RESEARCH ARTICLE

Antimicrobial Resistance and Biofilm Formation in Coagulase Negative Staphylococci Isolated from Bovine Clinical Mastitis

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Abstract

Bovine mastitis is a common and economically significant disease that affects dairy cows and buffaloes. Staphylococci are considered as a most common cause of bovine mastitis. The present research work was undertaken with the objective to isolate and identify coagulase-negative staphylococci (CoNS) by cultural, biochemical, and molecular methods from bovine mastitic milk samples, and to study their antimicrobial resistance patterns as well as the biofilm-producing ability. The investigation was carried out on 281 bovine clinical mastitis milk samples collected in and around Junagadh. Based on cultural and molecular methods, 34.87% (98/281) samples yielded *Staphylococcus* isolates. Through the conventional standard tube coagulation test, 16.72% (47/281) isolates were identified as CoNS. Mannitol fermentative patterns revealed 36.17% (17/47) CoNS isolates as fermentative, while 63.82% (30/47) CoNS isolates as non-fermentative. CoNS isolates showed high resistance against penicillin-G (64.95%), followed by erythromycin (57.34%), while the least resistance was observed against oxytetracycline (17.02%). Out of 47 CoNS isolates, 76.59% (36/47) were biofilm producers, while 23.40% (11/47) were non-biofilm producers.

Key words: Biofilm, Bovine, Clinical mastitis, CoNS.

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Introduction

Mastitis is defined as inflammation of parenchyma of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues (Radostits *et al*., 2000). In cattle and buffalo, mastitis is considered as major economic important disease worldwide including India (Das *et al*., 2018). Mastitis can be classified as clinical mastitis or subclinical mastitis (SCM) according to degree of inflammation (Viguier *et al*., 2009). Clinical mastitis is distinguished by sudden onset, changes in milk appearance and composition, decreased milk production and the presence of inflammation. Most cows with clinical and subclinical mastitis have milk samples that frequently contain the bacteria, *Staphylococcus* spp. (Pitkala *et al*., 2004; Tenhagen *et al*., 2006). They have been connected to the cause of subclinical and clinical mastitis and are categorized as coagulase positive staphylococci (CoPS) and coagulase negative staphylococci (CoNS) (De Buck *et al*., 2021). CoNS have been often considered as opportunistic minor pathogens, causing only mild clinical or subclinical mastitis, with only a marginal increase in somatic cell count (SCC) (Taponen *et al.,* 2006). However, in recent years, as a group, CoNS have become the most prevalent bacteria associated with bovine mastitis in many countries (Tenhagen *et al.,* 2006) and could be described as "emerging mastitis pathogens".

Antimicrobial therapy is commonly implemented for mastitis control and prevention. Unfortunately, despite using the most effective antibacterial remedies, failures of bacteriological cure are common due to antimicrobial resistance (Gomes and Henriques, 2016). CoNS are more resistant to antibiotics and are more prone to multi drug resistance. Only option for designing and implementing a therapeutic regimen is, to assess the antibiogram profile of the strains (Preethirani *et al*., 2015). Bacterial biofilms are the result of a genetically coordinated series of events that include initial surface attachment, microcolony formation and community expansion. Bacterial cells attach to the mammary gland epithelial cells and grow into colonies surrounded by an extracellular matrix, forming biofilms in bovine and ovine mastitis caused by staphylococci (Patel *et al*., 2014).

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Staphylococci are the most common cause of biofilm associated infections (Melchior *et al*., 2007). The biofilms formation was related to the virulence and pathogenicity of bacteria (Parasana *et al*., 2023). The aim of this investigation was to isolate and identify CoNS by cultural, biochemical, and molecular methods from bovine mastitic milk, and to study their antimicrobial resistance patterns as well as the biofilm-producing ability.

Materials and Methods

Sample Collection

A total of 281 milk samples were aseptically collected from bovine clinical mastitis cases in and around Junagadh district. Out of 281 milk samples collected, 103 were from dairy cows, while 178 were from buffaloes.

Isolation and Identification of *Staphylococcus* spp.

All the mastitic milk samples were cultured for primary isolation. Initially 0.1 mL of milk sample was enriched in brain heart infusion (BHI) broth for 6 h at 37 °C and then streaked on brain heart infusion agar and incubated at 37°C for 48 h. The yellow/white colonies grown on BHI agar were examined for its cultural characters. Bacteria on BHI agar were identified tentatively according to conventional methods including Gram staining, colony characters and catalase test. For molecular identification of *Staphylococcus* spp. primers targeting *16S rRNA* gene described by Mason *et al*. (2001) were used.

Coagulase Production

The coagulase production by staphylococci was detected using tube coagulase test. All the isolates to be tested for coagulase production were incubated overnight in BHI broth at 37°C. The tube coagulase test was conducted by adding 0.1 mL of the overnight BHI broth culture to 0.5 mL of rehydrated coagulase plasma (the contents of one vial of coagulase plasma [FD248] rehydrated aseptically with 3 mL of sterile distilled water) taken in a small test tube. After gentle mixing, the tubes were incubated at 37°C and observed for the formation of fibrin clots at intervals of 1 h, 4 h, and after overnight incubation. Simultaneously negative control was maintained to interpret the results. The test was considered positive, if there was any degree of clot formation and if the plasma was converted into stiff gel that remained in place when tube was tilted or inverted. No degree of clotting was considered as negative. The formation of fibrin clots indicates coagulase positive *Staphylococcus* spp. and those with no fibrin clot formation were identified as coagulase negative *Staphylococcus* spp.

Mannitol Fermentation

Mannitol fermentation was detected by culturing coagulase negative staphylococci on Mannitol salt agar (MSA). The yellow coloration of the colonies along with media was considered as mannitol fermenters.

Antimicrobial Sensitivity Test

All the CoNS isolates obtained from the clinical mastitis cases were subjected to *in vitro* antibiotic sensitivity testing as per Kirby-Bauer method. Zones of inhibition were measured in millimeter (mm) and compared with zone size interpretative table furnished by manufacturer as per the Clinical Laboratory Standards Institute guidelines. Total 8 antimicrobials from various antibiotic classes, *viz*., oxytetracycline, gentamicin, ceftriaxone, penicillin G, ampicillin-sulbactum, erythromycin, levofloxacin and methicillin were used to perform antimicrobial sensitivity testing. The discs were procured from Hi Media Lab Pvt Ltd., Mumbai.

Microtiter Plate Assay for Detection of Biofilm Formation

The experiment was performed using polystyrene flat bottom microtiter plates based on the techniques described by Ebrahimi *et al*. (2013). The mean optical density (OD) of the negative control +3 standard deviations of negative control was considered as the cut-off (ODc). The biofilm producers were categorized as: Non-biofilm former: ODs ≤ ODc, Weak biofilm former: ODc < ODs ≤ 2 × ODc, Moderate biofilm former: 2 × ODc < ODs ≤ 4 × ODc, Strong biofilm former: ODs > 4 × ODc, Where ODc = cut-off OD and ODs = Mean OD of sample.

Results and Discussion

In present study, out of 281 mastitic milk samples, 98 isolates were identified as *Staphylococcus* spp. based on cultural and molecular methods. Out of 98 *Staphylococcus* isolates, 47 isolates were found negative for tube coagulase test, which were considered as a coagulase negative staphylococci (CoNS). In present study, we found overall 34.87% prevalence of *Staphylococcus* spp. and 16.72% prevalence of CoNS as a cause of bovine mastitis. Similar results were found by many scientists. Kudinha and Simango (2002) observed 22.9% prevalence of CoNS in clinical mastitis cases. Mahmoud *et al*. (2015) reported 22.6% *S. aureus* infections among 318 mastitic animals and 12.5% had CoNS infections. Singh *et al*. (2017) recorded 17.89% prevalence of CoNS from 95 bovine mastitic cases by analyzing all quarters of infected animals. Klibi *et al*. (2018) found 27.66% and 22.66% prevalence of staphylococci and CoNS, respectively, among 300 mastitic milk samples from cows.

Amongst the 47 CoNS isolates, 17 (36.17%) isolates were mannitol fermentative, whereas 30 (63.82%) isolates were mannitol non-fermentative (Fig. 1). Many scientists have reported CoNS as mannitol non-fermentative, but in our

investigation, we observed mannitol fermentative CoNS. Such findings are also supported by Kateete *et al*. (2010) and Thakur *et al*. (2017).

In the group of CoNS isolates, high level of antibiotic resistance was seen for penicillin G (65.95%), erythromycin (57.44%), methicillin (48.93%), amoxicillin/sulbactum (44.68%) and gentamicin (42.55%), while low level of resistance was seen against ceftrixone (38.29%) and levofloxacin (36.17%). The isolated CoNS showed least resistant against oxytetracycline (17.02%). Similar results were also found by many scientists. Moniri *et al*. (2007) reported that penicillin had the highest rate of resistance (56.6%) against CoNS among 96 isolates from mastitic milk samples. Bal *et al*. (2010) isolated and identified 100 coagulase negative staphylococci, which revealed the highest resistance to penicillin G (58%), ampicillin (48%), neomycin (20%) and oleandomycin (14%). Kenar *et al*. (2012) reported resistance to erythromycin, penicillin and gentamicin at 73.2%, 58.3% and 53.8%, respectively, among 67 CoNS isolates.

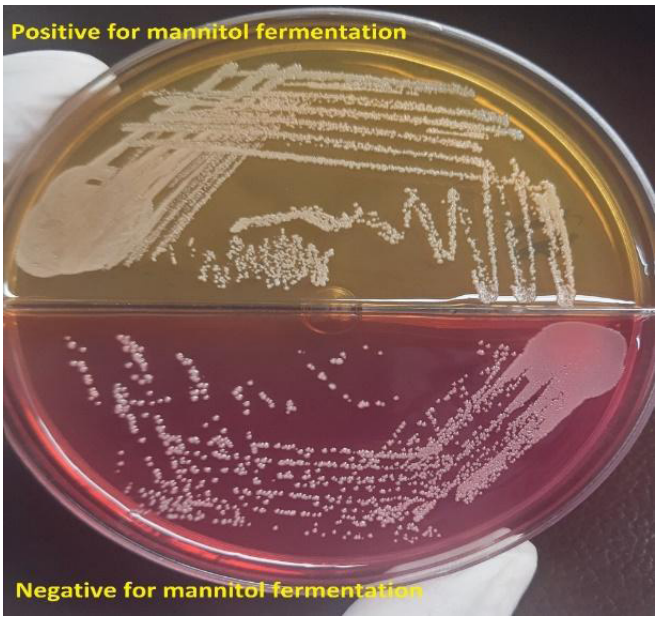

Fig. 1: CoNS on Mannitol salt agar (MSA) after 24 h of incubation at 37°C

In present study, out of 47 coagulase negative isolates, 11 (23.40%) were non-biofilm formers, 26 (55.32%) were weak biofilm formers, 07 (14.89%) were moderate biofilm formers and 03 (6.38%) were strong biofilm formers (Fig. 2). Overall, in our study around 76.59% isolates of CoNS demonstrated an ability to produce biofilm. Lee and Lee (2022) in their study of CoNS also observed 45.1% isolates as weak biofilm formers, 14.2% isolates as moderate biofilm formers and 19.1% isolates as strong biofilm formers. Tremblay *et al*. (2013) reported 85.1% of the CoNS isolates with biofilm forming ability, while 14.9% with no ability to form biofilm.

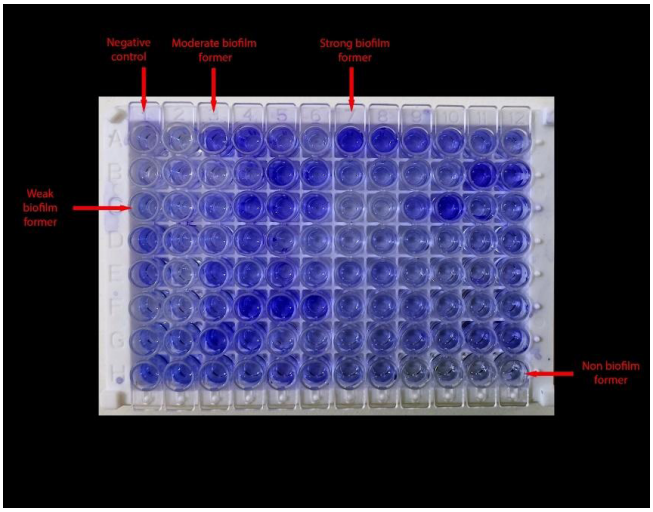

Fig. 2: Microtiter plate assay for biofilm

Conclusion

This study indicates the higher prevalence of biofilm formation amongst CoNS, which leads to higher antibiotic resistance. We recommend determination of biofilm formation for creating an effective treatment plan and prognosis of bovine mastitis.

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