



RESEARCH ARTICLE

Frequency and Antimicrobial Susceptibility of Methicillin and Vancomycin-Resistant *Staphylococcus aureus* from Bovine Milk

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ABSTRACT

The increase in resistance pattern of *Staphylococcus aureus* (*S. aureus*) has been an emerging threat in therapeutic areas of the dairy industry throughout the globe. The current study was conducted in bovines of district Faisalabad, Pakistan to investigate the phenotypic prevalence of methicillin-resistant (MRSA) and vancomycin-resistant *S. aureus* (VRSA) in milk samples positive for subclinical mastitis. The study further aimed to assess the associated risk factors and antimicrobial susceptibility pattern against MRSA and VRSA isolates. A total of 385 milk samples (n=193 cattle; n=192 buffalo) collected and screened for subclinical mastitis by surf field mastitis test (SFMT) were further subjected to standard microbiological techniques for the isolation of *S. aureus*. The positive isolates of *S. aureus* were phenotypically evaluated for MRSA and VRSA by the disc diffusion method. The study results revealed that out of 385 milk samples, 45.97% (177/385) samples were found positive for subclinical mastitis on SFMT while 37.14% (143/385) samples were confirmed for the presence of *S. aureus*. Out of these *S. aureus* isolates, MRSA and VRSA were confirmed in 17.48% (25/143) and 12.58% (18/143) samples respectively. The *in-vitro* trials of various antibiotics for MRSA and VRSA isolates showed 100% resistance towards Cefoxitin followed by 50% towards Gentamicin, Tylosin, and Trimethoprim + Sulfamethoxazole then 25% to Oxytetracycline, and Fusidic acid while ciprofloxacin, moxifloxacin, and linezolid were found sensitive against study isolates. The public health importance of *S. aureus* and emerging resistance against antibiotics like methicillin and vancomycin demands regular monitoring of effective use of antimicrobial agents against the isolates of VRSA and MRSA.

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INTRODUCTION

Bovine mastitis is considered a leading cause of a drastic decrease in milk production and tremendous financial losses to the dairy sector of any country (Abebe *et al.*, 2016). It not only decreases the quantity of milk but also deteriorates the milk quality by causing some physical, chemical, or microbiological changes which are considered undesirable and detrimental to a variety of dairy products (Heeschen, 2005). It causes loss of future

calf, milk reduction, discarded milk, replacement of animals, and decreased quarter-wise production (Khan *et al.*, 2019). About 135 different microbial pathogens have been isolated from mastitis milk. Among these pathogens, different species of *Streptococcus* and *Staphylococcus* are commonly associated with bovine mastitis. Concerning different *Staphylococci spp.*, *S. aureus* stands out as the most incriminated pathogen in the causation of this malady, and it has been considered a major threat to veterinary medicine throughout the world (Li *et al.*, 2017).

This pathogen has developed multi-drug resistance which helps to invade the host immune system (Altaf *et al.*, 2020).

Initially, penicillin was considered efficacious against many *staphylococcal* infections but in the mid-1940s, the strains of *S. aureus* had started to produce resistance against penicillin (Khan *et al.*, 2013). Due to its increased resistance towards penicillin, methicillin drugs were emerged, which were semi-synthetic beta-lactam antibiotics and resistant towards β -lactamase enzyme and were considered efficacious against penicillin-resistant *S. aureus*. The acquisition of the *mecA* gene causes methicillin resistance and this results in PBP2a, an alternative penicillin-binding protein with a decreased affinity for β -lactam antibiotics (Hamid *et al.*, 2017).

Vancomycin is considered a drug of last resort for severe mastitis caused by MRSA. The bactericidal effects of vancomycin occur by inhibiting peptidoglycan synthesis by binding to the D-ala-D-ala terminal of peptidoglycan Precursor lipid II (Blaskovich, 2018). Furthermore, D-Ala-D-Ala ends are highly conserved with Gram-positive bacteria including *Staphylococcus aureus*, making vancomycin effective against a wide range of gram-positive bacteria pathogens (Kim *et al.*, 2008).

The unchecked and enormous use of antibiotics (especially penicillin and cephalosporin groups) for the treatment of bovines in the field conditions of Pakistan, has led to the emergence of resistant strains of *S. aureus*. The resistance against methicillin and vancomycin produced by *S. aureus* has a bad impact in curing both

human and animal diseases. So, it is the need of the hour to check the status of resistant strains of *S. aureus* in bovines from the study area, because the responsible pathogen has a serious public health concern (Altaf *et al.*, 2019). Keeping in view the health and economic importance of pathogens, the current study was designed to find out the prevalence of subclinical mastitis in addition to the prevalence of MRSA and VRSA in association with assumed risk factors and antimicrobial susceptibility pattern of isolates obtained from bovine milk samples of district Faisalabad, Pakistan.

MATERIALS AND METHODS

Sampling strategy: The current study was conducted from June to September 2020 at various dairy farms located in different tehsils of district Faisalabad, Pakistan (Fig. 1). A total of 385 milk samples (n=193 cattle; n=192 buffalo) collected by convenient sampling method of non-probability sampling technique, were screened for subclinical mastitis by surf field mastitis test (SFMT) as narrated by (Muhammad *et al.*, 1995). The sample size was calculated based on a 50% prevalence of MRSA and VRSA at a 95% confidence interval by using the guidelines of Thrusfield (2007). The milk samples found positive on SFMT were collected in falcon tubes aseptically and were dispatched to Molecular Medicine Lab, Department of Veterinary Medicine, University of Veterinary and Animal Sciences (UVAS) Lahore for further processing.

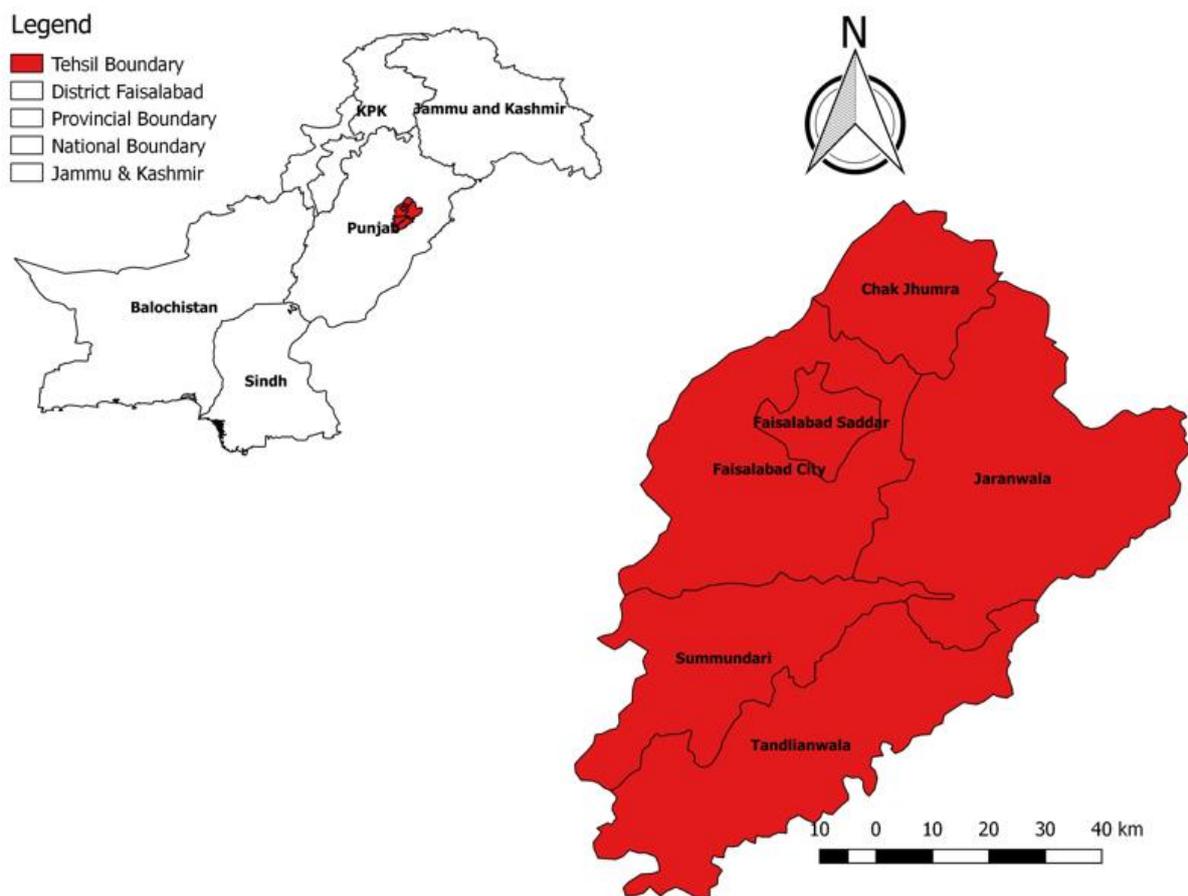


Fig. 1: QGIS map of study district Faisalabad, Punjab.

Analysis of Risk Factors: A data capture form was designed to assess the association of various risk factors related to animal and management practices with the occurrence of subclinical mastitis in bovines of district Faisalabad. The assumed risk factors considered for the data capture form include information like breed, animal health, grazing status, use of teat dips, parity status, physiological status, milk production, milkers' care during milking, presence of ticks, and use of antibiotics in general ailment and mastitis.

Isolation and identification of *S. aureus*: For the isolation and identification of *Staphylococci*, samples were subjected to standard microbial techniques (Cheesbrough *et al.*, 2006). Culturing of all milk samples (n=385) was carried out on blood agar and was incubated for 24-48 hours at 37°C. The bacterial colonies were further streaked to Mannitol salt agar with subsequent biochemical identification as per recommended protocols of Bergey's Manual of Systematic Bacteriology to confirm *S. aureus* (Holt *et al.*, 1994). *S. aureus* was further confirmed by microscopy, Gram staining, and biochemical testing such as catalase, coagulase, and mannitol fermentation test (Altaf *et al.*, 2020).

Phenotypic identification of MRSA and VRSA: For the identification of MRSA and VRSA, the oxacillin (1µg) and vancomycin (30µg) were placed on the activated growth of *S. aureus* (0.5 McFarland) on Muller Hinton agar plates respectively. The plates were incubated at 37°C for 24 hours. Zones of growth inhibition around antibiotics discs were measured by vernier calipers to compare with standard zones as described in Clinical and Laboratory Standard Institute (CLSI, 2020). The isolates showing resistance towards oxacillin discs were declared methicillin-resistant *Staphylococcus aureus* (MRSA) and those who were found sensitive were considered methicillin-sensitive *Staphylococcus aureus* (MSSA). Similarly, the isolates depicting resistance towards vancomycin discs were considered vancomycin-resistant *Staphylococcus aureus* (VRSA) while the remaining isolates were considered vancomycin-sensitive *Staphylococcus aureus* (VSSA) (CLSI, 2020).

In-vitro trial of MRSA and VRSA with other antibiotics: The *in vitro* susceptibility profile of VRSA/MRSA against various antibiotics including oxytetracycline (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Amikacin (30µg), Levofloxacin (5µg), Tylosin (30µg), Fusidic acid (10µg), Moxifloxacin (5µg), Cefoxitin (30µg), Linezolid (30µg) and Trimethoprim + Sulphamethoxazole (1.25µg, 23.75µg) was performed by placing antibiotic discs on Mueller Hinton agar streaked with activated VRSA/MRSA growth of 1×10^8 CFU/ml by giving incubation at 37°C for 24 hours. For this purpose, 3 groups were made (A, B, & C). Group A contained 4 isolates that were resistant to both vancomycin and oxacillin (VRSA/MRSA) and the group B contained those isolates that were only resistant to oxacillin but found sensitive to vancomycin (VSSA/MRSA) while group C was comprised of 4 isolates that were found sensitive to both vancomycin and oxacillin (VSSA/MSSA). To check the phenotypic prevalence of VRSA/MRSA against various antibiotics, the zones of

growth inhibition around the antibiotics discs were measured by vernier calipers and were compared with the standards of CLSI 2020.

Statistical analysis: The prevalence of MRSA and VRSA was calculated as per the formula narrated by Thrusfield, (2007). The risk factor analysis was done by non-probability testing using the chi-square method and those determinants which had ($P < 0.2$) were selected for the final multivariable logistic regression technique. The significance was checked at 5% probability ($P < 0.05$) using SPSS version 20 of the computer program.

RESULTS

The current study revealed an overall prevalence of subclinical mastitis of 45.97% (177/385) by surf field mastitis test (SFMT) from bovines of district Faisalabad. The prevalence was higher in cattle at 47.15% (91/193) as compared to buffaloes at 44.79% (86/192). Out of 385 milk samples swabbed on Mannitol salt agar, 37.14% (143/385) samples were confirmed as *S. aureus* based on its colony morphology and biochemical tests. The confirmed *S. aureus* isolates were further preceded to antimicrobial susceptibility testing on Mueller Hinton agar by applying antibiotic discs giving the incubation of 24 hours at 37°C. The phenotypic prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) 14.12% (25/177) and vancomycin-resistant *Staphylococcus aureus* (VRSA) was noted to be 10.16% (18/177) from 177 SFMT positive isolates while out of 143 confirmed *S. aureus* isolates, the prevalence of phenotypic VRSA and MRSA was found to be 12.58% (18/143) and 17.48% (25/143) respectively.

Risk factors associated with Subclinical Mastitis in Bovine: Risk factor-like hygiene during milking was found significantly ($p = 0.00$) associated with the occurrence of SCM (Table 1). Animals having bad hygienic conditions were observed to have more rate of SCM (54.43%) as compared to those with good hygiene (32.43%). The odds of having SCM in animals with poor hygiene during milking were 1.813 times higher than where hygiene was good (Table 2). A similar type of finding was observed for milkers' care during milking. Poor milkers' care during milking results in more prevalence (56.00%) of SCM as compared to animals with good milking care (31.87%) (Table 1).

The use of teat dip was found non-significant ($p = 0.06$) risk factor associated with disease occurrence. Disease management of SCM by self or veterinary professionals was also statistically assessed. Animals having veterinary care by the veterinary officers were found to be less suffered (33.33%) with SCM as compared to those who were treated on self-treatment (86.66%) by the owner. The parity of animals was found significant determinant towards disease occurrence. Animals in 1st, 2nd, and 3rd parity showed more prevalence of SCM occurrence, the odds of having the disease in 2nd and 3rd parity was found to be 1.327 and 1.079 times more prevalent than in first and more than 3rd parity. Contrarily, the presence of ticks, number of milking, physiological status and provision of feed and water were found statistically non-significant factors ($P > 0.05$) with the disease occurrence.

Table 1: Risk factors analysis for subclinical mastitis in bovines of district Faisalabad

Variable	Variable levels	No. of collected samples	Positive (%)	P-value
Specie	Cattle	193	91 (47.15)	0.64
	Buffalo	192	86 (44.79)	
Parity	1 st	45	28 (62.22)	0.04*
	2 nd	135	54 (40.00)	
	3 rd	116	50 (43.10)	
	>3 rd	89	45 (50.56)	
Physiological Status	Lactating	356	162 (45.50)	0.51
	Dry	29	15 (51.72)	
No. of Milking	Twice	339	161 (47.49)	0.10
	Thrice	46	16 (34.78)	
Milker's care during Milking	Good	160	51 (31.87)	0.00*
	Poor	225	126 (56.00)	
Hygiene during Milking	Yes	148	48 (32.43)	0.00*
	No	237	129 (54.43)	
Milk Yield	Low	345	159 (46.08)	0.89
	High	140	18 (12.85)	
Use of Teat dips	Yes	25	7 (28.00)	0.06
	No	360	170 (47.22)	
Presence of Ticks	Yes	50	29 (58.00)	0.06
	No	335	148 (44.17)	
Body Condition	Normal	354	162 (45.76)	0.90
	Thin	24	12 (50.00)	
	Emaciated	07	03 (42.85)	
Feed and Water	Well-fed	373	171 (45.84)	0.77
	Underfed	12	6 (50.00)	
Feeding System	Stall Feeding	152	73 (48.02)	0.52
	Grazing	70	28 (40.00)	
	Grazing + Stall Feeding	163	76 (46.62)	
Grazing Status	Mixed	266	89 (33.45)	0.00*
	Separate	119	88 (73.94)	
Disease Management	Veterinary Officer	168	56 (33.33)	0.00*
	Veterinary Assistant	187	95 (50.80)	
	Self	30	26 (86.66)	

P<0.05 Significant effect, P>0.05 Non-significant effect.

Table 2: Analysis of risk factors associated with occurrence of subclinical mastitis in bovines by logistic regression model

Variables	Variable levels	Odd ratio	95% C.I.		S.E	P-value
			Lower	Upper		
Parity	First	0.583	0.259-1.315	0.415	0.194	
	Second	1.327	0.725-2.429	0.308	0.358	
	Third	1.079	0.571-2.041	0.325	0.814	
	More than 3 rd	1				
No. of milking	Twice	0.639	0.309-1.320	0.370	0.226	
	Thrice	1				
Milkers' care during milking	Poor	0.579	0.359-0.935	0.244	0.025	
	Good	1				
Hygiene during milking	No	1.813	1.127- 2.918	0.243	0.014	
	Yes	1				
Use of teat dips	Yes	0.617	0.234- 1.630	0.496	0.330	
	No	1				
Presence of ticks	Yes	0.606	0.306- 1.199	0.348	0.150	
	No	1				
Grazing status	Mixed	4.652	2.801- 7.724	0.259	0.000	
	Separate	1				

Table 3: *In-vitro* efficacy of different antibiotics against VRSA/MRSA, VSSA/MRSA, and VSSA/MSSA isolates

Antibiotics discs	Group A			Group B			Group C		
	VRSA/MRSA isolates (%) N=4			VSSA/MRSA isolates (%) N=4			VSSA/MSSA isolates (%) N=4		
	S	I	R	S	I	R	S	I	R
Oxytetracycline (30 µg)	75	0	25	75	0	25	100	0	0
Ciprofloxacin (5 µg)	100	0	0	100	0	0	100	0	0
Gentamicin (10 µg)	50	0	50	50	0	50	75	0	25
Amikacin (30 µg)	50	0	50	50	0	50	75	0	25
Levofloxacin (5 µg)	75	0	25	75	0	25	100	0	0
Tylosin (30 µg)	50	0	50	75	0	25	100	0	0
Fusidic acid (10 µg)	75	0	25	75	0	25	100	0	0
Moxifloxacin (5 µg)	100	0	0	100	0	0	100	0	0
Cefoxitin (30 µg)	0	0	100	50	0	50	100	0	0
Linezolid (30 µg)	100	0	0	100	0	0	100	0	0
Trimethoprim + Sulfamethoxazole (1.25 µg, 23.75 µg)	50	0	50	75	0	25	75	0	25

Results of *in-vitro* trials of MRSA/VRSA with various antibiotics: The isolates of group A showed 100% resistance towards Cefoxitin followed by 50% towards Gentamicin, Amikacin, Tylosin, and Trimethoprim + Sulfamethoxazole then 25% to Oxytetracycline, Levofloxacin, and Fusidic acid while the isolates were found 100% sensitive to Ciprofloxacin, Moxifloxacin, and Linezolid. In group B, 50% resistance was also shown by Gentamicin, Cefoxitin, and Amikacin followed by 25% resistance towards Tylosin, Levofloxacin, Fusidic acid, Oxytetracycline, and Trimethoprim + Sulfamethoxazole while the isolates showed 100% sensitivity towards Ciprofloxacin, Moxifloxacin, and Linezolid as in group A. The susceptibility pattern of the group C isolates only showed 25% resistance towards Gentamicin, Amikacin, and Trimethoprim + Sulfamethoxazole while all other isolates were found sensitive against remaining antibiotics. It was also observed that Ciprofloxacin, Moxifloxacin, and Linezolid were found sensitive in all groups as none of the isolates from any group showed some resistance against these 3 antibiotics as shown in Table 3.

DISCUSSION

Antimicrobial therapy is a chief component of modern clinical practice but due to excessive use of antibiotics, the incidence of antibiotic-resistant strains of *S. aureus* has direfully increased and made the treatment process very complicated (Altaf *et al.*, 2020). The development of antibiotic resistance in pathogens has emerged as a serious public health concern as this pathogen can be transferred to human beings through improper handling or consumption of infected milk or meat products (Caruso *et al.*, 2016).

S. aureus is a major public health challenge in the dairy industry due to antibiotic resistance problems (Khan *et al.*, 2019). The current study showed a 45.97% (177/385) prevalence of subclinical mastitis and 37.14% (143/385) of *Staphylococcus aureus*. The prevalence of subclinical mastitis in dairy cattle and buffalo of Sindh, Pakistan was reported to be 26.95 % (Baloch *et al.*, 2016). Findings of subclinical mastitis and a higher rate of *Staphylococcus aureus* prevalence were also in line with recent studies (Aqib *et al.*, 2017).

In the current study, the prevalence of MRSA in bovine was 17.48% which was similar to the findings of (Spohr *et al.*, 2011) who reported a 16.7% prevalence of MRSA in bovine of Germany. The current findings were also similar to the prevalence reported in India (Kumar *et al.*, 2011) and Korea (Lim *et al.*, 2013) which was 13.1% and 6.3% respectively. The prevalence was lower than the study conducted in China (Pu *et al.*, 2014), and the previous study conducted in Pakistan (Aqib *et al.*, 2017) concluded 47% and 34% prevalence of MRSA in bovines.

The current study revealed that isolates who showed vancomycin resistance were 12.58% (18/143). This prevalence of VRSA found in the current study was close to the findings of (Omara, 2017) and Swetha *et al.* (2017) who reported VRSA 13 and 11.9% in milk samples in India. Contrary to the findings of the current study, a higher prevalence of VRSA (84.6%) was reported by (Maalik *et al.*, 2019). Similarly, a lower prevalence of 3.4% was also reported by Ateba *et al.* (2010) from South

Africa. Vancomycin resistance is a rising problem in clinical isolates of *S. aureus* and it is increasing progressively. It may be due to the acquired resistance as occurred in the case of methicillin (Marques *et al.*, 2013). *S. aureus* shows resistance against vancomycin due to acquired transposon Tn1546, from vancomycin-resistant *Enterococcus faecalis*, which is responsible for alteration in the cell wall structure and cellular metabolism of isolates (Gardete and Tomasz, 2014). Glycopeptide antibiotics, vancomycin is the last remedy to treat severe clinical infections of MDR *S. aureus* throughout the world. But the incessant use of vancomycin for treating MDR *S. aureus* infections is responsible for reduced vancomycin sensitivity in many countries

The current study reported a significant effect of parity on the occurrence of mastitis similar to (Muzammil *et al.*, 2021) and contradictory to the previous reports (Moges *et al.*, 2011). The occurrence of mastitis was observed higher in multiparous cows as compared to primiparous cows. Animals with tick infestation have poor body condition and immune response, so bacteria can easily attack them and produce infection. However, the current study has not reported a significant effect of tick infestation on mastitis which is in line with (Lakew *et al.*, 2019). Hygiene during milking and milkers' care during milking were considered as potential risk factors associated with the increased prevalence of mastitis. If proper hygienic measures are not adopted during milking, chances of mastitis occurrence become high due to dirty udder and legs as a result of the poor waste drainage system on dairy farms. Current study results were in line with previous studies in which a significant effect of udder hygiene on mastitis occurrence was noted (Altaf *et al.*, 2020). Direct contact with contaminated milk and the environment also contribute to the spread of MRSA. The situation is exacerbated by mismanagement on farms and the unjustifiable use of drugs in widespread diseases including mastitis (Nunang and Young, 2007; Juhász-Kaszanyitzky *et al.*, 2007). A person's unsanitary hands during milking are a potential cause of the spread of mastitis infectious agents during the milking process (Seifu and Tafesse, 2010).

MRSA/VRSA isolates of the current study showed 100% resistance towards Cefoxitin followed by Gentamicin, Amikacin, Tylosin, and Trimethoprim + Sulfamethoxazole while the isolates were found 100% sensitive to Ciprofloxacin, Moxifloxacin, and Linezolid. Current study findings are in line with the study conducted by (Aqib *et al.*, 2017) who also shared the results of 100% sensitivity of Ciprofloxacin, Moxifloxacin, and Linezolid towards the MRSA isolates while 90% of isolates were found sensitive towards Gentamicin and Levofloxacin followed by 60, 50 and 40% towards Tylosin, and Oxytetracycline. The findings of the present study related to the sensitivity of Linezolid and resistance towards Cefoxitin were also in agreement with (Nemeghaire *et al.*, 2014) while the sensitivity pattern of Ciprofloxacin differs from the current study in which only 84% of the isolates were found resistant. The findings of the current study were found contrary to the study designed by (Umaru *et al.*, 2014) who concluded that 10% of the isolates were found resistant towards Sulphamethoxazole/Trimethoprim and only 5% of the

isolates were resistant against Amikacin while the results of both studies were in agreement that Ciprofloxacin was found sensitive against all the isolates. Different researches around the globe have concluded that the occurrence of resistance against antimicrobial agents is natural in this era of antibiotic controlling problems due to the adaptability of evolutionary and genetic changes in the microorganisms. Other important aspects that are limiting the treatment of these multidrug-resistant bacteria include; reduced investment in the development of new antibiotics and increased economic costs (Gonzalez-Bello, 2017).

Conclusions: The milk samples positive for SCM collected from different tehsils of district Faisalabad, Punjab revealed a significant prevalence of methicillin-resistant and vancomycin-resistant *S. aureus* (MRSA/VRSA). *In-vitro* therapeutic efficacy showed that Linezolid, Moxifloxacin, and Ciprofloxacin were found sensitive against all study groups of bovine. However, other antibiotics were also found effective against the study isolates from bovine milk and concluded the promising treatment options against prevalent strains of both VRSA and MRSA from bovine milk. The global issue of multidrug resistance in bacteria particularly in MRSA/VRSA can be diminished by regular detection and treatment protocol, recurrent multidrug treatment, specificity for control, active surveillance, and by adopting proper control measures.

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Authors contribution: MI designed the study. MUJ and AG helped in study execution. AIA and AR contributed in data analysis and interpretation. MA and AA prepared the manuscript. AAA and ZF reviewed the manuscript. All authors gave final approval for the manuscript.

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