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Original Article

On-farm epidemiology and phylogenetic evaluation of methicillin and beta-lactam-resistant *Staphylococcus aureus* isolated from dairy cattle and buffaloes with endometritis

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Abstract

Background: *Staphylococcus aureus* is a potential emerging and prevailing multidrug-resistant (MDR) pathogen involved in bovine endometritis. **Aims:** Present research evaluated the prevalence and molecular characterization of methicillin-resistant *S. aureus* (MRSA) and beta-lactam resistant *S. aureus* (BRSA) and also analyzed the associated risk factors with endometritis along with antibiotic resistance patterns. **Methods:** A total of 384 uterine and vaginal fluid samples were collected from the adult dairy cattle and buffaloes showing the clinical signs of endometritis including foul-smelling vaginal discharge, fever, enlarged and thickened uterine horns on rectal palpation, and confirmation by ultrasonography findings. The collected samples were subjected to standard microbiological methods for the detection of *S. aureus*. The confirmed isolates were further subjected to the Kirby-Bauer disc diffusion test and the detection of the *mecA* and *blaZ* genes for the confirmation of MRSA and BRSA. **Results:** Study found an overall prevalence of 17.96% for *S. aureus* from bovine endometritis cases. Among *S. aureus* isolates, 50.72% and 37.68% isolates were confirmed MRSA while BRSA was found as 36.23% and 18.84%, based on phenotypic and genotypic methods, respectively. Phylogenetic analysis indicated the possibility of pathogen transmission within and between livestock animals. Risk factor analysis showed that the breed of animal, visible discharge from vagina, lactation number, insemination procedure, and calving place showed significant ($P<0.05$) association with *S. aureus*-associated endometritis. Antimicrobial susceptibility testing of study isolates showed the resistance to various commonly used antibiotics. **Conclusion:** The study concluded that *S. aureus* is found in 17.96% of bovines affected with endometritis and require further intensive research to elucidate the farm economic losses.

Key words: Antibiotic resistance, Endometritis, Molecular characterization, Risk factors

Introduction

Major early post-partum (first 30 days) issues of bovines especially cattle include the retention of placenta, ketosis, milk fever, abomasum displacement, and uterine infections which significantly disrupt the milk production and future reproduction of dairy animals (Adnane *et al*., 2017). Among reproductive disorders, uterine infections are of high significance and are classified into five different categories i.e. clinical and subclinical endometritis, pyometra, clinical metritis, and puerperal metritis (Sheldon *et al*., 2008). Endometritis is among the major hurdles in the profitable dairy industry due to the financial losses caused by reduced reproductive efficiency, treatment expenses, early culling, increased services per conception, and pathogen transmission hazards (Chen *et al*., 2020). The major causes of endometritis include various bacterial species that usually invade the reproductive tract while doing intervention during parturition, which leads to severe uterine infections and ultimately significant financial losses that jeopardize the health and productivity of the herd (Megahed *et al*., 2022).

Among potential pathogens isolated from reproductive infections in bovines, *Staphylococcus aureus*, a prime pathogen of veterinary and human health concern (Fluit, 2012; Foster, 2012; Tong *et al*., 2015; Lai *et al*., 2018) and is considered an important cause of clinical endometritis in cattle (Sheldon and Owens, 2018) causing significantly reduced reproductive performance (Gilbert *et al*., 2005). Antimicrobial resistance (AMR) is a prevailing global issue related to high morbidity and mortality (Anwaar *et al*., 2023; Ullah *et al*., 2023) leading to higher culling rates of animals.

Due to indiscriminate and undue usage of antibiotics in animals, the pathogens including *S. aureus* have developed antibiotic-resistant strains like beta-lactam-resistant and methicillin-resistant *S. aureus* (MRSA) (Dad *et al*., 2022; Li *et al*., 2023; Javed *et al*., 2024) that poorly responds to treatment of associated infections including endometritis (Eslami *et al*., 2015).

As all beta-lactams are resistant to methicillin, these isolates could also be known as multi-drug resistant (MDR), the capacity to generate persistent infections, and a significant level of antibiotic resistance (Gillespie *et al*., 2009; Cervinkova *et al*., 2013; Frey *et al*., 2013). Once established, these dreadful bacteria generally do not react to antibiotic therapy, and therapy is typically associated with poor results, leading to a disproportionately high culling rate (Hendriksen *et al*., 2008). Moreover, not most antimicrobials may reach all affected locations due to the generally unsatisfactory therapeutic efficacy against these bacteria (Güler *et al*., 2005; Pu *et al*., 2014). Infections of domestic animals with antibiotic-resistant *S. aureus* have been observed more frequently in recent years (Cuny *et al*., 2010). Over 40 antimicrobial resistance genes (ARGs) have been discovered on plasmids and transposons. It has been found that tetracycline, penicillin, erythromycin, clindamycin, and ciprofloxacin have all been reported to be ineffective against *S. aureus* (Wu *et al*., 2019). Moreover, these resistant staphylococci can serve as ARG donors (Kadlec *et al*., 2012). Therefore, *S. aureus* phenotypic characterization is no longer useful for managing this pathogen (Le Maréchal *et al*., 2011; Mai-Siyama *et al*., 2014). Targeting specific genes in the DNAs of bacteria by PCR has recently gained popularity as a molecular technique, particularly for the identification and detection of bacteria (Elsayed *et al*., 2015).

In Pakistan, beta-lactam and methicillin-resistant *S. aureus* (MRSA) have been testified from the bovine and caprine mastitis but molecular studies on associated resistant *S. aureus* infections responsible for endometritis in cattle and buffalo are scarce. Therefore, it was intended for the current investigation to characterize *S. aureus*, MRSA, and beta-lactam-resistant *S. aureus* (BRSA) molecularly and to determine their positive prevalence, associated risk factor analysis, and antibiotic resistance pattern from endometritis in cattle and buffaloes.

Materials and Methods

Study area and sampling strategy

The study was conducted on dairy cattle and buffaloes from 10 different dairy farms of district Lahore and Kasur, Pakistan (Fig. 1). The sample size was calculated based on a 50% prevalence of *S. aureus* at a 95% confidence interval using the online EpiTools software (epitool.ausvet.com). From November 2021 to December 2022, a total of 384 uterine and vaginal fluid samples (n=192 cattle samples; n=192 buffalo samples) were collected from the adult dairy cattle (Sahiwal, Holstein, and Mix breeds) and buffaloes (Nilli, Ravi, and Nilli Ravi breeds) showing clinical signs of endometritis including foul-smelling vaginal discharge, fever, enlarged and thickened uterine horns on rectal palpation, and confirmed by ultra-sonographic findings. After restraining the animal and securing its tail, the perennial region was washed. Sampling for bacteriological examination was performed immediately after the diagnosis of clinical endometritis. Vaginal aspirates were collected about 5-10 ml from vaginal fornix using a sterilized vaginal speculum and cotton swab sticks. The aspirates were poured into sterile test tubes containing physiological saline, were kept in ice box, and transported to the laboratory for further bacteriological and molecular analysis (Moges *et al*., 2013).

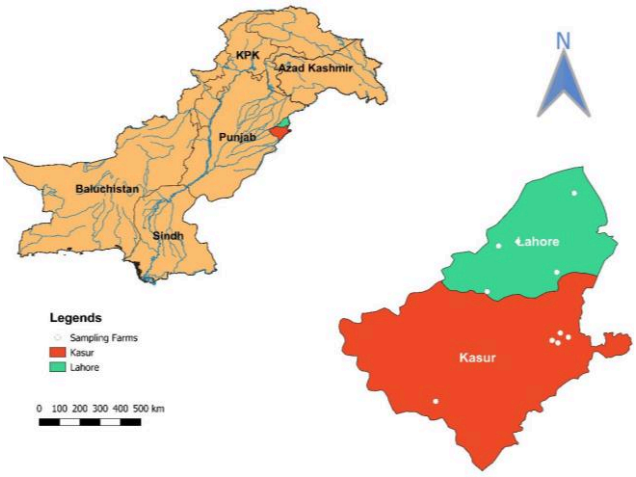

Fig. 1: QGIS map showing the study area

A pre-designed questionnaire was completed to collect data about various animal-related risk factors including body temperature (normal, high, low), visible vaginal discharge (mucous, bloody, pus), any systemic infection, inflammation on the perineal region (swelling, redness, and pain in the area between the anus and vagina), etc. and farm management-associated practices including farm settings and buildings (traditional, modern), feed type (ration, concentrate), operator of artificial insemination (veterinarian, technician, or quacks (an unqualified or unlicensed person fraudulently claiming to have veterinary expertise)). Collected data were later statistically analyzed to ascertain the association of presumed risk factors with the occurrence of *S. aureus*-associated reproductive tract issues in bovines (Kelly *et al*., 2020).

Isolation and identification of *S. aureus*

For bacteriological analysis of collected samples for *S. aureus*, the samples were first swabbed on blood agar, and incubated overnight at 37°C (Liu *et al*., 2023). Isolation of *S. aureus* was done by examining growth on differential media Mannitol Salt Agar (MSA) (Aqib *et al*., 2017; Altaf *et al*., 2020). The blood hemolysis pattern, Mannitol fermentation, and colony characters were considered to confirm *S. aureus* (Biswas *et al*.,

2015; Ijaz *et al*., 2023). Gram staining and biochemical techniques including catalase and coagulase assays were used to confirm the occurrence of *S. aureus* (Ahmed *et al*., 2022).

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

Molecular confirmation of presumptive phenotypically confirmed isolates of *S. aureus* was done by PCR, targeting the *nuc* gene by using forward primer 5´-GCG ATT GAT GGT GAT ACG GTT-3´ and reverse primer 5´-AGC CAA GCC TTG ACG AAC TAA AGC-3´ with an amplicon size of 270 bp. The PCR conditions of this reaction included an initial condition of 94°C denaturation for 5 min, followed by 94°C denaturation for 30 s, annealing at 55°C for 30 s, and 72°C elongation for 1 min (35 cycles). Then the last step of an extension was done at 72°C for 10 min (Javed *et al*., 2023).

Phenotypic and genotypic detection of methicillin-resistant and β-lactam resistant *S. aureus*

Phenotypic MRSA and β-lactam resistant *Staphylococcus aureus* identification were done by oxacillin and penicillin G discs diffusion tests, respectively (Altaf *et al*., 2019; Javed *et al*., 2023) as determined by the Clinical Laboratory Standards Institute (CLSI).

For genotypic identification of β-lactam-resistant and methicillin-resistant *S. aureus*, DNA was extracted from phenotypically positive β-lactam and methicillin-resistant *S. aureus* isolates by using a commercially available DNA extraction kit. After quantification of DNA by Nano drop (Shah *et al*., 2020), PCR was performed by targeting *blaZ* and *mecA* genes. Primers for *blaZ* gene were P1: 5´-CAA AGA TGA TAT AGT TGC TTA TTC TCC-3´, P2: 5´-TGC TTG ACC ACT TTT ATC AGC-3´, with a product size of 421 bp (Kaase *et al*., 2008) while the PCR conditions included a denaturation step at 95°C for 5 min was followed by 35 cycles of amplification (denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s), with a final extension at 72°C for 7 min.

For molecular confirmation of MRSA, the *mecA* gene was targeted using primers P1: 5´-TGG CAT TCG TGT CAC AAT CG-3´, P2: 5´-CTG GAA CTT GTT GAG CAG AG-3´ (Galdiero *et al*., 2003), with a product size of 310 bp. The conditions of the PCR reaction were the initial denaturation for 5 min at 94°C followed by 34 cycle denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 1 min at 72°C, and ended with a final extension step for 10 min at 72°C. The product amplified was then run on 1.5% agarose gel using ethidium bromide as a dye (Rasheed *et al*., 2023). The positive and negative controls were run alongside the samples to increase the reliability. PCR-positive samples were then sent for sequencing (Ghumman *et al*., 2023; Ishaq *et al*., 2022; Ghauri *et al*., 2021).

Molecular characterization of MRSA and beta-lactam-resistant *S. aureus*

All the PCR-positive samples were sent for sequencing and the representative samples were selected among the samples showing maximum homology with each other and were included in phylogenetic analysis. Molecular characterization of local MRSA and BRSA isolates was done by phylogenetic analysis with the appropriate bioinformatics tools using the gene sequences of *blaZ* and *mecA* genes (Wu *et al*., 2019). By using a Basic Local Alignment Search Tool (BLAST), the sequenced isolates of beta-lactam-resistant *S. aureus* and MRSA were compared to previously reported gene sequences. Then different bioinformatics tools were used such as the BLAST of NCBI and the ClustalW method of BioEdit software (version 7.5.0.3) to compare the nucleotide sequences and to align and analyze the sequences, respectively. After multiple sequence alignment, the MEGAX software constructed a phylogenetic tree using the method of maximum likelihood with bootstrap analysis of 1000 replicates (Ren *et al*., 2009).

In-vitro drug susceptibility of local MRSA isolates

The *in-vitro* susceptibility of defined MRSA to certain antibiotics was evaluated using a disc diffusion experiment (Anwaar *et al*., 2023). The Kirby-Bauer disc diffusion method was implemented to conduct an antibiotic sensitivity test. 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 amoxiclav (30 µg), cefoperazone (75 µg), ceftriaxone (30 µg), gentamicin (20 µg), ciprofloxacin (30 µg), cephalexin (30 µg), cefotaxime (10 µg), penicillin (10 µg), streptomycin (30 µg), and oxytetracycline (30 µg) (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 measures of the zone sizes (Indrayudha, 2021).

Statistical analysis

The formula presented by Thrushfield (2013) was used to calculate the incidence of *S. aureus.* Non-probability testing was used to assess the associated risk factor data using Chi-square and logistic regression approaches by using SPSS version 20.0 (Sohail *et al*., 2018). The significance was detected at a 5% probability ($P<0.05$). Moreover, descriptive statistics were used to statistically assess the susceptibility experiments.

Results

Status of *S. aureus*, MRSA, and beta-lactam resistant *S. aureus*

This study showed *S. aureus* prevalence of 17.96% in bovines suffering from endometritis. The cattle population showed a higher prevalence (20.83%) of *S.*

aureus compared to the buffalo population (15.10%). The results showed that among local *S. aureus* isolates, 50.72% of isolates were resistant to oxacillin discs and thus considered MRSA. While 36.23% of isolates were resistant to penicillin discs and thus considered BRSA. The confirmation of MRSA by *mecA* gene revealed that 37.68% of isolates were confirmed MRSA based on PCR (Fig. 2) whereas the confirmation of BRSA by *blaZ* gene (Fig. 3) revealed that 18.84% of isolates were confirmed BRSA (Table 1). Among bovines, the uppermost

Table 1: Frequency of *S. aureus*, MRSA, and BRSA from reproductive tract infections of bovines

Animal spp.	Breed	No. of animals	*S. aureus* (%)	P-value	MRSA (%)		Beta-Lactam (%)	
					Phenotypic (%)	Genotypic (%)	Phenotypic (%)	Genotypic (%)
Cattle	Sahiwal	87	12 (13.79)	0.027*	5 (41.67)	4 (33.33)	3 (25.00)	1 (8.33)
(n=192)	Holstein	63	20 (31.74)		11 (55.00)	7 (35.00)	8 (40.00)	5 (25.00)
	Mix breed	42	8 (19.04)		3 (37.50)	5 (62.50)	2 (25.00)	1 (12.50)
Buffalo	Nilli	56	10 (17.85)	0.351	6 (60.00)	4 (40.00)	5 (50.00)	3 (30.00)
(n=192)	Ravi	53	10 (18.86)		6 (60.00)	3 (30.00)	5 (50.00)	2 (20.00)
	Nilli Ravi	83	9 (10.84)		4 (44.44)	3 (33.33)	2 (22.22)	1 (11.11)
Total		384	69 (17.96)		35 (50.72)	26 (37.68)	25 (36.23)	13 (18.84)

* P<0.05 indicates significant results

Table 2: Risk factors analysis associated with *S. aureus* isolates from bovine endometritis

Variables	Variables level	*S. aureus* associated endometritis		
		Total	Positive (%)	P-value
Cattle breed	Sahiwal	87	12 (13.8)	0.034
	Holstein	63	20 (31.7)	
	Mix breed	42	8 (19.0)	
Buffalo breed	Nili	56	10 (17.9)	
	Ravi	53	10 (18.9)	
	Nili Ravi	83	9 (10.8)	
Body temperature	Normal	214	40 (18.7)	0.170
	High	97	12 (12.4)	
	Low	73	17 (23.3)	
Visible discharge from vagina	Mucous	141	21 (14.9)	0.003
	Bloody	125	15 (12.0)	
	Pus	118	33 (28.0)	
Any systemic infection	Yes	91	13 (14.3)	0.295
	No	293	56 (19.1)	
Inflammation on perennial region	Yes	121	21 (17.3)	0.832
	No	263	48 (18.3)	
Lactation number	<2	190	43 (22.6)	0.019
	>2	194	26 (13.4)	
	No	106	12 (11.3)	
Breeding procedure	Natural	133	31 (23.3)	0.047
	Artificial	251	38 (15.1)	
Farm settings and buildings	Traditional	181	34 (18.8)	0.694
	Modern	203	35 (17.2)	
AI procedure by	Veterinarian	119	26 (21.8)	0.412
	AI technician	239	39 (16.3)	
	Quacks	26	4 (15.4)	
Feeding system	Grazing	90	17 (18.9)	0.795
	Stall feeding	294	52 (17.7)	
Feed type	Green fodder	101	17 (16.8)	0.853
	Ration	187	33 (17.6)	
	Concentrate	96	19 (19.8)	
History of dystocia in last 3 months	Yes	211	37 (17.5)	0.807
	No	173	32 (18.5)	
History of reproductive disorders in past 6-12 months	Retained fetal membrane	132	34 (25.8)	0.009
	Endometritis	166	26 (15.7)	
	Pyometra	86	9 (10.5)	
Calving place	Outside	203	29 (14.3)	0.047
	Inside	181	40 (22.1)	
General use of B-lactam	Yes	297	50 (16.8)	0.285
	No	87	19 (21.8)	

phenotypic prevalence of MRSA was found in Nili buffalo (60%) and Ravi buffalo (60%), followed by Holstein cattle (55.00%), Nili Ravi buffalo (44.44%), Sahiwal cattle (41.67%), and mixed breed cattle (37.50%). Whereas, the highest phenotypic prevalence of BRSA was found in Nili buffalo (50.00%) and Ravi buffalo (50%) followed by Holstein cattle (40%), Nili Ravi buffalo (22.22%), Sahiwal cattle (25%), and mixed breed cattle (25%). Among cattle, the highest genotypic prevalence of MRSA was found in mixed breed (62.5%) followed by Holstein (35%), and Sahiwal (33.33%) while in case of buffaloes Nili (40%) showed high prevalence followed by Nili Ravi (33.33%), and Ravi (30%) breeds. Similarly, in cattle the highest genotypic prevalence of BRSA was found in Holstein (25%), mixed breed (12.5%), and Sahiwal (8.33%) while among buffalo breeds Nili (30%) showed higher prevalence as compared to Ravi (20%), and Nili Ravi (11.11%) (Table 1).

Risk factor analysis

In our study, the association of various assumed risk factors with *S. aureus*-associated reproductive tract issues was evaluated and the results showed a significant ($P<0.05$) association of different risk factors including the breed of animal (P=0.034), visible discharge from vagina (P=0.003), lactation number (P=0.019), insemination procedure (P=0.047), the history of reproductive disorders in past 6-12 months (P=0.009) and calving place (P=0.047) with *S. aureus*-associated reproductive issues (Table 2).

Molecular characterization of local isolates

Genetic characterization of MRSA-resistant study isolates, 18E, 11E, and 13E, and their comparison with already reported NCBI sequences was performed using MEGAX. Comparative analysis of our sequences with already reported sequences for *mecA* on NCBI revealed that our study 13E isolate showed more resemblance with an isolate of *mecA* from Pakistan (accession No.: MZ749753.1) than to isolates from other countries (accession No.: OK040767.1, KX024711.1, and KY467026.1). Furthermore, our study 11E isolate revealed a significant resemblance with the isolate of Pakistan (accession No.: MZ814972.1). Moreover, one of our study isolates, 18E revealed a significant resemblance with an isolates of *mecA* from Pakistan (accession No.: MZ814969.1) and showed the least resemblance with the isolates of *mecA* from Pakistan (accession No.: MZ814971.1) (Fig. 4).

Phylogenetic analysis of beta-lactam-resistant isolates; 4A, 10e, and 13e along with their comparison with already reported NCBI sequences of *blaZ* was performed using MEGAX. Our study 13e isolate exhibited more resemblance with an isolate from USA (accession No.: CP102976.1). While, our study isolate, 4A showed more resemblance with an isolate from Germany (accession No.: CP047810.1). When our sequences were compared with earlier published *blaZ* sequences on the NCBI, we found that our research isolate 10e significantly differed from *blaZ* isolates from various countries such as Myanmar (accession No.: MW453502.1), China (accession No.: FJ809758), and Italy (accession No.: U58139), then that of others (Fig. 5).

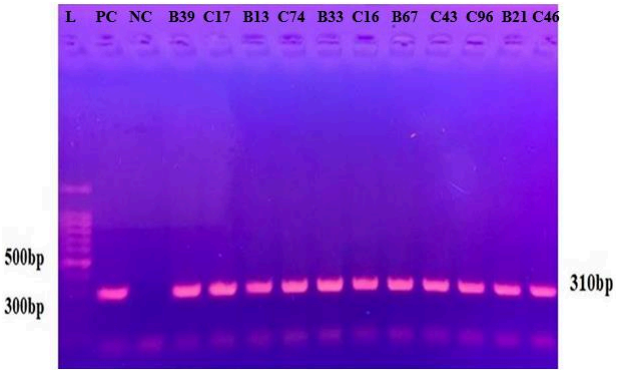

Fig. 2: PCR results of *S. aureus* positive for *mecA* gene. L: Ladder, PC: Positive control, NC: Negative control, B: Buffalo positive samples, and C: Cattle positive samples

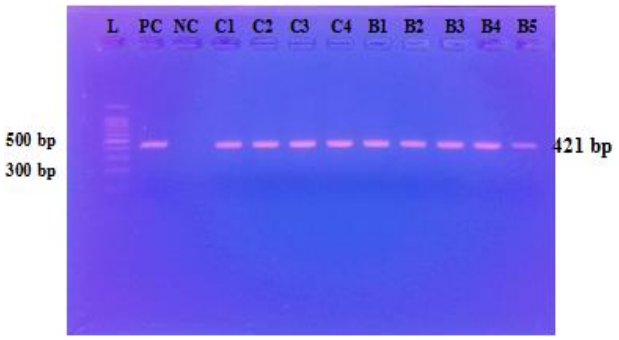

Fig. 3: PCR results of *S. aureus* positive for *blaZ* gene. L: Ladder, PC: Positive control, NC: Negative control, C: Cattle positive samples, and B: Buffalo positive samples

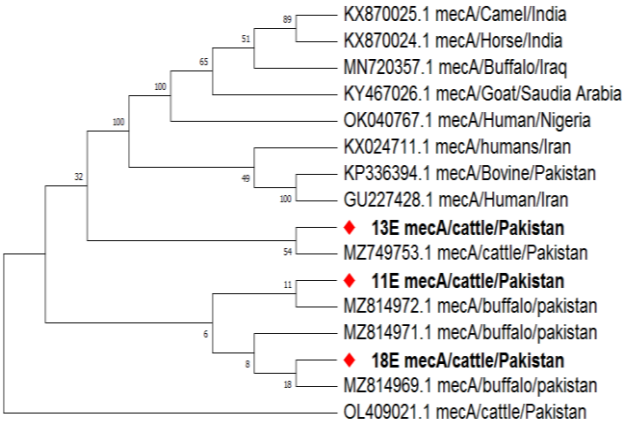

Fig. 4: Phylogenetic analysis of study isolates of MRSA with already reported sequences by Maximum Likelihood method

Antibiogram of MRSA and BRSA isolates from infected bovine reproductive tract

The isolates of MRSA and BRSA were examined for their antibiotic sensitivity. The overall resistance response rate was highest to cefotaxime (100%), ceftriaxone (88.46%), penicillin (84.62%), and

oxytetracycline (80.76%) while the highest sensitivity towards gentamicin (73.07%) and streptomycin (53.84%) were revealed, although ciprofloxacin (42.30%) showed a highest intermediate response Table 3. Overall resistant order of isolates was as: "cefotaxime > ceftriaxone > penicillin > oxytetracycline > amoxyclav > cefoperazone > cephalexin > streptomycin > ciprofloxacin > gentamicin" (Table 3).

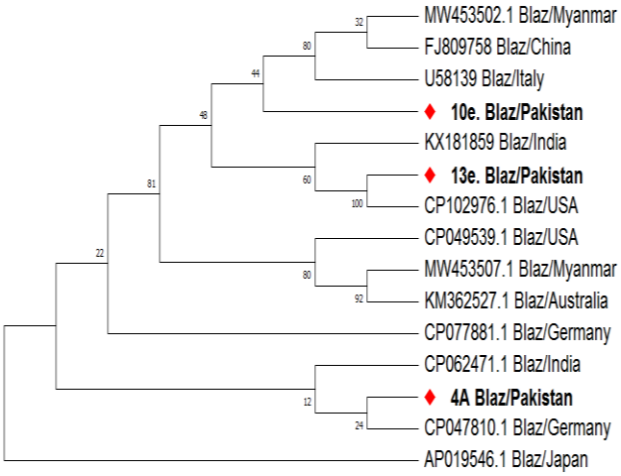

Fig. 5: Phylogenetic analysis of study isolates of beta-lactam resistant *S. aureus* with already reported sequences

Table 3: Antibiogram of MRSA isolates from infected bovine reproductive tract

Antibiotics	Potency (µg)	Resistant (%)	Intermediate (%)	Sensitive (%)
Penicillin	10 µg	22 (84.62)	0	4 (15.38)
Amoxiclav	30 µg	20 (76.92)	0	6 (23.07)
Ceftriaxone	30 µg	23 (88.46)	1 (3.84)	2 (7.69)
Oxytetracycline	30 µg	21 (80.76)	2 (7.69)	3 (11.53)
Ciprofloxacin	30 µg	6 (23.07)	11 (42.30)	9 (34.61)
Gentamicin	20 µg	5 (19.23)	2 (7.69)	19 (73.07)
Cefotaxime	10 µg	26 (100)	0	0
Streptomycin	30 µg	7 (26.92)	5 (19.23)	14 (53.84)
Cephalexin	30 µg	17 (65.38)	3 (11.53)	6 (23.07)
Cefoperazone	75 µg	19 (73.07)	5 (19.23)	2 (7.69)

Discussion

The results of this investigation showed that MRSA, BRSA, and other antibiotic-resistant strains of *S. aureus* exist in the bovine reproductive tract. In the present study, 384 samples were collected from numerous reproductive tract infectious cases of cattle and buffaloes. The overall prevalence of *S. aureus* was found to be 17.96% (69/384) while the phenotypic and genotypic prevalence of MRSA was found to be 50.72% (35/69) and 37.68% (26/69), respectively. The findings were consistent with those of earlier research that mentioned that 78.20% of bovine reproductive system samples were positive for *S. aureus*, while 26.92% of these were MRSA (Shafique *et al*., 2022). Unhygienic calving practices and a contaminated environment at the calving place contribute significantly to the higher infection rate. Another study reported that aborted bovines manifested 17.2% endometritis and 88.3% *S. aureus* from samples taken from aborted bovines (Petit *et al*., 2009) which is in accordance with our findings.

In a previous study, the prevalence of *S. aureus* isolated from different pyogenic conditions of bovines was observed as 38.1% (Yadav *et al*., 2018). *S. aureus* was frequently found in milk from mastitic cows, milk from mastitic buffaloes, and cow swabs with septic wounds was previously discovered to be 22.7%, 16.0%, and 22.0% respectively (El-Jakee *et al*., 2008). In a comparable study carried out in Iraq in 2012, *S. aureus* was found in 33.9% of samples from cow abscesses (AL-Tuffyli and Shekhan, 2012). The occurrence of *S. aureus* was also shown to decrease (36.22%) among clinical samples from a range of skin disorders in 2015 in buffaloes, cattle, camels, dogs, goats, horses, and sheep (Tiwari *et al*., 2015). Consequently, the current data demonstrates the rising incidence of *S. aureus*, albeit specifics may vary based on the sample type, the location, and the time of year. The phylogenetic analysis of current study isolates harboring *mecA* and *blaZ* genes showed similarity with the *mecA* and *blaZ* gene positive isolates of humans which represented the zoonotic potential of this pathogen that LA-MRSA can spread to humans (Elstrøm *et al*., 2019). Study isolates also represented similarity with already reported sequences of *mecA* and *blaZ* gene-positive isolates from buffalo, horse, camel, sheep, and cattle which represented the potential of the spread of pathogen between and within species (Grøntvedt *et al*., 2016).

A recent study reported a significant association between various risk factors and the prevalence of *S. aureus*-associated reproductive tract infections. Holstein and Friesian cows were at a greater risk of developing reproductive tract infections and the presence of retained fetal membrane (RFM) showed a high prevalence (25.8%) among Sahiwal and mix breeds, similar to a previous study in which retained fetal membranes were a well-established risk factor for reproductive tract diseases (Kim and Kang, 2003; Potter *et al*., 2010). As, retained fetal membranes delays the endometrium's involution and repair while keeping the cervix open, providing a point of entrance and a favorable environment for bacterial proliferation (Sheldon *et al*., 2008). In another study, the apparent prevalence of RFM was recorded at 1.8% (Kelly *et al*., 2020). According to previous researches, there is a link between parity and disorders of the reproductive tract; primiparous cows and those that are at least three lactations into their pregnancy are more likely to become ill than cows that are only in their second lactation (Markusfeld, 1984; Gröhn *et al*., 1990; Bruun *et al*., 2002) which is in accordance to our study in which higher infection rate was found in cattle in their second lactation. Many risk factors for reproductive tract infections are well established, and a broad selection of these risk factors was tested in this study.

During *in vitro* drug susceptibility testing for this study, a total of 10 antibiotics from various classes were observed. The greatest amount of resistance was seen to β-lactam antibiotics e.g., cefotaxime, ceftriaxone, and

penicillin. In a previous study, *S. aureus* isolates were also discovered to be completely resistant to β-lactam drugs including methicillin, penicillin, ampicillin, and sulfamethoxazole (Mohamed *et al*., 2011). In this study, antibiotics show a sensitivity response against gentamicin and streptomycin. In a previous investigation, 7.5% of the sample showed resistance to lincosamides (clindamycin), amphenicols (chloramphenicol), and aminoglycosides (amikacin, streptomycin, and gentamicin). These results differ from a prior study (Sen and Kilic, 2012), which revealed that *S. aureus* isolates had varying rates of sensitivity to amoxicillin/clavulanic acid, ofloxacin, and ceftriaxone of 57.1%, 80.9%, and 100%, respectively. Moreover, one of our isolates exhibited resistance to 13 different types of drugs. These results are consistent with past data that point to rising β-lactam antibiotic resistance (Mai-Siyama *et al*., 2014; Singh *et al*., 2016). The existence of the methicillin-resistance gene *mecA* is connected with resistance to beta-lactam drugs (Batabyal *et al*., 2012). The resistance was minimal or nonexistent against gentamicin, glycopeptide vancomycin, and amikacin. These results are consistent with a previous study (Sumathi *et al*., 2008) that discovered gentamicin to be effective against *S. aureus* infections based on *in vitro* trials. Moreover, it is also established that antimicrobial use is directly associated with bacterial resistance (Amit *et al*., 2010; Batabyal *et al*., 2012; Parmar *et al*., 2014; Tiwari *et al*., 2015).

The current study reported 17.96%, 37.68%, and 18.84% prevalence of *S. aureus*, MRSA, and BRSA in bovines suffering from endometritis. The similarity of isolates with *S. aureus* from other samples and even human isolates is indicative of the possibility of MRSA and beta-lactam-resistant *S. aureus* spill-over between and within species. The risk factor analysis revealed various management related practices and host factors that can contribute to *S. aureus*-associated infections. The findings of antibiotic susceptibility testing of study isolates showed higher resistance towards beta-lactam group (cefotaxime, ceftriaxone, and penicillin) while the aminoglycosides group (gentamicin and streptomycin) was found sensitive towards study isolates. The cumulative prevalence of resistant strains of *S. aureus* is suggestive of the fact that there is an essential need to stop the deliberate use of antibiotics to prevent MDR infections.

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Conflict of interest

All authors have declared no conflict of interest in the submission/publication of this data.

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