The Pharma Innovation Journal 2023; SP-12(8): 444-447

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(8): 444-447

www.thepharmajournal.com
Received: 22-05-2023
Accepted: 26-06-2023

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Investigation of tetracycline resistance in staphylococci isolated from bovine mastitis

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Abstract
The present study was conducted to evaluate the antimicrobial resistance profile of staphylococci isolated from bovine mastitis. In the current study, out of the 83 milk samples obtained from cows with clinical mastitis, 22 *S. aureus* and 27 coagulase negative staphylococci (CNS) were isolated based on the morphological, cultural, biochemical and molecular characteristics. On antibiogram by Kirby Bauer disc diffusion assay, 12 out of the 22 *S. aureus* (54.55 percent) and seven out of the 27 CNS (25.93 percent) were resistant to tetracycline. Molecular characterisation of tetracycline resistance by polymerase chain reaction (PCR) targeting *tetM* gene revealed positive amplicons in four isolates each of *S. aureus* (18.18 percent) and CNS (14.81 percent) respectively. This must be considered as an alarming situation because of the anticipated food safety and public health risks related to the transmission of milk-borne zoonotic diseases, antibiotic residues and organisms harbouring numerous toxins, resistance and virulence factors.

Keywords: Antimicrobial resistance, staphylococci, mastitis, tetracycline

1. Introduction
Mastitis is still the most prevalent and costly disease of dairy cattle, worldwide. It has a detrimental economic impact on farmers either directly or indirectly and ultimately endangers the national economy. Furthermore, due to the shedding of bacteria and their toxins in milk, milk from such animals poses a significant zoonotic threat, particularly if consumed raw or improperly pasteurised by vulnerable groups such as children, pregnant women, the elderly, or people suffering from immunosuppressive disorders. In spite of the multitude of aetiological agents, bacteria belonging to the genus staphylococcus are the most common pathogens causing mastitis in various dairy regions across the world. Although CNS were previously thought to be minor mastitis pathogens, they are now considered as emerging pathogens in bovine mastitis.

Mastitis has traditionally been controlled with chemical disinfectants, herbal teat dips or antiseptics and antibiotics (Maiti *et al*., 2004) [1]. For almost 50 years, antibiotics have been considered as the holy grail of mastitis therapy (Sharma *et al.*, 2007) [2]. However, antibiotic therapy was shown to be ineffective in lowering the incidence of mastitis. Indeed, various issues have arisen as a result of antibiotic misuse, which includes uncertain therapeutic effectiveness, emergence of resistance and the presence of antibiotic residues in milk. Antibiotic resistant udder pathogens are widespread, with resistance patterns varying by area. Antibiotic sensitivity testing will assist the veterinarian in identifying the sensitivity pattern of the organism and in selecting the suitable antibiotic for optimal mastitis therapy. This could also be beneficial in the long run since this could provide the veterinarian the best antibacterial choice.

Tetracyclines are one among the major broad-spectrum antibacterial agents that have been approved for use in bovine mastitis. They decrease bacterial protein synthesis by inhibiting the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Because of their favourable antibacterial capabilities and lack of significant side effects, these medicines are widely used in the treatment of both human and animal infections. Furthermore, in certain countries, tetracyclines are incorporated at subtherapeutic levels in animal feed to enhance growth and as a prophylaxis against many diseases. Although tetracyclines continue to play essential roles in human and veterinary medicine, the evolution of microbial resistance has restricted their efficacy and has contributed to the selection of resistant organisms (Chopra And Roberts, 2001) [3].

Under these circumstances, the present study was envisaged for the phenotypic and genotypic analysis of tetracycline resistance in *S. aureus* and other CNS from bovine mastitis.

2. Materials and Methods

2.1 Isolation and identification of bacteria

Midstream lacteal secretions of 83 lactating cows affected with clinical mastitis, in and around Thrissur district were collected aseptically in separate sterile screw capped plastic vials following the standard protocol.

All the milk samples were streaked on to brain heart infusion agar (M211, Himedia, India) for isolation of bacteria. The morphology and staining characters of the organism were studied by Gram's staining technique. Based on the cultural characteristics produced on different media, the organisms were classified and subjected to further biochemical tests as per Barrow and Feltham (1993) [4] and Quinn *et al*. (2013) [5].

2.2 Extraction of DNA

The DNA was extracted from the presumptively identified staphylococcus isolates using snap chill method (heat lysis method) as described by Vijayakumar and Jose, 2021 [6]. The concentration and purity of the DNA samples were estimated using a Nanodrop Spectrophotometer (Nanodrop™ 1000 Spectrophotometer). The DNA samples with 260/280 ratio greater than 1.8 were selected and used as template for the polymerase chain reaction (PCR).

2.3 Genotypic characterisation of *Staphylococcus spp.* and tetracycline resistance

The genotypic characterisation of *S. aureus* and CNS were done by PCR targeting the *23S rRNA* (Vijayakumar and Jose, 2021) [6] and *cns* gene (Okolie *et al*., 2015) [7] respectively. PCR products were analysed on 1.2 percent agarose gel stained with ethidium bromide, visualised under the ultraviolet transilluminator ((Genei™, Bengaluru) and photographed using GelDoc apparatus (Doc™ Gel EZ imager, BIO-RAD, USA).

The precise identification of resistance towards tetracycline was also attempted by PCR targeting the *tetM* gene. The PCR was performed in a total volume of 25 µl reaction mixture by combining the reagents as shown in table 1 using the programmable S1000 Thermal cycler, (BioRad, USA).

Table 1: Components of PCR reaction mix

Components	Volume (µl)
Master Mix (2X PCR Smart mix, Takara, Japan)	12.5
Forward Prime (100nM/ml, Sigma Aldrich)	1
Reverse Primer (100nM/ml, Sigma Aldrich)	1
Nuclease Free Water	5.5
Template DNA	5
Total	25

The details of the primers used and the PCR protocol are shown in table 2.

Table 2: Primer used for genotypic characterisation of *S. aureus*, CNS and *tetM* gene

Gene	Primer	Amplicon Size	Annealing temperature and time	Reference
23SrRNA	F: GGA CGA CAT TAG ACG AAT CA R: CGG GCA CCT ATT TTC TAT CT	1381 bp	55.8 ºC, 45 s	(Vijayakumar and Jose, 2021) [6]
cns	F: TAT CCA CGA AAC TTC TAA AAC AAC TGT TAC T R: TCT TTA GAT AAT ACG TAT ACT TCA GCT TTG AAT TT	204 bp	56.3 ºC, 1 min	Okolie *et al*. (2015) [7]
tetM	F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC	403 bp	61.5 ºC, 1 min	Archana, 2018 [8]

2.4 Phenotypic characterisation of tetracycline resistance in *Staphylococcus* spp.

All the *Staphylococcus* spp. were subjected to *in vitro* antibiotic sensitivity testing by Kirby Bauer Disc diffusion assay (Bauer *et al.,* 1966) [9]. Antibiotic discs with known concentration in microgram (mcg) or international unit (IU) per disc were used in the study. Pure culture was used as inoculum. A cell suspension of organism equivalent to 1.5 x 10^8 CFU/ml or 0.5 McFarland standard was used. For this, fresh broth cultures were spread over sterile Mueller Hinton Agar plates and the antibiotic discs were impregnated at about 2.4 mm apart. The plates were then incubated for 12 to 24 hours and zones of inhibition were measured and interpreted as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018) [10].

2.5 Statistical analysis

Statistical analysis of the phenotypic and genotypic tetracycline resistance of the *S. aureus* and CNS was performed by McNemar's test using IBM-SPSS software 24.0.

3. Results and Discussion

Out of 83 clinical mastitis (CM) samples examined by the standard bacteriological tests, 26 samples (31.33 percent) did not yield any growth. Remaining 57 samples yielded bacterial isolates, of which Gram positive cocci were the most numerous (88.06 percent). This was consistent with the studies of Kulangara *et al*. (2017) [11], who found that the most common pathogen causing chronic infections in dry bovine udders were Gram positive cocci (70.4 percent).

The Gram positive cocci which gave positive catalase and negative oxidase test were presumptively identified as *Staphylococci* spp. The staphylococcus organisms isolated were further characterised as *S. aureus* and CNS based on coagulase test and Voges Proskauer test. Coagulase negative staphylococci (32.53 percent) was the most frequently isolated bacteria followed by *S. aureus (26.5 percent)* based on the morphological, cultural and biochemical characterisation as well as by monoplex polymerase chain reaction. Similar findings were also reported by Hosseinzadeh and Saei (2014) [12], who identified 113 (71.5 percent) staphylococcus isolates from a total of 158 mastitic milk samples on the basis of biochemical and cultural characteristics as well as by genus specific PCR. Saidi *et al.* (2013) [13] and Udegbunam *et al*. (2014) [14] found *Staphylococcus* spp. (80 percent) as the major causative agent of mastitis in cattle.

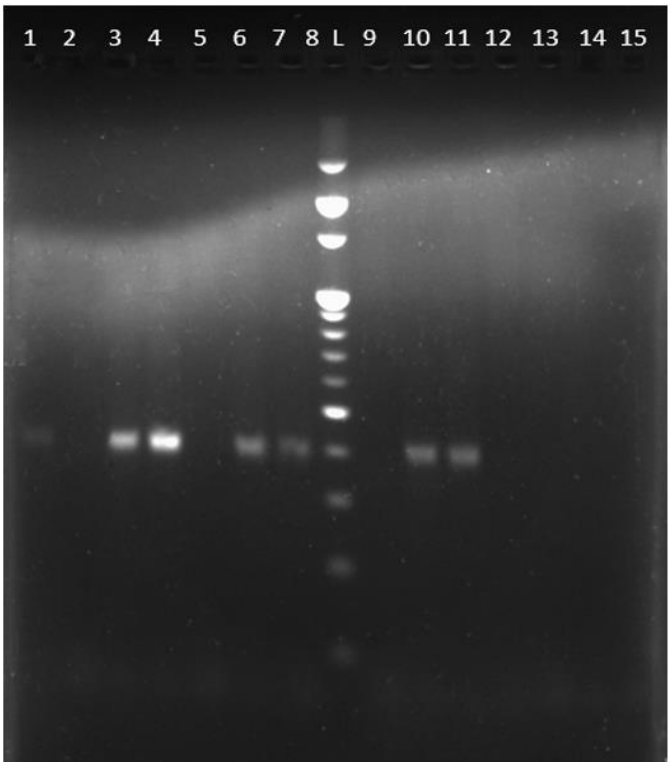
Bjork *et al*. (2014) [15] documented that CNS were found to be the common pathogens from subclinical mastitis among the

dairy cattle in Uganda. In a study by Kaliwal *et al*. (2011) [16], out of 310 milk samples screened for bovine mastitis, 180 (58 percent) isolates were confirmed as CNS. Similar observations were documented in our study where majority of the isolates were CNS. However, Barkema *et al*. (2009) [17] and Li *et al*. (2009) [18] documented *S. aureus* as the predominant agent isolated from bovine mastitis. Contrary to this, we could isolate only 22 *S. aureus* out of the total 49 staphylococcus isolates.

In this study, the 22 *S. aureus* and 27 CNS isolates obtained were subjected to antibiogram employing amoxicillin-sulbactam, ceftriaxone, ceftriaxone sulbactam, cotrimoxazole, enrofloxacin, gentamicin, methicillin, penicillin G and tetracycline that are frequently being used in mastitis. It was concluded that majority of the staphylococci were multidrug resistant. The results revealed that 12 out of the 22 *S. aureus* (54.55 percent) and seven out of the 27 CNS (25.93 percent) were resistant to tetracycline. With other antibiotics, the isolates showed varying degrees of susceptibility.

Tetracyclines are broad-spectrum, bacteriostatic antibacterials that acts at ribosomal level by inhibiting protein synthesis. Bacterial resistance to tetracycline is mediated either by drug efflux which restricts the access of tetracycline to ribosome or by ribosomal protection proteins that binds to ribosome leading to a conformational change in drug binding site that prevents effective drug binding (Donhofer *et al*., 2012) [19]. The genetic determinants of tetracycline resistance were allocated to four groups, namely, K, L, M and O. The *tetM* or *tetO* determinants are chromosome or transposon located and protects ribosomes from the inhibitory effect while *tetK* or *tetL* determinants are plasmid-borne and they specify membrane associated drug efflux systems (Warsa *et al*., 1996) [20]. Both the *tetM* and *tetK* determinants are inducible *in vitro* in S. *aureus*. The *tetM* gene imparts resistance to all drugs that are available in the group, including minocycline, a second generation tetracycline, while *tetK* mediates inducible resistance only to tetracycline but not to minocycline (Trzcinski *et al*., 2000) [21].

The previous study by Kulangara *et al*. (2017) [11] on AMR patterns of staphylococcal isolates from dairy cows in the same locality could not confirm the genotypic resistance against tetracycline in any of the isolates, although they documented 85 percent and 76.3 percent phenotypic resistance among the *S. aureus* and CNS isolates, respectively. This might be because their study targeted on the plasmid encoded, *tetK* gene only. Moreover, Schmitz *et al*. (2001) [22] reported a higher prevalence of *tetM* among MRSA isolates. Consequently, the present study was conducted to determine *tetM* mediated tetracycline resistance that has not been previously studied. The present study demonstrated *tetM* mediated genotypic resistance to tetracycline in four among the 22 *S. aureus* isolates (18.18 percent) and 27 CNS isolates (14. 18 percent) respectively (Figure 1). This was relatively higher than the findings from Northwest China (Feng *et al*., 2016) [23] that recorded a lower occurrence of the *tetM* (2.27 percent) mediated tetracycline resistance as compared to the *tetK* (22.73 percent). However, contradictory findings were reported by Duran *et al*. (2012) [24] from human samples of a teaching hospital in Turkey, where 42.4 percent of the *S. aureus* and 39 percent of the CNS were found to carry the *tetM* or *tetK* genes. Jamali *et al*. (2014) [25] also reported a higher prevalence (39.5 percent) of *tetM* gene among *S. aureus* from bovine mastitis in Malaysia.



Lane L : DNA marker 100 bp
Lane 8 : Positive control (403 bp)
Lane 9 : Negative control
Lane 3, 4, 6, 7, 10, 11 : Positive samples
Lane 1, 2, 5, 12- 15 : Negative samples

Fig 1: Agarose gel electrophoresis of *tetM* specific PCR

In *S. aureus* isolates, the p value is less than 0.05 as indicated by the value with superscript b (table 3). Thus, there is significant difference between phenotypic and genotypic tetracycline resistance in *S. aureus*. However, no statistically significant difference could be noted in CNS. One of the probable reasons behind the disparities among different studies might be the inaccurate identification of resistance mechanisms owing to the involvement of a number of plasmid and chromosome encoded genes. Resistance encoded by different genes must therefore be investigated in order to provide useful information for the epidemiological studies to trace strains harbouring tetracycline resistant determinants in the future (Warsa *et al*., 1996) [20].

Table 3: Comparison of phenotypic and genotypic tetracycline resistance in *S. aureus* and CNS

McNemars test	Tetracycline & *tetM*	
	S. aureus	**CNS**
N	22	27
Exact Sig. (2-tailed)	.008[b]	.508[b]
Exact Sig. (1-tailed)	0.004	0.254
Point Probability	0.004	0.164
5% level of significance Rows with superscript b indicates p value; p value < 0.05 – significant difference		

4. Summary

It was concluded that *Staphylococcus* spp. is most common cause of clinical mastitis among the dairy cows of the study population. They are emerging as potent multidrug resistant bacteria with majority of the isolates being resistant to tetracyclines. Most of them are harbouring the *tetM* gene coding for tetracycline resistance. The higher prevalence of

tetracycline resistance among *Staphylococcus* spp. suggests that the therapeutic and prophylactic use of tetracyclines should be carried out with great caution due to the possible transmissibility of specific resistant clones or the AMR determinants to human pathogens or to healthy animals which leads to a decrease in efficacy of the drug. This is of paramount importance as a vast majority of drugs used in veterinary practice are listed by the World Health Organisation as critically important drugs in human medicine

5. Acknowledgement
The authors were thankful to the Kerala Veterinary and Animal Sciences University (KVASU) and the Department of Veterinary Epidemiology and Preventive medicine, College of Veterinary and Animal Sciences, Mannuthy for providing the facilities needed for carrying out the research work.

6. Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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