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Emergence of Pathogenic Strains of *Staphylococcus aureus* in Goat Milk and Their Comparative Response to Antibiotics

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MIS, AIA and SUR designed the research. AA and SAM supervised the research. IKS, RS and JA did sampling. IM and MAN did lab work and wrote the article. MS analyzed the data statistically. FLL and QT review the article.

Key words
Goat, Mastitis, *S. aureus*, Biofilm, Risk factors, Antibiogram.

ABSTRACT

The nutraceutical milk of goat in agrobased countries is at risk of contamination with pathogenic strains of *Staphylococcus aureus*. The current study was designed to investigate prevalence of pathogenic strains of *S. aureus*, assessment of risk factors, and *in-vitro* antibiogram of non-biofilm producing *S. aureus* (nbpSA) and biofilm positive *S. aureus* (bpSA) from mastitic goats. The purposive sampling technique was applied to collect n=200 milk samples from different regions of goat populated areas of district Faisalabad-Pakistan. Using surf field mastitis test, collected milk samples were screened for subclinical mastitis at the spot for subsequent identification of pathogenic strains of *S. aureus* through microbiological examination in the laboratory. Non-probability statistical tools conferred 42% (84/200, CI=35.37-48.93) prevalence of subclinical mastitis, 38.1% *S. aureus* (32/84, CI=28.45-48.79), 15.6% MRSA (5/32, CI=6.87-31.76), 46.9% haemolytic *S. aureus* (15/32, CI=30.87-63.56) and 34.4 % biofilm producing *S. aureus* (11/32, CI=20.41-51.69). Earthen floor type (OR=1.75, p=0.0996), poor drainage system (OR=7.33, p=0.002), pond as source of drinking water (OR=2.05, p=0.179), stall feeding (OR=7.27, $p<0.001$), 4-6 years of age of goat (OR=4.2, p=0.0874), and teat injury (OR=13.74, $p<0.001$) were potential risk factors for subclinical mastitis. The *in-vitro* findings of current study revealed 100% sensitivity of *S. aureus* against gentamicin, oxytetracycline, amoxicillin, and linezolid while 80% of biofilm negative *S. aureus* (nbpSA) showed sensitivity against amoxicillin+clavulanic acid. None of the isolate from bpSA and nbpSA was resistant against linezolid, gentamicin, and oxytetracycline in this study. bpSA were highly resistant against amoxicillin and vancomycin. The study found higher prevalence of pathogenic strains of *S. aureus*, higher number of potential risk factors, and diversified responses to antibiotic.

INTRODUCTION

Livestock plays an important role in the agriculture sector of Pakistan. The total goat population of Pakistan is up to 74.1 million to produce 0.915 million tons of milk and 0.717 million tons of mutton annually (Anonymous, 2018). Milk production in goats is an active and emergent business in harsh climate areas where large ruminants cannot be reared or are difficult to rear and it largely contributes to the mainstream dairy milk production (Silanikove *et al*., 2010). Milk of goats has certain properties like better digestibility, alkalinity, buffer capacity and medicinal importance which make goat milk better than human and cow milk (Park, 2001). Mastitis can be illustrated as a result of pathological alterations in mammary glands resulting in elevation in somatic cell count of milk (Contreras *et al*., 2003). Mastitis occurs as clinical and/or subclinical form (Aqib *et al*., 2018). In clinical mastitis, signs of inflammation, redness, heat and pain are present, whereas there are no obvious indications of swelling in subclinical mastitis except decrease in milk production and increased somatic cell count (Sarker and

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Samad, 2011). Sub-clinical mastitis has occurrence of 45% in goats in Punjab whereas 53.3% in Khyber-Pakhtunkhwa (KPK), Pakistan (Najeeb *et al*., 2013; Ali *et al*., 2010).

Subclinical mastitis in goats is predominantly caused by transmissible bacteria *e.g. Staphylococcus* spp., *Streptococcus* spp., *Pasteurella* spp. and *E. coli* (Persson and Olofsson, 2011; Contreras *et al*., 2007). *Staphylococcus aureus* is the major causative agent where its frequency of isolation and identification vary from 4-40% of the entire isolated pathogens (Leitner *et al*., 2007). Antimicrobial resistance is reported in *S. aureus* probably due to excessive administration of antimicrobials (over-prescription, suboptimal termination of treatment regimen and/or insufficient dose administration of antimicrobials) resulting in lateral gene transfer (transformation, transduction and conjugation) of DNA from resistant strain for survivability (Castro-Sánchez *et al*., 2016). *S. aureus* can produce biofilm which act as a protective layer for the pathogen and provides continuous persistence via development of resistant genes *e.g. mecA, vanA, icaA, icaB etc*. (Jyothi *et al*., 2018). Production ability of coagulase enzyme is considered a significant phenotypic determinant in *S. aureus* linked with pathogenicity (Moreillon *et al*., 1995). Multiple drug resistance (MDR) is one of additional challenges in bacterial mastitis (Hameed *et al*., 2007). In 1972, first MRSA was isolated from dairy mastitic milk (Devriese *et al*., 1972). MRSA is now becoming major bacterial etiology of mastitis in addition to its isolation from vaginal and nasal swabs of animals (Cortimiglia *et al*., 2015).

Treatment with broad spectrum antibiotics along with anti-inflammatory drugs is used to treat mastitis in goats. Mechanism of development of drug resistance in bacteria associated with goat mastitis is very important to understand transmission frequency, better management strategies and developing valuable remedial interference (Aqib *et al*., 2018a; Merz *et al*., 2016). Therefore, epidemiological studies of pathogenic strains of *S. aureus* along with their response to antibiotics are necessary for prevention and treatment protocols. Subclinical mastitis in goats remained as neglected issue despite of its increasing prevalence and antimicrobial resistance of bacterial etiologies. Current study was thus designed to investigate the occurrence of different pathogenic strains of *S. aureus*, associated risk factors, and *in-vitro* antibiogram of biofilm positive and biofilm negative *S. aureus* in mastitic goats from Faisalabad, Pakistan.

MATERIALS AND METHODS

Sampling plan and screening for subclinical mastitis

Faisalabad is the second biggest city of province Punjab and the third most populated city of Pakistan having a total area of 5,856km². Purposive sampling technique (Thrusfield, 2007) was applied to collect milk samples (n= 200) from dairy farms located in district Faisalabad (Samundri, n= 62; Rasoolpur, n= 60; Livestock Farm of University of Agriculture Faisalabad, n= 36; Jhapal, n= 42) depending upon the willingness of the farmers to participate in the study and accessibility to Mastitis Research Laboratory, University of Agriculture Faisalabad, Pakistan. Milk samples were collected by strictly following the guidelines of National Mastitis Council of the USA (Reyher and Dohoo, 2011). Samples were screened by using Surf Field Mastitis test (SFMT) proposed by Muhammad *et al*. (2010). The SFMT positive milk samples, maintained in cold chain (4°C), were transferred to Mastitis Research Laboratory, University of Agriculture Faisalabad, Pakistan and preserved at -20°C till further process (Cengiz *et al*., 2015).

Risk factor analysis

A questionnaire comprising information like age of animal, type of housing, type of drainage system, type of floor, condition of floor, farm hygiene, source of drinking water, feeding system, vaccination against diseases, deworming, mastitis control program, parity number, stage of lactation, body condition score, milk consistency, milk yield, and teat injury was filled at the time of sampling to assess risk factors associated with mastitis. The risk factors were assumed based on the previous studies conducted by Amin *et al*. (2011) and Megersa *et al*. (2010).

Identification of pathogenic strains of S. aureus

SFMT positive samples were cultured on blood agar at 37°C for 24 h. Characteristic pinpoint colonies were further cultured on Mannitol Salt Agar, selective and differential medium for *S. aureus*, following the same incubation conditions. Series of biochemical tests were performed following guidelines of Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994).

Isolates were identified for their expression as α, β and γ haemolysis on blood agar by inoculating *S. aureus* on blood agar at 37°C for 24 h. Methicillin resistant *S. aureus* were identified by their resistance against oxacillin disc following standard protocol described in clinical and laboratory standard institute. Biofilm was identified by Congo Red Agar (CRA) method, a previously established method (Freeman *et al*., 1989). For biofilm identification, fresh culture of *S. aureus* was grown on CRA and incubated for 24 h at 37°C. After incubation, colour of colonies indicated strength of biofilm *i.e.* pinkish red colonies - no biofilm production; slight blackish -weak production; black sheeting - moderate production; and jet black dry

sheeting - strong biofilm production (França *et al*., 2012; Mathur *et al*., 2006).

In-vitro *drug response against bpSA and nbpSA*

In-vitro drug response was evaluated using the Kirby Bauer disc diffusion test. The positive isolates were subjected to testing against various antibiotics such as vancomycin (30µg), chloramphenicol (10µg), oxytetracycline (30µg), trimethoprim+sulphamethoxazole (25µg), gentamicin (10µg), linezolid (30µg), amoxicillin-clavulanic acid (20µg), amoxicillin (10µg), and oxacillin (1µg). Fresh cultures adjusted at 1.5×10^8 CFU were swabbed on Muller Hinton Agar whereas antibiotic discs were aseptically placed at equal distances from each other following the guidelines of CLSI (2015). Incubation was given at 37°C for 18-20 h and zone of inhibitions were measured by Vernier Callipers in millimetres and compared with provided standards.

Statistical analysis

The obtained data were analysed by descriptive statistics for occurrence of *S. aureus* and antibacterial activity of antibiotics whereas risk factor analysis was assessed by odd's ratio at 5% probability using IBM SPSS (version 20).

RESULTS

Prevalence of subclinical mastitis and pathogenic strains of S. aureus

The present study found overall 42.0% (84/200) prevalence of subclinical mastitis from goats based on Surf Field Mastitis Test (SFMT). The prevalence of subclinical mastitis was found higher in Jhapal (59.5%) followed by Samundri (38.7%), Rasoolpur (38.3%) and UAF Livestock Farm (33.3%) while there was non-significant association ($p> 0.05$) among different areas. The overall prevalence of *S. aureus* was found 38.1% while among *S. aureus* there was 15.6% MRSA, 46.9% haemolytic *S. aureus,* 34.4% biofilm producing *S. aureus* during current study. The higher prevalence of *S. aureus* and MRSA was noted from livestock farm (50.0% and 33.3%) followed by Samundri (33.3% and 12.5%), Rasoolpur (39.1% and 11.1%) and Jhapal (36.0% and 11.1%). The percentage of haemolytic *S. aureus* and biofilm producing *S. aureus* was 83.3% and 83.3% from UAF livestock farm, 44.4% and 33.3% from Rasoolpur, 37.5% and 12.5% from Samundri, and 33.3% and 22.2% from Jhapal, respectively. The study found non-significant difference ($p>0.05$) for *S. aureus*, MRSA and haemolytic *S. aureus* while significant difference was noted for biofilm producing *S. aureus* among different areas of study (Table I; Fig. 1).

Table I.- Prevalence of subclinical mastitis, *Staphylococcus aureus*, methicillin resistant *S. aureus*, hemolytic and biofilm producing Staphylococci from different areas of Faisalabad.

Area		Subclinical mastitis (SM) on SFMT basis*	*Staphylococcus aureus*[#]	Methicillin resistant *S. aureus* within *S. aureus*[‡]	Haemolytic *S. aureus*[@]	Biofilm producing *S. aureus*[†]
Samundri	No. observed	24/62	8/24	1/8	3/8	1/8
	Prevalence (%)	38.7	33.3	12.5	37.5	12.5
	CI (95%)	27.58-51.15	17.97-53.29	2.24-47.09	13.68-69.43	2.24-47.09
Rasoolpur	No. observed	23/60	9/23	1/9	4/9	3/9
	Prevalence (%)	38.3	39.1	11.1	44.4	33.3
	CI (95%)	27.09-50.98	22.16-59.21	1.99-43.50	18.87-73.33	12.06-64.58
UAF livestock farm	No. observed	12/36	6/12	2/6	5/6	5/6
	Prevalence (%)	33.3	50.0	33.3	83.3	83.3
	CI (95%)	20.21-49.66	25.38-74.62	9.68-70.00	43.65-96.99	43.65-96.99
Jhapal	No. observed	25/42	9/25	1/9	3/9	2/9
	Prevalence (%)	59.5	36.0	11.1	33.3	22.2
	CI (95%)	44.49-72.95	20.25-55.48	2.24-47.09	12.06-64.58	6.32-54.74
Total	No. observed	84/200	32/84	5/32	15/32	11/32
	Prevalence (%)	42.0	38.1	15.6	46.9	34.4
	CI (95%)	35.37-48.93	28.45-48.79	6.87-31.76	30.87-63.56	20.41-51.69

$p<0.05$ indicate significant difference. Among different areas subclinical mastitis, *, $p=0.072$; [#], $p=0.799$ and [‡], $p=0.623$; [@], $p=0.244$; [†], $p=0.034$.

Table II.- Risk factors associated with spread of mastitis in dairy goats.

Factor	Variables	No. positive	Percentage (%)	Odds ratio	C.I (95%)	*p*-value
Housing type	Open	29/80	36.25	1.093	0.49-2.46	0.829
	Street	13/38	34.21	1	-	-
	Backyard	42/82	51.21	2.019	0.91-4.48	0.084
Floor type	Earthen	40/80	50.00	1.75	0.90-3.41	0.099
	Bricks	20/54	37.03	1.029	0.49-2.17	0.939
	Cemented	24/66	36.36	1	-	-
Condition of floor	Even	56/120	46.67	1.625	0.91-2.91	0.102
	Uneven	28/80	35.00	1	-	-
Drainage system	Poor	16/24	66.67	7.333	2.54-21.21	0.0002
	Partially controlled	56/120	46.67	3.208	1.54-6.67	0.002
	Well formed	12/56	21.42	1	-	-
Farm hygiene	Very poor	9/32	28.13	0.671	0.24-1.85	0.44
	Poor	17/40	42.5	1.267	0.51-3.15	0.61
	Normal	44/90	48.89	1.64	0.75-3.57	0.213
	Good	14/38	36.84	1	-	-
Source of drinking water	Pond	9/16	56.25	2.047	0.72-5.82	0.179
	Underground	21/44	47.72	1.454	0.73-2.88	0.282
	Bucket	54/140	38.57	1	-	-
Feeding	Grazing	27/100	27.00	1	-	-
	Stall feeding	35/48	72.92	7.279	3.35-15.80	<0.0001
	Mixed	22/52	42.31	1.983	0.98-4.01	0.057
Vaccinated against diseases	Yes	25/124	20.16	2.467	1.23-4.95	0.011
	No	59/76	77.63	1	-	-
Deworming	Yes	53/128	41.41	1	-	-
	No	31/72	43.05	1.069	0.60-1.92	0.82
Mastitis control measures	Yes	30/76	39.47	1	-	-
	No	54/124	43.55	1.183	0.66-2.11	0.571
Age	Up to 2 years	13/42	31.0	3.138	0.62-15.85	0.166
	2-4 years	29/102	28.4	2.781	0.59-13.01	0.194
	4-6 years	12/32	37.5	4.2	0.81-21.77	0.087
	6-8 years	2/16	12.5	1	-	-
	Above 8 years	1/8	12.5	1	0.08-13.02	1
Parity	1-2 kidding	27/49	55.10	0.859	0.28-2.63	0.79
	2-4 kidding	20/34	58.82	1	0.31-3.26	1
	>5 kidding	10/17	58.82	1	-	-
Stage of lactation	Early	23/42	54.76	1	-	-
	Mid	12/23	52.17	0.901	0.33-2.50	0.841
	Late	22/35	62.86	1.398	0.56-3.49	0.473
Body condition score (BCS)	Poor	29/47	61.70	1.381	0.40-4.77	0.609
	Normal	20/40	50.00	0.857	0.24-3.00	0.809
	Good	7/13	53.84	1	-	-
Milk consistency	Thin	70/165	42.42	1.228	0.51-2.97	0.648
	Thick	9/24	37.50	1	-	-
	Purulent	5/11	45.45	1.389	0.33-5.90	0.656
Milk yield	Decreased	27/45	60.00	1.25	0.56-2.78	0.584
	Not Decreased	30/55	54.54	1	-	-
Teat injury	Yes	59/76	77.63	13.743	6.86-27.55	<0.0001
	No	25/124	20.16	1	-	-

C.I, confidence interval set at 95%; *$p< 0.05$ indicate significant difference.

Risk factor analysis

The findings of the current study presented type of drainage system, type of feeding, vaccination against diseases, and teats injury as potential risk factors ($p< 0.05$) of subclinical mastitis. Poor type of drainage system ($p= 0.0002$) and stall feeding ($p< 0.0001$) showed higher odds of getting mastitis compared to partially controlled drainage system ($p= 0.0018$) and mixed feeding ($p= 0.0572$), respectively. The risk factor analysis revealed backyard type of housing showing higher odds of getting mastitis ($p= 0.0843$) as compared to open type of housing ($p= 0.8289$). Similar findings were found in case of earthen type of floor ($p= 0.0996$) as compared to brick floor ($p= 0.9393$). Mid stage of lactation ($p= 0.8414$), 1-2 kidding ($p= 0.7900$), normal body condition scoring ($p= 0.8096$) were not proved to be potential risk factors of subclinical mastitis (Table II).

Antibiotic Susceptibility testing against S. aureus *and biofilm producing* S. aureus

The *in-vitro* findings of current study revealed 100% sensitivity of *S. aureus* against gentamicin, oxytetracycline, amoxicillin, and linezolid while 80% of biofilm negative *S. aureus* (nbpSA) showed sensitivity against amoxicillin + clavulanic acid (Table III; Fig. 1). Higher percentages of goat milk based resistant isolates were noted from bpSA and nbpSA against vancomycin, chloramphenicol, oxacillin, amoxicillin+clavulanic acid and amoxicillin. None of the isolate from bpSA and nbpSA was resistant against linezolid, gentamicin, and oxytetracycline in this study. In case of oxacillin, amoxicillin clavulanate and trimethoprim+sulphamethoxazole there was significant ($p<0.05$) difference at intermediate cadre, and same was observed at sensitive cadre of isolates. The antibiotics did not differ significantly in efficacies between sensitive bpSA strains and sensitive nbpSA strains (Table III).

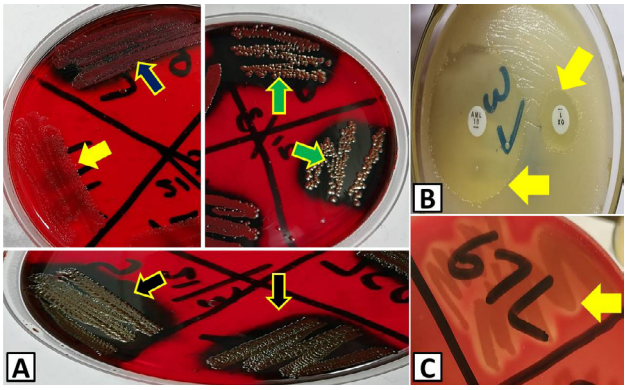

Fig. 1. Biofilm producing strains, response of *S. aureus* against antibiotics and haemolytic strains. **A**, arrows point out different kinds of biofilm: yellow arrow, no biofilm; blue arrow, weak biofilm; green arrows, moderate biofilm; black arrow, strong biofilm. **B**, zone of inhibitions produced by antibiotics against *S. aureus* (yellow arrows, zones of inhibition). **C**, haemolysis on blood agar produced by *S. aureus* (yellow arrow, partial haemolysis).

DISCUSSION

Prevalence of subclinical mastitis, S. aureus*, MRSA, hemolytic and biofilm producing* S. aureus

The prevalence of subclinical mastitis in current study was in line with findings of Najeeb *et al*. (2013) who reported 45% subclinical mastitis from goats. On the other hands, 37.5% and 53% subclinical mastitis in goats was also noted in previous studies by Abo-Shama (2014) and Ali *et al*. (2010). *S. aureus* has been formerly described

Table III.- Comparative response of antibiogram of biofilm producing and non-biofilm producing *S. aureus* isolated from mastitic dairy goats.

Antibiotic	Resistant %			Intermediate %			Sensitive %		
	NBPSA	BPSA	p-value	NBPSA	BPSA	p-value	NBPSA	BPSA	p-value
Vancomycin	20	57.14	0.021	40	42.86	0.014	40	0	0.064
Oxacillin	40	0.000	0.383	40	14.29	0.036	20	85.71	0.057
Amoxicillin+Clavulanic acid	0	42.86	0.322	20	0	0.024	80	57.14	0.026
Linezolid	0	0	N/A	0	28.57	0.787	100	71.43	0.035
Gentamicin	0	0	N/A	0	0	N/A	100	100	N/A
Trimethoprim + Sulphamethoxazole	20	0	0.689	40	0	0.044	40	100	0.056
Oxytetracycline	0	0	N/A	0	0	N/A	100	100	0.457
Chloramphenicol	40	14.29	0.047	40	14.29	0.047	2	71.43	0.037
Amoxicillin	0	71.43	0.057	0	0	N/A	100	28.57	0.479

NBPSA, non-biofilm producing *S. aureus*; BPSA, biofilm producing *S. aureus*; NA, not applicable.

as one of the most significant causative agent in caprine mastitis (Ali *et al*., 2010; Najeeb *et al*., 2013). Higher biofilm positive *S. aureus* in current study was in contradiction with findings of França *et al*. (2012) who reported 7.6% bpSA based on CRA from caprine milk.

Hemolysins are involved in various pathological processes. Kenny *et al*. (1992) reported that haemolytic toxins can develop clinical signs in mastitis cases, and Ebrahimi *et al*. (2007) reported that the udder of mastitic goats contain hemolytic Staphylococci. In the current study, 15.6% of *S. aureus* were found to be resistant to methicillin which was in line with the previous results of 9.2% as discussed by El-Deeb *et al*. (2018), 20% by Bochev and Russenova (2005), and 28.57% by Ebrahimi *et al*. (2007). The methicillin-resistant Staphylococci cannot be successfully treated with beta-lactam antibiotics as discussed by previous studies (Aqib *et al*., 2018b; Dar *et al*., 2006).

Risk factors

Potential risk factors of current study were in line with findings of previous studies conducted in Pakistan. Feeding system is significant factor for subclinical mastitis. Poor drainage system or farm hygiene can lead to occurrence of mastitis (Ali *et al*., 2010; Aqib *et al*., 2019; Najeeb *et al*., 2013). Teat injury is also strongly associated with mastitis (Ferdous *et al*., 2018). Wound on the teats and udder facilitates the entry of microbes into the glands, leading to mastitis (Gebrewahid *et al*., 2012). The findings of current study were in line with those of previous trials conducted on prevalence of subclinical mastitis in goats by Ali *et al*. (2010) and Najeeb *et al*. (2013).

Antibiogram

The results of current study were in line with those reported by Ali *et al*. (2010) and Saleem *et al*. (2018) who found 80-100% of *S. aureus* sensitive against these antibiotics. The decreased use of gentamicin in the late 1990's and obvious shift in strains of clinical isolates of *S. aureus* were major factors for increased gentamicin sensitivity (Klevens *et al*., 2006). Oxytetracycline is used as first line treatment by field workers. Oppliger *et al*. (2012) also suggested that *S. aureus* isolated from farm workers were 100% sensitive to oxytetracycline.

Vancomycin resistance is a rising problem in *S. aureus* isolates and their number is increasing day by day which may be due to the acquired resistance as occurred in case of methicillin (Marques *et al*., 2013). Glycopeptide antibiotics such as vancomycin are last choice for the severe clinical infections of MRSA throughout the world. But the continuous use of vancomycin for handling of MDR *S. aureus* infections has caused a decrease in vancomycin sensitivity in many countries (Hiramatsu *et al*., 1997; Rağbetli *et al*., 2016). Vancomycin resistance in *S. aureus* when studied at genomic level shows that the development of *van*A gene is associated with this behaviour (Akpaka *et al*., 2017). Mastitis is well known for its deterioration and lack of response to treatment chiefly due to resistance by bacteria against antibiotics (Shamila-Syuhada *et al*., 2016).

CONCLUSION

The present study found overall higher prevalence of subclinical mastitis (42%) in goats with increased percentage of *S. aureus* (3.8.1%) and pathogenic strains of *S. aureus* (MRSA 15.6%, hemolytic *S. aureus* 46.9%, and biofilm producing *S. aureus* 34.4%). Risk factor analysis revealed type of drainage system, type of feeding, and teats injury as potential risk factors of mastitis. The *in-vitro* drug trial indicated higher sensitivity of *S. aureus* against oxytetracycline, trimethoprim + sulphamethoxazole, gentamicin and linezolid against *S. aureus* and biofilm producing *S. aureus*. Biofilm producing *S. aureus* were highly resistant against amoxicillin and vancomycin. Current study reports higher prevalence of pathogenic strains of *S. aureus*, larger number of potential risk factors, and diversified response of antibiotic susceptibilities which suggest extensive molecular studies and development of effective preventive measures.

Statement of conflict of interest

The authors have declared no conflict of interest.

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