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Detection of Altered Pattern of Antibiogram and Biofilm Character in *Staphylococcus aureus* Isolated From Dairy Milk

Muhammad Aamir Naseer[1], Amjad Islam Aqib[2]*, Ambreen Ashar[3], Muhammad Ijaz Saleem[1], Muhammad Shoaib[4], Muhammad Fakhar-e-Alam Kulyar[1], Khurram Ashfaq[1], Zeeshan Ahmad Bhutta[1] and Shagufta Nighat[5]

[1]*Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan-38000*
[2]*Department of Medicine, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan-63100*
[3]*Government College Women University, Faisalabad*
[4]*Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan-38000*
[5]*Department of Zoology, Rawalpindi Women University, Rawalpindi, Pakistan*

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MAN, AIA, and MIS conceived idea. MAN and MS did research work. MAN, AIA, MFAK and MS analyzed the data. MIS and KA provided research assistance and supervision. MAN and ZAB wrote initial draft. AIA, AA and SN finalized the article.

Key words
***S. aureus*, Biofilm, Cattle, Buffalo, Antibiogram, Risk factors**

ABSTRACT

Dairy milk is overwhelming with biofilm producing *Staphylococcus aureus* (bpSA), whereas response to commonly used antibiotics is not only becoming worrisome in bpSA but also in non-biofilm producing *S. aureus* (nbpSA). Current study was planned to detect bpSA from dairy milk, confirmation of presumed risk factors, and comparative analysis of antibiogram of bpSA and nbpSA at various cadre. Milk samples (n=250) from cattle (n=90) and buffalo (n=160) were aseptically collected from various dairy farms and put to biofilm detection and antibiogram. Based on collected data with statistical inferences, the study found 61.60% of *S. aureus* from subclinical samples, while 72.73% of *S. aureus* were positive for biofilm with uniform hike in samples from cattle (77.55% bpSA) and buffalo (70.48% bpSA). Udder condition/consistency, teat dip, teat abnormality, tick infestation, body condition, mastitis knowledge, treatment approach, and therapeutic drug use were significantly ($p<0.05$) associated with rise in *S. aureus* in dairy milk. All the tested isolates were found 100% resistant to Cefotaxime, Fusidic acid, and Ampicillin while 60-80% of these isolates were found sensitive to Cefoxitin, Gentamicin, Trimethoprim + Sulphamethoxazole, and Oxytetracycline. Except Trimethoprim + Sulphamethoxazole, non-significant differences ($p>0.05$) of isolates at resistant, intermediate, and sensitive cadre were noted against Vancomycin, Oxacillin, Amoxy clavulanate, and Linezolid. Same pattern was observed when tested against Oxytetracycline, Gentamicin, Cefotaxime, Fusidic acid, and Ampicillin. The study concluded hiked biofilm character in *S. aureus* with prevailing significant risk factors and heightened change in antimicrobial resistance by all isolates which demands immediate action plans to be taken.

INTRODUCTION

Staphylococcus aureus has emerged as superbug of animal and human that is compromising health and economy (Aqib *et al*., 2018). *S. aureus* has various pathogenic attributes major of which are multidrug resistance and biofilm production (Munita *et al*., 2015). The latter becomes more of concern due to its ability to minimize antibiotics' effect, colonization to epithelial lining, longer persistence, evading immune response, and boosting of pathogenesis (Melchior *et al*., 2006). Such resistant strains are distinguished by systemic heterogenicity, genetic variety, interactions between complex community and the extracellular matrix of macromolecular substances (Begum *et al*., 2007). Studies report it to be second most etiology accounting to 17 million annual human deaths, while on the other hands it stands to be pertinent global problem in dairy milk production (Cosandey *et al*., 2016).

The emergence of resistive *S. aureus* strain in dairy has tuned to 61% in some of countries with fear to go rise as in case of prevailing risk factors (Aqib *et al*., 2018). It seems to be mushrooming as a pandemic. Such devastating

* Corresponding author: amjadwaseer@gmail.com
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scenario is presumed to be due to be multifactorial (Marques *et al*., 2007). From which, mainly in concern is biofilm production (Melchior *et al*., 2006). The ability of biofilm production may be strain specific or genetical trait of strain. Bacteria in biofilms use dense extracellular matrix to protect themselves from antibiotics (Vancraeynest *et al*., 2004). The resistance to antimicrobials ranges between 100-1000 times in biofilm enclosed pathogenic strains than those of planktonic cells (Begum *et al*., 2007). Moreover, the strains are responsible for transfer of resistance to the interacting bacteria within biofilm (Munita *et al*., 2015). Biofilm is reported to be well established even in case of lower number of somatic cell count representing biofilm microbes surviving in udder and contributing in prolong sustainment of pathogen at farm (Melchior *et al*., 2006).

Commonly practiced antibiotics in mammary infections are macrolides, fluoroquinolones, streptogramins, beta-lactam, lincosamides and beta-lactams that are now facing resistance. Usage of these antibiotics at subinhibitory level makes the scene worsen (Kumar *et al*., 2010). It is reported that the production of biofilm can be enhanced by sub-inhibitory concentration of antibiotics. Also, the recurrence of mastitis has been attributed to the sub-inhibitory concentrations of antibiotics that induces biofilm character to get established (Vancraeynest *et al*., 2004).

Strategies to cop this scenario can be implemented more effectively by understanding the prevalence of genetic patterns, availability and susceptibility of genes expression to antibiotics which are facing resistance, addressing the risk factors associated with spread of biofilm producing microbes in routine dairy analysis (Aqib *et al*., 2018). Similarly, antibiotics must be evaluated against biofilm producing *S. aureus*. Current study was planned to estimate prevalence and prevailing risk factors of biofilm producing *S. aureus* of dairy origin, and to find comparative evaluation of antibiotics' efficacy against biofilm *S. aureus*.

MATERIALS AND METHODS

Sampling

The sampling areas included were various small animal holders (having 1-5 animals) and accessible farms located in the jurisdiction of district Nankana Sahib, district Okara and district Faisalabad. These districts were selected based on higher dairy population and accessibility to dairy animals. A Total of n=250 milk samples were collected from dairy animals (n=90 cattle, n=160 buffalo) that were positive for subclinical mastitis using purposive sampling method of non-probability sampling technique. These samples were screened by Surf Field Mastitis Test for subclinical mastitis, as the test has been used in recent studies (Aqib *et al*., 2018).

Risk factors analysis

Predesigned dichotomous questionnaires having questions of udder condition and consistency. Use of teat dip, teat abnormalities, age, parity number, lactation stage, system of rearing, tick infestation, body condition, feeding, owner knowledge about mastitis, use of therapeutic drug and treatment approach were filled on-spot to access the potential risk factors.

Isolation and identification of Staphylococcus aureus

Positive samples were processed for isolation of characteristics yellow pinpoint round colonies of *S. aureus*. The confirmation was done using gram's staining, selective media growth and biochemical tests (i.e. Catalase test, Coagulase test) (Aqib *et al*., 2018).

Detection of biofilm producing Staphylococcus aureus

Biofilm production was identified by Tissue culture plate method. Briefly stating, optical density at 570nm of overnight incubated culture (150µl) in tryptic soy broth was determined using tissue culture plate reader. The culture of bacteria was poured in wells and further incubated for 24 h at 37°C. Negative control with only broth and positive control with broth seeded with strong biofilm producing standard strain was also run. Optical density at was measured after washing the wells with PBS thrice, and staining with crystal violet. Optical density < 0.12 indicate None/ Weak, 0.12-0.24 show moderate, while > 0.24 did show high biofilm production (Hassan *et al*., 2011).

Comparative analysis of antibiogram

Biofilm producing *S. aureus* and non-biofilm producing *S. aureus* isolated from similar sources were put to antibacterial susceptibility against various commercially available antibiotics (Oxoid™) vis-à-vis Vancomycin (30µg), Cefoxitin (30µg), Linezolid (30µg), Amoxy-clavulanate (20µg), Oxacillin (1µg), Oxytetracycline (20µg), Gentamicin (10µg), and Trimethoprim plus Sulfamethoxazole (1.25/23.75µg). Fresh culture of both strains adjusted at 1.5×10^8 CFU/ml were swabbed on Muller Hinton agar whereas antibiotic discs were aseptically placed at equal distance from each other following guidelines of CLSI (2015). Zones of inhibition around antibiotic discs were measured following 24 hours' incubation at 37 °C, and were compared with standards provided in CLSI (2015) for result interpretation.

Statistical analysis

The data obtained was analyzed by descriptive statistics for antibiotics while association of risk factors

were analyzed by chi-square at 5% probability using SPSS statistical computer program (version 20).

RESULTS

Prevalence of biofilm producing Staphylococcus aureus *in cattle and buffalo*

The present study showed that amongst the 250 subclinical mastitis samples, 61.60% (154/250) were positive for *S. aureus*. However, the prevalence of *S. aureus* was found to be higher in buffalo milk samples (65.62%,105/160) than in cattle milk samples (54.44%,49/90) which was non-significant difference ($p<0.05$) (Table I). There were 72.73% of *S. aureus* isolates positive for biofilm production. Biofilm producing stains of *S. aureus* isolated from cattle and buffaloes were noted to be 77.55% and 70.48%, respectively.

It was found that all the isolates from biofilm producing *S. aureus* (bpSA) and non biofilm producing *S. aureus* (nbpSA) of cattle and buffalo milk were 100% resistant to Cefotaxime, Fusidic acid, and Ampicillin. The general trend of sensitivity fell into Cefoxitin, Gentamicin, Oxytetracycline, and Trimethoprim + Sulphamethoxazole presenting 60-80% range of sensitive isolates in current study.

Comparison of antibiogram between bpSA and nbpSA

The study found overall (cattle and buffalo milk) higher resistant isolates against Vancomycin, Oxacillin, Amoxy clavulanate presenting >70% resistance while against Cefotaxime, Fusidic acid, Ampicillin 100% resistant strains from bpSA and nbpSA were noted (Table III). The general higher trend of resistance was noted in bpSA isolates at non-significant difference ($p>0.0\%$) against all antibiotics while comparison of bpSA and nbpSA differed significantly ($p<0.05$) against Trimethoprim + Sulphamethoxazole at resistant, intermediate and sensitive cadre. In case of Trimethoprim + Sulphamethoxazole, significant ($p<0.05$) higher percentage of resistant bpSA and intermediate bpSA while significantly ($p<0.05$) lower sensitive bpSA strains were noted.

Higher percentages of cattle milk based resistant isolates were noted from bpSA and nbpSA against Vancomycin, Oxacillin, Amoxy clavulanate, Cefotaxime, Fusidic acid, and Ampicillin. All the isolates from bpSA and nbpSA were resistant against the latter three antibiotics while among former three although higher percentages of bpSA were resistant but difference with nbpSA was non-significant ($p>0.05$). Linezolid, Cefoxitin, Gentamicin, and Trimethoprim + Sulphamethoxazole were the antibiotics that proved equally effective against both bpSA and nbpSA. Statistical analysis of comparison of bpSA and nbpSA at resistant, intermediate and sensitive cadre of isolates against all the antibiotics were non-significant ($p>0.05$) except Trimethoprim + Sulphamethoxazole where nbpSA showed significantly ($p<0.05$) higher percentage of sensitive strains than to that of bpSA isolates. The study noted most of the p values as NA (not applicable) on account of either 100% or 0.00% response at resistant, intermediate and sensitive cadre of strains against various antibiotics. The analysis did reveal that higher resistance to antibiotics existed in those strains that were even not producing biofilm.

The buffalo milk-based study showed higher percentages of sensitive strains of both biofilm producing *S. aureus* (bpSA) and non-biofilm producing (nbpSA) against Cefoxitin, Gentamicin, Trimethoprim + Sulphamethoxazole, Oxytetracycline in current study. While higher resistance was observed against Vancomycin, Oxacillin, Amoxy clavulanate, Linezolid with percentages to be >90, >60, 60-88, and 44%, respectively. All the isolates from both bpSA and nbpSA were 100% resistant to Ampicillin, Cefotaxime, and Fusidic acid. Statistical comparison of antimicrobial response of biofilm producing *S. aureus* (bpSA) and non-biofilm producing (nbpSA) against a list of 11 antibiotics at resistant, intermediate, and sensitive cadre was quite variable depending upon isolates' origin and the kind of antibiotic. The bpSA and nbpSA isolates obtained from buffalo milk did show non-significant difference against Vancomycin, Oxacillin, Amoxy clavulanate, and Linezolid at all three cadre i.e. resistant, intermediate, sensitive. The bpSA did show significant ($p<0.05$) higher percentage of resistant strains against combination of Trimethoprim + Sulphamethoxazole while significant higher sensitive strains of nbpSA were noted.

Comparison of resistant, intermediate, and sensitive strains of bpSA *against antibiotics*

Biofilm producing *S. aureus* did present significant difference among all antibiotic resistant, intermediate and sensitive strains except in case of Trimethoprim + Sulphamethoxazole where these strains did non-significantly differ ($p>0.05$) (Fig. 1). The trend of resistant, intermediate, and sensitive strains against different antibiotics was like that of nbpSA indicating that spectrum of antimicrobial resistance has been expanded.

Biofilm producing *S. aureus* of cattle origin did significantly differ in resistant, intermediate and sensitive strains of all antibiotics except Cefoxitin where non-significant ($p>0.05$) difference existed among Cefoxitin resistant, intermediate and sensitive strains of cattle milk based bpSA. All bpSA strains were resistant to

Table I. Prevalence of *S. aureus* and biofilm producing *S. aureus* in cattle and buffalo milk.

Sample source	Prevalence of *Staphylococcus aureus*					Prevalence of biofilm producing *Staphylococcus aureus**				
	Total	No. positive	(%)	C.I (95%)	*p*-value	Total	No. positive	(%)	C.I (95%)	*p*-value
Buffalo	160	105	65.62	44.18-64.34	0.081	105	74	70.48	61.16-78.36	0.359
Cattle	90	49	54.44	55.44- 67.41		49	38	77.55	64.12-86.97	
Total	250	154	61.60	57.98-72.55	-	154	112	72.73	65.21-79.15	

C.I, indicate confidence interval set at 95%; P< 0.05 indicate significant difference; * biofilm detected by tissue culture plate method.

Table II. Risk factors' association with spread of *Staphylococcus aureus* in cattle and buffalo.

Parameters	Levels	Total number	Positive	(%)	C.I	*p*-value
Udder condition and consistency	Normal	218	126	57.80	0.5116-0.6417	0.001
	Swollen	12	8	66.67	0.3907-0.8619	
	Fibrosed	20	20	100	0.8389-1.0000	
Teat dip	Yes	120	58	48.33	0.3958-0.5718	0.000
	No	130	96	73.85	0.6569-0.8064	
Teat abnormality	Normal	222	128	57.66	0.5108-0.6398	0.001
	Injured	4	4	100	0.5101-1.000	
	Stenosis	4	2	50.00	0.1500-0.8500	
	Fibrosed	20	20	100	0.8389-1.000	
Age group	2-3 year	96	58	60.42	0.5042-0.6962	0.351
	4-7 year	116	70	60.34	0.5124-0.6877	
	8-10 year	18	10	55.55	0.3372-0.7544	
	>10 year	20	16	80.00	0.5840-0.9193	
Parity number	1-2	180	108	60.00	0.5271-0.6688	0.371
	3-4	40	24	60.00	0.4460-0.7365	
	≥5	30	22	73.33	0.5555-0.8581	
Lactation stage	Early	160	98	61.25	0.5352-0.6845	0.977
	Mid	52	32	61.54	0.4796-0.7353	
	Late	38	24	63.16	0.4729-0.7662	
System of rearing	Dairy farm	160	92	57.50	0.4975-0.6490	0.076
	Small scale (1-5)	90	62	68.89	0.5872-0.7752	
Tick infestation	Yes	126	90	71.43	0.6300-0.7859	0.001
	No	124	64	51.61	0.4290-0.6022	
Feeding management	Underfed	168	102	60.71	0.5316-0.6778	0.680
	Overfed	82	52	63.41	0.5260-0.7302	
Body condition	Weak	120	84	70.00	0.6128-0.7747	0.004
	Normal	70	32	45.71	0.3457-0.5730	
	Over weight	60	38	63.33	0.5068-0.7438	
Mastitis knowledge	Basic	48	32	66.67	0.5254-0.7833	0.000
	Quackeries	82	74	90.24	0.8191-0.9497	
	Professional	120	48	40.00	0.3168-0.4894	
Treatment approach	Self	130	98	75.38	0.6732-0.8199	0.000
	Professional consultancy	120	56	46.67	0.3799-0.5556	
Therapeutic drug use	B-lactam	130	108	83.08	0.7571-0.8855	0.000
	Other antibiotics	120	46	38.33	0.3012-0.4726	

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

Table III. Overall comparative antibiogram of biofilm positive and biofilm negative *Staphylococcus aureus* of cattle and buffalo milk.

Antibiotic	Resistant %			Intermediate %			Sensitive %		
	*nbpSA	[a]bpSA	*p*-value	*nbpSA	[a]bpSA	*p*-value	*nbpSA	[a]bpSA	*p*-value
Vancomycin	80.00	95.00	0.151	15.00	5.000	0.292	5.000	0.000	0.311
Oxacillin	70.00	75.00	0.723	20.00	15.00	0.677	10.00	10.00	1.000
Amoxy clavulanate	75.00	70.00	0.723	15.00	15.00	1.000	10.00	15.00	0.633
Linezolid	35.00	60.00	0.113	20.00	20.00	1.000	45.00	20.00	0.091
Cefoxitin	5.000	20.00	0.151	25.00	15.00	0.429	70.00	65.00	0.736
Gentamicin	0.000	0.000	N/A	0.000	0.000	N/A	100.0	100.0	N/A
Trimethoprim + Sulphamethoxazole	0.000	40.00	0.002	0.000	25.00	0.017	100.0	35.00	0.000
Oxytetracycline	0.000	0.000	N/A	0.000	0.000	N/A	100.0	100.0	N/A
Cefotaxime	100.0	100.0	N/A	0.000	0.000	N/A	0.000	0.000	N/A
Fusidic acid	100.0	100.0	N/A	0.000	0.000	N/A	0.000	0.000	N/A
Ampicillin	100.0	100.0	N/A	0.000	0.000	N/A	0.000	0.000	N/A

NbpSA, biofilm negative *S. aureus*; bpSA, biofilm positive *S. aureus*; NA, not applicable.

Vancomycin, Cefotaxime, Fusidic acid, and Ampicillin, while Gentamicin and Oxytetracycline sensitive strains were found 100% from bpSA of cattle milk origin.

The study noted significant difference of resistant, intermediate, and sensitive isolates of bpSA to different antibiotics except Oxacillin, Amoxy clavulanate, and Linezolid where non-significant difference (p>0.05) was observed. bpSA did show 80 percent resistant isolates against Trimethoprim + Sulphamethoxazole which was very high percentage as compared to those of cattle milk-based isolates. Resistance to Vancomycin and Linezolid was also reduced compared to that of cattle milk bpSA.

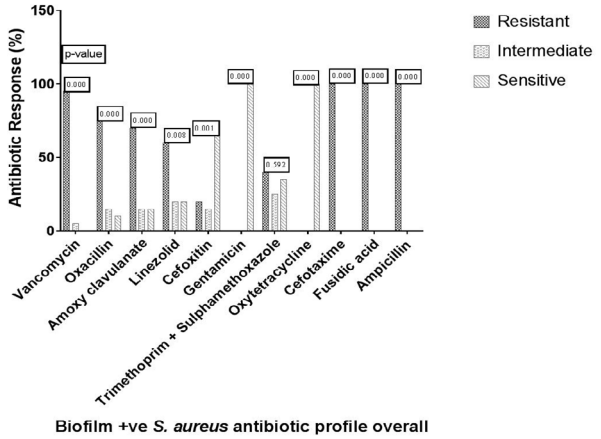

Fig. 1. Comparison of resistant, intermediate, and sensitive biofilm positive *S. aureus* strains of each antibiotic (overall milk samples).

Comparison of resistant, intermediate, and sensitive strains of nbpSA against antibiotics

Statistical analysis of overall (cattle and buffalo milk) nbpSA resistant, intermediate and sensitive strains to different antibiotics showed significant difference (p<0.05) presenting >70% Vancomycin, Oxacillin, Amoxy clavulanate while 100% resistant strains to Cefotaxime, Fusidic acid, Ampicillin were noted (Fig. 2). Linezolid resistant, intermediate, and sensitive strains of nbpSA did show non-significant difference (p>0.05) presenting 5, 25, and 75% of strains, respectively. Gentamicin, Trimethoprim + Sulphamethoxazole, and Oxytetracycline sensitive strains of nbpSA were found to be 100% in current study. Cefoxitin as exceptional to that of oxacillin presented 70 and 25% sensitive and intermediate strains of nbpSA.

Cattle milk based nbpSA resistant, intermediate, and sensitive strains to various antibiotics significantly differed (p<0.05). Relative to those of buffalo milk based nbpSA isolates, the ones from cattle were lower in percentages of resistant cadre. Vancomycin resistant nbpSA were 20 unites while those of Amoxy clavulanate, Linezolid and Cefoxitin were 10 units lower in percentages compared to nbpSA of buffalo milk. Rest of resistant and sensitive cadre were like that exhibited by buffalo milk based nbpSA.

Comparison of resistant, intermediate, and sensitive strains of nbpSA to different antibiotics showed significant difference (p <0.05) in buffalo milk except that of Linezolid where non-significantly (p >0.05) higher percentage of resistant isolates was noted. In addition to 100% resistant

isolates to already described antibiotics were the isolates resistant to Vancomycin (90%), Oxacillin (70%), and Amoxy clavulanate (80%). Cefoxitin sensitive strains of nbpSA were 70% of all tested from buffalo milk while 100% sensitive isolates were noted against Gentamicin, Trimethoprim + Sulphamethoxazole, and Oxytetracycline.

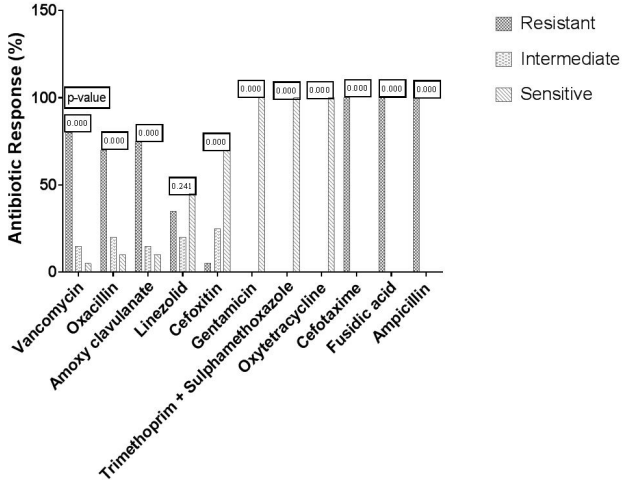

Fig. 2. Comparison of resistant, intermediate, and sensitive biofilm negative *S. aureus* strains of each antibiotic (overall milk sample basis).

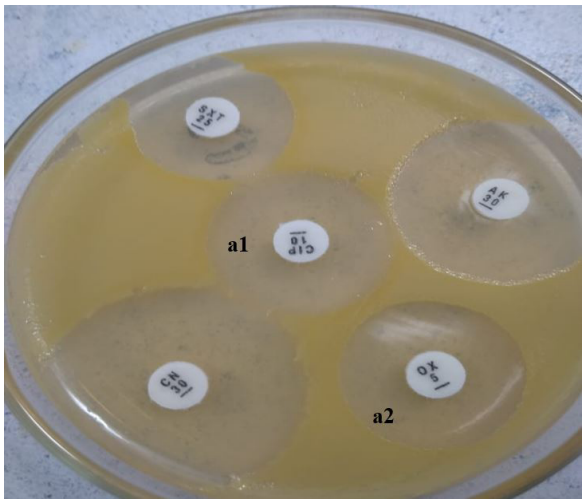

Fig. 3. Zones of inhibition of different antibiotics against biofilm positive *S. aureus* (1a=Ciprofloxacin which is usually used as standard effective drug in various studies, while oxacillin (a2) is showing comparable zones of inhibition even in case of biofilm character.

Risk factor analysis

Statistical analysis of assumed risk factors showed significant (p<0.05) association of teat dipping, tick infestation, body condition, and therapeutic drug use in causing mastitis with spread of *S. aureus* in dairy milk. On other hands, age, lactation stage, system of rearing, and feeding management did not show significant (p>o.05) association with the spread of *S. aureus* isolated from mastitis milk. All fibrosed udders presented 100% involvement of *S. aureus* while the normal udder presented 57.66% of cases associated with bacterial spread. Animals having weak body condition, greater number of parities, had higher percentages of *S. aureus* involvement (Table II).

DISCUSSION

Staphylococcus aureus continues to pose major public health challenges in many areas because of antibiotic resistance problems. Findings of higher rate of Staphylococcus prevalence in subclinical mastitis was in line with recent studies (Aqib *et al*., 2018).

Prevalence of biofilm character

Higher prevalence of *S. aureus* in current study could be related to higher number of significant risk factors in field condition. The salient of contributing factors included previous mastitis disease history, lack of knowledge about disease, breed, lactation stage, udder anomalies, tick infestation, and lack of teat dipping which prone the animal to infection and aggravate the pathogen persistence in the udder. Significant rise in biofilm characters has been in notice of (Marques *et al*., 2007) who do report that buffalo is more likely to get heaped biofilm character in milk as reported in study where *icaA* and *icaD* genes were found in 100% of tested animals. Rising biofilm was justifiable by microbial resistance, longer stay of organism in environment, lack of professional approach to deal infection, irrational antibiotics use against resistant micro-organism (Begum *et al*., 2007). Higher resistance to penicillin, and ampicillin by *S. aureus* in current study is line with reports by (Kong *et al*., 2016) who found 85% and 77% of isolates resistant to antibiotics. Both the biofilm production and beta lactamase coding genes group has been reported to enhance resistance against antibiotics (Marques *et al*., 2007). Continuous genetic variations and exotic genes uptake by *S. aureus* results in new phylogenetic categories in some of pathogens like those belonging to agr allele groups which encode increasing resistive pattern of this pathogen against different classes of antibiotics (Jarraud *et al*., 2002).

Response to antibiotics

Higher percentages of intermediate or sensitive strains to trimethoprim + sulfamethoxazole, amoxy

clavulanate and oxacillin was also reported by (Jarraud *et al*., 2002). Some studies reported very lower percentages of resistant isolates as conducted by (Carfora *et al*., 2015) found 1.3% of resistant isolates. The higher percentage of resistance strains may also appear even in the absence of biofilm due to various factors inclusive of which are the high frequency gene islands like sec-seg-sei (Cosandey *et al*., 2016). Multiple pathogenic factors when combine simultaneously may predispose higher resistance. Zhang *et al*. (2018) reported that a higher portion (83.8%) of *S. aureus* isolates from animals show biofilm character positive for *agr* alleles. Most biofilm-producing isolates were positive for microbial surface component recognizing adhesive matrix molecule (MSCRAMM), variant capsule type and *ica* group genes. The results illustrate a significant association between the prevalence rate of MSCRAMM, capsule type and *ica* group genes among isolates producing weak, moderate and strong biofilms. Deceasing multidrug resistance in community clinical isolates especially in MRSA is due to successful identification and treatment protocol, frequent multidrug therapy, specificity for control, contact precautions, active surveillance and adjunctive control measures adoption (Aqib *et al*., 2018). Vancomycin resistance shown in the isolates is in line with previous studies. Vancomycin resistance is an emerging issue in clinical isolates of *S. aureus* and their number is increasing day by day. This might be due to the acquired resistance as happened in case of methicillin (Marques *et al*., 2013). Vancomycin resistance in *S. aureus* is due to acquired transposon Tn*1546,* from vancomycin-resistant *Enterococcus faecalis*, causing changes in the structure of cell wall and cellular metabolism of isolates (Gardete and Tomasz, 2014). Glycopeptide antibiotics such as Vancomycin are last resort for the severe clinical infections of MDR *S. aureus* in whole world. But the continuous use of Vancomycin for handling of MDR *S. aureus* infections has caused a decrease in Vancomycin sensitivity in many countries. Following the identification of Vancomycin intermediate-resistant *S. aureus* (VISA) strains for the first time in Japan in 1997, glycopeptide-resistant staphylococci strains have been major concern for the researchers as well as clinicians. A new Vancomycin resistance defined as hetero resistant VISA (hVISA) was also identified in the same year as the VISA strains (Rağbetli *et al*., 2016). Vancomycin resistance in *S. aureus* when investigated at genomic level shows that the development of *van*A gene is encoding this resistive behavior (Marques *et al*., 2013). The excellent response to gentamicin observed during the study is supported by observations in previously conducted trial. The decease uses of gentamicin in late 1990's and apparent shift in strains of clinical isolates of *S. aureus* are major factors for increased gentamicin susceptibility (Gardete and Tomasz, 2014). Ampicillin resistance in clinical isolates has been reported in many studies as more than 90% isolates of animal origin are resistant to ampicillin and most susceptibility is observed in the case of tetracycline. Saba *et al*. (2017) reported that all *S. aureus* isolated from public places and hospitals are 100% resistant to ampicillin, oxacillin, tetracycline and trimethoprim + sulfamethoxazole. Marques *et al*. (2007) reported that *S. aureus* isolates are highly resistant to ampicillin and harboring *blaZ* gene encoding for such resistive behavior. Yılmaz and Aslantaş (2017) also reported the genes involved in antibiotic resistance. Kumar *et al*. (2010) reported that 96.6% of *S. aureus* isolates are resistant to ampicillin.

High resistance to Fusidic acid in clinical isolates of *S. aureus* can be explained on the basis of results of existing literature. Edslev *et al*. (2018) reported Fusidic acid in the category of antibiotics to which *S. aureus* isolates are highly resistant. Tremendous resistive response of *S. aureus* to Fusidic acid is due to mutations in *fus* gene islands resulting in amino acid substitutions of protein encoded. Due to this, 3 to 6 % increase in resistant clinical isolates per year is observed (Cosandey *et al*., 2016). Increasing Fusidic acid resistance in *S aureus* might be important for three reasons. First, it might mean that systemic Fusidic acid can no longer be used in situations where it is clinically indicated. Second, failure of topical treatment may be occurring, especially in primary care where treatment is often empiric, and third, resistance to Fusidic acid might be linked to other antibiotic resistances, therefore favoring spread of multiple antibiotic resistant *S aureus* such as MRSA (Dobie and Gray, 2004). Increasing trend of cefotaxime resistance in *S. aureus* isolates of animal origin has been reported that mutations in existing *S. aureus* isolates genome can result into extensive clinical resistance (Tomasz *et al*., 1989). Ishii *et al*. (1995) isolated and studied *Toho-1* gene which encodes for cefotaxime hydrolytic enzymes and reported that replacements in such genes specifies substrate molecules. Ishii *et al*. (1995) also reported that more than 80% of *S. aureus* isolates are resistant to cefotaxime. The main reason for this could be irrational exposure of pathogen to antibiotics in clinics.

We found deceasing susceptibility trends to potentiated penicillin which is due to genetic mutation in penicillin binding proteins encoding genes. This results in altering the binding capacity of drug to the receptor proteins, leading to higher MIC value of drug for required action (Munita *et al*., 2015). Rağbetli *et al*. (2016) reported 100% penicillin resistance in clinical isolates of *S. aureus*. Carfora *et al*. (2015) reported that *S. aureus* is developing abilities to hydrolyze penicillin, oxacillin and cephalosporins which is now being proven by molecular

studies and genes isolation of the enzymes playing important role in drug resistance. Bille *et al*. (1991) studied that the modified penicillin binding proteins affinity by clavulanic acid combination is the root cause of potentiated Amoxicillin spectrum maintenance which is losing its efficacy. The reasons for such response are irrational use, over and under-dosing, and continuous exposure of Amoxycillin clavulanate to microbes in the field. Oxytetracycline is one of the first line treatment choice of field workers. Rubin *et al*. (2011) also reported the same results showing that more than 85% *S. aureus* isolates are sensitive to tetracyclines. Oppliger *et al*. (2012) also reported that *S. aureus* isolates from farm workers and animal products handlers have 100% susceptibility to oxytetracycline.

CONCLUSION

Present study found higher prevalence of biofilm producing *S. aureus* in buffalo and cattle milk. Significant association of risk factors are also increasing which alarms emergence of resistant strains. The spectrum of antibiotic efficacy got narrowed. Some of antibiotics like Cefoxitin were found effective despite of the factor of biofilm which is prominent finding. On the other hands, resistant strains of non-biofilm *S. aureus* were noted against wider range of antibiotics. The pattern of antibiotics response is altering which requires immediate attention.

Statement of conflict of interest

The authors have declared no conflict of interest.

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