RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.1.153-159

Molecular characterisation of antimicrobial resistance in coagulase negative staphylococci isolated from bovine subclinical mastitis#

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Citation: Anaina,S., Vijayakumar,K., Justin Davis,K., Rathish,R.L. and Mani,B.K. 2023. Molecular characterisation of antimicrobial resistance in coagulase negative staphylococci isolated from bovine subclinical mastitis. *J. Vet. Anim. Sci.* **54**(1):153-159
DOI: https://doi.org/10.51966/jvas.2023.54.1.153-159

Received: 30.10.2022 *Accepted: 16.12.2022* *Published: 31.03.2023*

Abstract

Coagulase negative staphylococci (CNS) are emerging as the most prevalent causative agent of bovine mastitis. They are resistant to many commonly used antibiotics due to the presence of antimicrobial resistance (AMR) genes. A study was conducted to evaluate the AMR profiling of CNS isolated from bovine subclinical mastitis.Coagulase negative staphylococci were isolated from 49 (44.95 per cent) of the subclinical mastitis samples. Disc diffusion assay revealed that highest resistance was shown against gentamicin (42.85 per cent) followed by methicillin (32.6 per cent), ceftriaxone – tazobactam (24.48 per cent), enrofloxacin (20.4 per cent), tetracycline (16.32 per cent) and least resistance to cotrimoxazole (4 per cent). Genotypic characterisation of AMR genes such as mecA, aacA-aphD and norA by PCR was done for determining resistance to methicillin, gentamicin and fluroquinolone resistance. The CNS carried aacA-aphD, norA and mecA in 44.89 per cent, 32.65 per cent and 14.28 per cent, respectively. Comparison of phenotypic and genotypic characterisation of AMR in CNS was carried out by McNemar test and it was found that there was significant difference between the presence of mecA gene and methicillin resistance. There was no significant difference noticed forcharacterisation of phenotypic and genotypic AMR of CNS for gentamicin and fluroquinolone resistance.

Keywords: *Coagulase negative Staphylococci, antimicrobial resistance, methicillin, gentamicin, fluroquinolones*

#Forms part of the MVSc thesis submitted by the first author to the Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala.

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Subclinical mastitis (SCM) is a major concern as it leads to considerable economic loss in organised dairy farms, compared to clinical mastitis.A high prevalence of SCM (54 per cent) was reported from organised farms in Kerala (Amrithapriya *et al*., 2021). Coagulase negative staphylococcal outbreaks have to be closely monitored because they were known to be more resistant to antibiotics than *S. aureus* (Taponen and Pyorala, 2009). Majority of the resistance phenotypes had been found in several CNS and had been linked to resistance genes on mobile genetic elements, suggestive of the possibility of horizontal gene transfer across species borders (Huber *et al*., 2011). According to Tiwari *et al*. (2013), despite the emerging antibiotic resistance, empirical therapy should be replaced by selection of antibiotics based on the results of culture and sensitivity testing. Antimicrobial resistance is one of the reasons for high persistence of SCM in dairy farms.The present study was envisaged to detect the presence of antimicrobial resistance genes and antibiogram of CNS isolated from bovine subclinical mastitis.

Materials and methods

The present study was conducted in 100 apparently healthy milch cattle from University farm and an organised private farm in Thrissur district. Milk samples were collected aseptically.Individual quarter sample were directly streaked on to brain heart infusion agar. The bacterial isolates were identified using morphological, colony and biochemical characteristics (Barrow and Feltham, 1993; Quinn *et al*., 2013).

Antibiotic susceptibility of the CNS was tested based on Kirby Bauer disc diffusion assay (Bauer *et al.,* 1966) according to the regulations of Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). Antibiotic discs of six antibiotics with known concentration in microgram (mcg) or international unit (IU) per disc were used in the study,*viz*. methicillin, enrofloxacin, gentamicin, cotrimoxazole, tetracycline and ceftriaxone tazobactam. Spread of antibiotic resistant bacteria can be found out by calculation of multiple antibiotic resistance (MAR) index. The MAR index values for each isolate and antibiotic can be calculated separately (Krumperman, 1983).

All the isolates of *Staphylococcus* spp.obtained in the study were subjected to genotypic characterisation by amplification of *16SrRNA* and *cns* genes for molecular confirmation of *Staphylococcus* spp. and CNS respectively. The presence of AMR genes in CNS,*viz*. *mecA* for methicillin resistance, *aacA – aphD* for gentamicin resistance and *norA* for fluroquinolone resistance were determined by polymerase chain reaction (PCR) using the primers specific for them (Table 1).

Table 1. Details of primers and conditions used for PCR

Organism / AMR	Genes	Primer sequence	Annealing temperature	Amplicon Size(bp)	Reference
Staphylococcus spp.	*16S rRNA*	F:AACTCTGTTATTAGGGAAGAA CA	55.8^{0}C	756 bp	Ciftci *et al*. (2009)
		R:CCACCTTCCTCCGGTTTG TCA CC			
Coagulase negative Staphylococci	*cns*	F:TATCCACGAAACTTCTAAAACAACTG TTACT	56.3^{0}C	204 bp	Okolie *et al*. (2015)
		R:TCTTTAGATAATACGTATACTTCAGCT TTGAATTT			
Methicillin resistance	*mec A*	F:TGGCTATCGTGTCACAATC	60^{0}C	303 bp	Archana (2018)
		R:CTGGAACTTGTTGAGCAGAG			
Gentamicin resistance	*aacA-aphD*	F: TAA TCC AAG AGC AAT AAG GGC	55^{0}C	227 bp	Strommenger*et al*. (2003)
		R:GCC ACA CTA TCA TAA CCA CTA			
Fluroquinolones resistance	*norA*	F:TTCACCAAGCCATCAAAAAG	45^{0}C	620bp	Couto *et al*. (2008)
		R:CTTGCCTTTCTCCAGCAATA			

The results obtained by phenotypic and genotypic characterisation of AMR was compared using McNemar test with the help of SPSS version 24.0.

Table 2. Comparison of phenotypic and genotypic characterisation of antimicrobial resistance in CNS isolates

	M (P) & *mecA*	G(P) & *aacA-aphD*	E(P) & *norA*
N	49	49	49
Exact Sig. (2-tailed)	0.004**	1.000ns	0.180ns

** represents significance at 1 per cent level, ns-no significant difference

Results and discussion

About 109 samples yielded pure bacterial culture and 83 of them were found to be Gram positive cocci. Based on the colony characteristics and biochemical tests, 49 isolates (44.95 per cent) were CNS in this study. It was in accordance with Walid *et al*. (2021), who conducted a study on subclinical mastitis of cattle in Egypt, and revealed that CNS was isolated in 56.63 per cent of the positive samples. Similarly, Krupa (2020) found that 56.25 per cent of the isolates from bovine SCM in Kerala were CNS. Mahmoud *et al*. (2015) reported a lower percentage (8.9 per cent) of CNS from SCM samples in Egypt, which is contrary to the findings of this study. Variations in the findings of AMR in this study might be due to the heterogeneity in agro climatic conditions, management techniques and ecology of pathogens in the herd environment.

Highest resistance was shown against gentamicin by 21 isolates (42.85 per cent) followed by methicillin with 16 isolates (32.6 per cent), ceftriaxone - tazobactam with 12 isolates (24.48 per cent), enrofloxacin with ten isolates (20.4 per cent), tetracycline with eight isolates (16.32 per cent) and least resistance was shown to cotrimoxazole by two isolates (4 per cent) (Fig.1). Similar to this findings, Krupa (2020) reported that CNS isolated from bovine SCM in Kerala showed resistance towards enrofloxacin, gentamicin, cotrimoxazole, tetracycline and methicillin with 48.15 per cent, 37.04 per cent, 29.63 per cent, 25.93 per cent and 14.81 per cent respectively. Walid *et al*. (2021) reported that CNS isolated from bovine mastitis in Egypt was found to be susceptible to gentamicin, ciprofloxacin, amoxicillin/clavulanic acid, chloramphenicol, tetracycline, penicillin, vancomycin, and erythromycin, which is contrary to the findings in this study.

All the 49 phenotypically identified CNS were subjected to PCR and in all of them, *16S rRNA* and *cns* gene were amplified (Fig.2, 3). All the phenotypically identified CNS yielded positive signals for *cns* gene. As per Okolie *et al*. (2015), PCR assay for *cns* gene could identify the CNS with 100 per cent specificity. Similarly, Oliveira and Cunha (2010) reported that 82 per centwas positive by PCR among the 100 CNS isolates tested.

Methicillin resistance gene (*mecA*) was detected in seven (14.28 per cent) of the CNS isolates (Fig. 4). Piessens *et al*. (2012) reported that *mecA* was found in 11.7 per cent of CNS. This was in contrast to Nayel *et al*. (2020), who claimed that 73.33 per cent of CNS isolated from mastitis milk carried *mecA* gene. Gentamicin resistance gene (*aacA-aphD*) was detected in 22 (44.89 per cent) of the CNS isolates (Fig. 4). Similarly, Xu *et al*. (2015) reported *aacA-aphD* responsible for aminoglycoside resistance in 32.1per cent of staphylococci isolated from bovine SCM in China. In contrast to this, El-Ashker *et al*. (2020) detected *aacA-aphD* gene in 4 per cent of the CNS isolates. Sixteen (32.65 per cent) of the CNS isolates were detected with fluoroquinolone resistance gene (*norA*) (Fig. 4). It was not in agreement with the results of Patel and Trivedi (2018), who claimed that *norA* was the major AMR gene in staphylococcal isolates.

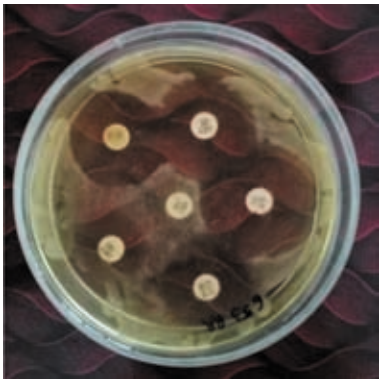

Fig. 1. Antibiotic susceptibility test by disc diffusion assay

There was significant difference between the characterisation of resistance by phenotypic and genotypic methods for methicillin, while no significant difference noticed for gentamicin and enrofloxacin (Table 2). The findings of the present studywas in accordance with that of Krupa (2020), who found a similar significant difference in the characterisation of resistance by phenotypic and genotypic methods for methicillin resistance from staphylococcal isolates.

The most likely explanation was that MRS susceptibility testing using standard microbiological techniques was difficult because the phenotypic expression of resistance depends largely on growth conditions (like osmolarity and medium temperature) (Ibrahim *et al*., 2022). Bogado *et al*. (2001) also supported the discrepancies between molecular and phenotypic classifications of methicillin resistance.

Apart from *mecA* gene, other genes like *mecC* gene also contributed to methicillin resistance. Additionally, Moon *et al*. (2007) demonstrated that isolates lacking in the *mecA* gene had methicillin resistance phenotypes. Due to the mobile nature of SCCmec components, there could be spread of resistance among the *Staphylococci* spp. including *S. aureus* (Santos *et al*., 2016). Results are depicted in fig. 2, 3 and 4.

Conclusion

Coagulase negative staphylococci are major pathogens causing bovine subclinical mastitis. They are resistant to commonly used antibiotics and possess AMR genes that can be transferred to other species.Majority of the isolates in the present study had resistance against gentamicin followed by methicillin, ceftriaxone - tazobactam, enrofloxacin, tetracycline and cotrimoxazole. *MecA* gene for methicillin resistance was detected in14.28 per cent of the CNS isolates in this study while 32.65 per cent and 44.89 per cent of the isolates were detected with fluoroquinolone resistance gene (*norA*) and gentamicin resistance gene (*aacA-aphD*). Routine screening for SCM and judicious use of antibiotics based on antibiogram is necessary to control the infection at the herd level.

Acknowledgement

The authors are thankful to the Kerala Veterinary and Animal Sciences University for providing the facilities needed for carrying out the research.

Conflict of interest

The authors declare no conflict of interest.

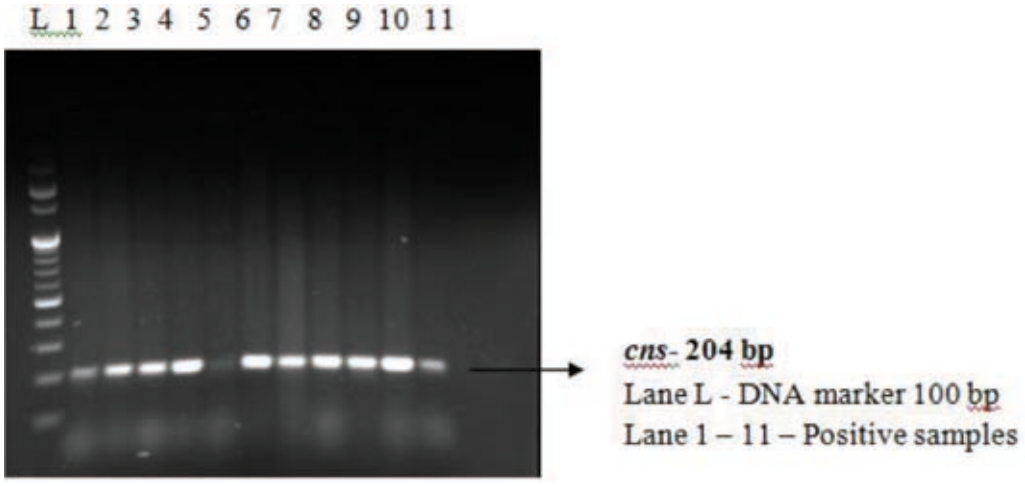

Fig. 2. PCR for detection of *Staphylococcus* spp.

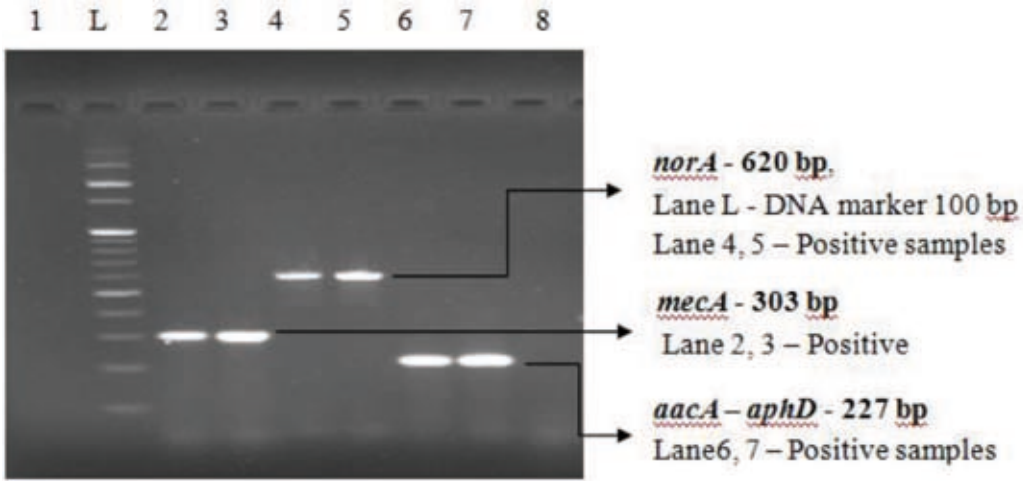

Fig. 3.PCR for detection of CNS

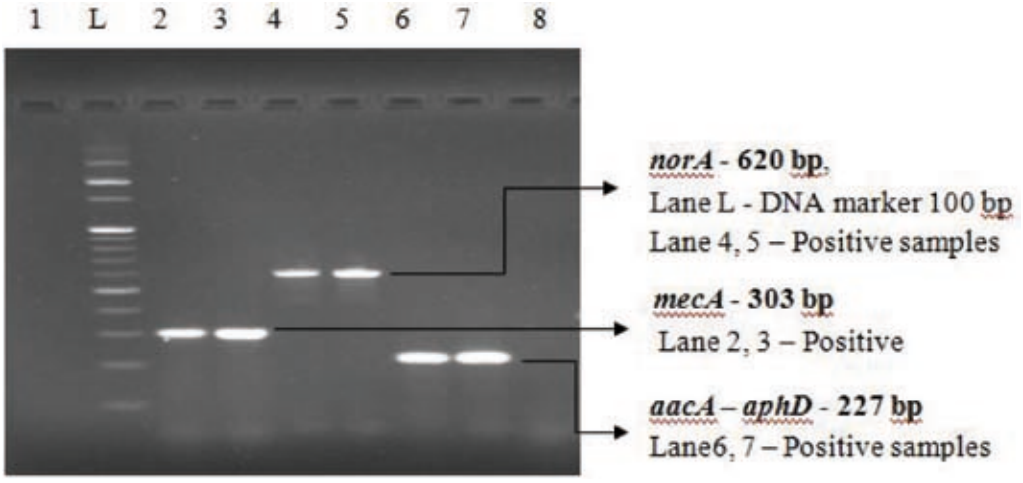

Fig. 4.PCR for detection of *mecA, norA* and *aacA-aphD*in CNS

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