poured in petridishes. It was left to dry and solidify for about 10min with lid of the plates removed partially and kept biosafety cabinet. In the bunsen burner, the inoculation loop was disinfected by placing it over the flame until it was red hot, then allowed it to cool. One secluded discrete colony from the agar plate was picked with the help of sterilized inoculation loop and a big circular smear was made in one top corner of the plate and parallel lines were streaked up to a total of 10 lines and last 3-4lines were the individual lines which were not taken from the smear to form the first quadrant by (approximately 1/4th of the plate). Likewise, 3quadrants were made to get the individual colony forming units.

Morphological characteristics

Incubation of material from nutrient broth into MSA plate was considered positive for *Staphylococcus aureus* with the growth of circular, small, convex yellow colonies.

3.3.4. Culture on nutrient agar

Isolates from mannitol salt agar were cultured on nutrient agar to get the pure isolate.

3.3.5. Culture on blood agar

Readymade blood agar plates were procured from Sigma Aldrich, India. It contains sheep blood, peptone as protein source, beef extract, agar, salt and distilled water. Differentiation of bacteria was done based on haemolysis pattern which was comprised of β-haemolysis causes complete haemolysis, α- haemolysis causes partial haemolysis and γ- haemolysis causes no haemolysis.

Procedure of inoculation

One isolated colony from the pure cultured agar plate was picked with the help of sterilized inoculation loop and a big circular smear was made in one top corner of the plate and parallel lines were streaked up to a total of 10 lines, likewise 3quadrants were made to get the individual colony forming units and kept at the incubator at 37^0C. Colonies were examined for haemolysis.

3.4. Antimicrobial susceptibility test

Pure culture which was made previously in nutrient agar grown on nutrient broth, kept overnight incubation at 37^0C. Sterile swab was dipped in the nutrient broth containing pure isolates to make lawn culture in nutrient agar. About 10 sterile antimicrobial discs such as Cefoperazone (CPZ), Tetracycline (TE), Ampicillin (AMP), Neomycin (N), Amoxycillin (AMX), Penicillin (P), Gentamicin (GEN), Streptomycin (S), Doxycycline (Do), Amikacin (AK) were put to the surface of the inoculated plates aseptically. Plates were then placed in the incubator in an inverted manner with temperature maintained at 37^0C for 24hours. After incubation that was checked for diameter of zone of inhibition with the help of meter ruler.

Table 1. Standard zone of inhibition

Antimicrobial discs with disc concentration	Diameter of the zones observed (mm)	
	Sensitive	Resistance
Cefoperazone (75mcg)	21	15
Tetracycline (30mcg)	19	14
Ampicillin (10mcg)	17	13
Neomycin (30mcg)	17	12
Amoxycillin (30mcg)	19	13
Penicillin (10units)	29	28
Gentamicin (10mcg)	15	12
Streptomycin (10mcg)	15	11
Doxycycline (10mcg)	23	21
Amikacin (10mcg)	17	14

3.5. Molecular identification of bacteria

3.5.1. Extraction of genomic DNA from gram positive bacteria

DNA from the genome of bacteria was extracted by referring the standard protocol provided in the kit (Nucleopore gDNA fungal/bacterial mini kit). 2-3loopful of pure isolates was taken from pure culture and put into sterilized test tube containing 200 µl of autoclaved water. Then 200 µl sample was taken in eppendrof tube. 20 µl proteinase K and 200 µl lysis buffer was added to it then vortexed, centrifuged at 8000rpm for 1minute. Eppendrof tube was then placed over the thermosetter with temperature maintained at 56^0C for 10minutes. 200µl of absolute ethanol was added, vortexed, centrifuged at 8000rpm for 1minutes and kept in room temperature at 25^0C. The total solution was 620 µl transferred to the extraction spin column, centrifuged at 8000rpm for 1minute. The flow through obtained in collection tube after centrifugation was discarded. Thereafter extraction column was moved to a new collection tube. 200 µl AW_1 buffer was added, centrifugation done at 8000rpm for 1minute. Repeat flow through discard and extraction spin column was then transferred to the new collection tube. Supernatant was removed. 200 µl AW_2 buffer was added

and centrifuged at 14000rpm for 2minutes. Collection tube with flow through was discarded extraction column was transferred to eppendrof tube. 81 µl of elution buffer was added and kept at room temperature for 3minutes, centrifuged 8000rpm for 1minutes. Extraction was discarded Sediment containing DNA was measured in nanodrop and recorded. DNA was stored at -20^0C in refrigerator till further use.

3.5.2. Identification of bacteria by 16S rDNA sequencing

Amount of PCR was determined for the 7 samples. Ready-to-use PCR master mix was procured from Sigma Aldrich, India. For the needed number of PCR tubes, an aliquot of master mix was made.

PCR reaction mixture was 25µl volume which was comprised of 12.5 µl master mix, 5 µl template DNA, 1 µl each forward and reverse universal primers were used. Volume was adjusted to 25 µl by adding 5.5 µl of nuclease free water (NFW). Gently shaked and amplification of DNA was done in thermal cycler (Proflex). Temperature was set for initial denaturation at 95^0C for 5min. 35cycles were run at the following temperature set up: denaturation at 95^0C, annealing for 55seconds, and extension at 65^0C for 1.5minutes.

The samples were sent to HKP scientific, India for Sanger's sequencing. Then the sequences were submitted to NCBI for accession number.

3.5.3. Agarose gel electrophoresis

Agarose gel 1% was made by taking 1gram agarose powder in a sterilized beaker 99.0 ml distilled water was poured into it. 1.0 ml of 50XTAE buffer (Hi-Media) was added into it. Mouth of the beaker was covered with aluminium foil. Beaker was boiled for few minutes. Ethydium bromide stain was added to the beaker. Gel was poured over the plate allowed to cool. Thereafter, gel was visualized under a UV transilluminator (E-gel imager, Life technology. Invitrogen, India). 1000bp DNA ladder (3B Black Biotec) was used.

3.6. Detection of resistance gene against antibiotics

Primers for the four antibiotic resistance genes *blaZ, aacA-aphD, tetK, tetM* were procured from Sigma Aldrich, India

Table 2. Primer sequences of antibiotic resistance genes against three antibiotic groups

Resistance Genes	Oligonucleotide sequences	Product size (bp)	Name of antibiotics	References
blaZ	F-TAAGAGATTTGCCTATGCTT R- TTAAAGTCTTACCGAAAGCAG	377	Penicillin	Olsen *et al.* (2006)
aacA-aphD	F-GAAGTACGCAGAAGAGA R- ACATGGCAAGCTCTAGGA	491	Gentamicin	Strommenger *et al.* (2003)
tetK	F- GTAGCGACAATAGGTAATAGT R- GTAGTGACAATAAACCTCCTA	360	Tetracycline	Strommenger *et al.* (2003)
tetM	F- AGTGGAGCGATTACAGAA R- CATATGTCCTGGCGTGTCTA	158	Tetracycline	Strommenger *et al.* (2003)

3.6.1. Molecular detection of resistance genes

Readymade PCR master mix was procured from Sigma Aldrich, India. For the needed number of PCR tubes, an aliquot of master mix was made.

The genomic DNA were isolated as described in the section 3.5.1 and sample DNA were subjected to PCR using specific primers (Table 2) for the detection of antibiotic resistance genes of beta lactam group, aminoglycoside group and tetracycline group for *blaZ, aacA-aphD, tetK, tetM* respectively. The mixture's volume was reduced to or adjusted to 25 µl. 14 µl mastermix, 0.5 µl each forward and reverse primers, 2 µl template DNA, 5 µl NFW constitutes 25 µl. Here gradient PCR was allowed to determine an optimal annealing temperature. Total of 30cycles were run at the following temperature set up: initial denaturation at 94^0C, 5minutes and denaturation at 94^0C for 30seconds, ten different annealing temperatures ranging from 45^0C to 55^0C for 45 seconds such as 45 ^{0}C, 48 ^{0}C, 52 ^{0}C and 55 ^{0}C for 45 seconds and extension at 72^0C for 5 seconds and 4^0C for infinity. Best condition was found in well 8 and well 10 where the temperatures were 52^0C and 55^0C.

3.6.2. Agarose gel electrophoresis preparation

1.2% agarose gel was prepared by taking 1.2gram agarose powder in a sterilized beaker 98ml distilled water was poured into it. 2ml of TAE buffer was added. Mouth of the beaker was covered with aluminium foil. Beaker was heated to

50^0C. Gel was observed under a UV transilluminator (E-gel imager, Life technology. Invitrogen, India) after electrophoresis, 1000bp DNA ladder (Sigma Pvt. Ltd) was used.

3.6.3. Nucleotide sequences and accession numbers

Six sequences of PCR samples (STP 1-6) used in this study were submitted to GenBank database for accession numbers.

3.7. Phylogeny

Phylogenetic tree was constructed using software MEGA 11 by neighbor joining method. Multiple sequence analysis of isolated strains was done by using Clustal W v 1.6. Bootstrap analysis of neighbor-joining data sets based on 1000 replications was used to evaluate the resulting tree topology.

CHAPTER-IV

RESULTS

520 lactating cows were screened from different herds in Malkangiri district of Odisha, of which 15 cows had clinical mastitis. Pooled milk samples from 15 cows were collected aseptically in sterile containers. Collected milk sample had poor consistency with bloody discoloration and/or presence of clots and flakes. The samples were transported to the lab in an ice box. These were inoculated into nutrient broth, kept overnight in the incubator at 37^0 C. Thereafter phenotypic susceptibility was observed by doing ABST using 10 antibiotic discs which belonged to three antibiotic classes. Seven isolates were further subjected to 16S rDNA identification by using universal primer. Samples were sent to HKP scientific Pvt. Ltd., India for Sanger's sequencing. All seven isolates of bacteria were further subjected to PCR for amplification of four antibiotic resistance genes (*blaZ* against β –lactam group*, aacA-aphD,* against aminoglycoside group, *tetK, tetM* against tetracycline group) by using specific primers. Out of 7gram positive bacteria 6 *Mammaliicoccus sciuri* isolates were submitted for Accession number in NCBI. Phylogentic tree was constructed.

4.1. Isolation and identification of bacteria through microbiological culture

Turbidity appeared in nutrient broth which indicated bacterial growth. Thereafter they were subjected to gram's staining. Among 15 samples 12 were gram positive and rest showed mixed infection with gram's staining. Out of 12 samples seven samples were gram positive cocci with bunch of grapes like structure suggestive for the presence of *Staphylococci*. Then seven samples which were grown over selective media i.e. mannitol salt agar (MSA), golden yellow color colonies appeared in all the seven samples which indicated positive for *Staphylococcus* spp. Further pure isolates was obtained by growing them on nutrient agar. They were grown on blood agar to rule out the presence of *Staphylococcus aureus*.

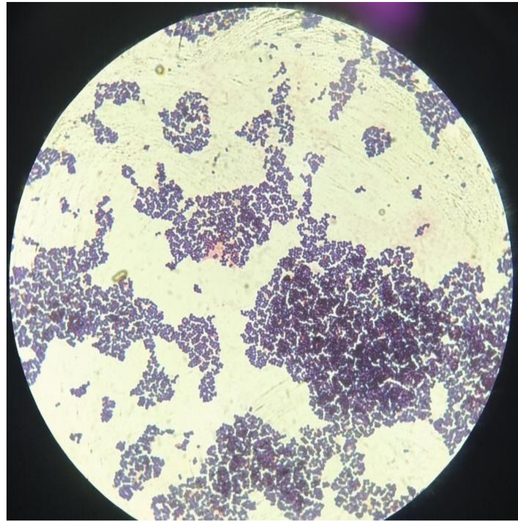

Fig 4. Bunch of grapes like cocci suggestive for the presence of *Staphylococci*

Fig 5. Yellow colored colonies on MSA

Fig 6. Pure culture on nutrient agar

4.2. *In-vitro* Antibiotic Susceptibility Test

All the 7 isolates were tested for Antibiotic sensitivity test (Table no.3) by using 10 antibiotics such as amikacin (AK), neomycin (N), cefoperazone (CPZ), gentamicin (GEN), penicillin (P), tetracycline (TE), streptomycin (S), doxycycline (Do), penicillin (85.7%), streptomycin (28%), cefoperazone and tetracycline showed (14.2%) resistance.

Table 3. Antibiotic sensitivity test

Sl. No.	Name of antibiotics	MK 6	MK 7	MK 9 (1)	MK 9 (2)	MK 11	MK 12 (1)	MK 12 (2)	% R	% S	% IS
1	Amikacin	S	S	S	S	S	S	S	0	100	0
2	Neomycin	S	S	S	S	IS	S	S	0	85.7	14.2
3	Cefoperazone	S	S	S	S	R	IS	S	14.2	71.4	14.2
4	Gentamicin	S	S	S	S	S	S	S	0	100	0
5	Penicillin	R	R	R	S	R	R	R	85.7	14.2	0
6	Tetracycline	S	S	S	S	R	S	S	14.2	85.7	0
7	Ampicillin	S	S	S	S	S	S	S	0	100	0
8	Streptomycin	S	S	S	S	R	R	S	28.5	71.4	0
9	Doxycycline	S	S	S	S	S	S	S	0	100	0
10	Amoxicillin	S	S	S	S	S	S	S	0	100	0

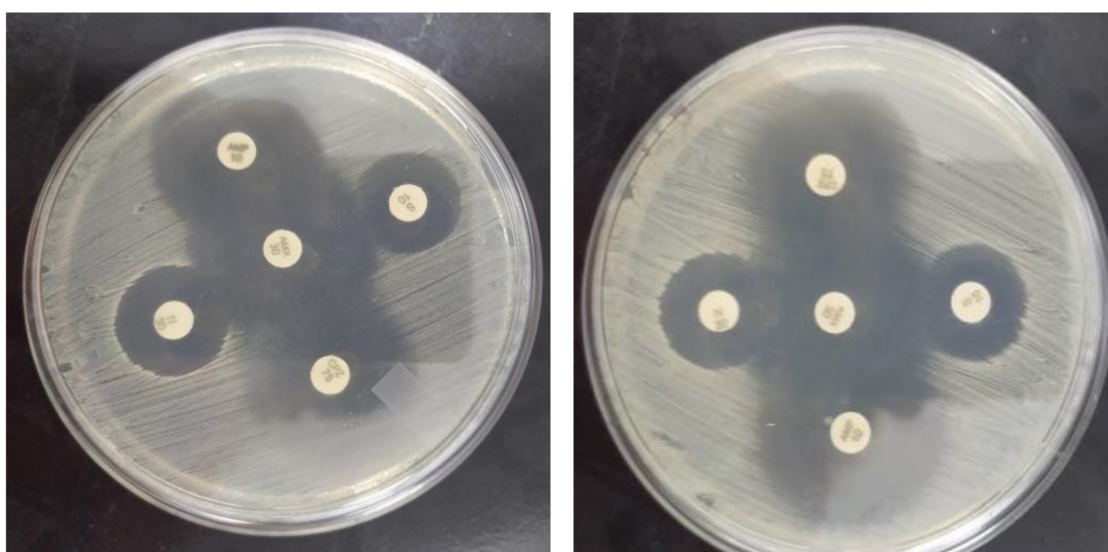

Fig 7. *In-vitro* antibiotic sensitivity test

4.3. Molecular identification

4.3.1. Identification of bacteria

Of the 16S rDNA sequencing of 7 isolates, 6 isolates were *Mammaliicoccus sciuri. Staphylococcus sciuri* was reassigned to the other genus of Staphylococcoceae family and renamed as *Mammaliicoccus sciuri*, (Madhaiyan *et al.,* 2020) and other one is *Enterococcus faecium*.

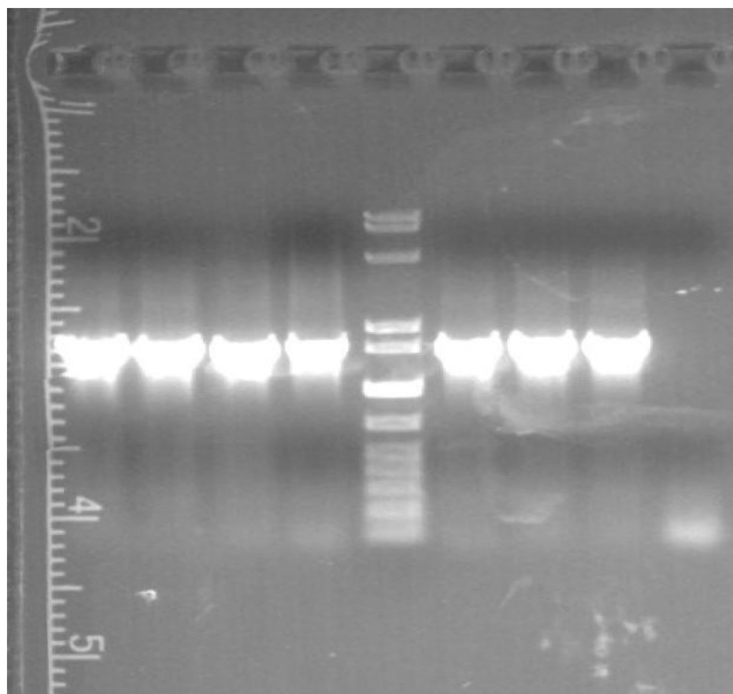

Fig 8. Molecular idetification of bacteria using 16S rDNA sequencing

4.3.2. Nucleotide sequences and accession numbers

Six sequences of PCR samples (STP 1-6) used in the study were deposited in GenBank database for accession numbers: OK412723, OK614118, OK6114108, OK614113, OK614117, OK614103 respectively.

4.3.3. Detection of resistance genes

Out of four resistance genes one isolate i.e. one *Mammaliicoccus sciuri* had amplified tetracycline resistance gene i.e. *tetK* at annealing temperature *55*^{0}C. *Enterococcus faecium* amplified both the resistance genes against tetracycline *tetK* at annealing temperature 55^{0}C and *tetM* at 52^{0}C. Rest was negative for other resistance genes.

Table 4. Amplification of resistance genes by 16S rDNA sequencing

Sample name	Species of organisms	Resistance genes			
		blaZ	***aacA-aphD***	***tetK***	***tetM***
MK 6	*M. sciuri*	N	N	N	N
MK 7	*M. sciuri*	N	N	N	N
MK 9 (1)	*M. sciuri*	N	N	N	N
MK 9 (2)	*E. faecium*	N	N	P	P
MK 11	*M. sciuri*	N	N	N	N
MK 12 (1)	*M. sciuri*	N	N	N	N
MK 12 (2)	*M. sciuri*	N	N	P	N

blaZ- penicillin resistant gene, *aacA-aphD*- aminoglycoside resistant gene, *tetK, tetM*- tetracycline resistant genes

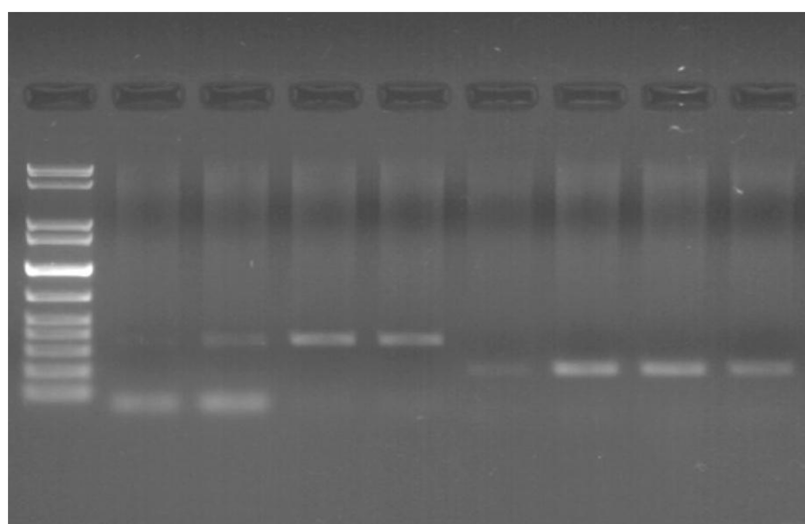

Fig 9. Amplification of *tetK* gene (360-bp) and *tetM* (158-bp) gene performed using specific primers

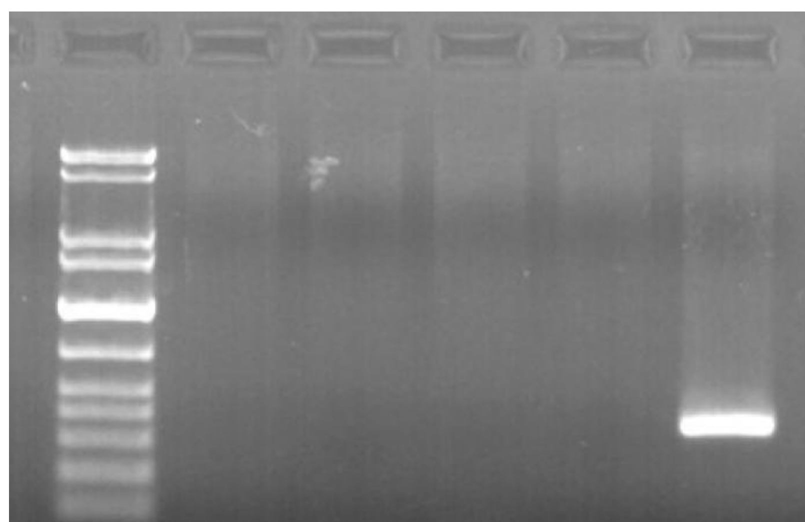

Fig. 10. Amplification of *tetK* (360-bp) gene performed using specific primers

4.4. Phylogenetic analysis

The construction phylogenetic tree was done by gathering all our *M. sciuri* isolates (STP 1-6) with the 24 similar reference gene sequences. Our recovered isolate's sequencing was compared to *M. sciuri* isolate sequences acquired from GenBank. Our sequences have a high level of similarity to those in the GenBank, ranging from 74.00 to 100%.

Table 5. List of *M. sciuri* strains used in the current study

Sl. No.	Name of isolates	Strain	Origin	Country	Accession no.
1	STP- 1	*M. sciuri*	Cow milk	India	OK412723
2	STP-2	*M. sciuri*	Cow milk	India	OK614118
3	STP-3	*M. sciuri*	Cow milk	India	OK614108
4	STP-4	*M. sciuri*	Cow milk	India	OK614113
5	STP-5	*M. sciuri*	Cow milk	India	OK614117
6	STP-6	*M. sciuri*	Cow milk	India	OK614103
7	CT12(2)	*M. sciuri*	Midgut of Culex tritaeniorhynchus	Sri-Lanka	MT072175
8	B9-58B	*M. sciuri*	Retail pork	USA	CP041879
9	CG1(3)	*M. sciuri*	Midgut of Culex gelidus	Sri-Lanka	MT072194
10	190306H232 (2)	*M. sciuri*	-	China	MT225726
11	AA1(4)	*M. sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
12	DN8(2)	*Staphylococcus* spp	Brine	China	MT433878
13	AA1(3)	*M.sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
14	YY1	Bacterium YY1	Blowhole material	China	KT759041
15	S189E	*M. sciuri*	Salt affected soil	Karnataka, India	JF513127
16	AA1 (5)	*M. sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
17	190306H232 (3)	*M. sciuri*	-	China	MT225726
18	CG1(4)	*M. sciuri*	Midgut of Culex gelidus	Sri-Lanka	MT072194
19	CT12(3)	*M. sciuri*	Midgut of Culex tritaeniorhynchus	Sri-Lanka	MT072175
20	DN8 (3)	*Staphylococcus* spp	Brine	China	MT433878
21	AA (6)	*M. sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
22	190306H232 (4)	*M. sciuri*	-	China	MT225726
23	CG1(5)	*M. sciuri*	Midgut of Culex gelidus	Sri-Lanka	MT072194
24	DN8	*Staphylococcus* spp	Brine	China	MT433878
25	RTE-S2	*M. sciuri*	Ready-to-eat foods	Bangladesh	LC572266
26	AA1	*M. sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
27	AA1(2)	*M. sciuri*	Midgut of Aedes albopictus adults	Sri-Lanka	MT275460
28	190306H232	*M. sciuri*	-	China	MT22572
29	CG1 (2)	*M. sciuri*	Midgut of Culex gelidus	Sri-Lanka	MT072194
30	CT12	*M. sciuri*	Midgut of Culex tritaeniorhynchus	Sri-Lanka	MT072175

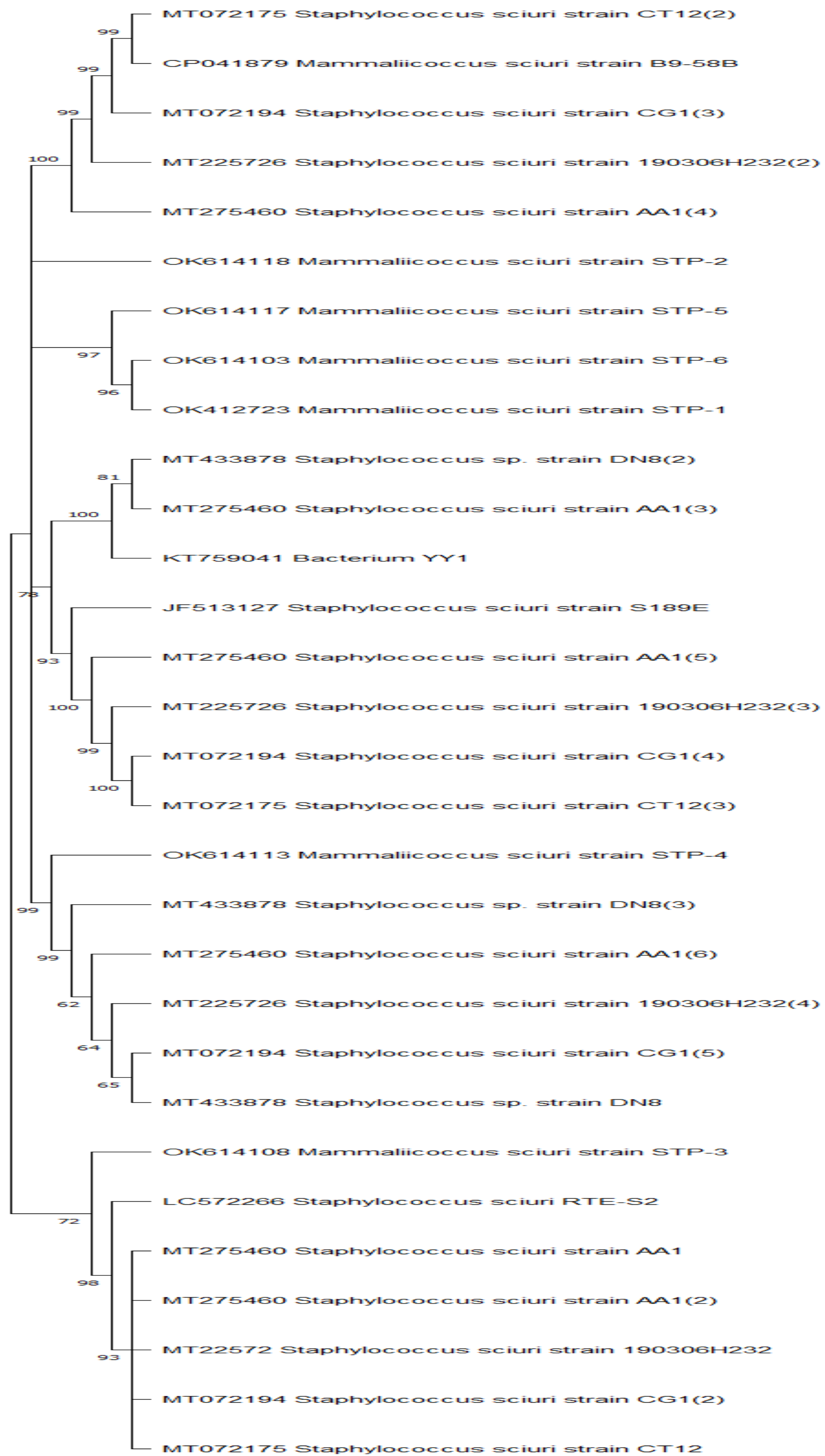

Fig 11. Phylogenetic relationship among *M. sciuri* sourced from GenBank

CHAPTER-V

DISCUSSION

Mastitis is a complex disease of dairy cows. Disease is manifested by inflammation of the mammary gland accompanied by various clinical signs and symptoms. A wide spectrum of microorganisms causes mastitis, which predominantly include bacteria (*Staphylococcus aureus* with some CoNS such as *S. sciuri*, *S. epidermidis*, *Aerobacter aerogenese*, *Campylobacter jejuni*, *Cornybacterium bovis*, *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumonia, Listeria monocytogenes, Mannheimia haemolytica, Mycobacterium bovis, Pasteurella multocida, Pseudomonas aerogenese, Serratia marcescens, Streptococcus agalactiae, Streptococcus pyogenese, S. dysgalactiae, S. pyogenese, S. uberis, S. zooepidemicus, Trueperalla pyogenese* etc.), actinomycetes (*Nocardia asteroides*), fungi *(Aspergillus nidulans, A. fumigates, A. flavus, A. niger, Candida tropicalis, C. albicans, C. guillermondii, C. krusei, C. parapsilosis, Cryptococcus neoformans, Debarryomyces hansenlii, Geotrichum candidum, Pichia farinose, Rhodotorula glutinis, Saccharomyces fragillis* and *Trichosporonbeigelii*), *Mycoplasma* spp, viruses and algae (*Prototheca* spp) are implicated in the etiology of mastitis of dairy animals as reported by Pal (1979), Pal and Mehrotra (1983), Watts (1988), Jensen *et al*. (1996), Pal (1997), Pal and Lee (1997), Wellenberg *et al*. (2002), Quinn *et al*. (2006), Kulkarni and Kaliwal (2011), Pal (2015), Pal *et al*. (2017) and Pal (2018). Pathogens invade the udder tissue, proliferates, release toxins that will be reflected on milk by alteration of physical and chemical properties of milk. Animals with clinical mastitis exhibit systemic signs such as fever, depression, loss of appetite, swelling of udder and progressive reduction in milk yield with reduced milk quality. Disease has been reported throughout the world where high yielding dairy cows especially exotic (*Bos taurus*) and crossbred cows are reared. Microorganisms that most frequently cause mastitis can be divided into two groups. contagious pathogens, that transmits between cows mainly during the milking process and the other category is environmental pathogens, that are found in dairy cows' natural surroundings and a third minor category i.e. teat skin opportunistic pathogens takes advantage of any chance to cause mastitis (Radostits *et al.,* 2007).

The treatment of mastitis is mainly dependent on antibiotic therapy using β-lactams, cephalosporin, aminoglycoside, tetracycline and macrolide, etc. be it for prevention by dry cow therapy or for treatment or for production as per the report of Chandrasekaran *et al*. (2014), Kaliwal *et al*. (2011) and Awandkar *et al*. (2013). Despite vigorous treatment by antibiotics, bacteriological cure rates seldom exceed 50% particularly in *Staphylococci* infection. As per the reports of many scientists antimicrobial resistance (AMR) is the most common reason for therapeutic failures and thus antibiotic sensitivity test is a crucial stage in the treatment process at the farm level before prescribing medicines. Little research has been done on detection antimicrobial resistance gene in mastitis milk of cows in this part of India. Current study was done to detect the presence of resistance genes against bacterial pathogens of mastitis milk of cows in Malkangiri district of Odisha. It is one of the few studies in India on recognition of resistant gene against most commonly used antibiotics in mastitis.

15 pooled milk samples from clinical mastitis affected cows were collected aseptically. Milk had poor consistency with discoloration and there was presence of clots and/or flakes. Sample had undergone microbiological culture and staining followed by 16S rDNA sequencing. Of 7 bacterial isolates, 6 isolates were *Mammalicoccus sciuri* (previously named as *Staphylococcus sciuri*) which are coagulase negative *Staphylococci* (CoNS) and the other one was *Enterococcus faecium*. Our findings is in close approximation to the earlier studies of Frey *et al*. (2013), Levison *et al*. (2016) and Lange *et al*. (2015) where the prevalence of CoNS was 65%. As per the available reports of Rajala-Schultz *et al*. (2009), Pyorala and Taponen (2009), De Vliegher *et al*. (2012) and Piessens *et al*. (2011). CoNS are becoming well recognized as significant mastitis pathogen. CoNS may have antimicrobial resistance elements such as the staphylococcal cassette chromosome element according to the reports of Wielders *et al*. (2001), Barbier *et al*. (2010) and Tsubakishita *et al*. (2010) and arginine catabolic mobile element (ACME) according to the reports of Diep *et al*. (2006) and Miragaia *et al*. 2009) that can be transferred to *S. aureus*. Since *S. aureus* considered as a major pathogen causing mastitis and the organisms have antimicrobial resistance element with them if this organism carries and disseminates antimicrobial resistance elements, there may be serious havoc in the future owing to development of resistance against most of the antibiotics.

Treatment of mastitis through antibiotics is the age-old practice. However due to indiscriminate use and overuse of antibiotics with lack of discovery of novel antibiotics microorganisms grabs this opportunity to evolve antibiotic resistance. AMR is a serious concern worldwide now raising national and global health threat. Many bacterial pathogens have evolved as MDR (Multidrug resistance) in human and animal disease outbreaks including CoNS (El Razik *et al.*, 2017 and Nemighaire *et al.*, 2014). If MDR in bacteria is based on self-adaptive mutation, it takes a long time to develop however gene transfer through conjugation, transduction or transformation allows quick dissemination of resistant determinant between microorganisms irrespective of the species as reported by Mathers *et al.* (2015) and Wang and sun (2015). Various studies conducted by Foster, 2007, Bjedov *et al.* 2003, Claverys *et al.,* 2006 and Velkov *et al.,* 1999 says that when bacteria undergo various stress-induced factors such as nutrient shortage, nucleic acid damage, temperature transitions, oxidative distress, and antibiotic exposure resulting to higher frequency of horizontal gene transfer and mutation.

Importantly, genotypes can provide information on a pathogen's current antibiotic susceptibility as well as its future potential for resistance and dissemination. Sequencing may reveal if a drug-resistant isolate has precursors to resistance genes in addition it is also beneficial to see whether resistance cassettes are just one mutation far from being even more resistant.

In the current study, we have processed clinical mastitis milk sample for isolation of bacteria and detection of four resistance genes against penicillin, gentamicin and tetracycline. Detection of resistant genes was done by conventional polymerase chain reaction by using their specific primer. Only one gene i.e. *tetK* gene for tetracycline was amplified in one *M. sciuri* isolate whereas amplification of both the resistance genes of tetracycline i.e. *tetK and tetM* occurred in *E. faecium*. However *in-vitro* sensitivity test of that isolate revealed tetracycline is sensitive in both the isolate. This key discrepancy of presence of resistance gene but not showing resistance ($P^{-}G^{+}$) might be due to two reasons (1) presence of multiple resistance genes having similar resistance characteristic responsible for the development of resistance against a particular antibiotic as reported by Davis *et al.,* 2011. A good number of resistance genes discovered against tetracycline *viz tetK, tetM*, *tetL, tetO,*

tetA, tetB, tetC, tetE etc. (2) expression of resistance of a gene depends on the stress it receives. In the present study we have used 30mcg concentration of tetracycline, were increased the concentration of antibiotic, resistance genes would have been expressed with higher concentration of antibiotic.

Also we may correlate the genotypic-phenotypic discrepancy with the findings of Davis *et al*. (2011). They have hypothesized and proved that lesser use of antibiotics would have resulted in more P^-G^+ isolates than higher antibiotic use. The P^-G^+ isolates harbors pseudogenes or false genes means those genes which are inactive but present as steady component or "mutation in DNA sequences analogous to known genes removed their ability to be expressed" (Hartl and Clark, 2007). Presence of multiple genes having similar resistance characteristics might have underestimated the frequency of pseudogenes which was proved by Davis *et al*. (2011). Moreover when resistance genes found in pairs (in our study the gene pair we have taken was aacA-aphD) if one of the two genes is not expressed due to start codon and a structural obstacle to the Shine-Dalgarno sequence and then also there is every possibility of not expression of resistivity against an antibiotic.

In the present study *M. sciuri* (gram positive, CoNS) found as the major pathogen causing clinical mastitis (Khazandi *et al.,* 2010). Our findings is in close relation to the findings of El Razik *et al.* (2017) where their observation revealed CoNS have distinctly high resistant to tetracycline. Larger usage of tetracycline could have expressed the resistance gene and spread the genetic resistance dynamics within CoNS isolates and among all *Staphylococci.* Subsequently there would be no effect of tetracycline against *Staphylococcus* spp. As per the research of El Razik *et al.* (2017) there is high degree of homology between nucleotide sequences of tetracycline resistance genes of CoNS isolates and same resistance genes against the same drug in *S. aureus* isolates of Egypt, which evidenced the spread of tetracycline resistance genes between CoNS and CoPS. This would be a major concern for the treatment of mastitis with tetracycline since *S. aureus* is a major pathogen associated with mastitis (Gillispie *et al.,* 2009).

Few researches have looked at antibiotic resistance differences among CoNS species (Sampimon *et al.,* 2009). In the present study of *in vitro* antibiotic susceptibility test, 6 (85.7%) CoNS isolates revealed resistance to penicillin but there

was no amplification *blaZ* resistance genes. This might be due to the presence of another resistance gene against β-lactam antibiotic i.e. *mecA, femA, femB* which might confered resistance characteristic. This is in contrast to most of the study on *Staphylococcus aureus* conducted by Yang *et al*. (2016), Ramaswamy *et al.* (2020), Girmay *et al.* (2020) and Chandrasekaran *et al.* (2014) where they have found both genotypic and phenotypic resistance for penicillin.

Aminoglycoside remains sensitive in most in vitro antibiotic sensitivity study and also there was absence of resistance genes (Yang *et al.,* 2016 and Gow *et al.,* 2008). Report says amikacin is sensitive phenotypically also lesser number of isolates amplified aminoglycoside resistance gene. But isolates are emerging resistance to kanamycin both phenotypically and genotypically. variety of mechanisms can be the cause for aminoglycoside antibiotic resistance, which includes (1) enzymatic modification by the enzymes aminoglycoside acetyltransferases (aacA), nucleotidyltransferases, or phosphotransferases (*aphD*) that are common to both G +ve and G -ve bacteria (Ramirezz and Tolmasky. 2010 and Shaw *et al* (1993) (2) enhanced release of antibiotic (3) decreased permeability to the bacteria and (4) modifications of the mechanism of action of aminoglycoside antibiotics by interfering with binding of aminoglycosides targeting 30S ribosomal subunit.

El Razik *et al*. (2017) explained the tetracycline resistance of CoNS in mastitis milk of buffaloes. Antibiotic sensitivity testing and multiplex PCR to identify tetracycline (tet) resistance genes were used to screen CoNS isolates for tetracycline resistance. Subsequently sequencing of PCR positive samples and phylogenetic analysis was done. Tetracycline resistance is very common in CoNS isolates. Tetracycline overuse for mastitis therapy causes the spread of genetic resistance mechanisms in CoNS strains and Staphylococcus spp.

Despite comprehensive study on resistance characteristic of mastitis milk by *in vitro* sensitivity test, there is quite a large percentage of treatment failure. The key discrepancies are inadequate knowledge and proficiency in isolation of diverse microbes, their proper identification, genetic diversity, varying mechanisms of resistance, little insight into genetic characterization of antimicrobial resistance for different organisms.

Cycle of AMR circulates among the human, animal and environment. The World Health Organization (WHO) produced a world's priority pathogen list that was divided into three categories: critical, high, and medium antibiotic resistance microorganisms. Gram-positive bacteria, particularly multidrug-resistant bacteria such as MRSA, Vancomycin-resistant *Enterococcus faecium* (VRE) and β-lactamase-resistant Streptococcus pneumonia, are considered a special concern and a health risk among these pathogens (Cornaglia, 2009 and Asokan *et al.*, 2019).

An estimated 2 million drug-resistant infections have been reported in the United States each year, resulting in an additional 8 million hospital days, 23,000 deaths, and $20 billion in direct healthcare expenses (CDC, 2013). By 2050, if the current AMR trend continues, there might be 10.0 million yearly drug resistant-related fatalities from a variety of illnesses (Review on antimicrobial resistance, 2016). WHO included AMR in top 10global health threat (WHO, 2019).

Some recent highlighted examples of the threat of emergence of AMR are New Delhi Metallo-beta-lactamase 1 (NDM-1) and Mobilized Colistin Resistance-1(MCR-1) and for the first time in the United States, plasmid-mediated carbapenem-resistant Enterobacteriaceae have emerged in swine. After the discovery of the NDM-1 in 2008, whose origin was in Indian subcontinent subsequently disseminated to the UK due to medical tourism (Yong *et al.,* 2009). Thereafter in 2010 it was found in surface and tap water samples in India. Then global spread of this gene and its variants reported (Walsh *et al.,* 2011, Islam *et al.,* 2017 and Khan *et al.,* 2017). The NDM-1 gene was discovered from mineral-rich Arctic soil on a Norwegian island, increasing fears that it may be transmitted by migrating bird faeces (McCann *et al.,* 2019). In 2014, A plasmid carrying Colistin resistance gene i.e. MCR-1 (Mobilized Colistin Resistance-1) was detected in chinese pigs (Liu *et al.,* 2016) subsequently disseminated to other countries (Wang *et al.,* 2018 and Marston *et al.,* 2016).

In order to tackle AMR, a collaborative and multi-sectoral one-health strategy is required. In particular there is a need of amalgamation of aquatic, environmental and wildlife issues to combat AMR (Thakur and Gray, 2019). If we are unable to control the drug resistance, very soon the world is going to face a serious pandemic by 2050 with an estimated loss of 10 million human lives (O'Neill *et al.,* 2016). Between 2000 and 2015, there was 65% rise in drug consumption in humans in 76 countries,

and in 2010, 63,000 tonnes of antimicrobial administration in animals. With a peak 67% rise in consumption by 2030, there could be sudden increase in AMR burden.

Findings of Juhász-Kaszanyitzky *et al*. (2007) indicates MRSA is transmitted directly from cows to people. They had isolated and confirmed MRSA from bovine subclinical mastitis milk and throat swab sample of a person who worked with those animals and concluded that they were both indistinguishable. Thereafter the strains of MRSA isolates were tested for drug sensitivity and detected the presence of *mecA* both in human and in animal sample.

As revealed in the present study, the tetracycline treatment in case of mastitis is a matter of concern. There will be higher chance of tetracycline resistance in that region also it may spread to other geo-locations. There is high level of similarity observed between our isolates and the same species/isolates taken from other countries and it also facilitates to know the rise of resistance to other antibiotics of the same class.

Phylogenetic analysis of *M. sciuri* performed in the present study showed high level of similarity between our isolate to the isolates obtained from GenBank i.e. 74-100%. This implies probability of transfer of tetracycline resistance gene among isolates of *M. sciuri*. Findings of El Razik *et al*. (2017) says that there is chance of transfer of tetracycline resistance gene between CoNS isolates including *M. sciuri* and *S. aureus* isolates as the nucleotide sequences of *tetK* resistance gene of *M. sciuri* is similar to that of *S. aureus* with minor variation.

Our study would encourage the preparedness and surveillance system to predict, identify and respond to the upcoming AMR problem in the nearby areas. Present research would help to determine the emerging trend, persistence and potential spread of AMR bacteria within the farm animals.

More research needed for the evidence of direct transmission of resistance gene from animal to human or vice- versa as there is little work done in this regard. To combat AMR, a complete One Health Research program that includes people, animal, ecosystem, ecological, and wildlife standpoint is required.

CHAPTER-VI

SUMMARY AND CONCLUSION

Mastitis is the commonly encountered illness in dairy cattle. Present study was carried out during March 2021 to October 2021 to recognize the drug resistance genes in gram positive bacteria found in mastitis milk of cows in Malkangiri district of Odisha.

A total of 520 lactating animals were screened, of which 15 crossbred jersey cows had signs of clinical mastitis such as fever, anorexia, depression, inflamed udder along with visible abnormality in milk such as bloody discoloration and presence of clots and flakes in milk. Pooled milk samples were collected aseptically in sterile vials and taken to the laboratory in ice box. Samples were inoculated into nutrient broth followed by gram staining positive samples were further by cultured on selective media i.e. MSA and blood agar. Seven isolates of *Staphylococcu*s spp found. Further 16SrDNA sequencing revealed 6 isolates were *Mammalicoccus sciuri* and the other one was *Enterococcus faecium*. *In vitro* antibiotic susceptibility test showed variable range of antibiotics resistance topenicillin (85.7%), tetracycline (14.2%) and streptomycin (28.5%). 14.2% strains showed intermediate sensitive to cefoperazone and neomycin antibiotics.

Resistance gene was detected by conventional polymerase chain reaction by using specific primer. Only one *Mammalicoccus sciuri* isolate amplified *tetK* resistance gene and *Enterococcus faecium* strain amplified both *tetK* and *tetM* resistance genes of tetracycline. None of the isolates amplified *blaZ* and *aacA-aphD* resistance gene meant for penicillin, gentamicin and tetracycline antibiotic respectively. *In vitro* antibiotic test results indicated that *Mammalicoccus sciuri* strain showed more than 85% resistant to penicillin but there was no amplification of penicillin resistant gene i.e. *blaZ* Whereas tetracycline showed 14.2% resistance but amplified resistance gene against the same antibiotics. In the present study *M. sciuri* (gram positive, CoNS) found as the major pathogen causing clinical mastitis and the isolate had amplified tetracycline resistance genes. Our finding is in close approximation to the earlier findings.

Phylogenetic analysis was done on our recovered isolates with the 24 similar isolates from GenBank. Our sequences have a high level of similarity to those obtained from GenBank, ranging from 74.00 to 100%.

Conclusion of the study

- *Mammalicoccus sciuri*, is a coagulase negative staphylococci (CoNS) isolated on culture and 16S rDNA sequencing in 40% milk samples from cows affected with clinical mastitis in Malkangiri district of Odisha. Other pathogens include *Enterococcus faecium, Bacillus sporothermodurans, Bacillus toyonensis*.
- *In vitro* antibiotic sensitivity test for the opportunistic pathogen *M. sciuri* isolates showed higher resistance to penicillin (85.7%) followed by, streptomycin (28%), cefoperazone and tetracycline (14.2%). Doxycycline, amoxicillin, gentamicin and amikacin were 100% sensitive.
- Molecular detection of resistance genes through 16S rRNA sequencing using specific primers, *M. sciuri* amplified one resistant genes of tetracycline i.e. *tetK*. *Enterococcus faecim* amplified two resistance genes i.e. *tetK and tetM.*
- It is the antimicrobial resistance study in mastitis, first ever in backyard region of Odisha, provide baseline data for the global threat.

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