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Genotypic and Phenotypic Characterization of *Mammaliicoccus sciuri* - A MDR Strain causing Clinical Mastitis in Cows of Odisha, India

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ABSTRACT

Background: *Mammaliicoccus sciuri* is a multidrug resistant human pathogen. Literature on its role causing bovine mastitis is scarce. Detection of *M. sciuri* from a cow with clinical mastitis in Malkangiri district of Odisha stimulated to ascertain its status in a larger population and to study its characteristics in genotypic and phenotypic levels.

Methods: 520 lactating cows from various herds of Malkangiri district of Odisha, India were screened for presence of clinical mastitis. Milk samples were collected aseptically from 15 cows with clinical mastitis.16S rRNA amplification was performed to know presence of bacterial pathogen(s). Conventional PCR was carried out to detect resistance genes against β-lactam (*blaZ*), aminoglycoside (*aacA-aphD*) and tetracycline (*tetK, tetM*). *In vitro* sensitivity test was studied against *M. sciuri* isolates using nine antibiotic discs. Phylogenetic analysis was performed to compare its origin.

Result: *M. sciuri* was isolated in 6(40.0%) mastitis positive milk samples. *In vitro* antibiotic sensitivity test against *M. sciuri* isolates showed highest degree of resistant to penicillin (100%) followed by, cefoperazone (33.3%), streptomycin (33.3%), tetracycline (16.0%). One isolate of *M. sciuri* had *tetK, tetM* resistance gene. Phylogenetic analysis showed (75-100%) similarity with other isolates obtained from GeneBank. Present study highlights *tetK* gene in *M. sciuri.*

Key words: *aacA-aphD, blaZ*, Cows, *Mammaliicoccus sciuri*, *tetK*, *tetM.*

INTRODUCTION

Mastitis is an economically important and common infectious disease of high yielding cows. Having its world-wide distribution, the disease is exhibited in two forms *viz*., clinical or subclinical. Of more than 200 pathogens causing bovine mastitis, coagulase-positive staphylococci, *Staphylococcus aureus,* constitute as one of the major etiological agents. Coagulase negative staphylococci (CoNS), once considered commensals, are now considered as a prevalent pathogen for bovine mastitis in many countries, (Rajala-Schultz *et al*. 2009; Pyorala and Taponen, 2009; Piessens *et al*. 2011; De Vliegher *et al*. 2012; Khazandi *et al*. 2018; Frey *et al*. 2013; Dabele *et al*. 2021) and isolated as an emerging multidrug resistant pathogen (Bogni *et al*. 2011; Park *et al*. 2012; Dabele *et al*. 2021).

About 30 species of staphylococci have been recognized on the basis of biochemical and molecular analysis. *Staphylococcus sciuri,* now classified as *Mammaliicoccus sciuri,* is a gram positive clustered CoNS in the novel *Mammaliicoccus* genus of Staphylococcoceae family (Madhaiyan *et al*. 2020). It is ubiquitous in nature and isolated from soil, hospital environment, tannery effluent, pus sample, human ear, gut of birds and mosquitoes. They are also found in a variety of domestic and wild animals (Kloos *et al*. 1997; Devriese *et al*. 1990). It may pose serious public health issue with risk of transmission to humans from animals (Juhász-Kaszanyitzky *et al*. 2007). In humans, the pathogen causes peritonitis, endocarditis, urinary tract infection, septic shock, pelvic inflammatory diseases and secondary soft tissue infections (Adegoke *et al*. 1986; Kolawale *et al*. 1997; Hedin *et al*. 1998; Wallet *et al*. 2000). A study was planned to unveil the status of *M. sciuri* causing bovine mastitis in Malkangiri district of Odisha and to determine antimicrobial resistance profiles in genotypic and phenotypic levels.

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MATERIALS AND METHODS

Sample collection and isolation of bacteria

The study was conducted during the period that stretched from March 2021 to October 2021. A total of 520 lactating cross-bred Jersey cows were screened from private dairy farms located in Malkangiri district of Odisha. 2.0 ml milk samples pooled from all four quarters of each cow were collected aseptically into sterile vials and transported to laboratory in ice box. At the time of collection the cows were exhibiting one or more clinical signs of acute mastitis such

as inflamed udder, engorged teats, discoloration of milk with watery milk discharge with presence of clots and flakes. Samples were initially inoculated into nutrient broth followed by mannitol salt agar and incubated overnight at 37°C (Yang *et al*. 2016). Pure colonies were subjected to haemolysis test on blood agar to identify *Staphylococcus* sp.

Molecular identification of bacteria

Genomic DNA of the bacteria colony was extracted as per the standard protocol provided in the Nucleopore gDNA fungal/bacterial mini kit of Qiagen make. The extracted DNA was used to amplify 16S rRNA gene using universal primers and the product was Sanger sequenced. The16S rRNA gene sequence was used for identification of bacteria and submmited to GenBank database.

Nucleotide sequences and accession numbers

Six sequences of PCR samples were submitted to GenBank database for accession numbers.

***In vitro* antibiotic sensitivity test of pure isolates**

In vitro antibiotic sensitivity test was performed on Mueller Hinton Agar using commercially available antibiotic discs (N=9) following standard protocol. Three groups of antibiotics such as β-lactam (Penicillin, Cefoperazone, Ampicillin, Amoxycillin), Aminoglycoside (Gentamicin, Neomycin, Streptomycin, Amikacin) and Tetracycline were included in the trial.

Results were recorded as resistant and sensitive. Intermediate sensitive isolates were considered as resistant. Inhibition zone diameter was measured in accordance with reference standard of clinical and laboratory standards institute.

Phylogenetic analysis

Phylogenetic tree was constructed by gathering all our *M. sciuri* isolates (STP 1-6) with the 24 similar reference gene sequences, using MEGA 11 software by neighbor joining method Clustal W v 1.6 was used for multiple sequence analysis. Bootstrap analysis of neighbor-joining data sets based on 1000 replications was used to evaluate the resulting tree topology.

Amplification of antibiotic resistance genes

Readymade PCR master mix was procured from Sigma Aldrich, India. Extracted genomic DNA utilized for the antibiotic resistance gene detection by conventional PCR using specific primers (Table 1) (Yang *et al*. 2016). The mixture's volume was adjusted to 25 µl. 14 µl mastermix, 0.5 µl each reverse and forward primers, 2 µl template DNA, 5 µl NFW constitutes 25 µl. Gradient PCR was allowed to determine an optimal annealing temperature. Total of 30 cycles were run at the following temperature set up: initial denaturation at 94°C for 5 minutes and denaturation at 94°C for 30 seconds, ten different annealing temperatures ranges. Best conditions were found with temperatures 52°C and 55°C and were analyzed by electrophoresis on 1.2% agarose.

RESULTS AND DISCUSSION

Examination of the 520 pooled milk samples from cows' in the Malkangiri district, we detected clinical mastitis in 15 (2.8%) heads. Microbiological and molecular study of the isolates unveiled *M. sciuri* from 6(40%) of clinical mastitic cows (Fig 1). *M. sciuri,* earlier known as *S. sciuri,* is a gram positive clustered CoNS having its pathogenesis both in animal and man. The remaining 9 (60%) samples in our study were *Enterococcus faecium* (3), *Bacillus sporother modurans* (2) and *Bacillus toyonensis* (4). Gram positive spore forming bacteria of *Bacillus* species *viz*., *B. sporother modurans* and *B. toyonensis* were detected either single or mixed with *M. sciuri*. Dabele *et al.* (2021) reported 1.6% prevalence of *M. sciuri* in mastitic cows in Ethiopia. *S. intermedius* and *S. xylosus* were reported as predominant CoNS and isolated to a tune of 40.0% and 44.4% by El Razik *et al.* (2017) and El Ashker *et al* (2015). Current study emphasized the importance of CoNS, *M. sciuri* as a major pathogen causing mastitis in cows of Malkangiri district.

In vitro antimicrobial sensitivity test of six *M. sciuri* isolates against tetracycline showed sensitivity in 16% cases (Table 3). The probable hypothesis of our results could be due to massive and persistent use of tetracycline in intra-mammary preparations for curative treatment of mastitis and/or its parenteral use against other systemic infectious diseases.

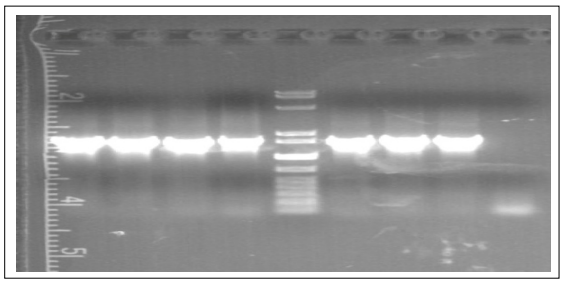

Fig 1: Idetification of bacteria using 16S rRNA sequencing.

Table 1: Oligonucleotide sequences for the corresponding antibiotic resistance genes.

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

One isolate of *M. sciuri* amplified both 360-bp fragment of primer specific for *tetK* gene (Fig 2) and and 158-bp fragment of *tetM* gene (Table 2). This finding corroborated with Osman *et al*. (2015), El Razik *et al*. (2017) and Dabele *et al*. (2021) where *M. sciuri* isolates were found resistant to tetracycline.

This key discrepancy in tetracycline (P^-G^+) might be due to two reasons: (1) presence of multiple resistance genes having similar characteristic responsible for the development of resistance against a particular antibiotic as reported by Davis *et al*. (2011) and (2) expression of resistance of a gene that relates to the stress it receives. In the present *in vitro* study, tetracycline concentration was 30 mcg. Resistance genes would have been expressed with higher concentration of antibiotic. Davis *et al*. (2011) 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 pseudo genes or false genes. It 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).

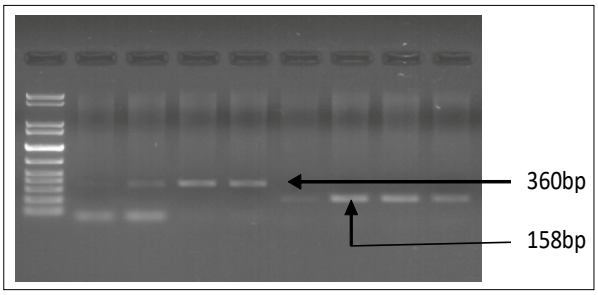

Fig 2: Amplification of 360-bp fragment of *tetK* gene and 158-bp fragment of *tetM* gene.

All 6 (85.7%) CoNS, *M. sciuri* isolates revealed resistance to penicillin but there was no amplification *blaZ* resistance gene. This might be due to the presence of another resistance gene against β-lactam antibiotic *i.e*., *mecA, fem* and *femB* which might have conferred resistance characteristic. This is in contrast to most of the study on *Staphylococcus aureus* conducted by Chandrasekaran *et al.* (2014); Yang *et al*. (2016) and Girmay *et al*. (2020) and where they have recorded both genotypic and phenotypic resistance against penicillin. In our *in vitro* study, aminoglycoside was sensitive with absence of resistance genes. Yang *et al*. (2016) and Gow *et al*. (2008) recorded similar type of sensitivity. Antimicrobial resistance determinants of *M. sciuri* have not been intensively studied so far. Genotypes can provide information on a pathogen's current drug sensitivity as well as its future potential for resistance and dissemination.

Six sequences of PCR samples (STP 1-6) of *M. sciuri* isolates were deposited in GenBank database with accession numbers: OK412723, OK614118, OK614108, OK614113, OK614117, OK614103, respectively.

Phylogenetic relationship between our recovered *M. sciuri* isolates showed 75-100% similarity with the *Staphylococcus* spp isolates obtained from GenBank (Fig 3). This implies there is probable transfer of tetracycline resistance gene within *M. sciuri* isolates and *Staphylococcus* spp. This supported by the findings of El Razik *et al*. (2017)

Table 2: Amplification of resistance genes by conventional PCR.

Sample name	Name 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	N
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	P

NB: *blaZ*-penicillin, *aacA-aph D*-aminoglycoside, *tetK* and *tetM*-tetracycline.

Table 3: *In vitro* antibiotic sensitivity test.

Name of antibiotics	Name of samples							
	MK 6	MK7	MK9	MK11	MK12 (1)	MK12 (2)	R%	S%
Penicillin	R	R	R	R	R	R	100	0
Cefoperazone	S	S	S	R	R	S	33.3	66.6
Ampicillin	S	S	S	S	S	S	0	100
Amoxicillin	S	S	S	S	S	S	0	100
Gentamicin	S	S	S	S	S	S	0	100
Neomycin	S	S	S	R	S	S	16	84
Streptomycin	S	S	S	R	R	S	33.3	66.6
Amikacin	S	S	S	S	S	S	0	100
Tetracycline	S	S	S	R	S	S	16	84

R: Resistance, S: Sensitivity.

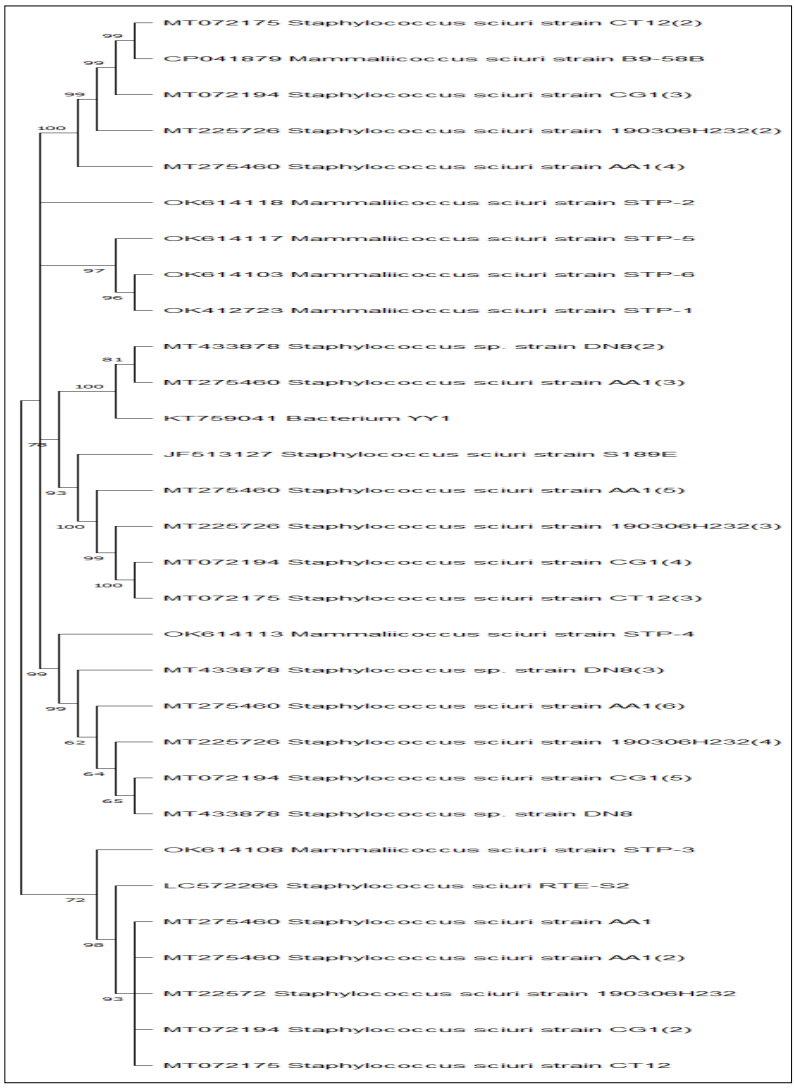

Fig 3: Phylogenetic relationship of *M. sciuri* isolates with the isolates obtained from Gen bank.

who proves the chance of transfer of tetracycline resistance gene between CoNS isolates and *S. aureus* isolates due to minor variation between their *tetK* nucleotide sequences. Current study provides baseline information about a zoonotic pathogen *M. sciuri* causing bovine mastitis in Malkangiri district that in turn would encourage collaborative, multi-sectoral one-health approach to address AMR problem in the region.

CONCLUSION

Mammaliicoccus sciuri, a coagulase negative *staphylococcous* sp., was isolated in 40% (6/15) milk samples from cows affected with clinical mastitis in Malkangiri district of Odisha. *In vitro* antibiotic sensitivity test for the *M. sciuri* isolates showed highest degree of resistance to penicillin (100%) followed by, streptomycin (33.3%), cefoperazone (33.3%) and tetracycline (16%). All six isolates were sensitive to amoxicillin, gentamicin and amikacin. *M. sciuri* isolates amplified both resistant genes of tetracycline *i.e. tetM* and *tetK*. The finding elevated *M. sciuri* to the status of an emerging major multidrug resistant pathogen causing bovine clinical mastitis.

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Conflict of interest: None.

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