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Antimicrobial Resistance and Biofilm Production Potential of *Staphylococci* from Bovine Mastitis in Andhra Pradesh

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ABSTRACT

Bovine mastitis is a frequent cause of economic loss to dairy farmers. This study is aimed at investigating the biofilm formation ability and antimicrobial resistance of *Staphylococci* from bovine mastitis. Among a total of 125 *Staphylococcal* isolates obtained from cows and she buffaloes with clinical and subclinical mastitis, 45 were coagulase positive (CPS) and 80 were identified as coagulase negative (CoNS) by tube coagulase test. Considerably high proportion of *Staphylococcal* isolates (56/125, 45%) formed biofilms on Congo red agar and when these isolates were screened for biofilm genes (*icaA, bap, icaAB, aap*), only four (7.2%) were found to possess *bap* gene. The *icaA, icaAB* and *aap* genes were not detected in any of the isolates. Majority of the CPS and CoNS isolates from our study (around 96%) were susceptible to ciprofloxacin and ceftriaxone, but most of them were resistant to gentamicin (100% of CPS and 92.5% of CoNS). The isolates (49/125, 39.2%) that showed resistance to cefoxitin were phenotypically identified as methicillin resistant, out of which 10 were MRSA and 39 were CoNS. In PCR for *mecA* and *mecC* genes, only eight isolates (8/125, 6.4%) of *Staphylococci* were found to possess *mecA* gene. None of the isolates carried *mecC* gene. The results suggest that the CoNS isolates (44.8%) from bovine mastitis had the potential to form biofilms and has considerably high (49%) methicillin resistant phenotype though only 6.25 per cent of them carried *mecA* gene and could be confirmed as MRCoNS.

HIGHLIGHTS

- Only 6.4% of the *Staphylococcal* isolates possessed *'mecA'* gene that confers methicillin resistance.
- 44.8% of the CoNS isolates showed biofilm production ability on Congo red agar.
- Only in 7.2% of the *Staphylococcal* isolates *'bap'* gene was detected.

Keywords: Bovine mastitis, CoNS, biofilm, methicillin resistance, *mec A, bap.*

Mastitis affects the health of dairy cows resulting in decreased milk production and quality. *Staphylococci* are the major cause of mastitis in different countries around the world. CoNS have traditionally been considered to be of minor pathogenicity, but in recent years their role as cause of bovine mastitis is increasingly evident. Infections with CoNS results in tissue damage, decrease in milk production and persistent intramammary infections, and CoNS have been isolated from milk samples collected from cows with clinical and subclinical mastitis in several countries (Srednik *et al.,* 2015). Virulence of *S. aureus* and CoNS in mastitis and other infections is attributed to biofilm formation, which confers resistance to antibiotics and offers protection against hostile environments. Biofilm production is dependent on the presence of genes such as *icaA, icaB, icaAB, icaC, icaD, bap, aap, fbe, embp* and *atlE* that code for biofilm-associated proteins and extracellular polysaccharide substances (Tremblay *et*

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al., 2013). The intracellular adhesion locus '*icaABCD*' encodes the proteins responsible for the synthesis of poly-N-acetyl glucosamine (PNAG), '*bap*' gene encodes the biofilm-associated protein, and '*aap*' the accumulation-associated protein. Several antibiotic resistance mechanisms have been described among CoNS strains from bovine mastitis, including β-lactamase production encoded by *blaZ* gene and production of low-affinity penicillin-binding proteins (PBP2a) encoded by *mecA* or *mecC*. The *mecA* gene confers methicillin resistance. Currently, *β*-lactam antimicrobials, aminoglycosides, and macrolides are commonly used to treat mastitis. Resistance to these antibiotics has been increasingly reported in CoNS associated with bovine mastitis. This study involves antimicrobial susceptibility testing, screening for biofilm production of the *Staphylococcal* isolates from bovine mastitis and detection of resistance and biofilm genes.

MATERIALS AND METHODS

Collection of samples

A total of 237 milk samples were collected from cows and she buffaloes with different parity, age and postpartum period affected with mastitis, randomly from different regions of Andhra Pradesh during the period from April 2019 to September 2019. Milk samples were streaked on Mannitol salt agar (MSA) and Mac Conkey agar (MAC) plates and incubated at 37ºC for about 48 hr. Single colonies were identified based on Gram's staining, colony morphology and catalase test. The *Staphylococcus* species were further differentiated by tube coagulase test.

Genomic DNA extraction

Genomic DNA was isolated using lysis method (boiling method). Three ml of overnight broth culture was taken and cells were pelleted by centrifugation at 10000 rpm for 10 min. The pellet was washed with PBS by centrifugation at 10000 rpm for 10 min. The pellet was resuspended in 200 µl of TE buffer by gentle mixing and boiled at 95 °C for 20 min. This was followed by snap chilling at -20 °C for 10 min. The suspension was centrifuged at 10000 rpm for 10 min and the resultant supernatant containing DNA was stored at -20 °C for use in PCR protocols.

Screening for biofilm production ability

All the *Staphylococcal* isolates were checked for their ability to produce biofilm using modified Congo red agar (Mariana *et al*., 2009). Congo Red Agar plates were inoculated with the *Staphylococcal* isolates and incubated at 37 °C for 48 hr initially and subsequently at room temperature for 2-4 days. The slime producing strains form strong black pigmentation to slightly black colonies, which indicates positive for biofilm production, where as non-slime producers develop red colonies which indicates negative for biofilm production.

Detection of biofilm genes by PCR

The S*taphylococcal* isolates that were found to form biofilm on Congo Red agar were screened for the presence of biofilm genes viz., *icaA, bap, icaAB* and *aap,* which are majorly associated with the biofilm production. The oligonucleotide primers, PCR cycle conditions and the corresponding amplicon sizes for the biofilm genes were mentioned in Table 1.

First the isolates were screened for *icaA* and *bap* genes in duplex PCR as per the method of Janeczko *et al*. (2014). Further, the *Staphylococcal* isolates that showed positive biofilm phenotype were also screened for the presence of *icaAB* gene (Iorio *et al*., 2011) and *aap* gene (Srednik *et al*., 2017) in two separate PCR tests.

Phenotypic characterization of antibiotic resistance

For all the *Staphylococcal* isolates, antimicrobial susceptibility test was performed by the standard Kirby Bauer disc diffusion method on Muller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). The isolates were tested for susceptibility to the following antibiotics viz., cefoxitin (CX30), ceftriaxone (CTR30), ciprofloxacin (CIP5), gentamicin (GEN10), oxacillin (OX1) and penicillin G (P10). CoNS isolates

Table 1: Oligonucleotide primer sequences for PCR

Target Gene	Primer sequence 5'-3'	Amplicon size (bp)	Reference & PCR conditions
icaA	*icaA*-F TCTCTTGCAGGAGCAATCAA	188	**Janeczko *et al*. (2014)**
	icaA-R TCAGGCACTAACATCCAGCA		95 ºC for 3 min, 35 cycles of 95 ºC for 30 sec, 52 ºC for 30 sec, 72 ºC for 1 min and finally 72 ºC for 5 min.
Bap	*bap*-F CCCTATATCGAAGGTGTAGAATTGCAC	971	
	bap-R GCTGTTGAAGTTAATACTGTACCTGC		
icaAB	*icaAB*-F TTATCAATGCCGCAGTTGTC	546	**Iorio *et al*. (2011)**
	icaAB-R GTTTAACGCGAGTGCGCTAT		94 ºC for 3 min, 30 cycles of 94 ºC for 1 min, 58 ºC for 1 min, 72 ºC for 1 min and finally 72 ºC for 5 min.
Aap	*aap*-F GAAGCACCGAATGTTCCAACTATC	289	**Srednik *et al*. (2017)**
	aap-R AGTTGGCGGTATATCTATTGTA		94 ºC for 6 min, 30 cycles of 94 ºC for 30 sec, 52 ºC for 30 sec, 72 ºC for 30 sec and finally 72 ºC for 10 min.
mecA	*mecA*-F GTAGAAATGACTGAACGTCCGATAA	310	**Vishnu Priya *et al*. (2014)**
	mecA-R CCAATTCCACATTGTTTCGGTCTAA		94 ºC for 5 min, 30 cycles of 94 ºC for 45 sec, 60 ºC for 30 sec, 72 ºC for 30 sec and finally 72 ºC for 10 min.
mecC	*mecC*-F CATTAAAATCAGAGCGAGGC	188	**Paterson *et al*. (2012)**
	mecC-R TGGCTGAACCCATTTTTGAT		94 ºC for 5 min, 36 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 30 sec, finally 72 ºC for 5 min.

were tested for susceptibility to novobiocin (NV5) also. Cefoxitin (CX30) is used as a surrogate for *mecA* mediated methicillin (oxacillin) resistance.

Screening for antibiotic resistance genes

For *Staphylococcal* isolates, *mecA* and *mecC* (*mecA* homologue) genes that confer resistance to the b-lactam antibiotics were targeted. The oligonucleotide primers, PCR conditions and the corresponding amplicon sizes for the antibiotic resistance genes were mentioned in Table 1. All the *Staphylococcal* isolates were screened for the presence of *mecA* gene as per the method of Vishnu priya *et al*. (2014). The isolates were further subjected to *mecC* PCR as per the method of Paterson *et al*. (2012).

RESULTS AND DISCUSSION

A total of 207 bacterial isolates were obtained from clinical and sub clinical mastitis cases together. Bacteria isolated were *Staphylococcus* species (125/207, 60.4%) and gram negative bacteria (82/207, 39.6%). Majority of the mastitis cases in this region were due to *Staphylococcus* (60.4%) and gram negative bacteria were the cause of infection only in 39.6% of the mastitis cases. CoNS account for 64% (80/125) of the mastitis cases due to Staphylococcus.

Screening for biofilm production ability

Biofilm formation on Congo red agar was observed in 44.8 per cent (56/125) of the *Staphylococcal* isolates from the present study. The incidence of biofilm phenotype was relatively higher in CoNS isolates (37/80, 46.25%) than CPS (19/45, 42.22%) (Table 2). In contrast to this, Arciola *et al*. (2001) reported that the incidence of biofilm formation tested with CRA was higher in *S. aureus* (61%) than *S. epidermidis* (49%) isolates.

Table 2: Biofilm production ability among the *Staphylococcal* **isolates**

Sl. No	Bacterial species	Bacterial colonies on CRA	
		Black colonies (biofilm producers)	Red colonies (non-biofilm producers)
1	**CPS (%)**	19 (42.2)	26 (57.8)
2	**CoNS (%)**	37 (46.3)	43 (53.8)
	Total	56 (44.8)	69 (55.2)

Detection of biofilm genes by PCR

Out of the 56 *Staphylococcal* isolates that were found to form biofilm on Congo red agar only four (7.2%) were found to possess the *bap* gene as they showed the amplified product of 971 bp (Fig. 1). The other biofilm genes *icaA*, *icaAB* and *aap* were not detected in any of these 56 isolates. Although high percentage of the *Staphylococcal* isolates were phenotypically positive for biofilm production on CRA, biofilm gene (*bap*) is detected only in four of these isolates.

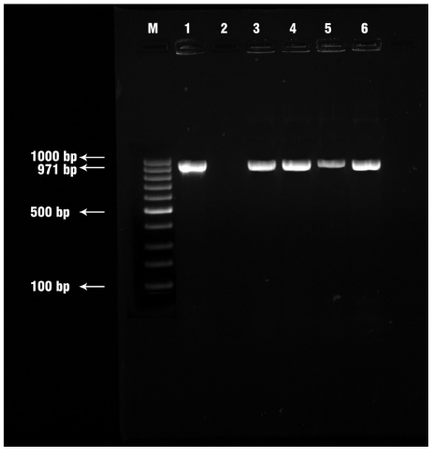

Fig. 1: Duplex PCR for biofilm genes. Lane M: Molecular weight marker (100bp); Lane-1: Reference strain of *bap* positive *S. aureus* from previous study as positive control; Lane-2: Negative control; Lane 3-6: Staphylococcal isolates from bovine mastitis showing 971 bp amplicon of *bap* gene

Phenotypic characterization of antibiotic resistance

Among the CPS isolates (45), resistance was high to gentamicin (45, 100%) followed by penicillin (13, 28.8%), cefoxitin (10, 22.2%) and oxacillin (4, 8.8%) and all of them were susceptible to ceftriaxone and ciprofloxacin. Among the CoNS isolates (80), resistance was observed to gentamicin (74, 92.5%), novobiocin (43, 53.8%), cefoxitin (39, 48.8%), penicillin (36, 45%), oxacillin (35, 43.8%). Most of the CoNS isolates were found susceptible to ceftriaxone and ciprofloxacin (Table 3). Resistance to novobiocin was observed in 53.8% (43/80) of the CoNS isolates. Among 45 CPS and 80 CoNS isolates, 10 CPS and 39 CoNS showed resistance to cefoxitin and hence they were regarded as methicillin resistant (MRSA and MRCoNS) as per CLSI recommendations.

Majority of the CPS and CoNS isolates from our study (around 96%) were susceptible to ciprofloxacin and ceftriaxone, but most of them were resistant to gentamicin (100% of CPS and 92.5% of CoNS). Resistance to cefoxitin was observed to be high among CoNS isolates (48.8%) when compared to CPS isolates (22.2%). Similarly, high percentage of CoNS isolates (43.8%) were oxacillin resistant than CPS isolates (9%). In contrast, Frey *et al*. (2012) observed low resistance to gentamicin (2.4%) and penicillin (23%) among CoNS isolated from bovine mastitis in Switzerland.

A previous study on bovine mastitis from our laboratory reported high sensitivity of *Staphylococcus* (CPS and CoNS) to ciprofloxacin, ceftriaxone, gentamicin and 50 per cent resistance to cefoxitin (Usharani, 2016). Contrastingly, Sumathi *et al*. (2008) observed that gentamicin was the most effective drug against *Staphylococci*, followed by ciprofloxacin. Chandrasekharan *et al*. (2014) also observed high sensitivity of *Staphylococcal* isolates to enrofloxacin followed by gentamicin and ceftriaxone. Development of resistance against a particular antibiotic in a specific region might be due to frequent and long term use and under dosage (Sabour *et al*., 2004, Moon *et al*., 2007 and Kumar *et al*., 2010).

Table 3: Antibiotic susceptibility of CPS and CoNS isolates

Antibiotic	CPS			CoNS		
	Resistant (%)	Intermediate (%)	Susceptible (%)	Resistant (%)	Intermediate (%)	Susceptible (%)
CX-30	10 (22.2)	—	35 (77.8)	39 (48.8)	—	41 (51.3)
P-10	13 (28.9)	—	32 (71.1)	36 (45)	—	44 (55)
GEN-10	45 (100)	—	—	74 (92.5)	—	6 (7.5)
CIP-5	—	1 (2.2)	44 (97.8)	2 (2.5)	1 (1.3)	77 (96.3)
CTR-30	—	2 (4.4)	43 (95.6)	3 (3.8)	13 (16.3)	64 (80)
OX-1	4 (8.9)	—	41 (91.1)	35 (43.8)	—	45 (56.3)
NV-5	—	—	—	43 (53.8)	—	37 (46.3)

Screening for antibiotic resistance genes

In PCR for *mecA* and *mecC* genes, only eight isolates (8/125, 6.4%) of *Staphylococci* were found to possess *mecA* gene with amplicons of 310 bp (Fig. 2) and they were confirmed as methicillin resistant. Out of these 8 *mecA* positive isolates, 3 were methicillin resistant *S. aureus* and the remaining 5 were methicillin resistant CoNS isolates. Out of these 8 isolates, 3 were coagulase positive and 5 were coagulase negative and in none of the isolates, the expected amplicon product of 188 bp was found and hence it is considered that they were all not carrying *mecC* gene.

Fig. 2: PCR for *mecA* gene. Lane M: Molecular weight marker (100bp); Lane-1: Reference strain of *mecA* positive *S. aureus* from previous study as positive control; Lane-2: Negative control; Lane 3-6: *Staphylococcal* isolates from bovine mastitis showing 310 bp amplicon of *mecA* gene

Out of the 49 *Staphylococcal* isolates that were phenotypically identified as positive for *mecA* mediated oxacillin resistance in the cefoxitin disc test, *mecA* gene was detected only in 6 isolates and other 2 isolates that were found to carry *mecA* gene were phenotypically susceptible to cefoxitin. None of the *Staphylococcal* isolates were positive for *mecC* gene indicating no prevalence of methicillin resistance by harboring *mecC* gene. All the eight *mecA* positive isolates were resistant to gentamicin and only six of them were resistant to cefoxitin and 5 to penicillin. But only 2 isolates were resistant to oxacillin, the other 6 *mecA* positive isolates (3 CPS, 3 CoNS) were found susceptible to oxacillin (1 mcg) disc.

In a similar study, Piessens *et al*. (2012) reported low incidence of *mecA* gene (11.7%) from 366 CoNS isolated from milk. Srednik *et al.* (2017) also reported low incidence of *mecA* gene (4) compared to *blaZ* gene (21) among CoNS (87) isolates from bovine mastitis. Srednik *et al.* (2015) also reported low incidence of *mecA* gene (6) when compared to *blaZ* gene (19) in CoNS (93) isolates from bovine mastitis.

According to the results of the present study, the prevalence of methicillin resistant phenotype was considerably high among CoNS (39/80, 49%) though only 6.25 per cent (5/80) of them carried *mecA* gene and could be confirmed as MRCoNS. As indiscriminate use of antibiotics was one of the reasons for development of resistance, there is necessity for the implementation of *in vitro* antibiotic susceptibility test prior to the use of antibiotics in the treatment and prevention of intra mammary

infections. Further investigation to establish the molecular basis of antibiotic resistance and biofilm potential is required.

CONCLUSION

The occurrence of biofilm producing and methicillin resistant phenotype is considerably high among the *Staphylococci* from bovine mastitis but, the *mecA* gene is detected only in 6.4% of them. Biofilm gene '*bap*'is detected in only 7.2% of the biofilm forming isolates.

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