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RESEARCH ARTICLE

Molecular Characterization and Therapeutic Insights into Biofilm Positive *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis

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ABSTRACT

The current study aimed to investigate the prevalence and molecular characterization of biofilm-positive *S. aureus* isolates from bovine subclinical mastitis. The study also highlights the role of commonly used NSAIDs and ivermectin to modulate the *S. aureus*-associated antibiotic resistance. The results found a 41.41% *S. aureus* prevalence, out of which 25.79% isolates were biofilm-positive based on Congo red agar, microtitre plate test, and presence of *icaA* gene. Phylogenetic analysis of study isolates showed a high similarity with Egyptian and Indian *icaA*-positive *S. aureus* isolates. The comparative antibiotic resistance profiling showed a significantly (p<0.05) higher resistance to gentamicin, oxytetracycline, and cotrimoxazole by biofilm-positive isolates compared to non-biofilm forming isolates. The prevalence of methicillin and vancomycin resistant *S. aureus* was 62.5 and 20.83%, respectively. Antimicrobial effects of non-antibiotics against study isolates accessed through well diffusion method showed higher zones of inhibition for meloxicam followed by flunixin, ketoprofen, and ivermectin. The combinations of resistant antibiotics with non-antibiotics were investigated using well diffusion method and checkerboard assay. The combinations of amoxicillin/meloxicam, cotrimoxazole/flunixin, cotrimoxazole/ketoprofen, and gentamicin/flunixin on well diffusion method and cotrimoxazole/flunixin, amoxicillin/ketoprofen and gentamicin/flunixin on checkerboard assay revealed synergistic interactions. The study concluded that biofilm positive *S. aureus* is an emerging and prevailing cause of bovine mastitis in dairy farms of Pakistan. The increasing antibiotic resistance in *S. aureus* can be modulated by combining the resistant antibiotics with NSAIDs, especially flunixin and ketoprofen.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*), a pathogen of veterinary and public health concern, is liable for a range of infections from mild skin issues to fatal endocarditis. In dairy animals, *S. aureus* is frequently associated with subclinical mastitis and leads to huge economic losses for the dairy industry (Abdeen *et al*., 2021; Aqib *et al*., 2021; Ijaz *et al*., 2021). Among several defensive mechanisms adopted by *S. aureus*, the biofilm production, mainly mediated by the *ica*A and *ica*D genes, is a potential virulence factor involved in bacterial evasion from host immune surveillance and leading to persistent udder infections in dairy animals (Thiran *et al*., 2018). Moreover, biofilm-producing *S. aureus* exhibits reduced antimicrobial susceptibility due to poor penetration of antimicrobials, slower growth of bacteria, and horizontal transfer of antibiotic resistance genes in biofilm (Shin *et al*., 2021).

The imprudent and indiscriminate usage of antibiotics in public and veterinary clinical practices has led to the development of multiple drug-resistant (MDR) pathogens (Yang *et al*., 2017; Javed *et al*., 2021). Commonly practiced antibiotics for bovine mastitis treatment i.e. beta-lactams, macrolides, lincosamides, streptogramins, and fluoroquinolones are facing resistance due to their undue and persistent usage in animals (Aqib *et al*., 2021). The number of effective antibiotics against MDR pathogens is rapidly declining and the advent of new antibiotics in clinical practice requires a prolonged time and has monetary challenges as well. It is a crucial need to either identify new antibiotics or modulate the resistance

of already available antimicrobials for the treatment of MDR pathogens (Kamble *et al*., 2022). Drug repositioning and synergy testing of non-antibiotics to augment the antibacterial activity of drugs against biofilm-producing MDR *S. aureus* seems to be a promising strategy for future implementations. The synergistic effects of combination therapy can improve anti-biofilm action and support the prevention or delay of antibiotic resistance development (Field *et al*., 2016). For drug repurposing or repositioning, the non-steroidal anti-inflammatory drugs (NSAIDs) are known to possess the anti-bacterial and anti-biofilm activity against numerous gram-positive and gram-negative bacteria. NSAIDs are routinely used with antibiotics in case of mastitis in dairy animals to cure the signs of inflammation. The possible antibacterial mode of action of these drugs may be associated with their ability to disrupt the cytoplasmic membrane of bacteria (Leão *et al*., 2020).

The current research was conducted to investigate the prevalence, molecular characterization and *in vitro* antistaphylococcal activity of three different NSAIDs and ivermectin alone and in combination with clinically important antibiotics against molecularly characterized biofilm-positive MDR *S. aureus*.

MATERIALS AND METHODS

Bacterial isolates: A total of 384 milk samples were aseptically collected from dairy farms of district Rawalpindi, Pakistan from July 2021 to February 2022 (Fig. 1). The milk samples were screened for subclinical mastitis using California mastitis test and positive samples were shifted to laboratory for further microbiological procedures. The *S. aureus* isolates from positive milk samples were confirmed microbiologically and biochemically based on colony characters and hemolysis pattern on blood agar, Mannitol fermentation test, Gram staining, catalase test, and coagulase test following the guidelines of Javed *et al*. (2021).

Assessment of biofilm-forming capability: All isolates were investigated for biofilm forming ability using different qualitative and quantitative assays. The isolates were subjected to Congo red agar (CRA) method for qualitative biofilm detection following the protocol of Freeman *et al*. (1989). Black color colonies with rough edges were declared biofilm producers isolates while red color colonies with smooth edges were indicative of non-biofilm producer isolates. The isolates were further checked by microtitre plate method (MTP) as per procedure mentioned in previous studies (Darwish and Asfour, 2013). The biofilm-forming ability was accessed by measuring the optical density (OD) of each well of microtitre plate at 570 nm using an ELISA reader. Cut-off OD (ODc) is the three standard deviations above the mean OD of the negative control. The isolates with OD value less than ODc were declared non-biofilm producer while those isolates that showed OD value greater than ODc were declared biofilm producers (Darwish and Asfour, 2013).

Molecular characterization of *icaA* gene: For molecular confirmation of biofilm-associated intercellular adhesion A (*icaA)* gene, the DNA was extracted from phenotypically confirmed isolates using DNA extraction kit method. The gene was detected using primers (P1: 5′-CCT AAC TAA CGA AAG GTAG-3′; P2: 5′-AAG ATA TAG CGA TAA GTG C) and conditions (5 minutes initial denaturation at 92℃ followed by 30 cycles of denaturation at 92℃ at 45℃, annealing at 56.5℃ for 45 seconds, elongation at 72℃ for 1 minute and final extension at 72℃ for 7 minutes) reported by (Vasudevan *et al*., 2003) and the isolates were declared *icaA* positive by observing the PCR product size of 1315 bp (Fig. 2). The positive PCR products were purified and sequenced. The sequences were submitted to GenBank and accession numbers were obtained as ON843647, ON843648, ON843649, ON843651, ON843652 and ON843653. The genetically diverse and representative sequences of study isolates were selected for phylogenetic analysis. The known sequences of *icaA* gene of *S. aureus* were retrieved from GenBank database for comparison purpose. Multiple sequences were aligned and analyzed by the *Clustal W* method of Bioedit software (version 7.5.0.3) and a phylogenetic tree was constructed on MEGA 6.0 software using Maximum Likelihood method with bootstrap analysis of 1000 replicates.

Antibiotic susceptibility of isolates: The antibiotic resistance profile of molecularly confirmed biofilm-positive and negative isolates was compared by the Kirby-Bauer disc diffusion test according to the already reported guidelines (Javed *et al*., 2021; Ghumman *et al.,* 2022). The used antibiotics include cefoxitin (30 µg), oxacillin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), vancomycin (30 µg), oxytetracycline (30 µg), tylosin (30 µg), chloramphenicol (10 µg), trimethoprim/ sulfamethoxazole (25 µg), fusidic acid (10 µg), and linezolid (30 µg). The zones of inhibition of antibiotics were compared with standard zones as given by Clinical Laboratory Standards Institute (CLSI) to declare the isolates resistant or sensitive. The isolates showing resistance to three or more antibiotics were declared multidrug resistant (MDR).

Synergy testing of NSAIDs and ivermectin: For *in vitro* trials, *icaA*-positive *S. aureus* isolates showing resistance to trimethoprim/sulfamethoxazole (cotrimoxazole), amoxicillin and gentamicin were selected.

Well diffusion method: The isolates were tested against NSAIDs (Flunixin meglumine, Ketoprofen, and Meloxicam) and ivermectin separately and in combination with cotrimoxazole, amoxicillin, and gentamicin using well diffusion method as per protocol followed by (Aqib *et al*., 2021). The resultant zones of inhibition (ZOI), the percentage increase in ZOI, and the modulation factor for ZOI was calculated (Aqib *et al*., 2021). The combinations of antibiotics with non-antibiotics showing modulation factor <0.5 were declared synergistic.

Broth micro-dilution method: Minimum inhibitory concentration (MIC) of cotrimoxazole, amoxicillin, gentamicin, NSAIDs (Flunixin meglumine, Ketoprofen, and Meloxicam) and ivermectin was determined using broth micro-dilution method using a 96 wells titration

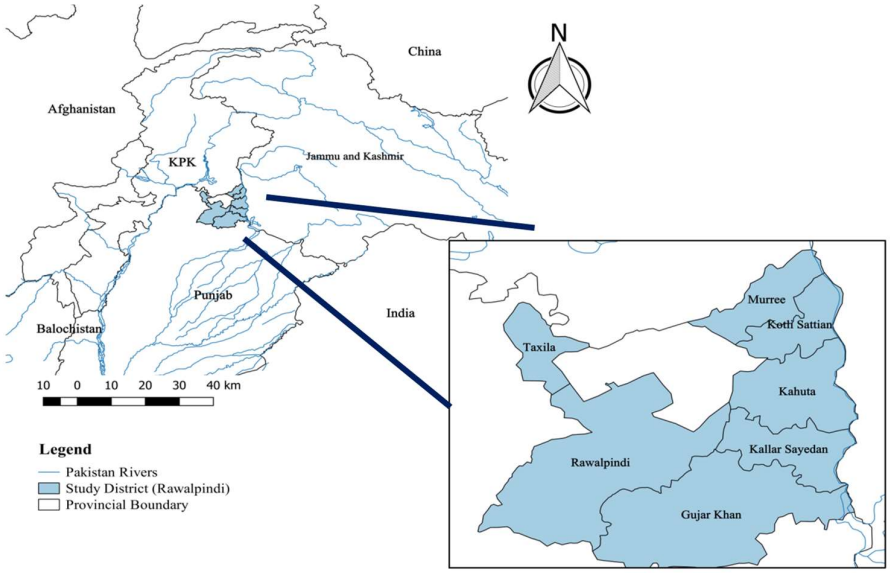

Fig 1: Map showing areas for collection of *S. aureus* isolates of dairy origin

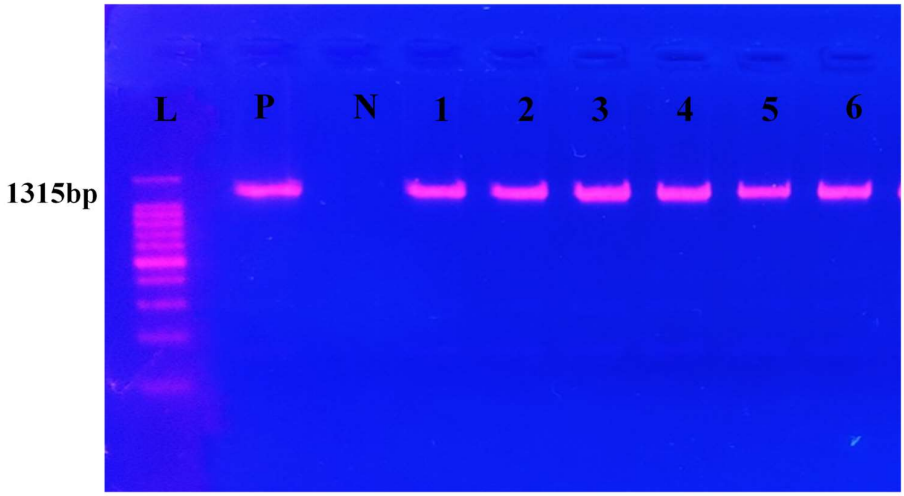

Fig 2: PCR results of biofilm-producing *icaA* gene positive *S. aureus* isolates showing bands at 1315bp. Lane L indicates ladder of 100bp molecular weight. Lane P = positive control, Lane N = negative control, Lane 1-6 = biofilm producing *S. aureus* isolates of bovine mastitis origin

plate. The combinations of these antibiotics with non-antibiotics were further subjected to the checkerboard method. In both procedures, serial dilutions of isolates were made in the wells and optic density (OD) was measured at 570 nm before and after 37ºC overnight incubation (Eman *et al*., 2016). Fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) of each combination was accessed as per the guidelines of (Altaf *et al*., 2019a).

Statistical analysis: The prevalence was calculated according to the formula described by (Thrushfield, 2013). The results of antimicrobial susceptibility trials were analyzed by descriptive statistics and the Chi-square test using SPSS (version 20). The difference with $p<0.05$ was considered significant.

RESULTS

Status of biofilm-positive *S. aureus*: The current study found a 41.41% (159/384) prevalence of *S. aureus*-associated subclinical mastitis from the study district with a higher prevalence in small dairy farms (58.95%) compared to medium (40.12%) and large dairy setups (29.51%). *In vitro* screening by Congo red agar and Microtitre plate method revealed biofilm-forming potential in 35.85 and 30.19% *S. aureus* isolates respectively. The *icaA* gene was confirmed molecularly in 45.28% of isolates while 41 isolates (25.79%) were positive to biofilm formation on both phenotypic as well as genotypic methods (Table 1).

Molecular characterization of *icaA* gene: The Phylogenetic analysis of *icaA* gene sequences of study isolates with already reported gene sequences was conferred by constructing a phylogenetic tree using Maximum Likelihood method. The results revealed that current gene sequences (Accession no. ON843649 and ON843653) showed high resemblance with *icaA* gene sequence of *S. aureus* isolated from bovine milk in India (MF346931) (Fig. 3). Similarly, other two study sequences (ON843652 and ON843651) also form same clad with *icaA* gene sequence from India (JX298872). However, the gene sequences (ON843647 and ON843648) lies in a same clad with sequence from Egypt (KT248386) (Fig. 3). In an overall scenario, the current study gene sequences resemble more with the sequences from neighboring countries.

Table 1: Status of biofilm-positive *S. aureus* from bovine subclinical mastitis in study district

Farm type	No. of samples	*S. aureus* (%)	Biofilm Detection Method (%)					
			CRA		MTP		*ica*A gene	
			Positive	%	Positive	%	Positive	%
Small	95	56 (58.95)	26	46.43	21	37.50	31	55.36
Medium	167	67 (40.12)	22	32.84	19	28.36	28	41.79
Large	122	36 (29.51)	09	25.00	08	22.22	13	36.11
Total	384	159 (41.41)	57	35.85	48	30.19	72	45.28

CRA = Congo red agar method; MTP = Microtitre plate method; *icaA* = intercellular adhesion gene: Small farm = < 50 animals; Medium farm = 50-200 animals; Large farm = >200 animals.

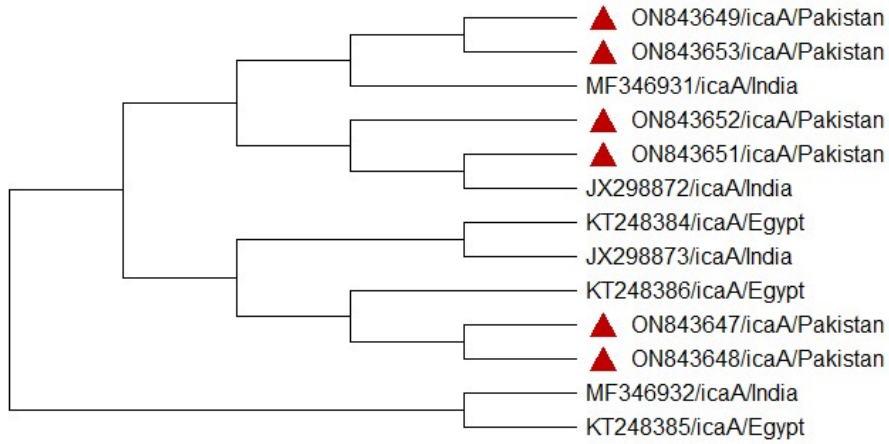

Fig. 3: Phylogenetic tree of *icaA* gene of *S. aureus* constructed by Maximum Likelihood method: Note: Red color indicates study isolates

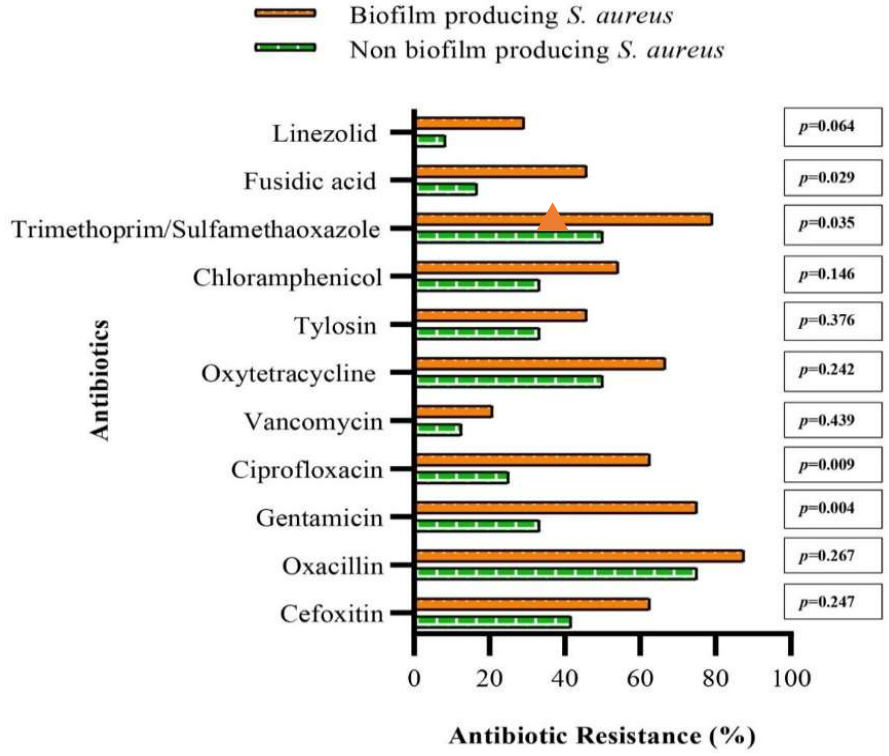

Fig 4: Comparison of antibiotic resistance pattern between biofilm-producing and non-producing *S. aureus* strains of dairy origin: Note: $p<0.05$ indicates significant difference

Comparative antibiotic resistance profile: Biofilm-positive isolates showed higher resistance against oxacillin (87.5%), followed by trimethoprim/ sulfamethoxazole (79.16%), gentamicin (75%), oxytetracycline (66.66%), and cefoxitin (62.5%). Vancomycin inhibited the bacterial growth but yet, 20.83% of isolates showed resistance against it. Fusidic acid and linezolid were among the least resistant antibiotics against biofilm-positive isolates. The comparative antibiotic resistance pattern showed that biofilm-positive isolates differed significantly from biofilm-negative isolates in showing resistance to gentamicin, ciprofloxacin, and trimethoprim/ sulfamethoxazole ($P<0.05$). Fusidic acid also showed a similar response ($P<0.05$) (Fig. 4).

Effect of NSAIDs and ivermectin on ZOI modulation: Among antibiotics, amoxicillin showed a higher ZOI compared to cotrimoxazole and gentamicin. Meloxicam and flunixin exhibited the highest while ivermectin

Table 2: Comparison of zones of inhibition (mm) of tested drugs (alone and in combination) against *icaA* positive *S. aureus*

Antibiotic, Non-antibiotic	Antibiotic alone	Non-antibiotic alone	Zone in combination	% increase in ZOI of antibiotic	Modulation factor
Cotrimoxazole, Flunixin Meglumine	12.21±0.68	7.21±1.23	26.47±1.02	116.79%	12.21/26.47 (0.46)
Cotrimoxazole, Ketoprofen	12.21±0.68	6.82±1.04	25.84±0.63	111.63%	12.21/25.84 (0.47)
Cotrimoxazole, Meloxicam	12.21±0.68	7.92±1.65	20.44±0.79	67.40%	12.21/20.44 (0.60)
Cotrimoxazole, Ivermectin	12.21±0.68	3.99±0.40	14.44±0.98	18.26%	12.21/14.44 (0.85)
Amoxicillin, Flunixin Meglumine	17.25±1.31	7.21±1.23	29.99±1.35	73.86%	17.25/29.99 (0.57)
Amoxicillin, Ketoprofen	17.25±1.31	6.82±1.04	25.91±1.05	50.20%	17.25/25.91 (0.67)
Amoxicillin, Meloxicam	17.25±1.31	7.92±1.65	35.51±0.98	105.86%	17.25/35.51 (0.49)
Amoxicillin, Ivermectin	17.25±1.31	3.99±0.40	18.92±0.82	09.68%	17.25/18.92 (0.91)
Gentamicin, Flunixin Meglumine	11.83 ±1.14	7.21±1.23	24.42±0.98	106.42%	11.83/24.42 (0.48)
Gentamicin, Ketoprofen	11.83 ±1.14	6.82±1.04	16.99±0.60	43.62%	11.83/16.99 (0.69)
Gentamicin, Meloxicam	11.83 ±1.14	7.92±1.65	19.27±0.74	62.89%	11.83/19.27 (0.61)
Gentamicin, Ivermectin	11.83 ±1.14	3.99±0.40	12.64±0.95	06.85%	11.83/12.64 (0.93)

Percentage increase in ZOI = ZOI of antibiotic in combination – ZOI of antibiotic alone / ZOI of antibiotic alone × 100: Modulation factor = ZOI alone / ZOI in combination: Modulation factor <0.5 indicates synergy.

Table 3: Synergy testing of non-antibiotics with antibiotics using broth micro-dilution method

Antibiotic + Non-antibiotic	MIC AB	MIC A	FIC A	MIC BA	MIC B	FIC B	FICI	Remarks
Cotrimoxazole + Flunixin	6.579	17.64558	0.37	3.4789	28.426	0.12	0.49	Synergistic
Cotrimoxazole + Ketoprofen	8.7589	16.639	0.53	45.9432	125	0.37	0.90	Additive
Cotrimoxazole + Meloxicam	3.277	7.8952	0.42	26.764	79.583	0.34	0.76	Additive
Cotrimoxazole + Ivermectin	4.57958	3.6527	1.25	75.4178	66.793	1.13	2.38	Indifferent
Amoxicillin + Flunixin	19.2739	21.5768	0.89	26.1398	16.724	1.56	2.45	Indifferent
Amoxicillin + Ketoprofen	6.78	18.925	0.36	10.625	98.547	0.11	0.47	Synergistic
Amoxicillin + Meloxicam	3.8795	8.4289	0.46	31.506	87.848	0.36	0.82	Additive
Amoxicillin + Ivermectin	32.6793	28.520	1.15	5.958	2.1593	2.76	3.91	Indifferent
Gentamicin + Flunixin	21.479	80.517	0.27	4.569	20.418	0.22	0.49	Synergistic
Gentamicin + Ketoprofen	9.4589	17.8313	0.53	24.8206	58.932	0.42	0.95	Additive
Gentamicin + Meloxicam	8.92056	13.591	0.66	19.4386	67.31935	0.29	0.95	Additive
Gentamicin + Ivermectin	3.8942	2.198	1.77	9.8467	16.7258	0.59	2.36	Indifferent

MIC = Minimum inhibitory concentration, FIC = Fractional inhibitory concentration, FICI = Fractional inhibitory concentration indices: An FICI of ≤0.5 was considered as synergistic, >0.5 but ≤1.0 as an additive, >1.0-4 as indifferent but >4.0 as antagonism.

revealed the lowest ZOI among all non-antibiotics (Table 2). Cotrimoxazole showed the highest percentage increase in ZOI when combined with NSAIDs compared to amoxicillin and gentamicin. Highest synergistic interaction (modulation factor <0.5) among antibiotic-NSAID combinations was observed in cotrimoxazole+ flunixin. meglumine (0.46), followed by cotrimoxazole+ ketoprofen (0.47), gentamicin + flunixin meglumine (0.48), and amoxicillin + meloxicam (0.49). Ivermectin combination with antibiotics revealed a lesser increase in ZOI (Table 2).

Resistance modulation by synergy testing: Synergy testing of antibiotics with non-antibiotics against *icaA* positive MDR *S. aureus* exhibited a synergistic interaction in combinations of gentamicin with flunixin meglumine, cotrimoxazole with flunixin meglumine and amoxicillin with ketoprofen (Table 3). The additive effect was shown by combinations of cotrimoxazole with ketoprofen and meloxicam, amoxicillin with meloxicam, and gentamicin with flunixin meglumine and ketoprofen. The remaining combinations revealed an indifferent response (Table 3).

DISCUSSION

Biofilm formation by *S. aureus* is chiefly accountable for the higher incidence of persistent udder infections in bovines as biofilms help bacteria to survive a wide range of hostile environments and resist antimicrobials even at a higher concentration (He *et al*., 2014). The prevalence (41.41%) of *S. aureus* in the current study was in agreement with the findings of previous studies in Pakistan reporting a prevalence between 10% to 65% (Aqib *et al*., 2021; Javed *et al*., 2021). Biofilm forming potential reported in 35.85% of study isolates was supported by previous studies reporting 11.42-91.42% biofilm-positive isolates on the CRA method (Fabres-Klein *et al*., 2015). The discrepancies in the biofilm detection rates among various studies might be associated with the criteria (morphology, color, or both) used to interpret the CRA test (Fabres-Klein *et al*., 2015). MTP method showed a biofilm detection rate of 30.19% which was lower compared to CRA method. These findings were in accordance with previous studies (Vasudevan *et al.,* 2003; He *et al.,* 2014). Despite the differences in detection rates, both two assays could be selected as appropriate tools for biofilm detection. As the phenotypic characters may develop from different genetic determinants, it is pertinent to investigate the biofilm formation at the genetic level (He *et al*., 2014). In this study, the *icaA* gene was confirmed in 45.28% of isolates. Previous studies have confirmed these genes in 56.25%-86.60% *S. aureus* isolates of dairy origin (Melchior *et al*., 2009; Melo *et al*., 2013; He *et al*., 2014; Aslantaş and Demir, 2016; Khoramrooz *et al*., 2016). The presence of *ica* locus in *S. aureus* strains is indicative of the biofilm-forming potential of bacteria liable for chronic intramammary infections.

Bacteria in a biofilm shows higher resistance to antimicrobials compared to planktonic form and are responsible for chronic or persistent infections (He *et al*., 2014). Early detection and evaluation of antimicrobial sensitivity patterns of biofilm-forming isolates are crucial for the selection of appropriate antimicrobials (Neopane *et al*., 2018). A significantly higher resistance (p<0.05) to ciprofloxacin, trimethoprim/sulfamethoxazole, and gentamicin exhibited by biofilm-positive *S. aureus* strains compared to biofilm-negative strains, as reported in this

study, is supported by the findings of previous studies (John and Murugan, 2013; Neopane *et al*., 2018; Naseer *et al*., 2020). The possible reason for this higher resistance may be associated with the frequent and undue usage of these antibiotics in the treatment of bovine mastitis as well as general ailments of animals in Pakistan. Vancomycin is considered a last resort drug and is highly effective against *S. aureus* and other gram-positive bacterial infections (Javed *et al*., 2021). Although lower, 20.83% of biofilm-positive study isolates revealed vancomycin resistance which was contrary to previous studies reporting a 100% sensitivity of isolates to vancomycin (John and Murugan, 2013; Neopane *et al*., 2018). Higher resistance in biofilm-positive isolates compared to biofilm-negative isolates, reported in this study, is indicative of the association of biofilm with higher antimicrobial resistance in isolates.

High antimicrobial concentration, required to eliminate biofilm-forming bacteria, may not show successful results *in vivo* due to the risk of toxicity and related side effects. Therefore, low concentration combination therapies may prove effective in biofilm-associated *S. aureus* infections (Neopane *et al*., 2018). NSAIDs have been reported to pose antibacterial activity according to various recent researches which may be associated with their ability to disrupt the cytoplasmic membrane or inhibition of DNA synthesis, replication, and repair of the bacterial cell membrane (Leão *et al*., 2020). Flunixin, meloxicam, and ketoprofen showed synergistic activity in combination with different antibiotics against biofilm-positive MDR *S. aureus* in this study. Previous studies have reported synergistic interaction when flunixin meglumine was combined with oxytetracycline and gentamicin to treat methicillin-resistant *S. aureus* infection (Altaf *et al*., 2019b). Meloxicam and ketoprofen have also shown great antibacterial and anti-biofilm efficacy in previous studies (Mohsen *et al*., 2015; Rehab and Sherein, 2016). Meloxicam and diclofenac sodium inhibit the polysaccharide intercellular adhesion, a major constituent of staphylococcal biofilm, by down regulating the expression of biofilm-associated *icaA* gene (Farouk Ahmed *et al*., 2017). NSAIDs are frequently used drugs along with antibiotics in treating mastitis and other animal diseases. The tested combinations of NSAIDs and antibiotics may be used in the field to uplift the antibacterial activity and reduce the infection load in animals. Similarly, the antistaphylococcal activity of ivermectin has also been reported in previous studies (Ashraf *et al*., 2018). The current study will help to reveal antibiotic resistance pattern of local biofilm-positive isolates and possible concomitant use of common NSAIDs to boost the antibacterial action of resistant antibiotics to treat bovine mastitis. The study will help in the antimicrobial stewardship program as well.

Conclusions: The study concluded that biofilm-positive MDR *S. aureus* is a prevalent cause of bovine subclinical mastitis in study district. The local biofilm-positive isolates showed a higher resistance to commonly used antibiotics compared to non-biofilm isolates. The study showed that antimicrobial effects of resistant antibiotics can be boosted by combining them with non-antibiotics of different classes to reverse the antibiotic resistance. The use of NSAIDs (flunixin meglumine, ketoprofen, and meloxicam) alone or as add-on therapy with conventional resistant antibiotics can give promising results in treating MDR *S. aureus* infections.

Authors contribution: AA did sampling and laboratory procedures. MI did conceptualization and write-up while JAK performed data analysis. AAA did reviewing, editing and proofreading of manuscript. All authors read and approved the final manuscript.

Competing interest: The authors declare no financial or non-financial interests to disclose.

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