SHORT COMMUNICATION
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.1.257-261

Isolation, identification and antibiogram of coagulase negative staphylococci from bovine clinical mastitis#

Krupa Rose Jose[1] and K. Vijayakumar[2]
Department of Veterinary Epidemiology and Preventive Medicine,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680651,
Kerala Veterinary and Animal Sciences University, Kerala, India

Citation: Krupa,R.J. and Vijayakumar,K. 2023. Isolation, identification and antibiogram of coagulase negative staphylococci from bovine clinical mastitis. *J. Vet. Anim. Sci.* **54**(1):257-261
DOI: https://doi.org/10.51966/jvas.2023.54.1.257-261

Received: 18.09.2022 *Accepted: 24.12.2022* *Published: 31.03.2023*

Abstract

The aim of this study was to identify the primary bacteria associated with clinical mastitis in dairy cows and to assess the antimicrobial resistance pattern of the commonest isolate using phenotypic methods. The milk samples were collected from 83 domesticated dairy cows suffering from clinical mastitis (CM), from an organised dairy farm as well as cows presented with CM at the University Veterinary Hospital (UVH), Mannuthy, Thrissur, Kerala. Microbiological analysis, using morphological, cultural and biochemical properties as well as molecular identification using polymerase chain reaction (PCR) revealed that coagulase-negative staphylococci (CNS) were the most predominant (40.31 per cent) bacteria, followed by Staphylococcus aureus (32.83 per cent), Micrococci spp. (11.94 per cent), Klebsiella spp. (7.46 per cent) Escherichia coli (4.48 per cent) and Streptococci (2.98 per cent). The majority of the bacteria were resistant to more than one class of antimicrobials (aminoglycosides, β lactams, fluoroquinolones or tetracyclines). Furthermore, our findings revealed that the CNS is highly resistant to β-lactam family of antibiotics and that CNS may play a significant role in clinical mastitis of dairy cows.

Keywords: *Antibiogram, coagulase negative staphylococci, mastitis*

Dairying forms a significant element of the Indian rural economy and provides a key source of income, food and nutritional security especially for the smallholder dairy farmers. Since 1998, India has been the world's largest producer and consumer of dairy products and according to the latest statistics from the Department of Animal Husbandry and Dairying (DAHD), India produced an all-time high milk yield of 209.96 million tonnes of milk during the period 2020-21, with more than 50 per cent contribution from cows(DAHD, 2022).Even though India possess the world's largest bovine population, milk production per animal is much lower as compared to other leading milk producers. One possible explanation is the increased occurrence of intramammary

 # Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

1. *Ph.D. Scholar*
2. *Dean, College of Veterinary and Animal Sciences, Mannuthy*
**Corresponding author: krupaputhuparampil@gmail.com, Ph: 8606719132*

infections, which is aggravated by the mounting tide of antibiotic resistance.

The disease had a constantly evolving aetiology involving numerous pathogens such as bacteria, fungi, algae, viruses, mycoplasma etc. Among the multitude of pathogens involved, bacteria constitute the most significant group pathologically as well as economically (Sharma *et al*., 2012). To date, most of the mastitis studies have been conducted in *Staphylococcus aureus* and *Escherichia coli*. However, reports from the recent past indicate that the members of CNS could be considered as emerging pathogens of bovine mastitis (Soares *et al*., 2012). Thus, it is of utmost importance to identify the CNS involved in bovine mastitis.

Antibiotics are the cornerstone of mastitis therapy. While antimicrobials improve the health and production of farm animals, their imprudent use exert tremendous selection pressure on bacteria residing in the body of cows and farm settings to be resistant to antimicrobials, that in turn compromises the cure rates (Sevenoet *al*., 2002; Oliver *et al*., 2011; Mukerji *et al*., 2017). Thus, it is of utmost importance to monitor the antimicrobial resistance (AMR) trends in bacterial isolates from mastitic animals and farm settings, not only for treatment choices but also to identify the potential resistome reservoirs on dairy farms. In this context, the current study was designed to identify the principal bacteria associated with clinical mastitis in dairy cows and to assess the phenotypic AMR pattern of the isolated bacteria.

The present study was carried out in the Centre for Mastitis Control laboratory, Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala 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 CM at the University Veterinary Hospital (UVH), Mannuthy.

Bacterial isolation was performed on milk samples. For this one millilitre of pre enriched milk samples were streaked on to brain heart infusion agar, incubated for 24h, and the colony morphology was examined by Gram's staining. All the Gram positive cocci were streaked on to mannitol salt agar (MSA; HiMedia Laboratories, Mumbai) and Gram negative bacilli to the Mac Conkey Agar (HiMedia Laboratories, Mumbai) and Eosin Methylene Blue (EMB) agar. The colony characteristics of each isolate on their selective media were analysed. Biochemical characterisation of the isolates was also carried out. *Staphylococcus* spp. were presumptively identified using catalase, coagulase, oxidase, Voges-Proskauer (VP) and nitrate reduction tests. The indole, methyl red (MR), VP, and citrate utilisation (IMViC) test was used to identify *E. coli* and *Klebsiella* spp. (Barrow and Feltham, 1993; Quinn *et al*., 2013).

The DNA was extracted from the 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. The DNA samples with a concentration ranging from 60-400ng/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 good quality and pure with least protein and reagent contamination and were selected for further the study.

The extracted DNA were then subjected to genotypic characterisation by amplification of *cns* gene using PCR. For this, five microlitres (µL) of genomic DNA template was used along with 10 pmol/L of each of the primers (F:TATCCACGAAACTTCTAAAAC AACTGTTACT and R:TCTTTAGATAATACG TATACTTCAGCTTTGAATTT) (Okolie *et al*., 2015) and 12.5 micro litres (µL) of Sapphire Amp Fast PCR master mix (2X PCR Smart mix, Takara, Japan) in a total reaction volume of 25 µL.The PCR products were analysed on 1.2 per cent 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).

The Kirby Bauer disc diffusion method (Bauer *et al*., 1966) was used to assess the *in*

vitro antibiogram profile of the CNS isolates, against nine commercially available antibiotic discs (HiMedia Laboratories, Mumbai). Following the criteria of the Clinical Laboratory Standards Institute (CLSI, 2017), the sensitivity or resistance was interpreted based on the zone of inhibition, which is inclusive of the disc diameter of six millimetres.

Among the 67 bacterial isolates obtained in the study, Gram positive cocci were the most numerous (88.06 per cent) compared to Gram negative bacilli (11.94 per cent). This was in consonance with the findings of Kulangara *et al*. (2017) who reported that Gram positive cocci (70.4 per cent) was the major pathogen responsible for persistent infections in dry bovine udders.Forty nine out of the 59 Gram positive cocci obtained resembled bunches of grapes and were catalase positive and oxidase negative suggestive of *Staphylococcus* spp. These isolates (n=49) were then streaked on to MSA for identification of *S. aureus* and CNS. After incubation, at 37ºC for 24 h, 22 isolates produced yellow colonies with yellow discolouration of media whereas, 27 isolates showed red colonies with no discolouration of media indicating 44.89 per cent mannitol fermenters and 55.10 per cent mannitol non-fermenters. Thus, the yellow colonies on MSA were apparently identified as *S. aureus* and the red colonies as other *Staphylococci* spp.All the presumptively identified staphylococci(n= 49) that were catalase positive and oxidase negative weresubjected to coagulase test to differentiate *S. aureus* and CNS and 27 isolates were found to be CNS.

Microbiological analysis, using morphological, cultural and biochemical characterisation revealed that CNS were the most predominant (40.31per cent) bacteria, followed by *Staphylococcus aureus* (32.83per cent), *Micrococci* spp. (11.94 per cent), *Klebsiella* spp. (7.46 per cent) *Escherichia coli* (4.48 per cent) and Streptococci (2.98 per cent).Singh and Kumar (2022) observed similar findings in cows with subclinical mastitis from three districts in the Bundelkhand region. The present study showed a substantial increase in the prevalence of CNS (40.31per cent) compared to those of Sebastian (2001) and Rathish (2014), who could isolate only 6.4 per cent and 23.81 per cent of CNS from all bacterial isolates from bovine mastitis cases, respectively.High prevalence of staphylococcal infections in dairy cattle udder has previously been recorded in large dairy farms(Song *et al*., 2020) which could be attributed to the contagious nature of staphylococcal infections, but high prevalence in isolated small holder dairy systems practising hand milking by individual milkers indicates that staphylococcal infection is endemic among the cattle population of the study area. However, the occurrence of coliform mastitis is a reflection of poor management practices and hygienic conditions as *E. coli* originates in the cow environment and infects the udder through the teat duct.

Due to the variable expression of its phenotypic characteristics, most phenotypic identification methods are unable to precisely identify the bacteria, but several PCR amplicon-sequencing-based techniques have been described for identification of bacteria (Heikens *et al*., 2005). According to Okolie *et al*. (2015), a PCR assay targeting the *cns* gene signal could selectively detect the CNS with 100% specificity; hence, the same primer pairs were used in the current work. In the present study, all the 27 phenotypically identified CNS isolates yielded a PCR product with amplicon size of 204 bp and were confirmed as CNS (Fig.1).

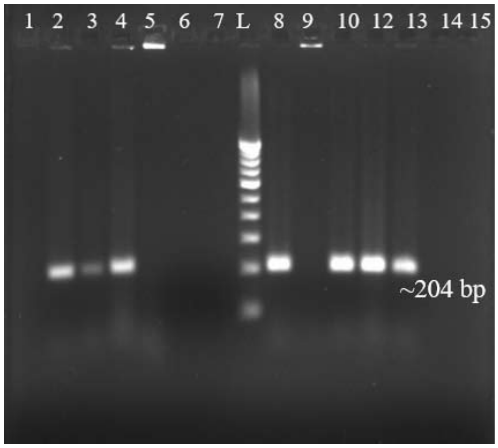

Fig 1. Agarose gel electrophoresis of *cns* gene
Lane L: DNA marker 100 bp
Lane 2,3,4,10,12,13: Positive samples **(204 bp)**
Lane 7:Negative control
Lane 8:Positive control
Lane 1, 5, 6, 9,14, 15: Negative samples

Monitoring the trends of antimicrobial resistance of bacterial isolates is critical for treatment decisions and prompt design of adequate resistance mitigation strategy design. It also aids in determining the emergence, persistence and possible risk of dissemination of antimicrobial-resistant bacteria and resistometo man, animals and the environment. In the present study, out of the 27 CNS, least number of isolates were found to be resistant to methicillin with four isolates (14.81 per cent) being resistant and a major proportion of the isolates were found to exhibit resistance towards penicillin (77.7 per cent) and amoxicillin-sulbactam (51.85 per cent). Thirteen isolates (48.15 per cent) were resistant to enrofloxacin, tenisolates (37.04 per cent) towards gentamicin and seven isolates (25.93 per cent) were found to be resistant to tetracycline. Moreover, eight isolates (29.63 per cent) each were being resistant ceftriaxone, ceftriaxone sulbactam and cotrimoxazole. Previous studies by Sebastian (2001) and Rathish (2014) on antimicrobial resistance pattern of CNS have also demonstrated poor sensitivity among the isolates to penicillin. Similar results were obtained by Soares *et al*. (2012) who found that CNS isolated from Brazilian dairy herds exhibited highest resistance towards penicillin (79 per cent), whereas least resistance was shown towards enrofloxacin (two per cent), 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 per cent of the CNS isolates were MDR with methicillin resistance being the most frequent resistant phenotype (47 per cent) followed by fusidic acid, tiamulin, penicillin, tetracycline, streptomycin and erythromycin.

The multidrug resistance among CNS strains is of tremendous concern as they could serve as a reservoir for resistance and virulence genes and results in considerable financial losses. Thisstudy also warrants the need for epidemiological and molecular studies, such as genotypic analysis for the precise identification of the organism and their antibiotic resistance genes.

Summary

Prudent use of antimicrobials for treatment and prevention of dairy cow mastitis is of paramount importance inorder to decrease the growth, persistence and transmission of drug resistant bacteria from dairy farms to humans, animals and farm environment. Alternative disease control strategies need to be further investigated owing to public health and food safety concerns about antimicrobial resistance and residues in meat and milk. Administration of core vaccines for prevention of endemic diseases, improved housing and management practices that lower the risk and impact of various infections, development of novel therapeutic strategies to combat drug resistance arethe alternate, yet effective strategies for preventing the emergence of antimicrobial resistant microbial pathogens in the dairy herd.

References

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, 331p.

Bauer, A. W., Kirby, M. M., Serris, J. S. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* **45**: 493-496.

Clinical and Laboratory Standards Institute. 2017. Performance standards for Antimicrobial Susceptibility testing (25th Ed.). Clinical and Laboratory Standards Institute, Wayne,15p.

DAHD [Department of Animal Husbandry and Dairying]. 2022. Annual Report 2021-22. Department of Animal Husbandry and Dairying. Ministry of Fisheries, Animal Husbandry and Dairying. Government of India. 183p.

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. *J. Dairy Sci*. **96**: 2247-2257.

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. *J. Clin. Microbiol.***43**: 2286-2291.

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.

Mukerji, S., O'Dea, M., Barton, M., Kirkwood, R., Lee, T. and Abraham, S.2017. Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact. *Essays Biochem.* **61**: 23–35.

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. *Mol. Cell Prob*. **29**: 144-150.

Oliver, Stephen P., Shelton E. M. and Bhushan M. J. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: Comprehensive review. *FoodbornePathog. Dis.* **8**: 337-355.

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

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

Sebastian, S. R., 2001. Clinicotherapeutic studies on bacterial mastitis in bovines. M.V.Sc. thesis, Kerala Agriculture University, Vellanikkara, Thrissur. 66p.

Sharma, N., Rho, G. J., Hong, Y. H., Kang, T. Y., Lee, H. K., Hur, T. Y. and Jeong, D. K., 2012. Bovine mastitis: An Asian perspective. *Asian J. Anim. Vet. Adv*.**7**: 454-476.

Singh, B. and Kumar, R. 2022. Comparative efficacy and validation of different diagnostic methods in detection of subclinical mastitis in farms of Bundelkhand. *J. Vet. Anim. Sci*. **53**: 226-234.

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. *Pesqui. Vet. Bras*.**32**: 692-696.

Song, X., Huang, X., Xu, H., Zhang, C., Chen, S., Liu, F., Guan, S., Zhang, S., Zhu, K. and Wu, C., 2020. The prevalence of pathogens causing bovine mastitis and their associated risk factors in 15 large dairy farms in China: an observational study. *Vet. Microbiol.***247**: p108757.

Vijayakumar, K. and Jose, K. R. 2021. Phenotypic and genotypic characterization of *Staphylococcus aureus* isolated from bovine mastitis for biofilm formation. *J. Anim. Res.* **11**: 249-255. ■