Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.157

RESEARCH ARTICLE

Molecular Epidemiology, Antibiogram Profiling, and Antimicrobial Resistance Modulation of *Streptococcus* spp. Isolated from Bovine Subclinical Mastitis

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ARTICLE HISTORY (26-461)

Received: April 24, 2026
Revised: May 31, 2026
Accepted: June 13, 2026
Published online: July 24, 2026

Key words:
Antimicrobial susceptibility profiling
Drug repurposing
Risk factors analysis
Streptococcus spp.

ABSTRACT

The mastitis caused by *Streptococcus* spp. in bovines is a major global issue for dairy farmers due to reduced milk production and high treatment costs. This study was designed to evaluate the molecular prevalence of *Streptococcus* spp. associated subclinical mastitis (SCM) along with risk factors analysis. The study also focused on the antimicrobial resistance profiling, resistance modulation strategy, and phylogenetic analysis of *Streptococcus* spp. A total of 384 milk samples were collected from bovines along with associated risk factor data. *Streptococcus* spp. isolates were phenotypically identified on the basis of hemolysis, catalase test, and esculin test on Edward's media. Genotypic confirmation was performed by PCR targeting the 16S *rRNA* gene. Antimicrobial resistance profiling and drug modulation were performed to evaluate the efficacy of antibiotics with non-antibiotics compounds. A total of 157 (40.89%) animals were positive for SCM, with *Streptococcus* spp. phenotypically confirmed in 32 (20.38%) milk isolates. Genotypic identification revealed that 31 (19.75%) isolates were positive for *Streptococcus* spp. The phylogenetic analysis revealed closer resemblance of study isolates to each other and with the isolates of other countries. Antibiotic resistance profiling showed high resistance against gentamicin and trimethoprim + sulfamethoxazole, while vancomycin and penicillin showed highest efficacy against streptococci isolates. Moreover, modulation therapy revealed a synergistic effect of gentamicin with flunixin meglumine and ketoprofen. However, trimethoprim + sulfamethoxazole and oxytetracycline exhibited synergism with ketoprofen and meloxicam, respectively. This study provided the key data regarding *Streptococcus* spp. associated with SCM and a better therapeutic regime against the rising issue of AMR in *Streptococcus* spp.

To Cite This Article: Shah MH, Ijaz M, Ahmed A and Awan F, 2026. Molecular epidemiology, antibiogram profiling, and antimicrobial resistance modulation of *Streptococcus spp.* isolated from bovine subclinical mastitis. Pak Vet J, 46(7): 1669-1677. http://dx.doi.org/10.29261/pakvetj/2026.157

INTRODUCTION

The dairy industry suffers significant financial losses as a result of bovine mastitis because of the higher treatment costs and decreased milk production. Mastitis is an economically significant disease in the dairy industry worldwide. About 40% of cows in herds suffer from this disease, making it one of the most common diseases in the dairy sector (Ahmed *et al.*, 2022). The estimated yearly economic losses from mastitis are 124€ (147$) per cow, with a global loss of 125 billion €. Numerous variables, such as decreased milk output, low milk quality, veterinary costs, and early cow culling, can be attributed to these losses (Cheng and Han, 2020).

Staphylococcus, Streptococcus, *Escherichia coli*, and *Klebsiella* spp. are the most commonly isolated pathogens from mastitis-affected cattle (Cheng and Han, 2020). Presence of bacterial pathogens in milk poses a public health concern too (Gohar *et al*., 2017). *Streptococcus* spp. is an important cause of contagious and chronic bovine mastitis that leads to a decrease in milk yield. *Streptococcus* spp. are among the most common bacteria that cause intramammary infections (Kabelitz *et al.*, 2021). Streptococci are Gram-positive bacteria that are commensal organisms of the cow's udder, skin, and mucosa, and are also widespread in the environment. Among *Streptococcus* spp., *S. agalactiae* (contagious agent), *S. dysgalactiae,* and *S. uberis* (environmental agent) are predominantly isolated from mastitis.

The early, fast, and effective identification of bovine mastitis increases the recovery rate and reduces the duration to retain normal milk production when

accompanied by appropriate antimicrobial treatment (Ahmed *et al*., 2022). Diagnostic methods being used include the California Mastitis Test (CMT), Christie-Atkins-Munch-Petersen (CAMP) test, and molecular detection by PCR. The bacteriological identification based on the gross colony morphology, Gram staining, and biochemical testing may not always accurately differentiate closely related other streptococcal species because of the close similarity in cultural and biochemical features. However, molecular detection on the basis of unique sequences of DNA within the *16S rRNA* (ribosomal RNA) gene is a fast, reliable, and accurate diagnostic method (Farzana *et al.*, 2023). *Streptococcus* spp. was identified by molecular methods as one of the most common infectious pathogens among the bacteria that cause contagious mastitis in Egypt. Therefore, due to high reliability and accuracy, the molecular method was used for the early detection of mastitis-causing pathogens in cattle (Abd El *et al.*, 2021).

The WHO (World Health Organization) recognizes antimicrobial resistance (AMR) as one of the top ten public health challenges that humanity is currently facing (Pinheiro *et al.,* 2020; Hannan *et al*., 2024; Rahman *et al.*, 2024; . *Streptococcus* spp. are notorious mastitis-causing pathogens due to the emergence of multidrug-resistant strains (Kabelitz *et al.*, 2021). The widespread and irrational use of antibiotics causes bacteria to mutate, resulting in antibiotic-resistant bacteria (Saman *et al.,* 2023; Yaseen *et al.*, 2025). Due to growing concern of AMR, drug repurposing strategies are investigated as a modified approach to tackle multidrug-resistant bacterial infections (Rasheed *et al.,* 2023). Recent studies have reported that non-antibiotics can broaden the antimicrobial spectrum and require a minimal amount of the antibiotic for efficient therapy, possibly reducing treatment-related complications (Ahmed *et al.*, 2022) . NSAIDs (non-steroidal anti-inflammatory drugs) work against mastitis-associated bacteria by altering the ion transporter, bacterial efflux pump, cell permeability, and the activity of vital enzymes (Muzammil *et al.*, 2022).

In Pakistan, numerous studies have investigated the molecular prevalence of mastitis-associated pathogens in bovines; however, the data regarding the molecular prevalence and resistance profiling of *Streptococcus* spp. associated subclinical mastitis in bovines remains limited. This study was designed to evaluate the prevalence of *Streptococcus* spp. associated subclinical mastitis in bovine, risk factors analysis, phylogenetic characterization, and antibacterial sensitivity profiling of study isolates. This study provides the key data regarding *Streptococcus* spp. associated with SCM and a better therapeutic regime against the rising issue of AMR in *Streptococcus* spp. Furthermore, synergy testing of antibiotics with NSAIDs was also carried out as a potential remedial option against genotypically confirmed isolates of *Streptococcus* spp.

MATERIALS AND METHODS

Ethical approval: The current study was approved by the Advanced Studies and Research Board (ASRB) of the University of Veterinary and Animal Sciences, Lahore, Pakistan, with letter No. DR/ 432 (Dated: 02-09-2024).

Study area and sample size determination: The study was performed in the dairy farms of district Multan (30.1951° N, 71.4774° E) and district Muzaffargarh (30.1392° N, 71.0973° E) (Fig. 1). Using the online EpiTools program (epitool.ausvet.com), the sample size was determined based on a 50% prevalence of *Streptococcus* spp. at a 95% confidence level (Altaf *et al.*, 2020).

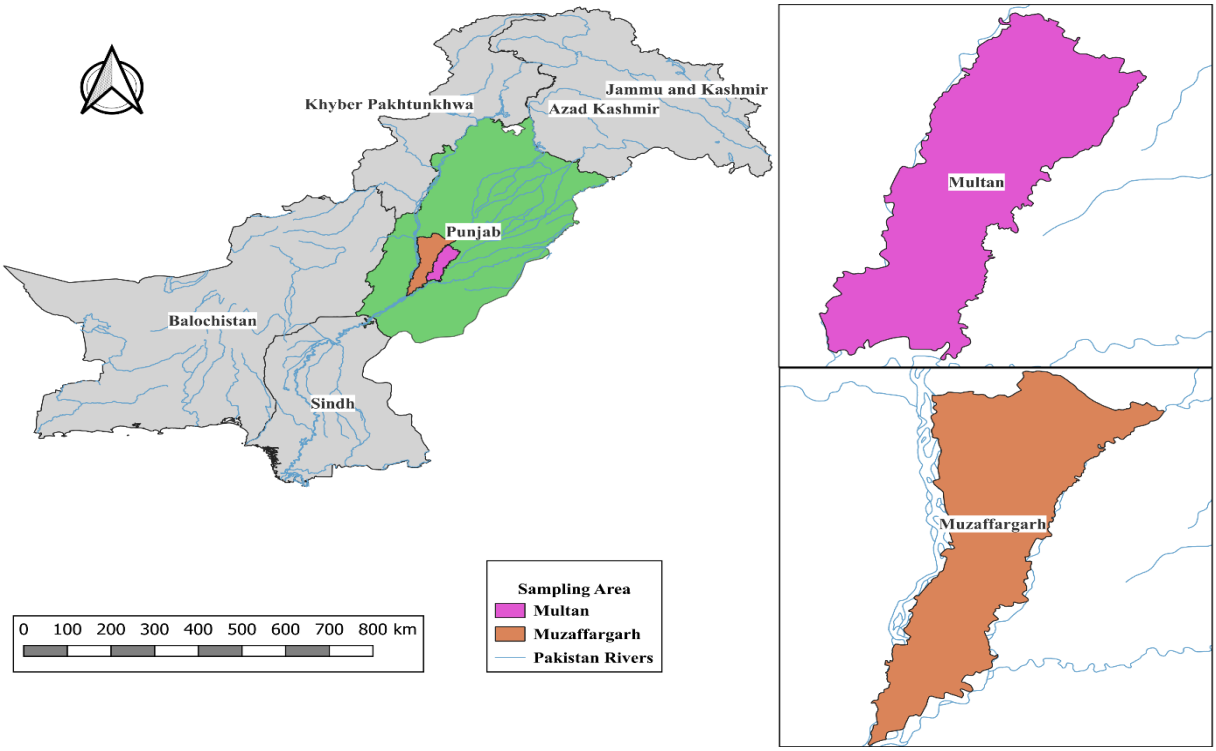

Fig. 1: QGIS map showing the study area.

Sampling Strategy: A total of 384 bovine milk samples, including 192 from cattle (indigenous Sahiwal, exotic Holstein, and mixed breeds) and 192 from buffalo (Nili, Ravi, and Nili Ravi breeds), were collected from both districts using a convenient sampling technique, irrespective of age, parity, and pregnancy status, from October 2023 to August 2024. A questionnaire was completed during sampling to evaluate the possible risk factors showing a strong association with the *Streptococcus* spp. associated SCM in bovines. Every variable was divided into several levels, like milk yield, which was categorized as high (≥ 10 L/day) and low (< 10 L/ day) on the basis of the milk production. Similarly, stages of lactation were classified as early (1-3 months), mid (4-6 months), and late (> 6 months). The initial two milk streams were discarded, and then milk samples were obtained aseptically in the falcon tubes and assessed for SCM through the California Mastitis Test (CMT) (Lakew *et al.,* 2019; Javed *et al.*, 2024). The milk samples showing visible gel formation on CMT were identified as SCM-positive samples, while samples with no gel formation were considered SCM-negative. SCM-positive milk samples were carried to the Medicine Research Lab, Department of Veterinary Medicine, University of Veterinary and Animal Sciences (UVAS) Lahore, for further bacteriological and molecular analysis.

Bacteriological isolation and identification: Isolation and identification of *Streptococcus* spp. were performed according to the procedures reported by (Lakew *et al.*, 2019). SCM positive milk samples were streaked on blood agar base enriched with 5% sheep blood and incubated at 37°C for 24 to 48 hours (Javed *et al*., 2025). *Streptococcus* spp. isolates were confirmed on the basis of hemolytic properties, gross colony morphology, and Gram staining. The catalase test was performed on suspected colonies, and catalase-negative isolates were considered *Streptococcus* spp. Positive colonies of *Streptococcus* spp. were selected and sub-cultured on modified Edward's media containing esculin and allowed to incubate in an aerobic environment at 37 °C for 24–48 h. The isolates exhibiting small, convex, and transparent to white colonies on Edward's agar were considered positive for *Streptococcus* spp.

Molecular investigation and confirmation of *Streptococcus:* Phenotypically positive isolates were grown in brain heart infusion (BHI) broth (da Silva *et al.*, 2017). The DNA was extracted through a ThermoScientific Gene JET Genomic DNA purification kit (Aqib *et al.*, 2018). Following DNA quantification by nano-drop (Nano Drop 2000 ThermoScientific), PCR was carried out for the molecular confirmation of *Streptococcus* spp. by targeting the *16S rRNA* gene using an already reported primer sequence (Mas-De-Xaxars and Garcia-Gil, 2009). Primers used for the *16S rRNA* gene were 1043F 5'-CACTCTAGCGAGACTGCCG-3', 1492R 5'-ACGGTTACCTTGTTACGACTT-3', having a product size of 450. PCR amplification was carried out in a final reaction volume of 20 µL, consisting of 10 µL of 2X PCR Taq Master Mix, 2 µL of template DNA, 0.7 µL of each primer, and 6.6 µL of nuclease-free water. The conditions for PCR were initial denaturation at 95 °C (for 10 minutes), followed by 30 cycles of denaturation at 94°C (for 30 seconds), annealing at 61°C (for 1 minute), extension at 72°C (for 1 minute), and final extension at 72°C (for 10 minutes) (Mas-De-Xaxars and Garcia-Gil, 2009). The amplified PCR products were run on a 1.5% agarose gel with dye added, named as ethidium bromide, using a voltage of 120 V for a time of 30 minutes (Javed *et al.*, 2024).

Sequencing of local isolates: The positive bands found on the gel electrophoresis were cut, purified using a gel extraction kit (ThermoScientific Gene JET), and sent to a commercial sequencing facility for Sanger sequencing (Altaf *et al.*, 2020). To obtain accession numbers, these sequences were submitted to NCBI (National Center for Biotechnology Information). After retrieving accession numbers, the BLAST (Basic Local Alignment Search Tool) was employed to compare the resulting sequences with those already reported from other countries. The *Streptococcus* spp. sequences reported from other countries, that have already been published on NCBI, were obtained, and their comparison with the study isolates was performed by constructing a phylogenetic tree using 1000 bootstrap replications and the Maximum Likelihood technique using MEGA XI software (Ahmed *et al.*, 2022).

Antimicrobial sensitivity pattern of isolates: Antimicrobial susceptibility testing of genotypically confirmed *Streptococcus* spp. isolates was carried out using the disc-diffusion antibiotic sensitivity test (Javed *et al.*, 2023). The antibiotics that were used include oxytetracycline (30 µg), tylosin (30µg), ciprofloxacin (5µg), levofloxacin (5µg), gentamicin (10 µg), penicillin (10 units), trimethoprim-sulfamethoxazole (1.25µg +23.75µg), cefoxitin (30 µg), vancomycin (30 µg), and moxifloxacin (5µg). Antibiotic discs were aseptically applied to Mueller-Hinton agar that was pre-swabbed with a bacterial suspension of $1x10^8$ CFU/ml. These plates were incubated for 24 hours at 37°C (Ahmed *et al.*, 2022).

After incubation, the antibiotic discs' surrounding bacterial growth inhibition zones were assessed by using a vernier caliper and compared with reference values from the Clinical Laboratory Standards Institute (CLSI) to determine whether the isolates were sensitive, intermediate, or resistant (CLSI, 2019; Nasir *et al.,* 2025). To ensure the reliability of the data and reproducibility of results, the experiment was conducted in triplets.

Resistance Modulation: The well diffusion assay was performed against selected NSAIDs (flunixin meglumine, meloxicam, and ketoprofen) alone and in combination with the three least effective antibiotics oxytetracycline, gentamicin, and trimethoprim-sulfamethoxazole (Aqib *et al.*, 2021). Serial dilutions of NSAIDs and resistant antibiotics, both separately and together, were added to the wells made on Mueller-Hinton agar plates that had already been inoculated with the tested bacteria. After incubation for 24 hours at 37°C, the growth inhibitory zones encircling the antibiotic discs were measured (Aqib *et al.*, 2021). The modulation factor was calculated by the zone of inhibition of the antibiotic alone divided by the zone of inhibition of the antibiotic in combination with the

NSAID. The combinations that exhibited a modulation factor of <0.5 were declared to exhibit synergism.

Determination of minimum inhibitory concentration: A 96-well microtiter plate was used in the microbroth dilution method for the determination of MICs (minimum inhibitory concentrations). The MICs of both NSAIDs and three least effective antibiotics, based on the Kirby-Bauer disc diffusion method, were recorded for the *Streptococcus* spp. isolates. Sequential dilutions of drugs being tested were added to the plate wells, while the negative controls were kept empty. Except for the negative control, all wells were filled with fresh bacterial cultures ($1.5–1.8\times10^5$ CFU/ml). The optical density value with a wavelength of 570 nm was recorded both before and after a 24 hours incubation period at a temperature of 37°C (Ahmed *et al.*, 2022).

Synergy testing of antibiotics and NSAIDs: The checkerboard method was used to perform synergy testing for several combinations against *Streptococcus* spp. isolates. A plate was filled with the chosen concentrations of both antibiotics and NSAIDs, and a serial dilution in the vertices and horizons was carried out. All wells were filled with the fresh bacterial culture ($1.5\text{-}1.8\times10^5$ CFU/ml) except the negative control. Before and after incubation for 24 hours at 37°C, the OD value at 570nm was recorded. FICI (Fractional inhibitory concentration indices) were calculated to assess the antibiotics and NSAIDs combinations to determine if the interactions were antagonistic, additive, synergistic, or indifferent (Muzammil *et al.*, 2022). FIC values <0.5 were considered synergistic interaction, while values ranging from 0.5 to 1.0 were identified as additive interaction.

Data Analysis: The prevalence of *Streptococcus* spp. was computed using descriptive statistics. Risk factors were evaluated using the chi-square test, followed by logistic regression analysis (Javed *et al.*, 2021). In logistic regression analysis, risk factors exhibiting a *P*-value <0.05; Odds Ratio >1 were considered as potential risk factors associated with subclinical mastitis. Descriptive statistics were applied to analyze susceptibility trials of antibiotics and non-antibiotics. Fractional inhibitory concentration indices were calculated using the formula, as demonstrated by (Muzammil *et al.*, 2022).

RESULTS

Status of *Streptococcus* spp. CMT was used to screen for SCM, and molecular confirmation of *Streptococcus* spp. was subsequently performed. This study found that out of the total 384 sampled animals (192 from cattle and 192 from buffalo), 157 animals were SCM-positive with a prevalence of 40.89%. The highest prevalence was found in Holstein cattle 26 (45.61%), compared to buffalo 26 (42.62%) of the Nili breed. From 157 SCM-positive milk samples, phenotypically *Streptococcus* spp. was detected in 32 (20.38%) samples (Table 1).

Phenotypically positive samples were analyzed by using the PCR technique for genotypic identification of the *Streptococcus* spp. by targeting the *16S rRNA*. Based on PCR, 31 (19.75%) isolates were found to be *Streptococcus* spp. (Table 1).

Table 1: Prevalence of *Streptococcus* spp. in the udder of bovines

Sr. No	Animal Species	Breed	No. of Animals	SCM (%)	*Streptococcus* spp. (%)	
					Phenotypic	Genotypic
1	Cattle	Sahiwal	91	37 (40.66%)	11 (29.73%)	11 (29.73%)
		Holstein	57	26 (45.61%)	6 (23.08%)	5 (19.23%)
		Mixed breed	44	18 (40.91%)	3 (16.67%)	2 (11.11%)
		Nili	61	26 (42.62%)	4 (15.38%)	3 (11.54%)
2	Buffalo	Ravi	53	18 (33.96%)	2 (11.11%)	3 (16.67%)
		Nili Ravi	78	32 (41.03%)	6 (18.75%)	7 (21.88%)
Total			384	157 (40.89%)	32 (20.38%)	31 (19.75%)

Risk factor analysis associated with subclinical mastitis in bovines: Risk factors were analyzed by Chi-square test to assess association of risk factors with bovine subclinical mastitis. Statistical evaluation of risk factors revealed that the floor condition, stock density, teat dip usage, milk yield, teat injury, and mastitic quarter were possible risk factors showing a significant relationship with subclinical mastitis (*P*-value < 0.05) and were further analyzed by logistic regression. In contrast, species, breed, season, age, type of housing, system of rearing, farm hygiene, floor type, general physical condition of animal, lactation (parity), and stage of lactation appear to be non-significant (*P*-value > 0.05) (Table 2).

The logistic regression analysis was performed to evaluate the potential risk factors associated with SCM in bovines. The logistic regression model's results showed that the conditions of floor, stock density, use of teat dip, milk yield, teat injury, and mastitic quarter were potential risk factors linked to the development of disease (*P*-value <0.05; Odds Ratio>1). It was observed that there were 3.626 times more chances of subclinical mastitis in animals that were kept on an uneven floor because of poor cleanliness and the risk of injury to the udder. Similarly, farms having more stock density were at 3.376 times more risk of developing subclinical mastitis than farms having low stock density. Similarly, the odds of having subclinical mastitis in bovines with a lack of teat dips (OR 2.596; $p = 0.003$), high milk yield (OR 1.777; $p = 0.007$), teat injury (OR 1.861; $p = 0.003$), and animals having already mastitic quarter (OR 2.319; $p = 0.001$) were noticeably greater than comparable levels of the variables (Table 3).

Phylogenetic characterization of *Streptococcus* spp.: The phylogenetic analysis was performed to analyze the evolutionary relationship of the *Streptococcus* spp. isolates. BLAST analysis showed that study isolates exhibited higher homology with the previously submitted isolates of *S. agalactiae.* Using MEGA XI, the evolutionary relationship of our study isolates (PP838542, PP837912, PP886253, PP837941, and PP838140) and previously documented isolates from countries such as India, China, Iran, USA, Bangladesh, Australia, France, and Norway were evaluated. Study isolates PP838542 and PP886253, which formed a tight clade exhibiting more homology with each other and the *S. agalactiae* isolate from Bangladesh, compared to other isolates. Similarly, the study isolates PP837912, PP837941, and PP838140,

which showed more similarity with each other compared to other isolates, indicating low genetic variations among these strains as well as suggesting a common ancestry. All indigenous isolates showed less homology with other *Str. agalactiae* isolates from Norway (MK364802), Australia (AF291419), Brazil (FJ555491), China (OQ940565), USA (AH006153), India (OP572129), France (DQ232512), Iran (OP688483), and Iraq (MK680052), suggesting the evolutionary differences among them (Fig. 2).

Table 2: Risk factors evaluation related to SCM

Variable	Variable level	Total	SCM		*P*-value
			Positive	%	
Specie	Buffalo	192	76	39.58	0.604
	Cow	192	81	42.19	
Breed	Nili-Ravi (Buffalo)	192	76	39.58	0.699
	Sahiwal (Cow)	14	4	28.57	
	Holstein (Cow)	104	45	43.27	
	Mixed (Cow)	74	32	43.24	
Season	Spring	65	27	41.54	0.938
	Summer	195	77	39.49	
	Autumn	78	34	43.59	
	Winter	46	19	41.3	
Age	1-4 years	34	12	35.29	0.067
	5-7 years	74	39	52.7	
	8-11 years	276	106	38.41	
Type of housing	Backyard	81	32	39.51	0.854
	Open area	138	59	42.75	
	Street	165	66	40	
System of rearing	Extensive	47	19	40.43	0.992
	Semi-intensive	155	63	40.65	
	Intensive	182	75	41.21	
Farm hygiene	Excellent	17	8	47.06	0.373
	Good	33	10	30.3	
	Normal	56	23	41.07	
	Poor	118	43	36.44	
	Very poor	160	73	45.63	
Floor type	Bricks	186	77	41.4	0.966
	Cemented	36	15	41.67	
	Earthen	162	65	40.12	
Condition of floor	Even	121	26	21.49	0.000*
	Uneven	263	131	49.81	
Stock density	High	301	140	46.51	0.000*
	Low	83	17	20.48	
Use of teat dip	No	324	143	44.14	0.003*
	Yes	60	14	23.33	
General physical condition	Excellent	8	3	37.5	0.957
	Good	26	9	34.62	
	Normal	94	38	40.43	
	Poor	218	92	42.2	
	Very poor	38	15	39.47	
Lactation (parity)	1	31	14	45.16	0.754
	2	47	17	36.17	
	3	94	40	42.55	
	4	84	31	36.9	
	5	81	32	39.51	
	6	47	23	48.94	
Stage of lactation	Early	44	20	45.45	0.696
	Mid	133	56	42.11	
	Late	207	81	39.13	
Milk yield	High	143	71	49.65	0.007*
	Low	241	86	35.68	
Teat injury	Yes	159	79	49.69	0.003*
	No	225	78	34.67	
Mastitic quarter	Yes	90	51	56.67	0.001*
	No	294	106	36.05	

* Indicates the significantly associated risk factors

Antimicrobial susceptibility profile of *Streptococcus* spp. Isolates: Antimicrobial susceptibility testing by using Kirby-Bauer disc diffusion was carried out to investigate the antimicrobial susceptibility profiling of confirmed *Streptococcus* spp. isolates. The results revealed that maximum resistance was observed against gentamicin (CN) (88.89%), followed by trimethoprim-sulfamethoxazole (TMP-SMX) (77.78%) and oxytetracycline (OTC) (55.56%). In the case of sensitivity, vancomycin (VAN) was found fully sensitive (100%) against *Streptococcus* spp. isolates followed by penicillin G (PEN) (88.89%), moxifloxacin (MXF) (77.78%), cefoxitin (CFX), ciprofloxacin (CIP), and levofloxacin (LEV) (55.56%) (Fig. 3).

Table 3: Risk factors analysis by logistic regression.

Variable	Response levels	Odds ratio	Confidence interval	Std. Error	*P*- value
Condition of floor	Uneven	3.626	2.207	0.253	0.000
	Even	Ref	5.958		
Stock density	High	3.376	1.892	0.296	0.000
	Low	Ref	6.025		
Use of teat dip	No	2.596	1.373	0.325	0.003
	Yes	Ref	4.909		
Milk yield	High	1.777	1.167	0.215	0.007
	Low	Ref	2.707		
Teat injury	Yes	1.861	1.229	0.212	0.003
	No	Ref	2.818		
Mastitic quarter	Yes	2.319	1.435	0.245	0.001
	No	Ref	3.749		

Antibacterial efficacy of antibiotics and NSAIDs: The well diffusion assay was conducted to determine the antibacterial efficacy of antibiotics alone and in combination with NSAIDs. The results indicated that gentamicin had the highest ZOI among antibiotics (15.5±0.53), while trimethoprim/sulfamethoxazole had the lowest (11.25±1.16). In NSAIDs, flunixin meglumine showed the highest ZOI (7.38±0.74), while ketoprofen showed the lowest ZOI when used alone (5.75±0.71). Oxytetracycline exhibited the greatest % rise in ZOI (125.90%) when combined with Meloxicam, followed by trimethoprim/sulfamethoxazole + ketoprofen (116.71%) and gentamicin + flunixin meglumine (111.29%). Synergistic effect (modulation factor <0.5) was observed by combining trimethoprim/sulfamethoxazole with ketoprofen (0.46), oxytetracycline with meloxicam (0.44), and gentamicin with both flunixin meglumine (0.47) and ketoprofen (0.49). The modulation factor of gentamicin + meloxicam, trimethoprim/sulfamethoxazole + flunixin meglumine, trimethoprim/sulfamethoxazole + meloxicam, oxytetracycline + flunixin meglumine, and oxytetracycline + ketoprofen is more than 0.5, so there may be an additive effect instead of being synergistic, and the ZOI of these combinations was close to the sum of their individual ZOI (Table 4).

Synergy Evaluation: The checkerboard method was used to evaluate synergy for several combinations against *Streptococcus* spp. isolates. The checkerboard assay showed that gentamicin exhibited a synergistic effect with ketoprofen and flunixin meglumine, trimethoprim/sulfamethoxazole with ketoprofen, and oxytetracycline with meloxicam. The additive effect was observed by combining gentamicin with meloxicam, trimethoprim/sulfamethoxazole with meloxicam and flunixin meglumine, and oxytetracycline with ketoprofen. The combination of oxytetracycline and flunixin meglumine showed an indifferent effect (Table 5).

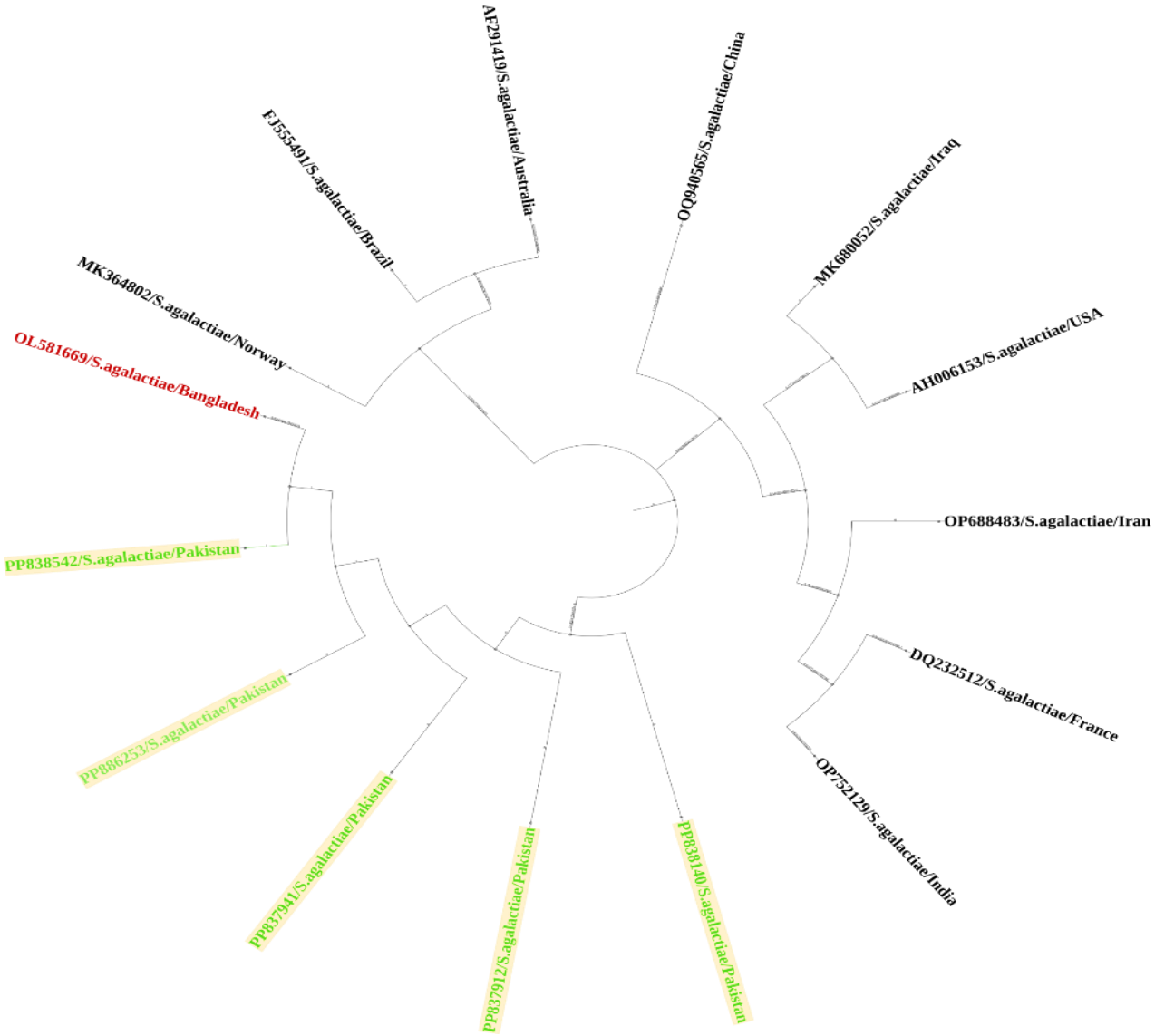

Fig. 2: Phylogenetic analysis of *Streptococcus* spp. isolates (accession numbers: PP838542, PP837912, PP886253, PP837941, PP838140).

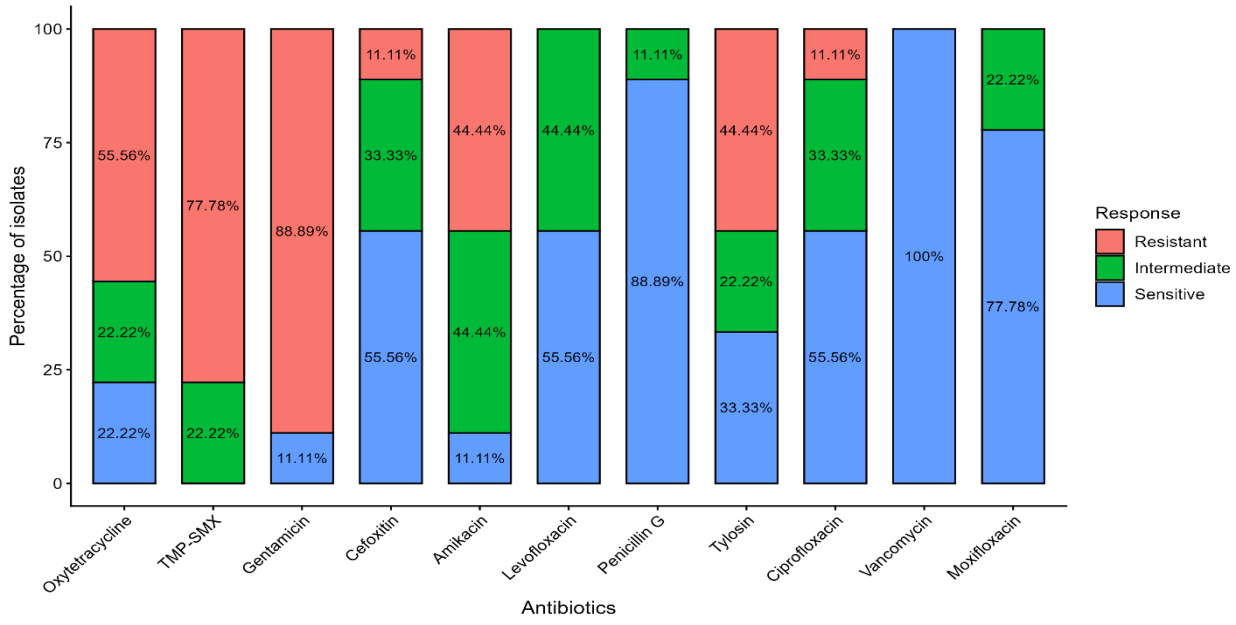

Fig. 3: Antibiogram of *Streptococcus* isolates.

Table 4: Inhibition zones (mm) of antibiotics alone and in combination with NSAIDs against *Streptococcus* spp. isolates

Antibiotic, NSAIDs	Antibiotic alone	NSAIDs alone	ZOI in combination	%increase in ZOI of antibiotic	Modulation factor
Gentamicin, Flunixin Meglumine	15.5±0.53	7.38±0.74	32.75±0.89	111.29%	0.47
Gentamicin, Ketoprofen	15.5±0.53	5.75±0.71	31.5±1.2	103.23%	0.49
Gentamicin, Meloxicam	15.5±0.53	7.25±0.89	28.62±1.06	84.65%	0.54
Trimethoprim/sulfamethoxazole, Flunixin Meglumine	11.25±1.16	7.38±0.74	20.62±1.19	83.29%	0.54
Trimethoprim/sulfamethoxazole, Ketoprofen	11.25±1.16	5.75±0.71	24.38±1.77	116.71%	0.46
Trimethoprim/sulfamethoxazole, Meloxicam	11.25±1.16	7.25±0.89	17.88±1.25	58.93%	0.63
Oxytetracycline, Flunixin Meglumine	11.62±1.19	7.38±0.74	19.12±1.12	64.54%	0.61
Oxytetracycline, Ketoprofen	11.62±1.19	5.75±0.71	22.11±1.36	90.28%	0.52
Oxytetracycline, Meloxicam	11.62±1.19	7.25±0.89	26.25±1.39	125.90%	0.44

% Rise in ZOI = Inhibition zones of antibiotic in combination – Inhibition zones of antibiotic alone / Inhibition zones of antibiotic alone × 100. Modulation factor = ZOI alone / ZOI in combination. A modulation factor <0.5 indicates synergism

Table 5: Synergy testing of NSAIDs and antibiotics

Antibiotics, NSAIDs	MIC AB	MIC A	FIC A	MIC AB	MIC B	FIC B	FICI	Remarks
Gentamicin + Flunixin meglumine	5.984	17.432	0.343277	4.0123	27.146	0.147804	0.491	Synergistic
Gentamicin + Ketoprofen	6.986	19.378	0.360512	3.968	30.683	0.129322	0.490	Synergistic
Gentamicin + Meloxicam	7.312	16.764	0.436173	5.013	28.693	0.174712	0.611	Additive
Trimethoprim/sulfamethoxazole + Flunixin meglumine	8.322	17.398	0.478331	4.97	28.765	0.172779	0.651	Additive
Trimethoprim/sulfamethoxazole + Ketoprofen	4.739	16.2124	0.292307	5.285	29.569	0.178734	0.471	Synergistic
Trimethoprim/sulfamethoxazole + Meloxicam	9.0324	17.519	0.515577	3.743	26.584	0.140799	0.656	Additive
Oxytetracycline + Flunixin meglumine	9.629	14.739	0.653301	9.863	20.179	0.488775	1.142	Indifferent
Oxytetracycline + Ketoprofen	6.498	16.259	0.399656	5.531	26.373	0.209722	0.609	Additive
Oxytetracycline + Meloxicam	4.976	18.274	0.272299	5.419	27.387	0.197868	0.470	Synergistic

MIC = Minimum inhibitory concentration, FIC = Fractional inhibitory concentration, FICI = Fractional inhibitory concentration indices. FIC <0.5 Synergistic*, 0.5–1.0 Additive, 1.0–4.0 Indifferent, >4.0 Antagonist

DISCUSSION

The most common bacterial pathogens causing mastitis include *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., and *Streptococcus* spp. (Gao *et al*., 2017; Yang *et al*., 2020; Rasheed *et al.,* 2023; Anwar *et al.,* 2026). Among these pathogens, *Streptococcus* spp.-associated mastitis in bovines is a major global issue for dairy farmers and the veterinary sector. It is one of the main causative agents of contagious mastitis in dairy animals (Kabelitz *et al.*, 2021). In this study, the status of *Streptococcus* spp. from the udder of bovines affected with SCM, associated risk factors, antibiogram profiling, and resistance modulation through the combination of resistant antibiotics and NSAIDs were evaluated.

In the current study, 157 (40.89%) of animals were found to be affected by SCM based on CMT. These findings are close to those reporting a 53% prevalence of SCM in cows (Michira *et al.*, 2023). This prevalence is lower than 74.49% SCM from bovine mastitis reported by Farzana *et al.*, (2023) but higher than 28.75% reported by Bhuiyan *et al.*, (2020). Prevalence of SCM may vary across studies as a result of variations in sample size and sampling techniques. The climate, husbandry techniques, animal rearing methods, geographic location, and hygiene may also contribute to variations (Karimuribo *et al.*, 2008).

On the phenotypic basis, 32 (20.38%) of isolates were identified as *Streptococcus* spp. These findings are close to the study (Farzana *et al.*, 2023), which disclosed a 19.86% prevalence of *Streptococcus* spp. in bovines affected with SCM based on a positive CAMP test and a negative esculin hydrolysis test. These findings are consistent with those of Raheel *et al.*, (2022), which outlined 20.5% of *Streptococcus* spp. from buffalo. Prevalence of bovine mastitis, which is caused by several causative bacteria, fluctuates since it is a complicated disease where several elements interact, including husbandry practices, environmental circumstances, milking practices, a mastitis control strategy, animal risk variables, and climate (Farzana *et al.*, 2023).

Based on PCR, this study found 31 (19.75%) of the samples to be *Streptococcus* spp. Another study (Yang *et al.*, 2020) reported a 14.1% prevalence of *Streptococcus* spp. from SCM samples**.** The study outlined 12% genotypic prevalence of *Streptococcus* spp. by targeting the 16S *rRNA* gene (Tuzcu *et al.*, 2023). Many factors, like differences in management practices between rural and urban areas, sample sizes, and geographic features, play their part in the significant variation of prevalence rates of the region (Graveland *et al.*, 2011).

In the current study, risk factors including floor condition, stocking density, use of teat dip, milk yield, teat injury, and mastitic quarter appeared significant after statistical analysis. These observations are in agreement with the recent study in which the teat injury ($p = 0.02$) and the type of floor ($p = 0.000$) were found to be significant in the development of mastitis on farms (Lakew *et al.*, 2019). The previous study on mastitis found that post-milking teat disinfection is a significant factor in controlling mastitis (Cheng and Han, 2020).

High sequence similarity was found when the 16S *rRNA* gene of typical PCR outputs of *Streptococcus* spp. was sequenced, and BLAST comparisons of these sequences against the NCBI showed higher similarity with *S. agalactiae*. The phylogenetic characterization showed the resemblance of study isolates with the previously reported isolates from other nations, including Bangladesh, Norway, USA, Australia, Brazil, India, Iran, and Iraq. This might be due to the international trading of dairy products, cross-border animal movements, animal trading, and international travel by people, all of which are important contributors to the proliferation of infections (Fèvre *et al.*, 2006).

This study reported the highest resistance against gentamicin, and no resistance was observed against vancomycin, which follows the findings of a formerly reported study (Liu *et al.*, 2018). Another study in

Thailand on bovine mastitis reported 100% resistance against gentamicin against *Streptococcus* spp. isolates (Wataradee *et al.*, 2024) and also reported penicillin and vancomycin as highly susceptible drugs. The recently published study also declared susceptibility to penicillin in 100% isolates of *Streptococcus* spp. while conducting a study on bovine mastitis in Denmark, which is also in line with the current study results (Chehabi *et al.*, 2019). In contrast, studies conducted on bovine mastitis in China declared higher resistance in *Streptococcus* spp. against beta-lactam (Han *et al.*, 2022). In this study, the resistance against oxytetracycline was found to be 55.56%, which is close to the findings of a study (da Silva *et al.*, 2017), which found 47.54% resistance against tetracycline in herds of cattle against *Streptococcus* spp. isolates. Another study (Rato *et al.*, 2013) also reported 64.8% resistance against tetracycline and 80.6% against aminoglycosides, while demonstrating sensitivity against vancomycin and penicillin. The past study also reported that most resistant antibiotics, 51% strains of *Streptococcus* spp. were resistant against gentamicin, 15% against sulfamethoxazole-trimethoprim, and 77% against tetracycline (Mesquita *et al.*, 2019). According to the results of a prior published study, a 95% prevalence of tetracycline resistance in *Streptococcus* spp. strains of bovine mastitis originating in China and Pakistan (Leghari *et al.*, 2023).

NSAIDs possess antimicrobial activity, evidenced by a number of studies, which is linked with their ability to interrupt the cytoplasmic membrane or inhibit the synthesis of DNA and replication (Leão *et al.,* 2020). Numerous studies have demonstrated that NSAIDs have antibacterial properties and lessen the bacterial load when used either alone or in combination with antibiotics that are resistant to them (Bakht *et al.*, 2024). The synergistic effect of antibiotics with NSAIDs has been well studied in the past (Leão *et al.*, 2020; Aqib *et al.*, 2021; Muzammil *et al.*, 2022; Ahmed *et al.*, 2022; Javed *et al.*, 2024). In the current study, synergism was seen between some antimicrobials and NSAIDs, like gentamicin with flunixin meglumine and ketoprofen, trimethoprim/ sulfamethoxazole with ketoprofen, and oxytetracycline with meloxicam. The synergism of gentamicin with flunixin meglumine is consistent with recent observations (Javed *et al.*, 2024). In the current study, oxytetracycline showed synergism with meloxicam and an indifferent effect with flunixin meglumine, which is consistent with the previous results (Muzammil *et al.*, 2022). The study also reported that NSAIDs significantly lowered the MICs of antibiotics against streptococci, and the therapeutic activity of tested antibiotics increased significantly when combined with NSAIDs, showing synergism (Javed *et al.,* 2024). Similar results were found in an investigation that NSAIDs have a synergistic effect with antibiotics (Chan *et al.*, 2017). To treat a variety of animal diseases, NSAIDs are commonly used in conjunction with antibiotics, including *streptococcal* mastitis. Therefore, the above-mentioned combinations of NSAIDs and antibiotics showing synergism could be applied to counter antimicrobial resistance and uplift antibacterial activity against *Streptococcus* spp. The emerging approaches, such as nanoparticle-based antimicrobials and bacteriophage therapy, may enhance antibacterial efficacy while reducing dependency on conventional antibiotics (Farzana *et al.,* 2022).

This study provides valuable insights into the prevalence of *Streptococcus* spp. associated subclinical mastitis in bovine, risk factors analysis, phylogenetic characterization, and antibacterial sensitivity profiling of study isolates. Furthermore, synergy testing of antibiotics with NSAIDs may act as a potential remedial option against genotypically confirmed isolates of *Streptococcus* spp. Future investigation may focus on a larger epidemiological study area, *in-vivo* trials, and the incorporation of whole-genome sequencing to better understand the molecular prevalence and treatment strategies for *Streptococcus* spp. associated bovine subclinical mastitis.

Conclusions: This study concluded that *Streptococcus* spp. is a notable prevalent cause of bovine subclinical mastitis along with the associated risk factors in the study districts. This study exhibited that resistant antimicrobial's bactericidal properties can be increased by simultaneous use of NSAIDS with these antimicrobials. This study emphasizes that improved management strategies, rational antibiotic use, and non-antibiotic alternatives should be encouraged to address the growing concern of AMR.

Credit authorship contribution statement: Maqbool Hussain Shah: Writing original draft, Muhammad Ijaz: Conceptualization, Supervision, Arslan Ahmed: Formal analysis, Furqan Awan: Reviewing and editing

Acknowledgment: The authors are thankful to the Medicine Research Laboratory (MRL) in the Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, for the provision of chemicals and laboratory goods.

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Declaration of competing interest: The authors declare that there are no conflicts of interest

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