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

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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, Amoxycillin, 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 heterogeneity, 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
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. Biofilm is reported to be well established even in case of interacting bacteria within biofilm (Munita 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., 2017, 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 (Aqib et al., 2017).

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., 2017, 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 1.5×10⁸ 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 (Aqib et al., 2017).

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 (p>0.05) (Table I). There were 72.73% of S. aureus isolates positive for biofilm production. Biofilm producing strains 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>0.05) 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 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 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 biofilm producing S. aureus

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 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>0.05) 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 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.

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

| Sample source | Total No. positive | Prevalence of *Staphylococcus aureus* (%) | C.I (95%) | p-value | Prevalence of biofilm producing *Staphylococcus aureus* (%) | 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               | 0.8389-1.000 |
| 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            | 33           | 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            | 70           | 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      | *bpSA          | p-value     | *nbpSA | *bpSA | p-value | *nbpSA | *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.

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*>0.05) 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., 2017, 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 (Aqib et al., 2017). 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 + sulframethoxazole, amoxy
clavulanate and oxacillin was also reported by (Jarraud et al., 2002). Some studies reported very low percentages of resistant isolates as conducted by (Carflora et al., 2015) found 1.3% of resistant isolates. The higher percentage of resistance strains may also appear in the absence of biofilm due to various factors inclusive of which are the high frequency gene islands like sec-seg-sef (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 Tn1546, 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 (Ragbetli et al., 2016). Vancomycin resistance in S. aureus when investigated at genomic level shows that the development of vanA 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 (Aqib et al., 2017). 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. Yilmaz and Aslantaş (2017) also reported the genes involved in antibiotic resistance. Aqib et al. (2017) reported 80% of S. aureus isolates are resistant to ampicillin. 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. Carflora 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 Amoxicillin 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 also reported the same results showing that more than 85% S. aureus isolates are sensitive to tetracyclines. 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.

REFERENCES

Begum, A., Uddin, H.S., Islam, M.J., Nazir, M., Islam, K.H.M.N.H. and Rahman, M.T., 2007. Detection of biofilm producing coagulase positive Staphylococcus aureus from bovine mastitis, their pigment production, hemolytic activity and antibiotic sensitivity pattern. J. Bangladesh Soc. Agric. Sci. Technol., 4: 97-100.

Aqb, A., Ijaz, M., Durrani, Z.A., Anjum, A.A., Hussain, R., Sana, S., Shahid, F., Hussain, K. and Saleem Ahmad, S., 2017. Prevalence and antibiogram of Staphylococcus aureus, a camel Mastitogen from Pakistan. Pakistan J. Zool., 49: 861-867. https://doi.org/10.17582/journal.pjz/2017.49.3.861.867

Aqb, A., Nighat, S., Ahmed, R., Sana, S., Jamal, M., Kulyar, M.F., Khan, U.N., Sarwar, S.M., Hussain, A.M., Asadullah, A., Rahman, A. and Rahman, U.S., 2018. Drug susceptibility profile of Staphylococcus aureus isolated from mastitic milk of goats and risk factors associated with goat mastitis in Pakistan. Pakistan J. Zool., 51: 307-315. https://doi.org/10.17582/journal.pjz/2019.51.1.307.315

Bille, J., Francioli, M., Glauser, M.R. and Moreillon, P., 1991. β-Lactam resistance mechanisms of methicillin-resistant Staphylococcus aureus. J. Infect. Dis., 163: 514–522. https://doi.org/10.1093/infdis/163.3.514

Carfora, V., Caprioli, A., Marri, N., Sagrufoli, D., Boselli, C., Giacinti, G., Giangolini, G., Sorbara, L., Dottarelli, S., Battisti, A. and Amatiste, S., 2015. Enterotoxin genes, enterotoxin production, and methicillin resistance in Staphylococcus aureus isolated from milk and dairy products in Central Italy. Int. Dairy J., 42: 12-15. https://doi.org/10.1016/j.idairyj.2014.10.009

CLSI (Clinical and Laboratory Standards), 2015. Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standard Institute. CLSI Document, Wayne, PA, pp. M100-S24.

Cosandey, A., Boss, R., Luini, M., Artursson, K., Bardiau, M., Brittenwieser, F., Hegenberger, E., Lam, T., Mansfeld, M., Michel, A., Mösslacher, G., Naskova, J., Nelson, S., Podpečan, O., Raemy, A., Ryan, E., Salat, O., Zangerl, P., Steiner, A. and Graber, H.U., 2016. Staphylococcus aureus genotype B and other genotypes isolated from cow milk in European countries. J. Dairy Sci., 99: 529–540. https://doi.org/10.3168/jds.2015-9587

Dobie, D. and Gray, J., 2004. Fusidic acid resistance in andlt; emandgt;Staphylococcus aureusandlt;/ emandgt; emandgt;Staphylococcus aureusandlt;/ emandgt; Arch. Dis. Child., 89: 74. https://doi.org/10.1136/adc.2003.019695

Edslev, S.M., Clausen, M.-L., Agner, T., Stegger, M. and Andersen, P.S., 2018. Genomic analysis reveals different mechanisms of fusidic acid resistance in Staphylococcus aureus from Danish atopic dermatitis patients. J. Antimicrob. Chemother., 73: 856–861. https://doi.org/10.1093/jac/dkx481

Gardete, S. and Tomasz, A., 2014. Mechanisms of vancomycin resistance in Staphylococcus aureus. J. Clin. Invest., 124: 2836–2840. https://doi.org/10.1172/JCI68834

Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A. and Iqbal, M., 2011. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz. J. Infect. Dis., 15: 305–311. https://doi.org/10.1590/S1413-86702011000400002

Ishii, Y., Ohno, A., Taguchi, H., Imaio, S., Ishiguro, M. and Matsuzawa, H., 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class
A beta-lactamase isolated from *Escherichia coli*. *Antimicrob. Agents Chemother.*, **39**: 2269. https://doi.org/10.1128/AAC.39.10.2269

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J. and Vandenesch, F., 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect. Immun.*, **70**: 631–641. https://doi.org/10.1128/IAI.70.2.631-641.2002

Kong, E.F., Tsui, C., Kucharíková, S., Andes, D., Van Dijck, P. and Jabra-Rizk, M.A., 2016. Commensal protection of *Staphylococcus aureus* against antimicrobials by *Candida albicans* biofilm matrix. *MBio*, **7**: e01365-16. https://doi.org/10.1128/mBio.01365-16

Kumar, R., Yadav, B.R. and Singh, R.S., 2010. Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Curr. Microbiol.*, **60**: 379–386. https://doi.org/10.1007/s00284-009-9553-1

Marques, J.B., Dalmolin, T.V., Bonez, P.C., Agertt, V.A., Campos, M.M.A. de and Santos, R.C.V., 2013. Detection of *Staphylococcus aureus* with an intermediate profile to vancomycin (VISA) isolate from Santa Maria, RS. *Brazilian J. Microbiol.*, **44**: 277–279. https://doi.org/10.1590/S1517-83822013000300029

Melchior, M.B., Vaarkamp, H. and Fink-Gremmels, J., 2006. Biofilms: A role in recurrent mastitis infections? *Vet. J.*, **171**: 398–407. https://doi.org/10.1016/j.tvjl.2005.01.006

Munita, J.M., Bayer, A.S. and Arias, C.A., 2015. Evolving resistance among Gram-positive pathogens. *Clin. Infect. Dis.*, **61 Suppl 2**: S48–S57. https://doi.org/10.1093/cid/civ523

Oppliger, A., Moreillon, P., Charrière, N., Giddey, M., Morisset, D. and Sakwinska, O., 2012. Antimicrobial resistance of *Staphylococcus aureus* strains acquired by pig farmers from pigs. *Appl. environ. Microbiol.*, **78**: 8010. https://doi.org/10.1128/AEM.01902-12

Rağbetli, C., Parlak, M., Bayram, Y., Guðucuoglu, H. and Ceylan, N., 2016. Evaluation of antimicrobial resistance in *Staphylococcus aureus* isolates by years. *Interdiscip. Perspect. Infect. Dis.*, **2016**: 9171395. https://doi.org/10.1155/2016/9171395

Rubin, J.E., Ball, K.R. and Chirino-Trejo, M., 2011. Antimicrobial susceptibility of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from various animals. *Can. Vet. J. La Rev. Vet. Can.*, **52**: 153–157. retrieved from internet: https://www.ncbi.nlm.nih.gov/pubmed/21532820.

Saba, C.K.S., Amenyon, J.K. and Kpordze, S.W., 2017. Prevalence and pattern of antibiotic resistance of *Staphylococcus aureus* isolated from door handles and other points of contact in public hospitals in Ghana. *Antimicrob. Resist. Infect. Contr.*, **6**: 44. https://doi.org/10.1186/s13756-017-0203-2

Tomasz, A., Drugeon, H.B., de Lencastre, H.M., Jabes, D., McDougall, L. and Bille, J., 1989. New mechanism for methicillin resistance in *Staphylococcus aureus*: clinical isolates that lack the PBP 2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. *Antimicrob. Agents Chemother.*, **33**: 1869. https://doi.org/10.1128/AAC.33.11.1869

Vancraeynest, D., Hermans, K. and Haesebrouck, F., 2004. Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. *Vet. Microbiol.*, **103**: 241–247. https://doi.org/10.1016/j.vetmic.2004.09.002

Yilmaz, E.S., and Aslantaş, Ö., 2017. Antimicrobial resistance and underlying mechanisms in *Staphylococcus aureus* isolates. *Asian Pac. J. Trop. Med.*, **10**: 1059–1064. https://doi.org/10.1016/j.aptm.2017.10.003

Zhang, Y., Xu, D., Shi, L., Cai, R., Li, C. and Yan, H., 2018. Association between agr type, virulence factors, biofilm formation and antibiotic resistance of *Staphylococcus aureus* isolates from pork production. *Front. Microbiol.*, **9**: Article Number: 1876. https://doi.org/10.3389/fmicb.2018.01876