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Research Article

Biofilm Formation, Hemolysin Production and Antimicrobial Susceptibilities of *Staphylococcus aureus* Isolated from the Mastitis Milk of Buffaloes in Udaipur, India

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ABSTRACT

Mastitis is one of the most common problems of dairy animals throughout the world. Mastitis causes heavy economic losses to the dairy industry worldwide. Many etiological agents are responsible for this disease. *Staphylococcus aureus* is one of the major causes for mastitis in cow and buffalo. Cow and buffalo mastitis is an inflammatory reaction in the udder and a main contagious disease. The aim of present research is to determine the biofilm forming ability and antibiotic resistance in *Staphylococcus aureus* strains isolated from clinical cases of cow and buffalo mastitis in Udaipur of Rajasthan, India. A total 182 clinical mastitis milk samples were examined for bacteriological studies, out of this total 40 (21.9%) *S. aureus* strains were isolated. Within total 40 *S. aureus* isolates 30 (75%) were displayed hemolytic activities on blood agar. For determination of biofilm forming ability 40 clinical isolates of *S. aureus* were investigated with Congo Red Agar (CRA) and Tube assay methods. Out of the 40 *S. aureus* isolates, 67.5% displayed biofilm positive by CRA method and 62.5% by Tube assay method. In CRA method, within 27 positive isolates, 10 (25%) showed high and 17 (42.5%) moderate biofilm producing ability were observed and in Tube method 8 (20%) showed high and 17 (42.5%) moderate strains. The results of Antibiotic susceptibility test revealed that biofilm forming *S. aureus* strains were 62.5, 60 and 45% resistance against ceftriaxone (30µg), ceftizoxime (30µg) and erythromycin (10µg) respectively, whereas non biofilm forming isolates were observed 10, 10 and 75% respectively. Present research work will be helpful in improve production of milk with quality and quantity, health and solve problems related to cow and buffalo mastitis and also increase annual income of dairy owners. Detection of biofilm forming ability in mastitis isolates may provide useful information for the establishment of a more adequate therapeutic treatment.

Key words: *Staphylococcus aureus*, Biofilm, Bovine mastitis, Congo Red Agar method and Tube assay method

INTRODUCTION

Mastitis is one of the most widespread and critical disease of cow and buffalo throughout the world. Many etiological agents are responsible this disease. *Staphylococcus aureus* is one of the major causes for mastitis in cow and buffalo. Cow and buffalo mastitis, an inflammatory reaction in the udder, is a main contagious disease. Mastitis shows physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues of the udder of cow and buffalo. Mastitis causes numerous problems in milk production, quality, quantity and products which results in heavy economic losses to the dairy industry worldwide. India, annual economic loss incurred by the dairy industry on account of udder infections is estimated to be Rs. 6053.21 crores. In another report from India, the annual economic losses due to mastitis have been calculated to be Rs. 7165.51 crores (Radostits *et al.*, 2000; Dua, 2001; Leblanc *et al.*, 2006; Pdadmas, 2011; Sharma *et al.*, 2012 and Raza *et al.*, 2013).

One Hundred Forty (140) species of microorganisms were identified as etiological agents of Mastitis out of these major mastitis incidences were due to Staphylococci, Streptococci and Coliforms (Watts, 1988; Tenhagen *et al.*, 2006; Piepers *et al.*, 2007; Malinowski and Klossowska 2010; Smulski *et al.*, 2011; Zenebe *et al.*, 2014 and Marama *et al.*, 2016).

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Biofilm is structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert and living surface which enhance the secure growth in the environment. Many constant and frequent infections have been attributed to the formation of biofilm. Some strains of *Staphylococcus aureus* are capable of producing biofilm, a complex aggregation of microorganisms growing on a solid substrate. Biofilm production by Staphylococci is an important virulence factor. The key step in pathogenesis of Staphylococcal infection is the colonization and the formation of stable biofilm, which speculated that testing for biofilm formations could be a useful marker for the pathogenicity of Staphylococci (Grosserode and Wenzel 1991; Nickerson, 1993; Mulder and Degener 1998; Costerton *et al.,* 1999; Begum *et al.,* 2007; Krukowski *et al.,* 2008; Dhanawade *et al.,* 2010; Smith *et al.,* 2010; Nayak *et al.,* 2011 and Ebrahimi *et al.,* 2013).

Staphylococci, the ability to produce biofilm are the most important reason for unusual problems with eradication of infection and recurrent infection of mammary glands. Production of slime enables the adhesion of bacteria to the epithelium of mammary glands. It also facilitates persistence of micro-organisms in the host tissue by protecting the bacterial cells against the mechanisms of the host defense. Importantly, it causes the evident reduction of susceptibility to antibiotics, due to altered growth rate and delayed penetration of antimicrobial agents within the biofilm structure (Prakash *et al.,* 2003; Turkyilmaz and Eskiizmirliler 2006; Melchior *et al.,* 2006 a & b; Melchior *et al.,* 2007; Ciftci *et al.,* 2009 and Al-Rubaye *et al.,* 2016).

However, in the Rajasthan Mastitis disease is untouched by the scientists. So the study has been designed for detection of biofilm formation and antibiotics resistance in *Staphylococcus aureus* strains clinically isolated from milk of cow and buffalo mastitis in Udaipur. Present research work will be helpful in solving problems related to cow and buffalo mastitis, improving milk production, health of cows and buffalos and also increase annual income of dairy owners. Detection of biofilm forming ability in mastitis isolates may provide useful information for the establishment of a more adequate therapeutic regimen.

MATERIALS AND METHODS

Isolation and characterization of *S. aureus* from clinical mastitis

Total 182 clinical mastitis milk samples of cows and buffaloes were examined in bacteriological studies. Forty (40) *S. aureus* isolates recovered from 182 clinical mastitic milk samples. The isolates were identified by their cultural characteristics and colony morphology on blood agar, mannitol salt agar and microscopic appearance in Gram stained preparations, positive catalase reaction, OF (Oxidative-Fermentative) test, hemolysis. Further pathogenicity was determined by coagulase test assay with coagulase plasma (From rabbit) HiMedia.

Coagulase test

The coagulase test was performed with rabbit plasma 0.1 gm per vial (HiMedia Cat. No. FD 248). Rehydrate the contents of one vial aseptically with 3 ml sterile distilled water. Add 0.5 ml of rehydrated rabbit plasma in a tube. To this added approximately 0.05 ml of overnight broth culture of test organisms. Mix gently & incubates at 37°C in the incubator for up to 4 hours. Observe for clot formation in the tube at regular intervals. Any degree of clotting within 4 hours is considered as positive results.

Biofilm forming assay

Congo red Agar assay: Production of slime was studied by cultivation of all *S. aureus* strains on Congo red Agar media (Freeman *et al.,* 1989). Briefly, the medium was composed of Brain Heart Infusion Agar (37gm/ml), sucrose (50gm/ml), and Congo red stain (0.8gm/ml). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121 ºC for 15 min and was added when the agar had cooled to 55ºC. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. A Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the center of the colonies and background of medium black was observed. Red colony with no medium change considered as no biofilm producer strain.

Tube assay: A loopful of test organisms inoculated in 5 ml of trypticase soy broth with 1% sucrose in test tubes. Incubate the tubes at 37ºC for 48 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%), wash excess stain with distilled water and dried in inverted position. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate and 3-strong (Christensen *et al.,* 1982).

Antibiotic Susceptibility Testing

For Antibiotic susceptibility testing, isolates were incubated in Brain-heart infusion broth at 37˚C for 24 hours. The suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. Antibiotic Susceptibility was checked by the disc diffusion assay (Bauer *et al.,* 1966). About 100 µl of inoculums was spread on sterile Muller Hinton agar plates and antibiotics discs were placed on the inoculated surface. Then the plates were incubated at 37˚C for 24 hours.

All the clinical isolated *S. aureus* were tested for their sensitivity to 9 different antibiotics commonly used in the treatment of cow and buffalo mastitis. These included Amikacin (10µg), Azithromycin (30µg), Cefoperazone-sulbactam (2.5µg), Ceftriaxone (30µg), Ceftizoxime (30µg), Colistin (10µg), Enrofloxacin (10µg), Erythromicin (10µg), Gentamicin (50µg).

RESULTS

In present study 40 (21.9%) clinical isolates of *S. aureus* recovered from 182 milk samples of mastitis affected cow and buffalo. These isolates were gram positive and show hemolytic activity on blood agar (Fig.

1). On Mannitol Salt agar, *S. aureus* ferment mannitol and produce yellow colored colonies surround by yellow zone (Fig. 2). All strains found catalase positive test (Fig. 3). *S. aureus* strains showed positive coagulase activity (Fig. 4)

Biofilm production

In present study, out of the 40 *S. aureus* isolates, 67.5% showed the ability of biofilm formation by CRA method and 62.5% by the Tube assay method. The comparative analysis performed with proportion test concerning frequency of slime-producing strain incidence subject to a procedure employed, revealed that both experimental methods demonstrate a similar sensitivity. Still, 7 strains (17.5%) were found to produce biofilm only when the CRA method used while 5 strains (12.5%) only with the Tube method. In total, 32 strains showed the capacity to form slime (Table 1 and Table 2).

Table 1: Biofilm production by *Staphylococcus aureus* isolates

Strain	Number	Biofilm producing strains in			
		CRA method		Tube method	
		N	%	N	%
S. aureus	40	27	67.5	25	62.5

(N: Number of isolates of *S. aureus*, CRA method: Congo red agar method).

In CRA method, out of 27 positive isolates, 10 (25%) displayed black or almost black colonies with dry crystalline morphology which were considered as high biofilm producer (Figs. 5 and 7A), 17 (42.5%) displayed pink colony with center black pigmentation and a corner of colonies black (Figs. 6 and 7B) which were considered as moderate biofilm producer. Out of 40 isolates, 13 (32.5%) isolates showed red colony who considered as no biofilm producer (Figs. 5 and 7C).

In the Tube assay method, out of 25 positive isolates, 8 (20%) showed a high visible film lined the wall and bottom of the tube with ring formation considered as high biofilm producer and 17 (42.5%) showed visibly lined the wall and bottom of the tube considered as moderate biofilm producer. By this assay 15 (37.5%) isolates did not show a visible film considered as non-biofilm producer (Fig. 8 and Table 3).

Hemolytic activity

Out of 40 clinical isolates of *S. aureus*, 30 (75%) strains show hemolytic activity on blood agar. In present research biofilm producer strains showed high hemolytic activity compare to non-biofilm producer strains. Among 32 biofilm producer strains, 26 (81.25%) isolates exhibit hemolytic activity and out of 8 non biofilm producer strains, 4 (50%) showed hemolytic activity (Fig. 1 and Table 4).

Antibiotic Susceptibility

Antibiotic susceptibility of *S. aureus* revealed that 90, 80 and 77.5% strains show sensitivity to Gentamicin (50µg), Amikacin (10µg), Azithromycin (30µg) antibiotic, respectively and 62.5, 60 and 45% strains show resistance to Ceftriaxone (30µg), Ceftizoxime (30µg), Erythromicin (10µg) antibiotic, respectively (Table 5).

Table 2: Comparison of biofilm production ability of *S. aureus* by two methods

Strain	Number	Biofilm producing strains							
		Only in CRA method (a)		Only in Tube method (b)		In both (c)		Total (a+b+c)	
		N	%	N	%	N	%	N	%
S. aureus	40	7	17.5	5	12.5	20	50	32	80

(N: Number of isolates of *S. aureus*, CRA method: Congo red agar method).

Table 3: Screening of 40 *S. aureus* isolates for detection of biofilm formation by CRA and Tube assay method

No. of isolates (N)	Biofilm formation	Screening methods			
		CRA		Tube assay	
		N	%	N	%
	High	10	25	8	20
40	Moderate	17	42.5	17	42.5
	Weak/ non	13	32.5	15	37.5

(N: Number of isolates of *S. aureus*, No: Number, CRA method: Congo red agar method).

Table 4: Screening of 40 *S. aureus* strains for Hemolytic Activity

Biofilm producing strains			Non biofilm producing strains			Total no. of strains		
N	Hemolytic strains	%	N	Hemolytic strains	%	N	Hemolytic strains	%
32	26	81.25	8	4	50	40	30	75

(N: Number of isolates of *S. aureus*, No: Number, CRA method: Congo red agar method).

Table 5: Antibiotic Susceptibility pattern of 40 clinical isolates of *S. aureus*

Antibiotics	Concentration of antibiotics	Sensitive		Intermediate		Resistance	
		N	%	N	%	N	%
Gentamicin	50 µg	36	90	2	5	2	5
Amikacin	10 µg	32	80	1	2.5	7	17.5
Azithromycin	30 µg	31	77.5	1	2.5	8	20
Enrofloxacin	10 µg	29	72.5	7	17.5	4	10
Cefoperazonesulbactam	2.5 µg	27	67.5	3	7.5	10	25
Colistin	10 µg	24	60	0	0	16	40
Erythromycin	10 µg	19	47.5	3	7.5	18	45
Ceftizoxime	30 µg	16	40	0	0	24	60
Ceftriaxone	30 µg	11	27.5	4	10	25	62.5

(N: Number of isolates of *S. aureus*, µg: microgram).

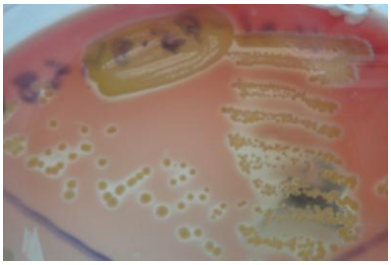

Fig. 1: Hemolysis on Blood agar.

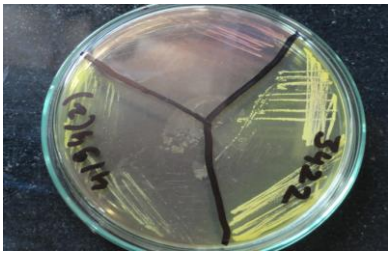

Fig. 2: Yellow colour colony of *S. aureus* on Manitol salt agar.

Fig. 3: Bubbling show Catalase positive*S. aureus*strain.

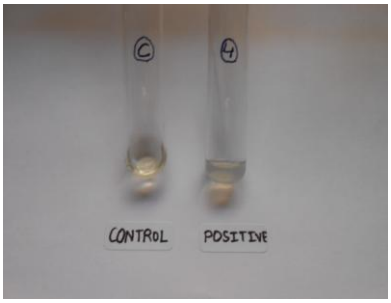

Fig. 4: Coagulase activity of *S. aureus* strain.Positive strain shows clot formation.

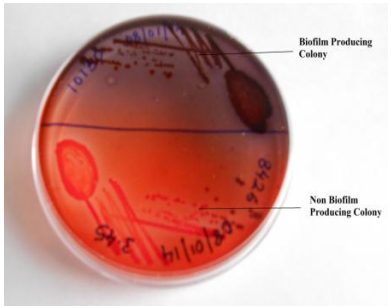

Fig. 5: On Congo Red agar Black colony indicate Biofilm producing colony and Red colony indicate Non biofilm producing colony.

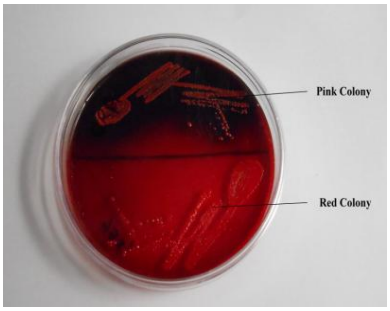

Fig. 6: On Congo red agar Pink colony turns medium colour to black and Red colony remains colour red.

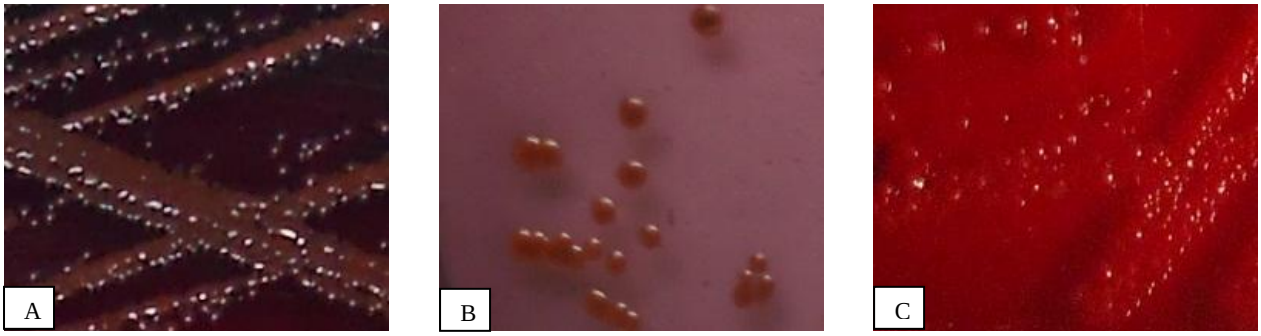

Fig. 7: Colony Morphology on CRA: (A) Black colony, (B) Pink colony with center black pigmentation, (C) Red colony.

In the present study revealed that biofilm producing strains displayed more resistance to antibiotics compared to non-biofilm producing strains. Out of total resistance isolates, 55, 50 and 37.5% biofilm producing strains resistance to Ceftriaxone (30µg), Ceftizoxime (30µg) and Erythromicin (10µg), respectively, where non-biofilm producing strains displayed 10, 10 and 7.5% resistance to respectively antibiotics (Table 6).

DISCUSSION

The result of present work showed that *S. aureus* showed higher in-vitro biofilm forming ability and able to produce hemolysin which responsible for hemolysis of blood cells. This finding indicates of higher pathogenicity of *S. aureus* isolates and concerning about spreading of mastitis in dairy animals. *S. aureus* belongs to the major pathogenic group of bovine mastitis. This pathogen can severely damage the bovine mammary gland as it secretes toxins that destroy secretory epithelial cells similar observation were also observed by other researchers (Watts, 1988; Nickerson, 1993; Malinowski and Klossowska, 2010; Smith *et al.*, 2010; Nayak *et al.*, 2011; Ebrahimi*et al.*, 2013; Zenebe*et al.*, 2014 and Marama *et al.*, 2016). Biofilm is a structural complex of bacteria in which they are enclosed and composed of a self-made polymeric matrix. These make connections to inert and free living surface which enhance the secure growth in the environment (Prakash *et al.*, 2003; Melchior *et al.*, 2006b; Nayak*et al.*, 2011 and Ebrahimi *et al.*, 2013).

The present observation of biofilm forming ability of *S. aureus* were showed 54.23% strains displayed biofilm forming ability by both determination methods. However, the present research work showed the higher biofilm forming ability of *S. aureus* strains in contrast to results of research work done by other scientists they observed 80% strains showed biofilm forming ability by CRA and Tube method (Turkyilmaz and Eskiizmirliler 2006; Begum *et al.*, 2007; Krukowski *et al.*, 2008 and Dhanawade *et al.*, 2010).

Table 6: Antibiotic Susceptibility of Biofilm forming (bf) and Non biofilm forming (Non bf) S. *aureus* isolates

Antibiotics	Concentration of antibiotics	No. of bf resistance isolates		No. of non bf resistance isolates		Total resistance isolates	
Ceftriaxone	30 µg	22	55	4	10	26	62.5
Ceftizoxime	30 µg	20	50	4	10	24	60
Erythromycin	10 µg	13	37.5	5	7.5	18	45
Colistin	10 µg	10	25	6	15	16	40
Cefoperazonesulbactam	2.5 µg	7	17.5	3	7.5	10	25
Azithromycin	30 µg	5	12.5	3	7.5	8	20
Amikacin	10 µg	5	12.5	2	5	7	17.5
Enrofloxacin	10 µg	4	10	0	0	4	10
Gentamicin	50 µg	2	5	0	0	2	5

(bf: biofilm formation, µg: microgram, No: Number).

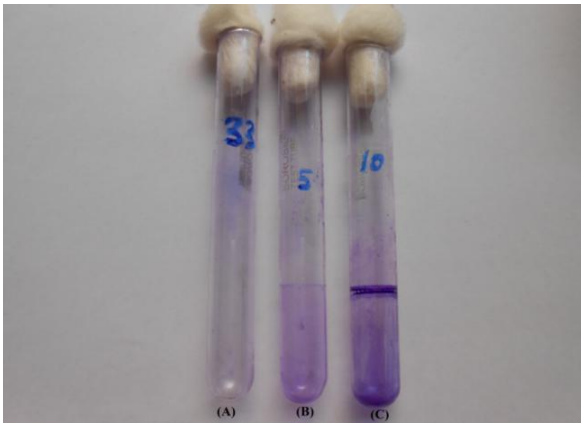

Fig. 8: Screening of biofilm producer by tube method. (A) Non biofilm producer (B) Moderate biofilm producer (C) High biofilm producer.

In this study, slime production was examined qualitatively, depending on colony morphology of 40 clinical isolates of *S. aureus* and produced on Congo Red Agar. Some differences between researchers were apparent with respect to interpretation of CRA test results. In that respect, both bright black colonies (Citak *et al.*, 2003) and black colonies (Jain and Agarwal, 2009) were considered as a positive result. However, previous research described the dry crystalline colony and turn medium red to black considered as higher biofilm producer and pink colony that turn medium red to the black and displayed center of the colony black pigmentation or colony corner black considered as moderate biofilm producer. Red colour colony with no colour change of the red colour of agar medium considered as non-biofilm producer strain. Screening of biofilm producing strains results displayed that out of 27 positive isolates, 10 (25%) higher biofilm producer and 17 (42.5%) moderate biofilm producer. So based on morphology of the colony and medium of Congo Red Agar researchers can easily differentiate biofilm producing and non-biofilm producing strains by CRA method.

Many researchers work done on biofilm forming ability of *S. aureus* and compare antibiotic resistance and hemolytic activity. The researcher's indicate that slime producing *S. aureus* displayed highly resistance to the antimicrobial agent's comparison to non-slime producing strains (Turkyilmaz and Eskiizmirliler, 2006). The present finding showed agreements with previous mentioned research work and make clear that biofilm forming *S. aureus* more resistance to antibiotics used. 71.43% Coagulase positive *S. aureus* produce hemolysis on blood agar and gentamicin inhibit the growth of all the isolates recovered from bovine mastitis were reported (Begum *et al.*, 2007). The present observation confirms that biofilm forming *S. aureus* more pathogenic compare to non-biofilm forming strains and shows high hemolytic activity. Present research work indicates that Gentamicin (50µg) reduce 90% the growth of *S. aureus* isolates recovered from clinical cases of cow and buffalo mastitis and Ceftriaxone (30µg) resistance to 62.5% strains. According to result of present research work suggested that during treatment of bovine mastitis ceftriaxone (30µg), ceftizoxime (30µg) and erythromycin (10µg) antibiotics could not recommend.

Conclusion

In conclusion, the result showed that the majority of *S. aureus* clinical isolates (80%) evaluated biofilm forming ability under *in vitro* conditions. Present research showed that isolates were more resistance to antibiotics and pathogenic when it forms biofilm. So results indicate that biofilm forming strains of *S. aureus* increase severe degree of inflammation in cow and buffalo and reduce antimicrobial sensitivity of antibiotics. The isolates showed low sensitivity to ceftriaxone (30µg), ceftizoxime (30µg) and erythromycin (10µg) antibiotics. This study will further allow for development of new strategies to a better management and use of antibiotics for reducing the frequency of cow and buffalo mastitis cause by pathogens.

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