Indian Journal of Animal Sciences **94** (4): 308–314, April 2024/Article
https://doi.org/10.56093/ijans.v94i4.129868

Antimicrobial resistance profiling of coagulase negative staphylococci isolated from bovine mastitis

KRUPA ROSE JOSE[1]✉ and K VIJAYAKUMAR[1]

Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala 680 651 India

Received: 4 November 2022; Accepted: 26 February 2024

ABSTRACT

Mastitis plays a crucial role in the economics of dairy industry by deteriorating the quality and quantity of milk produced, as well as endangering the animal health and welfare. The objective of this study was to investigate the antimicrobial resistance (AMR) profile of coagulase negative staphylococci (CNS) isolated from bovine mastitis. Microbiological evaluation by morphological, cultural and biochemical characterisation as well as by monoplex polymerase chain reaction (PCR) of the 83 quarter milk samples revealed that CNS were the most predominant bacteria (32.53%). It was also concluded that most of the CNS were resistant to multiple antibiotics. The phenotypic and genotypic AMR profiling was done using *in vitro* disc diffusion assay and PCR, respectively, to identify the resistance pattern towards penicillin, methicillin, tetracycline and enrofloxacin. The results depicted a significant difference between the phenotypic and genotypic resistance of CNS against penicillin, methicillin and tetracycline. This outcome on the interaction of phenotypic and genotypic AMR profiling is intriguing and opens a huge scope for future studies on the transcriptomic and proteomic aspects of drug resistance. A better knowledge of the AMR profile guides the dairy producers in developing suitable timely intervention strategies for the economic management of mastitis, which in turn helps in tackling AMR and reduces the threat of its zoonotic transmission.

Keywords: Antibiogram, Coagulase negative Staphylococci, Genotypic resistance, Mastitis, Phenotypic resistance

Bovine mastitis is a disease with multiple aetiology, that causes significant financial loss for farmers and the dairy industry worldwide (Morales-Ubaldo *et al.* 2023). Bacterial agents of the genus *Staphylococcus* are the most common pathogens causing mastitis in various dairy industries across the world. The coagulase negative staphylococci (CNS) were previously thought to be minor mastitis pathogens. Although not as pathogenic as other mastitis pathogens, CNS mostly remains subclinical and cause persistent infections and were currently considered as an emerging mastitis pathogen (Soares *et al.* 2012).

The most challenging task involved in mastitis is the treatment and recovery of animal. Prompt and timely therapy with an effective and responsive antimicrobial agent is an absolute necessity in the management of mastitis. However, antibiotic resistance, or the ability of microbes to resist the action of antibiotics is a major barrier to effective mastitis therapy across the world. Antibiotic-resistant udder infections can be found throughout the world, with varying resistance patterns depending on the region. With a wide range of antimicrobials available to treat mastitis and the rising incidence of therapy refractory infections, the necessity for antimicrobial resistance profiling of the bacterial isolates has become obvious. Moreover, it has a tremendous significance from public health point of view, due to the possibility of lateral transmission of resistant genes between different staphylococcal species or the direct transmission of resistant bacteria between man and animals (Walther and Perreten 2007, Reygaert 2018, Larsson and Flach 2022). Hence, antimicrobial resistance profiling might be useful for a variety of goals, including selection of effective therapeutic protocols, ensuring safe treatment with minimal toxicity, preventing the emergence of resistance and reducing the treatment cost.

The majority of investigations on mastitis have been undertaken in *Staphylococcus aureus*, but latest reports reveal that CNS are also responsible for both clinical and subclinical mastitis with a similar frequency. Despite this, research on bovine clinical mastitis caused by CNS is limited. In this context, the current work was carried out to assess the antimicrobial resistance profiles of CNS isolated from bovine milk to clinically relevant antimicrobials.

MATERIALS AND METHODS

Ethics statement: As per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), which regulates animal experimentation in India, it is not mandatory to obtain permission from or to notify the Institutional

Present address: [1]Kerala Veterinary and Animal Sciences University, College of Veterinary and Animal Sciences, Mannuthy, Kerala. ✉Corresponding author email: krupaputhuparampil@gmail.com

Animal Ethics Committee for the collection of mastitic milk samples from animals. However, oral consent was secured from the farmers who owned the animals, and the necessary results were delivered to the farmers as required.

Study population and sample collection: The present study was carried out in the Centre for Mastitis Control laboratory, Department of Veterinary Epidemiology and Preventive Medicine during the period from March 2019 to July 2020. Under aseptic precautions, milk samples were obtained from 83 domesticated dairy cows from an organised dairy farm as well as cows presented with clinical mastitis at the University Veterinary Hospital (UVH), Mannuthy. Prior to sample collection, the teat was thoroughly disinfected and the initial squirt of milk was discarded. The samples were transported on ice to the laboratory.

Isolation and identification of bacteria: The milk samples were inoculated on brain heart infusion agar (BHI; HiMedia Laboratories, Mumbai) for primary isolation of bacteria. For this, 1 ml of milk was inoculated into 5 ml of brain heart infusion broth and incubated for 6 h at 37°C. The enriched samples were then streaked onto brain heart infusion agar, incubated for 24 h, and the colony morphology was examined.

The CNS isolates were identified based on their colony morphology, biochemical characteristics and molecular identification techniques like PCR (polymerase chain reaction). Besides Gram's staining, all the Gram-positive cocci were streaked onto mannitol salt agar (MSA; HiMedia Laboratories, Mumbai) and the colony characteristics of each isolate was analysed. Biochemical characterisation of the *Staphylococcus* spp. was done using the catalase, coagulase, oxidase, Voges-Proskauer (VP) and nitrate reduction tests (Barrow and Feltham 1993, Quinn *et al.* 2013).

Extraction of DNA: The DNA was extracted from 27 phenotypically identified CNS using heat lysis/snap chill method (Vijayakumar and Jose 2021) and determined the yield and purity of the DNA isolates by Nanodrop Spectrophotometer™ 1000. DNA samples with a concentration ranging from 60-400 ng/µL at OD of 260 nm, absorbance ratios A260/280 and A260/230 in the range of 1.8-2.0 were considered to be of good quality and pure with least protein and reagent contamination and were selected for further study.

Genotypic characterisation of CNS and its antimicrobial resistance: All the isolates of CNS obtained in the study were subjected to genotypic characterisation by amplification of *cns* gene using PCR. Further, the template DNA from all the 27 genotypically confirmed CNS isolates were subjected to molecular characterisation of antimicrobial resistance genes such as *blaZ*, *mecA*, *tetM* and *gyrA* for determining the resistance towards β-lactamase, methicillin, tetracycline and fluroquinolone respectively. Confirmed isolates from our collection were used as reference strains for *mecA* (Accession No. MW195499), *tetM* (Accession No. MW258979) and *gyrA* (Accession No. MW364643). The primers and the annealing conditions are shown in Table 1.

The PCR products were analysed on 1.2% agarose gel stained with ethidium bromide, visualised at 300 nm with ultraviolet transilluminator (Genei™, Bengaluru) and documented using GelDoc apparatus (Doc™ Gel EZ imager, BIO-RAD, USA).

Phenotypic antibiotic susceptibility testing: Phenotypic antibiotic susceptibility test was done for all the 27 CNS isolates against the following antibiotics: amoxicillin sulbactam, ceftriaxone, ceftriaxone sulbactam, cotrimoxazole, enrofloxacin, gentamicin, methicillin, penicillin and tetracycline (HiMedia Laboratories, Mumbai) by modified Kirby-Bauer disc diffusion technique (Bauer *et al.* 1966) based on CLSI regulations (CLSI 2017).

Statistical analysis: Data were entered into a Microsoft Excel spread sheet, verified for correctness, imported into IBM-SPSS software 24.0 and was used for the statistical analysis. The association between the phenotypic and genotypic resistance for the four antibiotics under study were statistically analysed using McNemar's test.

RESULTS AND DISCUSSION

Isolation and identification of CNS: Primary criteria for identification of CNS were colony morphology of the isolates followed by microscopic examination using Gram's staining. Following overnight incubation of a total of 83 milk samples onto brain heart infusion agar, 57 samples (68.67%) yielded bacterial isolates. However,

Table 1. Primers and annealing conditions of CNS and its AMR genes

Organism and target gene	Primer sequence (5'-3')	Product size	Annealing conditions	Reference
Coagulase negative Staphylococci - *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°C; 1 min	Okolie *et al.* (2015)
Penicillin - *blaZ*	F: ACTTCAACACCTGCTGCT TTC R: TGACCACTTTTATCAGCAACC	173 bp	55 °C; 30 s	Martineau *et al.* (2000)
Methicillin - *mecA*	F: TGGCTATCGTGTCACAATC R: CTGGAACTTGTTGAGCAGAG	303 bp	60°C; 1 min	Archana (2018)
Tetracycline - *tetM*	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	403 bp	61.5°C; 1 min	Archana (2018)
Fluroquinolone - *gyrA*	F: GGC GGA TCC CAT ATG GCT GAA TTA CCT CA R: GGC GGA ATT CGA CGG CTC TCT TTC ATT AC	473 bp	69.8°C; 2 min	Ferrero *et al.* 1994

no growth could be detected in 31.33% samples. Out of the 57 samples which yielded bacterial growth, five were mixed cultures of two different organisms. Hence, a total of 67 bacterial isolates were obtained in pure culture.

Gram's staining revealed that 59 out of the total 67 isolates (88.06%) were Gram positive cocci and eight (11.94%) of them were Gram negative rods. Among the 59 Gram positive cocci obtained, 49 resembled bunches of grapes, eight were arranged in tetrads and the remaining two were arranged in chains or pairs. The 59 Gram positive cocci were further streaked on to mannitol salt agar (MSA; Himedia, India) for identification of *Staphylococcus* spp. Among the total 59 isolates, 27 produced pink coloured colonies with no colour change to mannitol salt agar, suggestive of CNS.

Genotypic characterisation of coagulase negative staphylococci: The phenotypic identification of CNS often appears to be unreliable, imprecise and irreproducible. Because of the varied expression of phenotypic features, commercial identification tools and automated systems are unable to distinguish various CNS species. Other identification tools, such as enzyme electrophoresis or cellular fatty acid composition analysis, have failed to provide an accurate identification. However, several PCR amplicon-sequencing-based approaches for identification of CNS have been published (Heikens *et al.* 2005). The genotypic differentiation of CNS from *S. aureus* has been based on the presumption that the detection of a *S. aureus* marker such as *coa*, *nuc* or *spa* rules out the presence of CNS (Okolie *et al.* 2015). However, this approach may be unsuitable for polymicrobial samples harbouring both *S. aureus* and CNS. According to Okolie *et al.* (2015) PCR assay targeting the *cns* gene signal could exclusively identify the CNS with 100% specificity and therefore the same primer pairs were employed in this study also. In the present study, out of the 49 genotypically identified *Staphylococcus* spp., 27 isolates yielded a PCR product with amplicon size of 204 bp and were confirmed as CNS (Fig. 1).

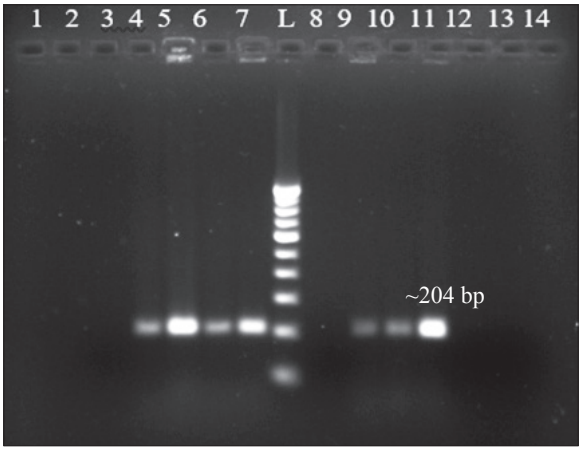

Fig. 1. Agarose gel electrophoresis of *cns* specific PCR of CNS [Lane L: DNA marker 100 bp; Lane 4,5,6, 9,10,11: Positive samples (204 bp); Lane 7: Positive control; Lane 8: Negative control; Lane 1, 2, 3, 12, 13, 14: Negative samples].

Genotypic characterisation of antimicrobial resistance in staphylococci: Rapid and early detection of the pathogen and the dynamics of its resistance mechanisms is an absolute necessity for addressing the challenges of AMR (Anjum *et al.* 2018). Though there are numerous low cost, easily adaptable, phenotype based resistance typing tools, several previous studies of bovine mastitis pathogens have reported *in vivo* resistance to certain antimicrobials even though they are sensitive *in vitro* and *vice versa* (Constable and Morin 2003, Kasravi *et al.* 2010), thereby limiting its practical utility in diagnosis and containment of resistant infections. This may be due to the fact that most *in vitro* susceptibility breakpoint data are not guided by host and mammary tissue factors that assist in clinical cure and are more likely to be derived from human pathogens and the pharmacokinetics of the drug in human serum (Constable and Morin 2003). Molecular approaches, such as those focussed on PCR, are therefore regarded as a method of choice for the rapid and reliable diagnosis of resistant mechanisms and epidemiological investigations of outbreaks involving resistant strains (Anjum *et al.* 2018). It also eliminates the need for cumbersome cultural procedures and combats for the heterogeneous nature of the phenotypic expression of resistance since they could be performed directly on clinical specimens (Tan 2003). Therefore, in the present study, PCR was employed to uncover the AMR among the CNS isolates.

Among the various antimicrobial agents which have been approved for use in bovine mastitis, β-lactams, such as cephalosporins and penicillins, tetracyclines, and fluroquinolones, were among the commonest antimicrobial agents that have been licenced for use in bovine mastitis. Staphylococcal resistance to β-lactams is mediated by either β-lactamases encoded by the *blaZ* gene or the *mecA* encoded alternative penicillin binding protein, PBP2a, which has a decreased binding to the β-lactam antibiotics presently available for mastitis therapy (Aarestrup *et al.* 2006). The current study revealed that 100% of the CNS isolates carried the *blaZ* gene. This was in consonance with the previous study by Amrithapriya (2019) and Jose (2017) which reported 100% prevalence of *blaZ* gene among staphylococcal isolates from mastitic cows of the same locality. However, Kulangara *et al.* (2017) had observed a comparatively low prevalence (90%) of *blaZ* gene among the dairy cows in early dry period.

The findings of the present study also revealed the presence of *mecA* gene in 11 (40.74%) out of the 27 CNS isolates. This was relatively higher when compared to the previous study by Kulangara *et al.* (2017) on dry bovine udders from the same locality wherein the presence of *mecA* gene was noticed only in 19.75% of the CNS isolates from dry cows. Varying trends regarding the presence of *mecA* gene was reported by Amrithapriya (2019) and Ciftici *et al.* (2009) who reported the presence of *mecA* gene in 18.18% and 30.7% of the staphylococcal isolates, respectively.

Tetracyclines are broad-spectrum, bacteriostatic antibacterial that acts at ribosomal level by inhibiting

protein synthesis. Bacterial resistance to tetracycline, 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 the effective drug binding (Donhofer 2012). The genetic determinants of tetracycline resistance were allocated to four groups, namely, K, L, M and O. The previous study by Kulangara *et al.* (2017) 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% and 76.3% 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) reported a higher prevalence of *tetM* among MRSA isolates. Consequently, the present study was conducted to determine t*etM* mediated tetracycline resistance that has not been studied previously. The present study demonstrated *tetM* mediated genotypic resistance to tetracycline in four among the 27 CNS isolates (14.18%). This was relatively higher than the findings from Northwest China (Feng *et al.* 2016) that recorded a lower occurrence of the *tetM* (2.27%) mediated tetracycline resistance as compared to the *tetK* (22.73%) mediated resistance. However, contradictory findings were reported by Duran *et al.* (2012) from human samples of a teaching hospital in Turkey, where 42.4% of the *S. aureus* and 39% of the CNS were found to carry the *tetM* or *tetK* genes. Jamali *et al.* (2014) also reported a higher prevalence (39.5%) of *tetM* gene among *S. aureus* isolates from bovine mastitis in Malaysia. 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).

Fluoroquinolones are a class of synthetic bactericidal agents that function by inhibition of bacterial type II topoisomerase, thereby preventing bacterial replication (Brown 1996). The present study was pursued to determine the fluroquinolone resistance mediated by the *gyrA* gene located on the quinolone resistant determining region. Even though phenotypic resistance against fluroquinolones could be documented, the results of the present study revealed that none of the isolates were positive for the presence of *gyrA* gene. Similar findings were reported by Osman *et al.* (2016) and Rasool *et al.* (2020), wherein the *gyrA* gene was not amplified in any of the isolates. This could be because certain bacteria can lose essential genes through various mechanisms such as deletions or acquisition of mobile genetic elements. This might also be due to the natural variation within a particular bacterial population. However, in order to confirm the absence of *gyrA* gene or to unravel the effects of mutations in specific gene additional approaches such as DNA sequencing of the *gyrA* gene is warranted.

Phenotypic characterisation of antimicrobial resistance in CNS: The disc diffusion assay is a simple, low-cost, easily interpretable qualitative typing method for phenotypic profiling of AMR in many bacterial isolates (Balouiri *et al.* 2016). In the present study, it was found that highest resistance was shown against penicillin with 19 isolates (70.37%) being resistant followed by amoxicillin-sulbactam and enrofloxacin with 14 (51.85%) and 13 (48.14%) isolates being resistant. Ten isolates (37.04%) were resistant to gentamicin, whereas seven isolates (25.93%) were resistant to tetracycline. Ceftriaxone, ceftriaxone sulbactam and cotrimoxazole showed a similar resistance pattern with eight isolates (29.63%) each being resistant. Least resistance was shown against methicillin with only four isolates (14.81%) being resistant. Poor sensitivity among CNS to penicillin has been reported by Sebastian (2001) and Rathish (2014). Similar results were obtained by Soares *et al.* (2012) who found that CNS isolated from Brazilian dairy herds exhibited highest resistance towards penicillin (79%), whereas least resistance was shown towards enrofloxacin (2%), ampicillin-sulbactam and cotrimoxazole. In contrast, a report by Frey *et al.* (2013) on the antibiogram of CNS obtained from cases of bovine mastitis in Switzerland revealed that, 15.1% of the CNS isolates were MDR with methicillin resistance being the most frequent resistant phenotype (47%) followed by fusidic acid, tiamulin, penicillin, tetracycline, streptomycin and erythromycin.

Statistical analysis of phenotypic and genotypic antimicrobial resistance: In the present study, the results from *in vitro* disc diffusion assay of CNS isolates revealed that 77.77%, 14.81%, 25.93% and 48.15% isolates were resistant to penicillin, methicillin, tetracycline and

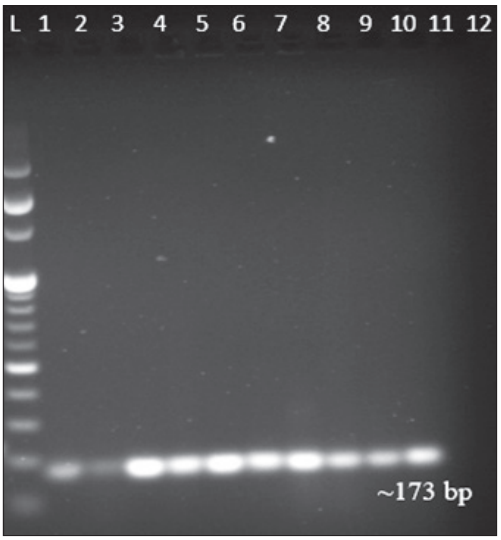

Fig. 2. Agarose gel electrophoresis of *blaZ* specific PCR of CNS [Lane L: DNA marker 100 bp; Lane 1: Positive control; Lane 12: Negative control; Lane 2 to 11: Positive samples (~173 bp)].

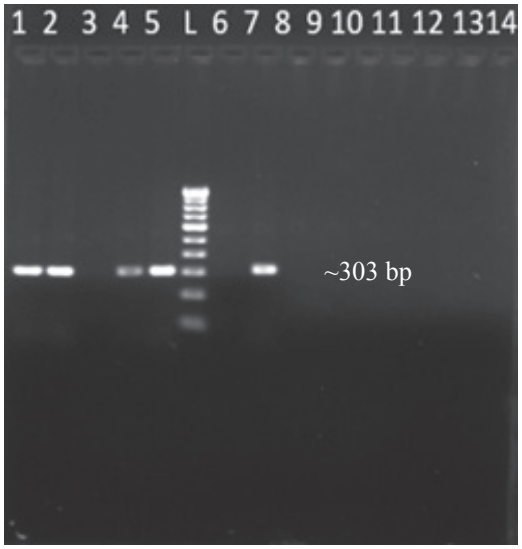

Fig. 3. Agarose gel electrophoresis of *mecA* specific PCR of CNS [Lane L: DNA marker 100 bp; Lane 6: Negative control; Lane 7: Positive control; Lane 1, 2, 4, 5: Positive samples (303bp); Lane 3,6,8,9,10,11,12,13,14: Negative samples].

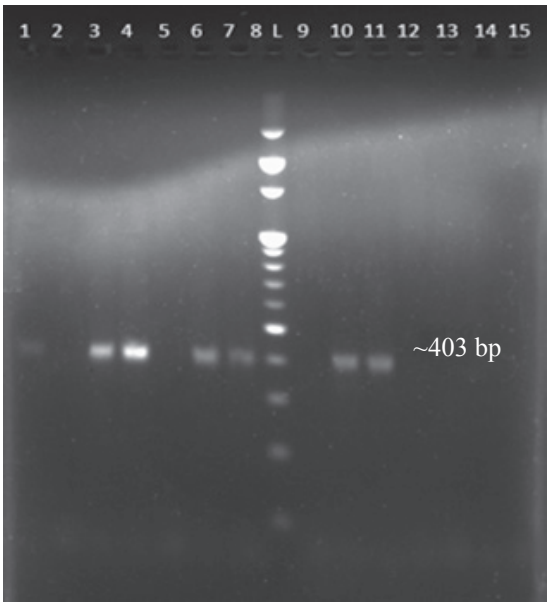

Fig. 4. Agarose gel electrophoresis of *tetM* specific PCR of CNS [Lane L: DNA marker 100 bp; Lane 8: Positive control (403 bp); Lane 9: Negative control; Lane 3, 4, 6, 7, 10, 11: Positive samples; Lane 1, 2, 5, 12-15: Negative samples].

enrofloxacin, respectively by disc diffusion assay whereas, all the isolates (100%) possessed *blaZ* gene, 40.74% had *mecA* gene and 14.81% had *tetM* gene (Figs. 2 to 4). None of the CNS were found to carry *gyrA*.

Statistical analysis using McNemar's test revealed that there is significant difference between the phenotypic and genotypic resistance of the CNS against the penicillin, methicillin and tetracycline (Table 2). Thus, it could be concluded that the isolates which showed the absence of AMR resistant genes were phenotypically resistant to the respective antimicrobial agent. Phenotypic sensitivity

Table 2. Comparison of phenotypic and genotypic antimicrobial resistance in coagulase negative staphylococci

McNemars test	Methicillin & *mecA*	Penicillin & *blaZ*	Tetracycline & *tetM*	Enrofloxacin & *gyrA*
N	27	27	27	27
Exact Sig. (2-tailed)	.039[b]	.008[b]	.508[b]	.000[b]
Exact Sig. (1-tailed)	0.020	0.004	0.254	0.000
Point Probability	0.018	0.004	0.164	0.000

to antimicrobial agent even in presence of related AMR genes were also noted. Similar findings were reported by Choi *et al.* (2003) and Ciftici *et al.* (2009) wherein the methicillin resistant, *mecA* negative strains was attributed to the non PBP-2a dependent mechanisms such as hyper production of β lactamase and alteration of PBP types or due to the involvement of *mecC*, a divergent homolog of *mecA* in the expression of resistance. Methicillin sensitive *mecA* positive strains is because the methicillin resistance is not consistently expressed and certain auxillary genes such as *femA*, *mecR* and the gene encoding β-lactamase plasmid may participate in the control of its expression.

Similar variations in phenotypic and genotypic AMR patterns in staphylococci have been documented in earlier studies also. The relatively high level of phenotypic resistance could also be attributed to the mutation or genetic variability in gene which may impair its detection by conventional PCR due to the potential barrier of primer mismatch (Costa *et al.* 2019) or due to the simultaneous presence of more than one genetic mechanism that determines the resistance as seen in case of tetracycline resistance (Warsa *et al.* 1996). Alternative bacterial resistance mechanisms such as bacterial biofilm formation may also contribute to this discrepancy between the phenotypic and genotypic resistance (Pantosti *et al.* 2007). Hence, it is recommended that diverse variants of the gene should be covered for genotypic profiling of AMR (Hamid *et al.* 2017).

It can be concluded that CNS (32.53%) was found to be the most common cause of clinical mastitis among the dairy cows of the study population. The study also reported an alarming level of resistance among the CNS isolates. The higher prevalence of AMR among CNS isolates suggests that the therapeutic and prophylactic use of antimicrobials should be carried out with great caution as it leads to the acquisition of resistance genes by the milk-borne bacteria, which leads to a decrease in efficacy of drug, when used to treat human infections. This is of paramount importance as a vast majority of drugs used in veterinary practice are listed by the World Health Organization as critically important drugs in human medicine.

ACKNOWLEDGEMENT

The authors are thankful to the Kerala Veterinary and Animal Sciences University for providing the facilities

needed for carrying out the research.

REFERENCES

Aarestrup F M. 2006. *Antimicrobial Resistance in Bacteria of Animal Origin*. ASM Press, Washington, DC. Pp. 187-206

Amrithapriya M G. 2019. 'Clinico-therapeutic studies of bovine mastitis caused by Staphylococcus aureus and its molecular typing.' MVSc thesis. Kerala Veterinary and Animal Sciences University, Pookode, 154p

Anjum M F, Zankari E and Hasman H. 2018. Molecular methods for detection of antimicrobial resistance. *Antimicrobial Resistance in Bacteria from Livestock and Companion Animals*. 50p.

Archana. 2018. 'Identification of biofilm forming staphylococci associated with otitis in dogs.' MSc thesis. Kerala Veterinary and Animal Sciences University, Pookode, 87 p.

Balouiri M, Sadiki M and Ibnsouda S K. 2016. Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **6**(2): 71–79.

Barrow C I and Feltham R K A. 1993. *Cowan and Steel's Manual for the Identification of Medical Bacteria*. (3rd Ed.). Cambridge University Press, Great Britain. Pp. 331.

Bauer A W, Kirby M M, Serris J S and Turck M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology* **45**: 493–96.

Brown S A. 1996. Fluoroquinolones in animal health. *Journal of Veterinary Pharmacology and Therapeutics* **19**: 1–14.

Choi S. M, Kim S H, Kim H J, Lee D G, Choi J H, Yoo J H, Kang J H, Shin W S and Kang M W. 2003. Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among *Staphylococcus* species. *Journal of Korean Medical Science* **18**: 631.

Ciftci A, Findik A, Onuk E E and Savasan S. 2009. Detection of methicillin resistance and slime factor production of Staphylococcus aureus in bovine mastitis. *Brazilian Journal of Microbiology* **40**: 254–61.

Clinical and Laboratory Standards Institute. 2017. *Performance Standards for Antimicrobial Susceptibility Testing* (25th Ed.). Clinical and Laboratory Standards Institute, Wayne. Pp. 15.

Constable P D and Morin D E. 2003. Treatment of clinical mastitis: Using antimicrobial susceptibility profiles for treatment decisions. *Veterinary Clinics: Food Animal Practice* **19**(1): 139–55.

Costa S S, Sobkowiak B, Parreira R, Edgeworth J D, Viveiros M, Clark T G and Couto I. 2019. Genetic diversity of *norA*, coding for a main efflux pump of *Staphylococcus aureus*. *Frontiers in Genetics* **9**: 710

Donhofer A, Franckenberg S, Wickles S, Berninghausen O, Beckmann R and Wilson D N. 2012. Structural basis for tetM-mediated tetracycline resistance. *Proceedings of the National Academy of Sciences, USA* **42**: 16900–05.

Duran N, Ozer B, Duran G G, Onlen Y and Demir C. 2012. Antibiotic resistance genes and susceptibility patterns in staphylococci. *Indian Journal of Medical Research* **135**: 389.

Feng Y A N G, Qi W A N G, Wang X R, Ling W A N G, Li X P, Luo J Y, Zhang S D and Li H S. 2016. Genetic characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis cases in Northwest China. *Journal of Integrative Agriculture* **15**: 2842–47.

Ferrero L, Cameron B, Manse B, Lagneaux D, Crouzet J, Famechon A and Blanche F. 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: A primary target of fluoroquinolones. *Molecular Microbiology* **13**: 641–53.

Frey Y, Rodriguez J P, Thomann A, Schwendener S and Perreten V. 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *Journal of Dairy Science* **96**: 2247–57.

Hamid S, Bhat M A, Mir I A, Taku A, Badroo G A, Nazki S and Malik A. 2017. Phenotypic and genotypic characterization of methicillin-resistant *Staphylococcus aureus* from bovine mastitis. Veterinary World **10**: 363.

Heikens E, Fleer A, Paauw A, Florijn A and Fluit A C. 2005. Comparison of phenotypic and genotypic methods for species level identification of clinical isolates of coagulase negative staphylococci. *Journal of Clinical Microbiology* **43**: 2286–91.

Jamali H, Radmehr B and Ismail S. 2014. Prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from bovine clinical mastitis. *Journal of Dairy Science* **97**: 2226–30.

Jose J. 2017. 'Phenotypic and genotypic characterization of *mecA* and *blaZ* gene mediated antibiotic resistance in *Staphylococcus aureus* and other coagulase negative Staphylococci associated with mastitis in bovines.' MSc Thesis, Kerala Veterinary and Animal Sciences University, Pookode, 47p.

Morales-Ubaldo A L, Rivero-Perez N, Valladares-Carranza B, Velázquez-Ordoñez V, Delgadillo-Ruiz L and Zaragoza-Bastida A. 2023. Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. *Veterinary and Animal Science* **21**: 100306–27.

Kasravi R, Bolourchi M, Farzaneh N, Seifi H A, Barin A, Hovareshti P and Gharagozlou F. 2010. Relationship between *in vitro* antimicrobial sensitivity of bovine subclinical mastitis isolates and treatment outcome in lactating dairy cows. *Iranian Journal of Veterinary Research* **11**: 249–54.

Kulangara V, Nair N, Sivasailam A, Sasidharan S, Kollannur J D and Syam R. 2017. Genotypic and phenotypic β-lactam resistance and presence of PVL gene in Staphylococci from dry bovine udder. *PloS ONE* **12**: p.e0187277.

Larsson D G and Flach C F. 2022. Antibiotic resistance in the environment. *Nature Reviews Microbiology* **20**(5): 257–69.

Martineau F, Picard F J, Grenier L, Roy P H, Ouellette M and Bergeron M G. 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. *Journal of Antimicrobial Chemotherapy* **46**: 527–34.

Okolie C E, Wooldridge K G, Turner D P, Cockayne A and James R. 2015. Development of a new pentaplex real-time PCR assay for the identification of poly-microbial specimens containing *Staphylococcus aureus* and other staphylococci, with simultaneous detection of staphylococcal virulence and methicillin resistance markers. *Molecular and Cellular Probes* **29**: 144–50.

Osman K, Badr J, Al-Maary K S, Moussa I M, Hessain A M, Girah Z M A, Abo-Shama U H, Orabi A and Saad A. 2016. Prevalence of the antibiotic resistance genes in coagulase-positive-and negative-*Staphylococcus* in chicken meat retailed to consumers. *Frontiers in Microbiology* **7**: 1846–52.

Pantosti A, Sanchini A and Monaco M. 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future Microbiology* **2**: 323–34.

Quinn P, Markey B, Carter M and Carter G R. 2013. *Clinical Veterinary Microbiology* (2nd Ed) Mosby, St. Louis. Pp. 514.

Rasool K H, Hussein N H and Taha B M. 2020. Molecular detection of gyrA gene in *Salmonella enterica* serovar *typhi*

isolated from typhoid patients in Baghdad. *Pakistan Journal of Biological Sciences* **23**(10): 1303–09.

Rathish R L. 2014. 'Clinico-therapeutic studies and experimental evaluation of a bacterin against common bacterial isolate of bovine mastitis.' MVSc thesis. Kerala Veterinary and Animal Sciences University, Pookode, 177p.

Reygaert W C. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology* **4**(3): 482–502.

Schmitz F J, Krey A, Sadurski R, Verhoef J, Milatovic D and Fluit A C 2001. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *Journal of Antimicrobial Chemotherapy* **47**: 239–40.

Soares L C, Pereira I A, Pribul B R, Oliva M S, Coelho S M and Souza M. 2012. Antimicrobial resistance and detection of *mecA* and *blaZ* genes in coagulase-negative *Staphylococcus* isolated from bovine mastitis. *Pesquisa Veterinaria Brasileira* **32**: 692–96.

Sebastian S R. 2001. 'Clinico-therapeutic studies on bacterial etiology of bovine mastitis.' M.V.Sc thesis. Kerala Veterinary and Animal Sciences University, Pookode, 57p.

Tan T Y. 2003. Use of molecular techniques for the detection of antibiotic resistance in bacteria. *Expert Review of Molecular Diagnostics* **3**: 93–103

Walther C and Perreten V. 2007. Letter to the editor: Methicillin-resistant *Staphylococcus epidermidis* in organic milk production. *Journal of Dairy Science* **90**: 5351.

Warsa U C, Nonoyama M, Ida T, Okamoto R, Okubo T, Shimauchi C, Kuga A and Inoue M. 1996. Detection of tet (K) and tet (M) in *Staphylococcus aureus* of Asian countries by the polymerase chain reaction. *Journal of Antibiotics* **49**: 1127–32.

Vijayakumar K and Jose K R. 2021. Phenotypic and genotypic characterization of *Staphylococcus aureus* isolated from bovine mastitis for biofilm formation. *Journal of Animal Research* **11**: 249–55.