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Molecular Insights and Comparative Antibiogram Profiling of Tetracycline and Aminoglycosides Resistant *Staphlococcus aureus* Strains Isolated from Bubaline Endometritis

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Buffalo, Antibiotic resistance, Genetic characterization, TRSA, ARSA

ABSTRACT

Staphylococcus aureus is an emerging and multidrug-resistant (MDR) bacterium associated with infections of the buffalo's reproductive system. The aim of current investigation was to calculate the prevalence and molecular characterization of tetracycline resistant (*tetk*) and aminoglycoside resistant (*aacA- aphD*) genes of *S. aureus* along with antibiotic resistant pattern against different antibiotics groups. For this purpose, a total of 192 vaginal swab samples were checked for *S. aureus* by using bacteriological, biochemical, and molecular approaches. The antibiogram profiling of tetracycline and aminogylcosides resistant isolates was also performed by Kirby-Bauer disc diffusion test. The current study repprted the prevalence of *S. aureus* in 15.10% of the isolates. The results depicted that 44.82% and 31.03% of isolates were positive for tetracycline and aminoglycoside resistance on phenotypic basis while 34.48% and 48.27% of the isolates found positive on molecular analysis, respectively. The phylogenetic analysis of the research isolates indicated the possibility of tetracycline-resistant *S. aureus* (TRSA) and aminoglycosides-resistant *S. aureus* (ARSA) transmission within and between livestock animals. The antimicrobial sensitivity testing of study isolates showed the resistance to several frequently used antibiotics. Among the tested antibiotics, the higher resistance was shown towards β-lactam group including penicillin group while ciprofloxacin was found hughly sensitive antibiotic againt TRSA and ARSA. The study concluded that *S. aureus* and its associated resistant strains are important pathogens involved in bovine reproductive tract infection and requires further intensive research to elucidate the farm economic losses.

INTRODUCTION

Buffalo has a crucial role in the agricultural economy of many evolving countries in terms of providing milk, meat, and draught power. According to estimates, there are 199 million buffaloes around the globe, with more than 96% of the population is present in Asia including 16.4% from Pakistan (Warriach *et al*., 2015). For farm owners and professionals, the productivity and, more importantly, reproductive efficiency of the buffalo species present a challenge because the health of the buffaloes' reproductive systems determines how much milk they can produce at their best. Uterine infections also negatively affect the economics of milk production, so a thorough understanding of reproductive issues at parturition is necessary (González-Lozano *et al*., 2020). Ascending uterus infections caused by a wide range of bacteria are common in buffalo after parturition (Dar *et al*., 2016). Bacteria are abundant in the bovine uterus after parturition, and postpartum uterine illness is caused by the invasion and proliferation of bacteria in the endometrium (Sheldon *et al*., 2019). The primary reproductive system disease in

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buffaloes that generates significant financial losses for the farmers is endometritis (Nischala and Sireesha, 2018), an inflammatory disorder of the endometrium with and without systemic symptoms. It is defined by a purulent to mucopurulent discharge from the vagina that results in reduced milk yield, longer time to pregnancy, increased feed consumption per lactation, decreased pregnancy per insemination, and an increased culling rate, among other economic losses (Eslami *et al*., 2015; Umar *et al*., 2021). Several germs have been discovered in dairy cows' uterus during calving, and it is believed that these microbes are the main reason for uterine infection (Földi *et al*., 2006). Based on the etiological analysis of Chinese dairy cows, one of the most common bacteria that cause endometritis is *S. aureus* (Dan *et al*., 2019). *S. aureus* is a facultative bacterium that can infect animals and humans, and cause a wide range of infectious disorders, including endometritis, a common disease of the reproductive system, and violently impaired reproductive performance (Li *et al*., 2020). As a result of widespread use of antibiotics in the therapeutic prevention and treatment of endometritis, antibiotic-resistant bacteria, particularly multidrug-resistant (MDR) bacteria, have become more prevalent (Li *et al*., 2020). The effective prevention and treatment of infectious diseases are threatened by antimicrobial resistance. The misuse and overuse of antibiotics in veterinary care, animal farming, and agricultural contexts accelerate the emergence of antimicrobial resistance (Kirn *et al*., 2023; Pirolo *et al*., 2019). It has been stated that *S. aureus* is resistant to ciprofloxacin (30%), tetracycline (43.5%), clindamycin (51.2%), penicillin (81%), and erythromycin (44.5%) (Wu *et al*. 2019). For the meantime, the traditional screening of new antimicrobial has suffered a significant decline. MDR "superbug" that no longer respond to the current treatment modalities and become resistant to number of antibiotics groups including penicillin, chloramphenicol, cephalosporins, tetracyclines, lincomycin, aminoglycosides, macrolides and sulfonamides which is an extremely challenging problem in clinical treatment (Guo *et al*., 2020). Moreover, due to its high rates of morbidity and death, MDR *S. aureus* has been linked to the world's major infectious diseases (Dad *et al*., 2022). This has drawn the attention of the international medical community and poses a serious threat to public health (Hassoun *et al*., 2017). Consequently, it is urgent to finding efficient medications to treat multidrug resistant bacteria as this MDR pathogen is of zoontic potential (Liu *et al*., 2023). The current study was carried out to find the prevalence of *S. aureus* associated endometritis, molecular confirmation of tetracycline resistant *S. aureus* (TRSA) and aminoglycosides resistan *S. aureus* (ARSA) and the antibiogram profiling of TRSA and ARSA against variety of commonly used antibiotics for the treatment of reproductive tract issues in buffaloes of Pakistan.

MATERIALS AND METHODS

Sample collection

The study was conducted on dairy buffaloes from different dairy farms in districts Lahore and Kasur, Pakistan during period of April 2022 to March 2023. A total of 192 buffaloes' uterine and vaginal fluid samples by using convenient sampling technique were collected from the animals showing clinical signs of infected reproductive tract (Thrushfield, 2013). The samples were collected from the infected bovine reproductive tract using a sterile cotton swab (Yadav *et al*., 2018) and the samples were immediately shifted to the laboratory by proper maintenance of the cold chain for further bacteriological and molecular analysis.

Bacteriological analysis of samples

For the bacteriological analysis of samples for *S. aureus*, the samples were first swabbed on 5% blood agar and incubated overnight at 37°C. Isolation of *S. aureus* was done by examining growth on differential media mannitol salt agar (MSA). The blood hemolysis pattern, mannitol fermentation, and colony characters were considered to confirm *S. aureus* while Gram's staining and biochemical techniques including catalase and coagulase assays were also used to further confirm the occurrence of *S. aureus* as per standard procedures (Ahmed *et al*., 2022).

Molecular confirmation of S. aureus *by* nuc *gene*

The amplification of the *nuc* gene, with primers P1= GCG ATT GAT GGT GAT ACG GTT and P2= AGC CAA GCC TTG ACG AAC TAA AGC, was used to confirm the molecular identification of *S. aureus*. The conditions were as follows: first denaturation at 94°C for 5 min, then 35 cycles each with a final denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, an initial extension at 72ºC for 1 min, and a final extension at 72°C for 10 min. The amplicon size was 270 bp as reported by Ghumman *et al*. (2022).

Phenotypic and genotypic detection of ARSA and TRSA

Phenotypic identification of aminoglycosides-resistant *S. aureus* (ARSA) and tetracycline-resistant *S. aureus* (TRSA) was done by discs diffusion test using gentamicin and tetracycline discs respectively as described by the CLSI (2016). The isolates were declared as resistant, sensitive, and intermediate on the basis of zones of inhibition around the antibiotic discs as per guidelines of CLSI (2016).

Table I. List of primers and cycling conditions used for the amplification of targeted genes.

Target gene	Fragment size (bp)	Primers (5' → 3')	Cycling conditions	Reference
nuc	270	P1: GCG ATT GAT GGT GAT ACG GTT P2: AGC CAA GCC TTG ACG AAC TAA AGC	Initial denaturation: 5 min at 94°C, 35cycles Final denaturation:30s at 94°C Annealing: 30s at 55°C Initial extension: 1min at 72ºC Final extension: 10min at 72°C	Ghumman *et al*., 2022
aacA-aphD	227	P1 : 5′ TAATCCAAGAGCAATAAGGGC -3′, P2: 5′- GCCACACTATCATAACCACTA -3	Initial denaturation: 3 min at 94 ◦C Final denaturation: 30 s at 94 ◦C Annealing: 30 s at 55 ◦C Initial extension: 30 s at 72 ◦C Final extension: 4 min at 72 ◦C 30 cycles	Strom-menger *et al*., 2003
tetK	697	P1: 5′-TTAGGTGAAGGGTTAGGTCC-3′, P2: 5′-GCAAACTCATTCCAGAAGCA3	Initial denaturation: 5 min at 94 ◦C Final denaturation: 1 min at 94 ◦C Annealing: 1min at 50.6 ◦C Initial extension: 2 min at 72 ◦C Final extension: 10 min at 72 ◦C 30 cycles	Bamidele *et al*., 2017

DNA was extracted by using a commercially available DNA extraction kit from phenotypically positive TRSA and ARSA isolates to molecular confirmation of *aacA-aphD* and *tetk* genes. After quantification of DNA by nano-drop, PCR was carried out by targeting *aacA-aphD and tetk* genes by using already reported primers (Table I).

Molecular characterization of ARSA and TRSA

Molecular characterization of local TRSA and ARSA isolates was done by phylogenetic analysis with the appropriate bioinformatics tools by using the gene sequences of *aacA-aphD and tetk* genes (Wu *et al*., 2019). By using a Basic Local Alignment Search Tool (BLAST), the sequenced isolates of TRSA and ARSA were compared to previously reported gene sequences. Subsequently, other bioinformatics tools were employed, including the NCBI's BLAST and BioEdit software's ClustalW technique (version 7.5.0.3), to compare and align nucleotide sequences and analyse the resulting data, respectively. Following multiple sequence alignment, the MEGAX programme used the maximum likelihood technique with 1000 repetitions of bootstrap analysis to create a phylogenetic tree (Abbas *et al*., 2023; Ren *et al*., 2009).

Antimicrobial susceptibility test

The *in-vitro* susceptibility pattern of defined ARSA and TRSA isolates were evaluated to certain antibiotics by using Kirby-Bauer disc diffusion method. All the isolates were inoculated in nutrient broth, incubated at 37 °C for 24 h. Bacterial isolates were suspended after being adjusted to the 0.5 McFarland standard, or 1.5×10^8 CFU/ml. Filter paper discs were employed, each containing a specific quantity of an antimicrobial agent from a different class, such as ceftriaxone (30µg), amoxiclav (30µg), cefoperazone (75µg), gentamicin (20µg), cephalexin (30µg), cefotaxime (10µg), penicillin (10µg), ciprofloxacin (30µg), streptomycin (30µg), and oxytetracycline (30µg) (Yadav *et al*., 2018). Using the CLSI recommendations (Wayne, 2019), media were incubated at 37°C for 24 h, and the findings were categorized as sensitive, moderate, or resistant depending on the zones of inhibition around the antibiotics discs (Indrayudha, 2021).

Statistical Analysis

The formula provided by Thrushfield (2013) was used to calculate the prevalence of *S. aureus*. Moreover, descriptive statistics was used to statistically assess the susceptibility experiments.

RESULTS

Prevalence of S. aureus *and its antibiotic resistant strains*

The current study elucidated a 15.10% (29/192) prevalence of *S. aureus* in buffaloes with reproductive tract infections. Among buffalo breeds, the Ravi buffalo showed the highest prevalence (18.86%) of *S. aureus* as compared to Nilli (17.85%) and Nilli Ravi (10.84%). The result showed that among *S. aureus* isolates, 44.82% of isolates were resistant to tetracycline and 31.03% isolates exhibited resistance to gentamicin and were declared TRSA and ARSA phenotypically, respectively. The genotypic prevalence of tetracycline was 34.48% by targeting the *tetK* gene. Nilli buffalo showed the highest (50.00%)

genotypic prevalence as compared to Ravi (30.00%) and Nilli ravi (22.22%). Whereas, the genotypic prevalence of aminoglycosides was observed (48.27%) by targeting the *aacA-aphD* gene. The highest genotypic prevalence of *aacA-aphD* gene was found in Nilli Ravi (55.55%) as compared to Nilli (50.00%) and Ravi (40.00%) (Table II).

Molecular characterization of tetk *gene of TRSA*

The evolutionary history was inferred by using the maximum likelihood method and the bootstrap consensus tree inferred from 1000 replicates. Molecular characterization of our study isolates 12HA and 16H and their comparison with already reported NCBI sequences was performed using MEGA X. Our study isolates showed significant variation with each other. Our study isolate 12HA exhibited more similarity to the tetk isolates from the USA (accession numbers: CP030702, CP030464), Denmark (accession number: CP119109), China (accession number: CP045473) and Germany (accession number: CP031671) than that of other reported gene isolates when compared to previously published sequences for *tetk* on the NCBI. Moreover, our study isolate 16H resembles with isolate from the China (accession number: CP048645) making an in-group than that of other isolates of *tetK* isolated from various species in different countries such as Nigeria (accession number:CP051484), France (accession number: CP033114), China (accession numbers: CP048645, 121204), Switzerland (accession number: CP094749) and USA (accession number: CP117243). Whereas, the isolate from Korea (Accession number: CP083259) significantly differs from our study isolates and makes an out-group (Fig. 1A).

Molecular characterization of aacA-aphD *gene of ARSA*

Molecular characterization of aminoglycoside-resistant isolates 21HA, 11e, and 9e along with their comparison with already reported NCBI sequences was performed using BioEdit and MEGA X. The isolates from our analysis, 21HA and 11e, revealed greater similarities than other isolates of *aacA-aphD* isolated from other countries. Our isolate 9e was more similar to the isolate from Egypt (accession number: CP113244) than that of the isolate from Japan and fell in the same clade. Whereas our isolate 11e showed more resemblance to the isolate from Japan (accession number: LC757492) and least resemblance with others.

Comparative analysis of our sequences with that of already reported sequences for *aacA-aphD* on NCBI revealed that our study isolate 21HA showed significant variation with *aacA-accD* isolates from different other countries such as India (accession numbers: CP102575, EF591791), China (accession number: CP121204), and Taiwan (accession numbers: CP113015, CP113009) than that of others (Fig. 1B).

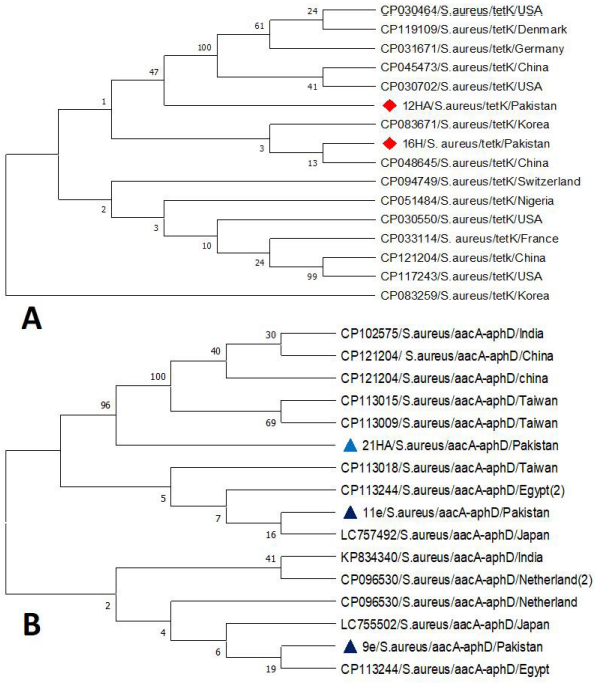

Fig. 1. Phylogenetic analysis of study isolates of tetracycline-treated (A) and aminoglycoside-resistant (B) *S. aureus* with already reported sequences

Table II. Frequency of *S. aureus*, TRSA, and ARSA from reproductive tract infection samples of buffalo.

Animal Spp.	Breed	No. of animals	*S. aureus* (%)	TRSA (%)		ARSA (%)	
				Phenotypic (%)	Genotypic (%)	Phenotypic (%)	Genotypic (%)
Buffalo (n=192)	Nilli Ravi	83	9 (10.84)	3(33.33)	3(33.33)	6(66.66)	5(55.55)
	Nilli	56	10 (17.85)	2(20.00)	4(40.00)	2(20.00)	2(22.22)
	Ravi	53	10 (18.86)	3(30.00)	3(30.00)	3(30.00)	2(22.22)
Total		192	29 (15.10)	8(27.58)	10(34.48)	11(37.93)	9(31.03)

TRSA, tetracycline-resistant *S. aureus*; ARSA, aminoglycosides-resistent *S. aureus*

Table III. Antibiogram of TRSA (tetracycline-resistant *S. aureus*) and ARSA (aminoglycosides-resistent *S. aureus*) isolates from buffalo reproductive tract infections.

Antibiotics	Potency	TRSA (n=13)			ARSA (n=13)		
		R	S	I	R	S	I
Ceftriaxone	30µg	7(53.84)	4(30.76)	2(15.38)	7(53.84)	6(46.15)	0
Amoxiclav	30µg	7(53.84)	3(23.07)	3(23.07)	6(46.15)	5(38.46)	2(15.38)
Cefotaxime	10µg	9(69.23)	3(23.07)	1(7.69)	7(53.84)	4(30.76)	2(15.38)
Oxytetracycline	30µg	6(46.15)	5(38.46)	2(15.38)	5(38.46)	6(46.15)	2(15.38)
Streptomycin	30µg	5(38.46)	7(53.84)	1(7.69)	6(46.15)	5(38.46)	2(15.38)
Cefoperazone	75µg	8(61.53)	5(38.46)	0	7(53.84)	5(38.46)	1(7.69)
Cephalexin	30µg	5(38.46)	5(38.46)	3(23.07)	6(46.15)	5(38.46)	2(15.38)
Gentamicin	20µg	6(46.15)	4(30.76)	3(23.07)	7(53.84)	3(23.07)	3(23.07)
Penicillin	10µg	9(69.23)	4(30.76)	0	8(61.53)	4(30.76)	1(7.69)
Ciprofloxacin	30µg	4(30.76)	3(23.07)	6(46.15)	3(23.07)	5(38.46)	5(38.46)

n, number of isolates used. R, Resistant. S, Sensitive. I, Intermediate.

Comparative antibiogram profiling of TRSA and ARSA isolates

Antibiotic susceptibility testing results revealed that the maximum resistance of 69.23% was seen against cefotaxime and penicillin in the instance of *S. aureus* with the *tetK* gene. This was followed by 61.53% against cefoperazone, 53.84% amoxiclav, and 46.15% oxytetracycline. Lowest resistance 30.76% was observed against ciprofloxacin. While in the case of ARSA isolates the greatest resistance (61.53%) against penicillin was found, the lowest resistance (24.07%) was observed in ciprofloxacin, followed by 53.84% against ceftriaxone, cefoperazone, gentamicin, and cefotaxime (Table III).

DISCUSSION

Antibiotic resistance is spreading worldwide among *S. aureus* bacterial infections and continuously caused severe problems in veterinary and human medicines (Bounar-Kechih *et al*., 2018). *S. aureus* is a common pathogen that can cause post-partum clinical and subclinical endometritis in animals (Zhang *et al*., 2017). The results of the current study showed that TRSA and ARSA exist in buffalo reproductive tract issues. A total of 192 samples were taken from an infection of the buffalo reproductive tract *S. aureus* was reported to be 15.10% (29/192) prevalent overall. An earlier investigation found that samples collected from aborted cattle included 88.3% *S. aureus* and 17.2% endometritis (Petit *et al*., 2009). The results were in line with other studies that found *S. aureus* in 78.20% of samples taken from the bovine reproductive system (Shafique *et al*., 2022). However, *S. aureus* (4.54%) was common in the endometritis case (Azawi *et al*., 2008). In a prior investigation, 38.1% of *S. aureus* isolates from various bovine pyogenic situations were shown to be present (Yadav *et al*., 2018). Consequently, the current data clearly demonstrates the rising incidence of *S. aureus*, albeit specifics may vary based on the sample type, the location, and the time of year. The research isolates differed significantly from one another and from isolates from various countries, including Nigeria, Germany, Denmark, Korea, China, Switzerland, and Nigeria, as revealed by the molecular characterisation of the *tetk* gene. Phylogenetic analysis of *aacA-aphD* gene exhibited significant alteration among ARSA isolates and with isolates from other countries such as USA, South Africa, Netherland, India, and Egypt. The reason for the unusual resemblance between the isolates from our study and those from other nations is the unregulated trade of animals and transborder movement, which promotes the spread of resistant microorganisms between nations. The discovery of these genes in *S. aureus* of animal origin raises serious concerns for public health and increases the possibility of treatment failure in both veterinary and human medicine because these genes are spread by mobile genetic elements and may facilitate the spread of several resistance genes (Pérez *et al*., 2020).

S. aureus has been reported to acquire resistance to antibiotics when used over an extended period of time (Pirolo *et al*., 2019). Antibiotic susceptibilty testing showed in case of TRSA , highest resistance 69.23% was found against cefotaxime and penicillin, followed by 61.53 against cefoperazone, 53.84% amoxiclav and 46.15% oxytetracyclinewhich is similar in findings to previous study (Alfeky *et al*., 2022; Rasheed *et al*., 2023). The *S. aureus* isolates were found to have a significant resistance

to tetracycline (85.71%), a popular antibiotic, in a prior investigation. Tetracycline and penicillin are typically used in most countries to treat *S. aureus* infections in bovines. Resistance to penicillin and tetracycline increases as a result of their widespread and ongoing usage as antimicrobial medicines (Shamila-Syuhada *et al*., 2016). As per the findings of Grossman (2016) study, 64% of *S. aureus* isolates exhibited resistance to tetracycline. The preferred antibiotic for inhibiting both Gram-positive and Gram-negative bacteria was tetracycline, which can also interfere with the process of protein synthesis. The beta-lactamase enzyme found in *Staphylococcus* bacteria is capable of degrading and rendering inactive the beta-lactam ring of antibiotics (Pieshesa, 2011). The process by which aminoglycoside converting enzymes inactivated antibiotics by attaching aminoglycosides to particular protein receptors of the 30S subunit on the bacterial ribosome, followed by the inhibition of the initiation complex of peptide formation, was the mechanism by which bacteria developed resistance to gentamicin (Garneau-Tsodikova and Labby, 2016).

The current rise of infectious strains resistant to other important antibiotics makes this trend of resistance concerning for the treatment of cattle diseases and productivity in general (Okpo *et al*., 2016). In the present study, isolates showed the highest intermediate response against ciprofloxacin (46.15%) as compared to the previous studies (Abdulrahman *et al*., 2018) revealed sensitive results towards ciprofloxacin. The antibiotic sensitivity tests revealed *S. aureus* as a multidrug-resistant pathogen carrying different genes. Our investigation indicates the multidrug resistance of *S. aureus* isolates found in buffalo reproductive tract issues samples. It is an important consideration for animals and public health. In order to decrease the spread of multidrug-resistant *S. aureus* in dairy bovines, it is necessary to devise countermeasures to encourage careful usage of antibiotics.

CONCLUSION

The present research revealed the prevalence of *S. aureus* and its resistant strains along with antibiotic susceptibility pattern of TRSA and ARSA in buffalo reproductive tract infections. The resemblance of isolates with *S. aureus* from other samples and even human isolates is indicative of the possibility of TRSA and ARSA spread between and within species. The increased prevalence of TRSA and ARSA against β-lactam and other antibiotics groups is suggestive of the fact that there is an essential need to stop the deliberate use of antibiotics and invent other resistance modulation strategies for providing potential therapeutic plans fight against this MDR pathogen. Moreover, there is need of hour to implement the proper infection control measures to prevent the spread of this pathogen.

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Ethics approval

The research work was performed in Medicine Research Laboratory, University of Veterinary and Animal Sciences, Lahore. This research was part of PhD study having DAS/1670 approved by Advanced Studies and Research Board (ASRB), UVAS on 04-08-2022.

Data availability statement

Data will be available on request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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