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Methicillin resistance genes and in vitro biofilm formation among *Staphylococcus aureus* isolates from bovine mastitis in India

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ABSTRACT

Introduction: Biofilms, an assemblage of microbial cells irreversibly associated with a surface and enclosed in a matrix of polysaccharide material pose serious health challenges, resulting in high economic losses. The emergence of methicillin-resistant *S. aureus* (MRSA) infections and ability to form biofilms in dairy animals is of emerging concern for livestock and public health owing to their association with serious infections. The present study was undertaken to examine the presence of methicillin resistance genes among the biofilm forming *Staphylococcus aureus* strains isolated from cases of acute and subacute bovine mastitis. A total of 150 mastitic milk samples referred to Veterinary Clinical Complex, Shuhama (Aulesteng) SKUAST-K were screened in present study. The methicillin resistant *Staphylococcus aureus* isolates were also screened for in vitro biofilm forming ability.

Results: A total of 80 (53.33%) *S. aureus* isolates were recovered from cases of bovine mastitis of which 20 (25%) were methicillin (*mec*A) gene positive. Of the 20 *mec*A positive isolates, 20% were positive for SCC*mec* I, 35% for SCCm*ec* IV and 45% for SCC*mec* V subtypes. In vitro antibiotic sensitivity testing of MRSA revealed complete resistance towards methicillin and other pencillin group of antibiotics.

Conclusion: A significant correlation was observed between in vitro biofilm formation and presence of methicillin resistance gene in *S aureus* isolates recovered from acute and subacute mastitis. The *Staphylococcus aureus* isolates positive for methicillin resistance gene (*mec*A) were either strong or moderate biofilm formers.

1. Introduction

Mastitis, a major disease for dairy cattle is characterized by decreased milk quality, production, reduced profitability due to cost of treatment and milk discard [1]. Many Gram positive and negative pathogens are implicated in etiology of bovine mastitis. In most cases *Staphylococcus aureus* has been associated with subclinical and persistent intra-mammary infection which responds poorly to antibiotic treatment [2]. Biofilms are microbially derived sessile communities, characterized by cells attached to a substratum interface, to each other, embedded in a matrix of extracellular polymeric substance [3]. The production of biofilms by *S aureus* is under the control of intercellular adhesion operon (*ica* operon) that codes for polysaccharide intercellular adhesion (PIA). PIA independent factors like biofilm associated protein (bap), clumping factor A and B (Clf A and B) and fibronectin binding proteins A and B (FNBpA and FNBpB) can also promote biofilm production [4]. Biofilm production in *S. aureus* mastitis can be associated with antimicrobial resistance [5]. The resistance is attributed to physical and chemical diffusion barrier formed by exopolysaccharide matrix, hindering antimicrobial penetration, the existence of microenvironments that antagonize the antibiotic action, the activation of stress responses that cause changes in bacterial physiology, the slow and stable growth of these microorganisms due to nutrient limitation and the absence of antimicrobial targets [6]. Biofilms resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free floating bacteria [7].

β-lactam antibiotics are preferred for treatment of *Staphylococcal* infections. The production of β-lactamase enzymes and low-affinity penicillin binding protein (PBP2a), has led to increase antimicrobial resistance globally [8]. Methicillin resistant *S. aureus* (MRSA) infections

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are life-threatening due to multidrug resistance of such strains and ability to form strong biofilms [7]. Presence of glycocalyx in biofilm protects the enclosed bacteria from host defenses and impedes delivery of antibiotics. The resistance to methicillin in *S. aureus* is mediated by *mec*A gene, carried on the mobile genetic element *Staphylococcal* cassette chromosome *mec* (SCC*mec*). Based on the structural organization and diversity these elements are classified as SCC*mec* type I- SCC*mec* XI. Methicillin susceptible strains (MSSA) show increased biofilm production by expressing PIA whereas MRSA form biofilm in an *ica*-independent manner by secreting surface proteins or release of extracellular DNA [9]. Studies have revealed that presence or absence of *mec*A gene influences the expression of biofilm phenotype. SCC*mec* typing (I–V) of MRSA has revealed expression of an altered penicillin binding protein, PBP2a, with decreased affinity for β-lactam antibiotics, thus allowing continuous cell-wall assembly.

There is a need to study the presence of methicillin resistance in biofilm producing *S aureus* strains. Identification and efficient control protocol against biofilm forming MRSA can be one of the essential steps towards the prevention of the most serious MRSA infections [10]. The present study was undertaken to screen the *Staphylococcus aureus* isolates recovered from cases of bovine mastitic (acute or subacute) for in vitro biofilm production and presence of methicillin resistance gene (*mec*A) and SCC*mec* I-V subtypes

2. Material and methods

2.1. Bacterial isolates

A total of 150 mastitis milk samples were collected from Veterinary Clinics of FVSc and A.H and local villages of Shuhama in sterile 10 ml polypropylene tubes and transported to the laboratory on ice. The samples were subjected to bromothymol blue (BTB) test as per protocol of [11] to confirm the clinical status of collected milk samples. The animals were presented by owners to Veterinary Clinics for treatment of mastitis and sampling was done as a routine diagnostic protocol. No intervention was done to collect the samples from animals.

2.2. Isolation and identification of S. aureus

The samples were inoculated in tryptic soy broth (TSB) and incubated at 37 °C for 24 h. Enriched samples were streaked on nutrient agar plates followed by incubation at 37 °C for 24–48 hrs. The *S. aureus* isolates were identified by Gram staining and standard biochemical tests. DNA was extracted using heat lysis method (snap chill method) and confirmed by *nuc* gene specific PCR [12].

2.3. Detection of methicillin resistance

S. aureus isolates were screened for the presence of methicillin resistant genes viz; *mec*A and SCC*mec* genes; SCC*mec* I, SCC*mec* II, SCC*mec* III, SCC*mec* IV and SCC*mec* V (Table1) using a PCR assay as per [13].The PCR reactions were carried out in a 25 µl reaction volume in 0.2 ml thin walled PCR tubes in a Master cycler gradient (Eppendorf, Hamburg Germany). The reaction consisted of 2.0 µl template DNA, 2.5 µl of 10X buffer, 0.2 µl of 25 mMdNTP mix, 2.5 µl of 25 mM $MgCl_2$, 1 U of Taq DNA Polymerase (Fermentas Life Sciences), 1 µl of each primer and sterile nuclease free water. The cyclic conditions for *mec*A gene comprised of initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, followed by final extension at 72 °Cfor 7 min where as cyclic conditions of SCC*mec* I, SCC*mec* II, SCC*mec* III, SCC *mec* IV and SCC*mec* V gene comprised of initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 51 °C for 1 min and 72 °C for 1.5 min, followed by final extension at 72 °Cfor 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

Table 1
Primers sequences for screening *mec*A *and* SCC*mec* I-V genes Atshan et al. (2012).

Gene	Primer Sequence	Size
mec A	F-GTG AAG ATA TAC CAA GTG ATT R- ATG CGC TATAGATTGAAA GGA	147bp
SCC*mec* I	F- GCTTTAAAGAGTGTCGTTACAGG R-GTTCTCTCATAGTATGACGTCC	613 bp
SCC*mec* II	F- CGTTGAAGATGATGAAGCG R- CGAAATCAATGGTTAATGGACC	398 bp
SCC*mec* III	F- CCATATTGTGTACGATGCG R-CCTTAGTTGTCGTAACAGATCG	280 bp
SCC*mec*IV	F-GCCTTATTCGAAGAAACCG R-CTACTCTTCTGAAAAGCGTCG	776 bp
SCC*mec* V	F-GAACATTGTTACTTAAATGAGCG R-TGAAAGTTGTACCCTTGACACC	325 bp

2.4. In vitro antibiotic sensitivity testing

Invitro antibiotic sensitivity assay for methicillin resistant *S aureus* (MRSA) and methicillin sensitive *S aureus* (MSSA) isolates was performed by disc diffusion method [14]. A panel of fifteen comprising of methicillin (10 µg), penicillin (10 µg), ampicillin (10 µg), salbactam (10 µg), amoxicillin (30 µg), cefotaxime (30 µg), sufadiazine (300 µg), amikacin (10 µg), tetracycline (30 µg), vancomycin (30 µg), kanamycin (300 µg), gentamicin (10 µg), ciprofloxacin(5 µg), enrofloxacin (10 µg) and chloramphenicol (30 µg) were tested for their activity against *S aureus* isolates.

2.5. Determination of biofilm forming ability of isolates

Twenty MRSA and sixty MSSA isolates were tested for biofilm production by Tube assay, Microtiter dish biofilm assay and Congo red assay.

2.6. Tube assay

S. aureus isolates were screened for biofilm production using tube assay of [15]. Briefly, 2 ml of TSB was inoculated with loop full of *S. aureus* colonies and incubated for 48 h at 37 °C. The contents were decanted and tubes were washed with PBS and left to dry at room temperature. The tubes were stained with 4% crystal violet solution and rotated gently to ensure uniform staining. Stain was decanted and tubes were observed for biofilm formation. Positive result indicated presence of visible film lining the wall and bottom of the tube. Results were interpreted on basis of a score card (0-Absent, 1-Weak, 2-Moderate, 3-Strong biofilm).

2.7. Micro titer dish biofilm assay

The protocol of [16] was followed. Isolates were grown overnight at 37 °C in Tryptic Soy Broth (TSB) and diluted in 1:100 ratio into fresh TSB medium. 100 µl of the dilution was added /well in a 96 well microplate (Nunc) and incubated for 4–24 hrs at 37 °C. The cells were dumped out after incubation by turning the plate over and shaking out the liquid. The plate was submerged in water followed by shaking to remove unattached cells and media components. 125 µl of a 0.1% crystal violet solution was added to each well and the plate incubated at room temperature for 10–15 min. The plate were rinsed 3–4 times with water and blotted vigorously on a filter paper stack to remove excess cells and dyes. The plate was turned and dried for few hours or overnight. 125 µl of 30% acetic acid was added to each well to solubilize crystal violet followed by incubation at room temperature for 10–15 min. 125 µl of solubilized crystal violet was transferred to new flat bottomed microtiter plate and plate was read at 550 nm in an ELISA plate reader (Helsinki, Finland) using 30% acetic acid in water as

control. The results were interpreted on basis of mean OD values (mean OD values of < 0.120: Non /weak, 0.120-0.240, Moderate biofilm former > 0.240: strong biofilm former)

2.8. Congo red assay

Isolates were screened for biofilm forming ability using Congo red assay as per protocol of [15]. Congo red agar plates (Hi Media) were inoculated with a loop full of *S.aureus* colony followed by incubation at 37 °C for 24 h. Formation of black colonies with a dry crystalline consistency indicated a strong biofilm formation while as formation of red/orange red color indicated no biofilm formation.

2.9. Statistical analysis

Chi Square test [17] was used to test whether or not there was a statistically significant association between in vitro biofilm formation and methicillin resistance in *S aureus* isolates.

3. Results

A total of 80 (53.33%) *S. aureus* isolates were recovered from 150 mastitic milk samples on the basis of their colony characteristics on mannitol salt agar (MSA) and biochemical tests. All 80 *S. aureus* isolates were positive for tube coagulase test, morphological and biochemical characteristics as well as presence of *nuc* gene corresponding to presence of an amplicon size 270bp.

The *S. aureus* isolates obtained in present study were screened for methicillin resistant gene (*mec*A) and *Staphylococcal* cassette chromosome *mec* (SCC*mec*) SCC*mec* I-V subtypes. It was observed that 20 (25%) out of 80 isolates were positive for *mec*A gene (methicillin resistant) whereas remaining 60 were *mec*A negative (methicillin sensitive isolates). These twenty MRSA isolates were further screened for regulatory genes SCC*mec* I-V subtypes. The study revealed that 4(20%) isolates were positive for SCC*mec* I, 7 (35%) for SCC*mec* IV 9 (45%) for SCC*mec* V Figs. 1–4). None of the isolate was positive for SCC*mec* II *and* SCC*mec* III. The distribution of *mec*A and SCC*mec* I-V subtypes in MRSA isolates is depicted in Table 2.

3.1. In vitro antibiotic sensitivity

In vitro antibiotic sensitivity analysis of *S. aureus* isolates showed high sensitivity against Chloramphenicol (91.4%), Vancomycin (88.57%), Gentamicin (84.2%), Ciprofloxacin (82.8%), Amoxicillin (72.85%), Tetracycline (68%), Amikacin (61.42%) and Enrofloxacin (60%). A higher resistance was recorded against Methicillin (100%), Penicillin (99.2%), Ampicillin/salbactam (85.7%), Kanamycin (71.42%), Cefotaxime (54.28) and Sulphadizine (51.48%). Antibiotic sensitivity pattern of MSSA and MRSA isolates are depicted in (Figs. 5 and 6)

3.2. Biofilm assay

Of 20 MRSA isolates, strong biofilm formation was observed in 18 (90%), 17 (85%) and 16 (80%) isolates by tube, microtitre dish and congo red assay, respectively. The tube assay revealed that of the 20 MRSA isolates 14(70%) were strong biofilm formers, 4(20%) were moderate biofilm formers and 2 (10%) were non-biofilm formers. In case of 60 MSSA isolates 2(3.34%) were strong biofilm formers, 16(26.6%) were moderate biofilm formers and 42 (70%) were non-biofilm formers (Fig. 7). The mean OD values in microtiter dish biofilm assay reflected that out of 20 MRSA isolates, 13 (65%) were strong biofilm formers, 4 (20%) were moderate biofilm formers and 3 (15%) were non-biofilm formers. In case of 60 MSSA isolates, 3 (5%) isolates were strong biofilm formers, 8 (13.34%) were moderate biofilm formers and 49 (81.66%) were non-biofilm formers (Fig. 8). Congo red assay revealed that among the 20 MRSA isolates, 16 (80%) were moderate biofilm formers and 4 (20%) were non-biofilm formers. And of the 60 MSSA isolates, 8 (13.34%) were moderate biofilm formers and 52 (86.6%) were non-biofilm formers (Fig. 9).

The number of strong, moderate and non-biofilm forming phenotypes among 20 MRSA and 60 MSSA isolates using three biofilm expression techniques viz; tube, microtitre dish biofilm assay and congo red technique are depicted in Table 3. The chi square test statistic and the P value for all the three techniques is also expressed in the table. Higher proportion of strong and moderate biofilm formers were found to be associated with MRSA while higher proportion of non-biofilm and moderate biofilm formers are associated with MSSA in all the three techniques. Statistically for all the three techniques used, chi square test revealed a highly significant relation between the biofilm formation and methicillin resistance.

4. Discussion

Bovine mastitis is considered as the most common, economically unbearable and complicated disease in dairy animals worldwide [18]. *S. aureus* is one of the significant causes of udder infection in dairy animals [19]. Intramammary infections (IMI) with this pathogen may lead to clinical and sub-clinical mastitis associated with the increase in somatic cell number (SCC). The prolonged infections are related to microbial growth as adhesive colonies are enclosed by a large exopolysaccharide matrix, establishing a biofilm [20]. The resistance to

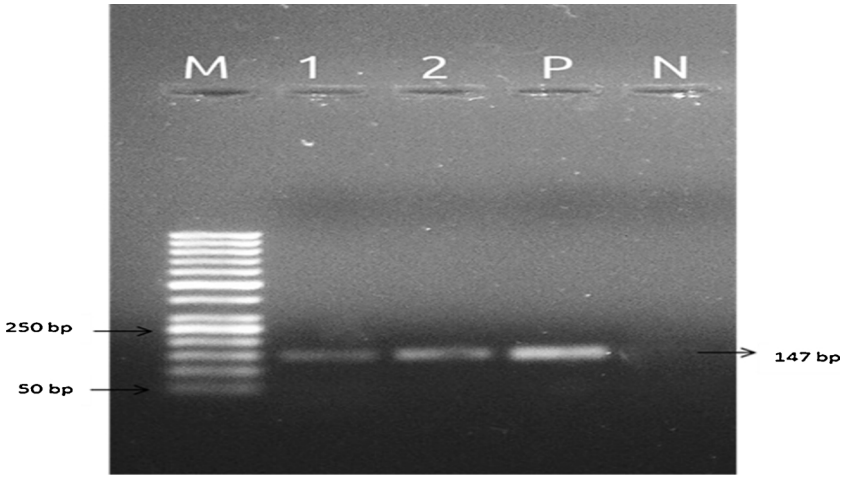

Fig. 1. *mec*A PCR. M: DNA marker, P: positive control, N: negative control, 1–2: positive isolates.

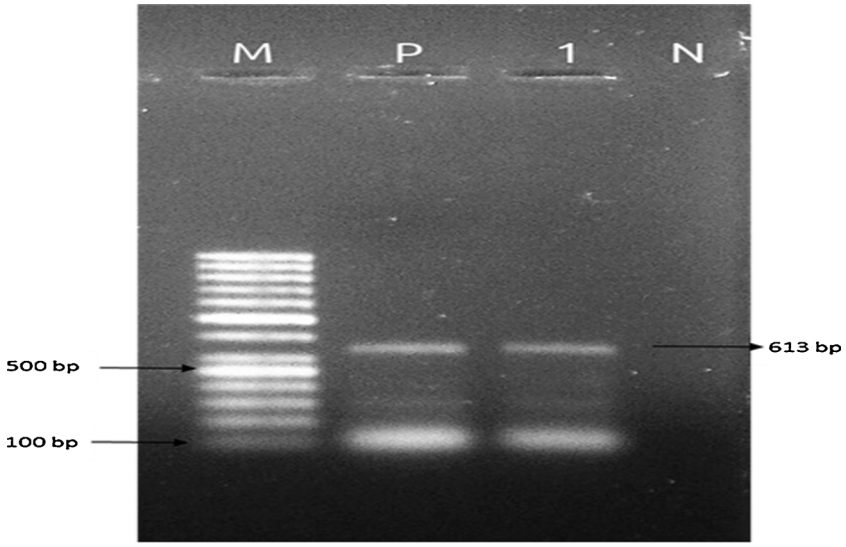

Fig. 2. SCC*mec* I PCR. M: DNA marker, P: positive control, N: negative control, 1–2: positive isolates.

antimicrobials is one of the most important striking features of biofilm forming infectious strains besides evasion of host defense mechanisms [21] which complicates the treatment of clinical cases [22].

In the present study biofilm production correlated well with methicillin resistance in present study as majority of MRSA isolates (85%) were strong to moderate biofilm formers while as only 25% of MSSA isolates were biofilm formers. A significant correlation ($P < 0.00001$) was found between biofilm formation and methicillin resisitance in Staphylococcus *aureus* isolates. Moreover, tube, microtitre and congo red techniques can be utilized to judge the biofilm forming abilities of *S. aureus* isolates in case of mastitis with microtire assay being most sensitive of the three biofilm assays. Biofilm formation has been attributed to severity of urinary tract infections, catheter infections, middle ear infections, dental plaques, gingivitis, endocarditis, cystic fibrosis and infections of joint prosthesis and heart valves [23]. Marchant et al. [24] concluded biofilm formation influences antibiotic resistance and infection persistence in association with central venous catheter (CVC) or other indwelling medical devices despite suitable antibiotic therapy.

A considerable increase in the prevalence of MRSA has been observed globally during the last decade [25]. β-lactam antibiotics usually target penicillin-binding proteins (PBPs) and prevent peptidoglycan synthesis. Methicillin / penicillin binding protein(PBP2a) is encoded by *mec*A gene, located in *Staphylococcal* cassette chromosome (SCC*mec*) at origin of replication shows low affinity towards β-lactam antibiotics and increases risk of biofilm formation.

The recent decade has witnessed an increasing incidence of MRSA and vancomycin resistant *S.aureus* strains besides an overall increase in resistance to many other antibiotics like penicilllin, ampicillin, streptomycin, tetracycline and oxy-tetracycline [26,27] thus making such infections life threatening. MRSA strains include both hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA) strains. MRSA has become a treatment challenge for veterinarians as well. The presence of persister cells in MRSA which are deeply embedded in biofilms where antibiotics fail to reach contribute to the severity of such infections [28].

Most commonly HA-MRSA strains carry *SCCmec* types I, II and III, while CA-MRSA strains carry *SCCmec* type IV and V [29]. Identification and efficient control protocol against biofilm forming MRSA is one of the essential steps towards the prevention of serious MRSA infections [8].

In present study 20 MRSA and 60 MSSA isolates were identified ob basis of *mec* A gene detection and antibiotic sensitivity test. These MRSA isolates were positive for regulatory genes SCC*mec* I, SCC*mec* IV and SCC*mec* V. SCC*mec* V was recorded as highest among the isolates followed by SCC*mec* IV. In a striking contrast to present study many researchers have reported that majority of MRSA isolates harbouring SCC*mec* V followed by SCC*mec* IV gene. Ghasemian et al. [30] screened

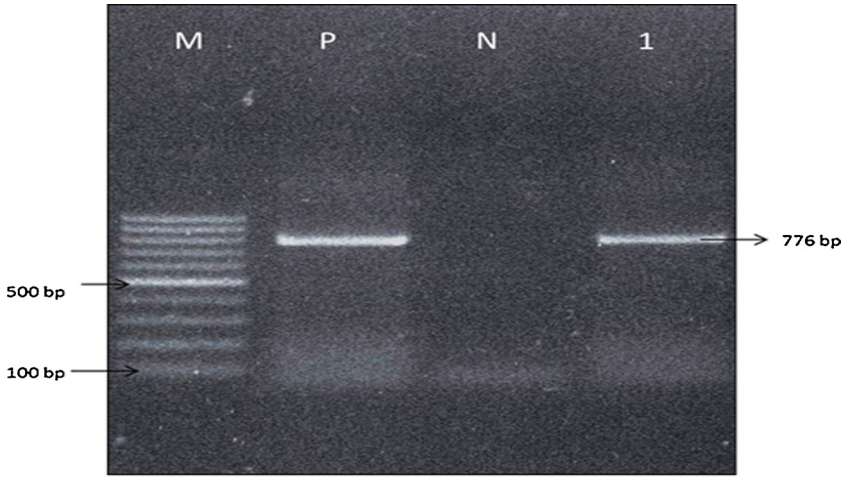

Fig. 3. SCC*mec* IV PCR. M: DNA marker, P: positive control, N: negative control, 1–2 : positive isolates.

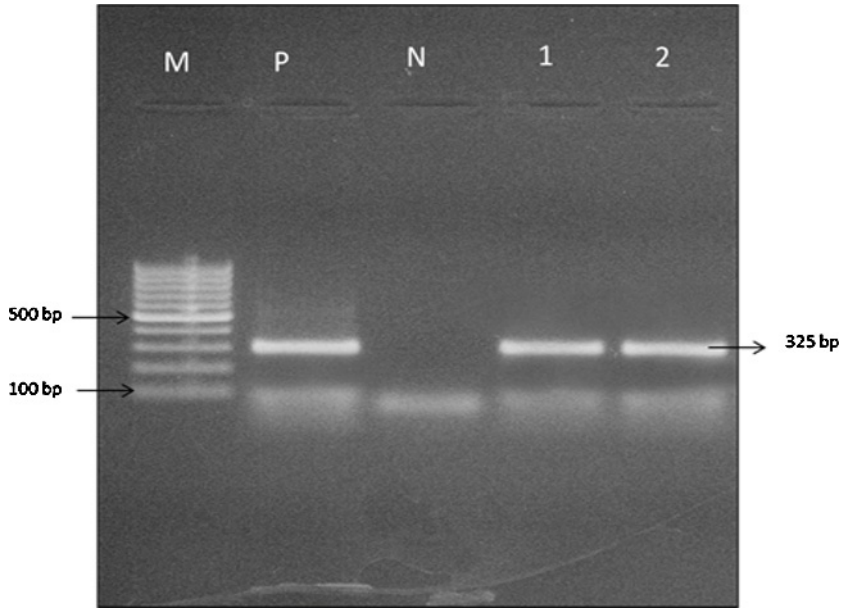

Fig. 4. SCC*mec* V PCR. M: DNA marker, P: positive control, N: negative control, 1–2 : positive isolates.

Table 2
Distribution of SCC*mec* I-V genes among MRSA isolates.

Staphylococcal cassette chromosome *mec* (SCC*mec*)	No. of *mec*A positive *S aureus* strains (n = 20)
SCC*mec* I	4/20 (20%)
SCC*mec* II	0
SCC*mec* III	0
SCC*mec* IV	7/20(35%)
SCC*mec* V	9/20(45%)

S. aureus isolates from 48 blood and wound isolates for methicillin resistant genes. It was found that 12 (25%) isolates harbored *SCC mec* III followed by *SCC mec* V. These biofilm positive *Staphylococcus aureus* isolates obtained from blood and wounds showed resistance towards ciprofloxacin, tetracycline, gentamicin, oxacillin, erythromycin and methicillin. Deurenberg et al. [31] performed molecular identifications of SCCmec type I-V I. The study revealed, types I, IV, V and VI encoded for resistance to β-lactam antibiotics only. In contrast, SCC*mec* types II and III possess multi resistance properties because these elements contained additional drug resistance genes carried on integrated plasmids such as pUB110 (kanamycin, tobramycin, and bleomycin resistance), pI258 (penicillin and heavy metals resistance), pT181 (tetracycline resistance), as well as a transposon Tn554 (carrying ermA gene, for inducible macrolide, lincosamide, and streptogramin (MLS) resistance). Zong et al. [32] found that SCC*mec* types III, IV and V isolated from clinical isolates were resistant to methicillin and other antibiotics. A strong association was observed between SCC*mec* type I and III and non- beta lactam antibiotics resistance where the overall resistance was found to be more common in type I isolates, due to the higher proportion of those particular isolates than isolates with type III.

5. Conclusion

Biofilms are of significant concern due to their involvement in many animal diseases, bovine mastitis being important owing to its great impact on livestock economy. The ubiquity of *Staphylococcus aureus* strains to form biofilms and inherent multi-drug resistance properties is a compelling force behind research for a better solution to combat antimicrobial resistance than use of antimicrobials (that have decreased penetration through biofilm matrix) and or disinfectants for treatment

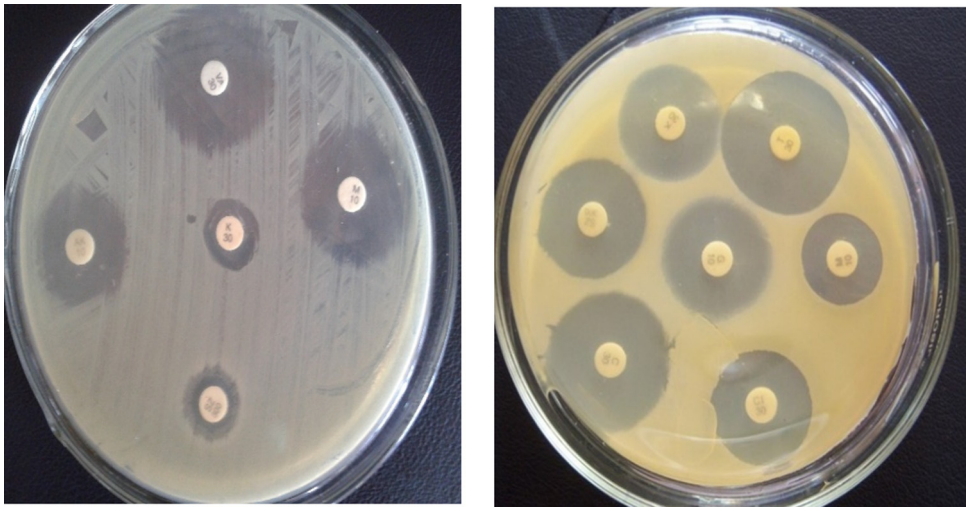

Fig. 5. In vitro antibiotic sensitivity (MSSA).

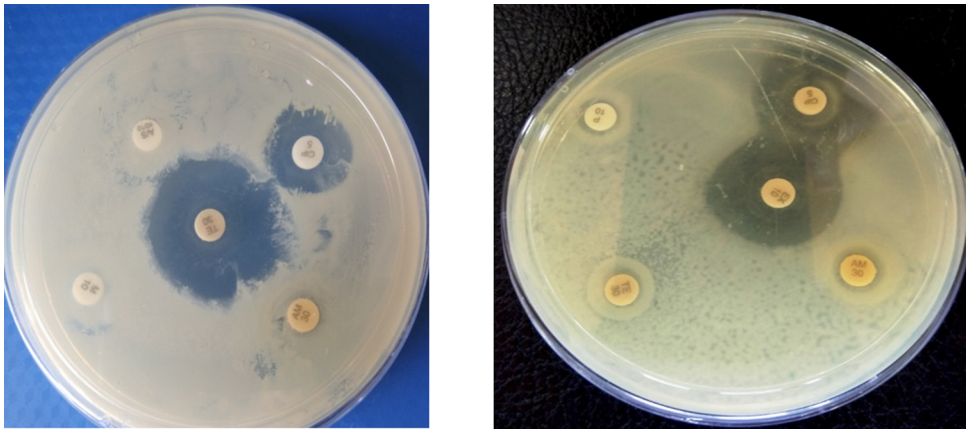

Fig. 6. In vitro antibiotic sensitivity (MRSA).

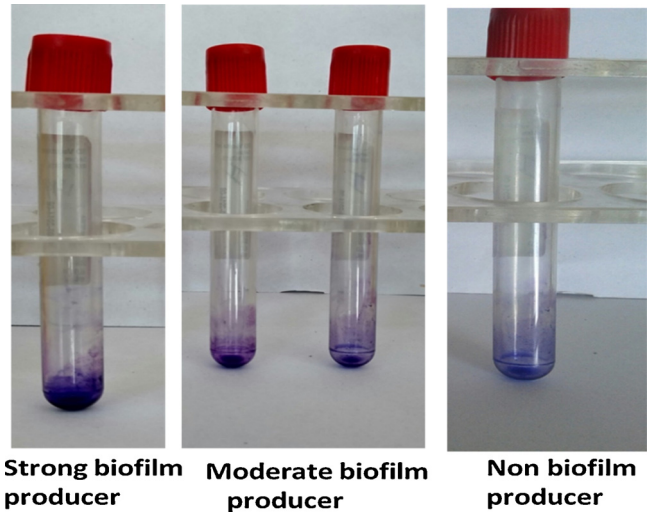

Fig. 7. In vitro biofilm formation in Tube assay.

of clinical and sub-clinical mastitis. Methicillin resistance among S. *aureus* strains restricts therapeutic options. The threat of MRSA infections results from not only the occurrence of multidrug resistance but also the emergence of bacteria that form strong biofilms. Strains of MRSA should be routinely screened for biofilm formation as well as biofilm formation studies may help us in understanding the resistance to antimicrobials in vivo. Studies that would determine the factors helping in colonization in teat would go along way in developing strategies for tackling the antibiotic resistance or alternatives for treatment for biofilm induced resistance.

Authors' contributions

SQ, ZK, SF were involved in designing, supervising and in interpreting the data; MSS,SMK, AH, BG, BA were involved in study execution. MSB was involved in the collection of data; SQ wrote the manuscript. SAW critically revised the manuscript. AAK performed the statistical analysis. All authors have read and approved the final manuscript.

Compliance with ethical standards

NA.

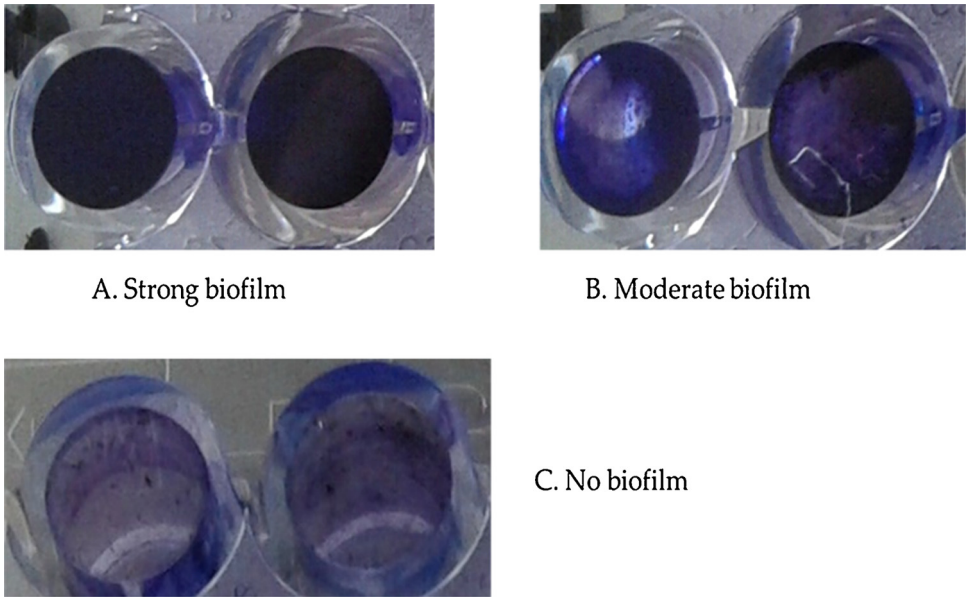

Fig. 8. In vitro biofilm formation in Microtiter dish biofilm assay.

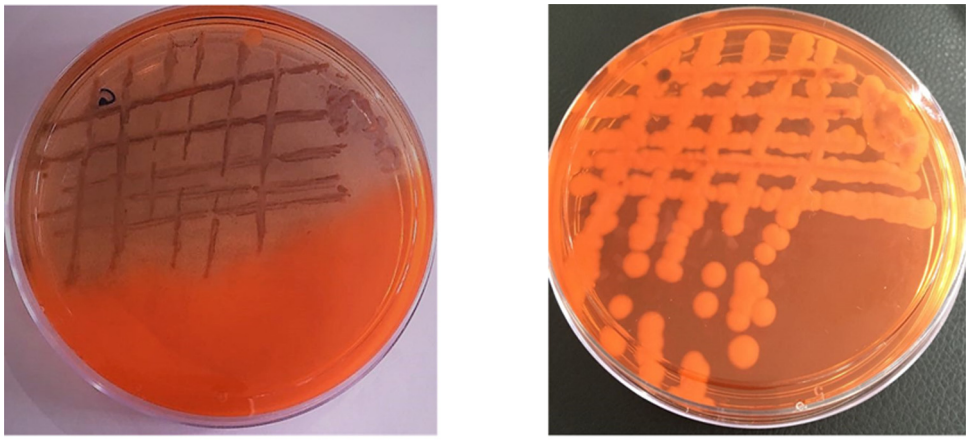

Fig. 9. Congo red assay.

Table 3
Comparative evaluation of three phenotypic tests in vitro biofilm expression techniques for MRSA and MSSA isolates.

Bio-film Characteristic	Tube Assay		χ^2	P value	Microtitre dish Biofilm Assay		χ^2	P value	Congo Red Assay		χ^2	P value
	MRSA isolates (n = 20) *mec*A positive	MSSA isolates (n = 60)			MRSA isolates (n = 20) mecA positive	MSSA isolates (n = 60)			MRSA isolates (n = 20) *mec*A positive	MSSA isolates (n = 60)		
Strong biofilm former	14 (70%)	2 (3.33%)	43.42	< 0.00001	13 (65%)	3 (5%)	37.70	< 0.00001	–	–	31.75	< 0.00001
Moderate biofilm former	4 (20%)	16 (26.66%)			4 (20%)	8 (13.34%)			16 (80%)	8 (13.34%)		
Non biofilm former	2 (10%)	42 (70%)			3 (15%)	49 (81.66%)			4 (20%)	52 (86.66%)		

Conflict of interest

The authors declare that they have no competing interests.

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