**Available online at www.most.gov.bd**
Volume 06, Issue 01, Pages: 01–12, 2024
DOI: https://doi.org/10.3329/jscitr.v6i1.77358

**Journal of Science and Technology Research**
**Ministry of Science and Technology**
Government of the People's Republic of Bangladesh
Bangladesh Secretariat, Dhaka

# Molecular Characterization of *Streptococcus* spp. Isolated from Milk, Feces and Farm Environment of Mastitic Dairy Cows

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**Abstract**

*Streptococci* are the primary cause of mastitis, significantly impacting the dairy industry economically. This study aimed to explore the molecular epidemiology, antimicrobial resistance, and virulence characteristics of *Streptococcus* spp. isolated from the milk, feces, and environment of dairy cows with mastitis. Sixty (60) samples (milk = 20, feces = 20 and soil = 20) from cows with clinical mastitis (CM) were purposively collected and examined. Samples were enriched in Luria Bertani broth (LB) and *Streptococcus* spp. was isolated on Modified Edwards Medium and confirmed by ribosomal (*16S rRNA*) gene sequencing. Twenty-two (36.67%) of the samples were positive for *Streptococcus* spp. (milk = 40.90%, feces = 31.82% and soil = 27.28%) by cultural and molecular examination. Phylogenetic analysis revealed 59.5, 30.5, 6.0% and 4.0 % of the *Streptococcus* isolates as *S. uberis*, *S. agalactiae*, *S. hyovaginalis* and *S. urinalis*, respectively. The milk samples had higher prevalence (42.75%) of *S. uberis* mastitis than feces (36.80%) and soil (20.45%) samples. Likewise, *S. agalactiae* was prevalent in 51.5%, 37.60% and 10.90% milk, feces and soil samples, respectively. The draft assembly sizes of G2M6 and G6M1 were 1,960,858 and 2,303,841 base pair (bp), respectively. The strains were typed as *S. uberis* sequence type 155 (ST155) and *S. agalactiae* sequence type 58 (ST58). The G2M6 genome contained 367 SEED subsystem features, 45 antimicrobial resistance genes (ARGs), and 160 virulence and virulence-related genes, whereas the G6M1 genome possessed 378 SEED subsystem features, 41 ARGs and 178 virulence genes. One plasmid replicon such as IncY of 4,012 bp, was identified in the G6M1 genome (with 95% identity and 60% coverage) while G2M6 genome harbored none. Genome completeness analysis using BUSCO revealed the presence of 100% complete BUSCOs in the hybrid assembly of both genomes. *Streptococcus* spp. associated with bovine mastitis exhibit a variety of genomic traits and harbor an array of ARGs and virulence genes. Further investigation is needed to identify specific traits that govern virulence fitness in the pathophysiology of mastitis.

*Received: 15.02.2024*
*Revised: 12.06.2024*
*Accepted: 22.06.2024*

**Keywords:** *Streptococcus* spp., Clinical Mastitis, Cow, Antimicrobial Resistance, Virulence, Whole Genome Sequencing.

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## Introduction

Mastitis is a critical issue in the dairy industry, leading to billions of dollars in annual losses globally, including in Bangladesh. This disease is caused by over 350 microbial species, including bacteria, archaea, and viruses (Hoque *et al.*, 2019). Among these, *Streptococcus* spp. is the most frequently isolated genus in dairy herds (Lundberg *et al.*, 2014; Silva *et al.*, 2021). The *Streptococcus* spp. is associated with both clinical and subclinical forms of bovine mastitis, making it a significant pathogen in the dairy industry (Lundberg *et al.*, 2014; Silva *et al.*, 2021). Within the *Streptococcus* genus, *S. uberis* and *S. agalactiae* are the most prevalent species associated with bovine mastitis (Rossi *et al.*, 2018; Silva *et al.*, 2021). *S. uberis* is a significant pathogen in bovine mastitis, causing both clinical and subclinical forms. It leads to visible symptoms like udder swelling and pain, as well as reduced milk production and changes in milk composition, including elevated somatic cell counts, impacting dairy productivity and udder health (Kester *et al.*, 2015; Silva *et al.*, 2021). Conversely, *S. agalactiae*, also known as group B *Streptococcus*, is a highly infectious pathogen that invades the mammary glands of dairy cows through the skin and teats, leading to mastitis (Kabelitz *et al.*, 2021). Mastitis caused by *S. agalactiae* is typically a chronic disease with few acute outbreaks and minimal clinical symptoms. However, it significantly reduces milk yield and has severe economic consequences for dairy farms (Alawneh *et al.*, 2020).

Recent studies have provided evidence that certain strains of *Streptococcus* spp. can be transmitted from cow to cow during milking, highlighting the potential for direct transmission as a route of infection in bovine mastitis (Davies *et al.*, 2016; Tomazi *et al.*, 2019). Moreover, *S. agalactiae* is known to cause serious infections in humans, including infant sepsis, endocarditis, meningitis, and pneumonia. The ability of *S. agalactiae* to cause both bovine mastitis and severe human infections underscores its importance as a pathogen of concern in both veterinary and medical settings (Hoque *et al.*, 2022a; Zheng *et al.*, 2020). In cows, the main route of entry for *Streptococcus* species is via the teat, but infection can also occur via the oral–fecal route and directly or indirectly trigger mastitis (Jørgensen *et al.*, 2016; Ruegg, 2017). *S. uberis* was also associated with persistent intramammary infections, which could be related to its ability to internalize in the mammary gland (Hoque *et al.*, 2022a; Ruegg, 2017), along with its increased resistance to antimicrobials (Cameron *et al.*, 2016; Tomazi *et al.*, 2019).

Currently, antibiotics are the primary treatment of choice for bovine mastitis. However, the increasing problem of antibiotic resistance and the emergence of resistant organisms are rendering antibiotics less effective over time (Hoque *et al*., 2020a; Hoque e*t al.*, 2022a). Furthermore, there is the matter of antibiotic residues, which pose a risk to public health (Al Amin *et al.*, 2020). The rise in drug-resistant strains has resulted in increased antibiotic use, contributing to environmental pollution and posing a threat to human health.

The pathogenicity of *Streptococcus* spp. depends on multiple virulence factors, such as strong adhesion and mechanisms to evade phagocytosis and the immune system (Desai *et al.*, 2017). A variety of surface proteins, endotoxins, and capsular polysaccharides can enhance these virulence factors promote survival and spread of bacteria and seriously compromise the health of both animals and humans (Hoque *et al.*, 2022a; Jørgensen *et al.*, 2016; Kabelitz e*t al.*, 2021). Despite several studies on the *Streptococcus* genus in recent years, its role in the epidemiology of mastitis remains incompletely understood.

The advent of powerful molecular methods, such as whole genome sequencing (WGS), now enables the detection of genetic antimicrobial resistance determinants and virulence factor genes (VFGs), which could shed more light on this pathogen's impact (Vélez *et al.*, 2017). Advancements in understanding the genetic features of *S. uberis* and *S. agalactiae* associated with mastitis outcomes—such as cure rates after antimicrobial treatment, death or culling due to mastitis, mammary quarter loss, and disease recurrence—can aid in developing efficient prevention and control strategies for these pathogens in dairy herds. Whole-genome analysis is the ideal approach to robustly build phylogenies of infectious pathogens, exploring their diverse backgrounds by identifying virulence factors, antimicrobial

resistance genes (ARGs), metabolic pathways, and other genetic variants (Yang *et al.*, 2019). However, genetic determinant analysis of bovine isolates is limited, and relatedness between human and bovine *Streptococcal* isolates remains unexplored. This study aimed to provide basic data on *Streptococcal* spp. causing mastitis in dairy cows in Gazipur, Bangladesh, assessing drug resistance, resistance gene carriage, and virulence gene distribution in isolated strains.

## Materials and Methods

### *Sample Collection, Isolation and Identification of Streptococcus spp.*

Different small-holding dairy farms were selected from the Gazipur district (24.09° N, 90.41° E) of Bangladesh. Cows with clinical mastitis (CM) was diagnosed through California Mastitis Test (CMT®, Original Schalm reagent, ThechniVet, USA) following manufacturer's instruction. A total of sixty samples (20 milk, 20 feces, and 20 soil) from CM cows were purposively collected and examined. Samples were enriched in Luria Bertani broth (LB) and *Streptococcus* spp. was isolated on Modified Edwards Medium (MEM, Himedia, India) and confirmed by *16S rRNA* gene sequencing. In brief, 100 µL enriched culture was evenly spread on the MEM followed by overnight incubation at 37 °C. Colonies with characteristics indicative of *Streptococcus* spp. (blue and/or black coloration) were first screened using Gram's staining. Subsequently, these colonies were purified through successive streaking on MEM to isolate single colonies (Hassan *et al.*, 2023b). DNA was extracted from the isolated colonies using the boiling method. Streptococcus spp. were then confirmed through PCR and *16S rRNA* gene sequencing using 8F (5′-AGAGTTTGATCMTGGC-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) primers (Hoque *et al.*, 2020b; Srinivasan *et al.*, 2015).

### *Antimicrobial Susceptibility Assay*

The antimicrobial susceptibility patterns of the confirmed *Streptococcus* isolates (n=42) were assessed using the disk diffusion method, in accordance with the guidelines provided by the Clinical Laboratory Standards Institute (CLSI) guidelines 2018 (Humphries *et al.*, 2018). This method allowed for the evaluation of the isolates' resistance or sensitivity to various antibiotics, providing crucial data on their antimicrobial profiles. Antibiotics were selected for susceptibility testing corresponding to a panel of antimicrobial agents (CM0337, OxoidTM, Thermo Scientific, UK) commonly used by veterinary practitioners in Bangladesh. The groups of antimicrobials used were- Beta-lactams (ampicillin, 10 µg/mL; oxacillin, 1 µg/mL), Monobactams (aztreonam, 30 µg/mL), Tetracyclines (doxycycline, 30 µg/mL; tetracycline, 30 µg/ML), Nitrofurans (nitrofurantoin, 300 µg/mL), Fluoroquinolones (ciprofloxacin, 10 µg/mL; nalidixic acid, 30 µg/mL), Cephalosporins (cefoxitin, 30 µg/mL), Carbapenems (imipenem, 10 µg/mL), Aminoglycosides (gentamycin, 10 µg/mL; streptomycin, 10 µg/mL), Chloramphenicol (chloramphenicol, 30 µg/mL), Macrolides (azithromycin, 15 µg/mL), and Sulphonamides (compound sulphonamide, 300 µg/mL). Resistance was defined according to CLSI 2018 guidelines (Humphries *et al.*, 2018).

### *Whole Genome Sequencing, Assembly and Annotation*

Genomic DNA was extracted from two multidrug-resistant (MDR) *Streptococcal* isolates: *S. uberis* G2M6 (from milk) and *S. agalactiae* G6M1 (from feces) using the boiling method (Hassan *et al.*, 2023a). Cultures were incubated in nutrient broth (Biolife™, Italy) at 37 ºC for 24 hours (h) before DNA extraction with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA purity and concentration were assessed using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher, Waltham, MA, USA). Libraries were prepared from 1 ng of DNA using the Nextera™ DNA Flex Library Prep Kit (Illumina, San Diego, USA) and sequenced on an Illumina MiSeq sequencer (2 × 250-bp protocol). Trimmomatic v0.39 (Bolger *et al.*, 2014) was used for read processing with parameters optimized for quality, confirmed by FastQC v0.11.7 (Andrews, 2010). SPAdes v3.15.5 (Bankevich *et al.*, 2012) facilitated de novo assembly of clean reads, and assembly quality was assessed using QUAST v5.0.2 (Gurevich *et al.*, 2013) and BUSCO v4.1.2 (Seppey *et al.*, 2019). The National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Annotation Pipeline (PGAP) annotated the genomes, while PlasmidFinder (Carattoli *et al.*, 2014), CRISPRimmunity (http://www.microbiome-bigdata. com/ CRISPRimmunity/index/home), and PHASTER (http://phaster.ca/) server were used to predict plasmid replicons, CRISPR arrays, and

phage-associated genes, respectively, providing insights into the genetic makeup and potential traits of these bacteria.

*Sequence Typing, Phylogenetic Analysis and Genomic Comparison*

BacWGSTdb 2.0 was used to carry out *in silico* multilocus sequence typing (MLST) analysis and bacterial source tracing using a core genome MLST (cgMLST) analysis (Feng *et al.*, 2021). Based on the cgMLST results, the study genomes (*S. uberis* strain G2M6 and *S. agalactiae* strain G6M1) and 20 reference genomes of *Streptococcus* spp. were used in phylogenetic analysis. Genomes were aligned with MUSCLE v5.0 (https://github.com/rcedgar/muscle) (Edgar, 2021), and a phylogenetic tree was created using PhyML v3.0 (Guindon *et al.*, 2010), and finally visualized through iTOL (v3.5.4) (http://itol.embl.de/) (Letunic and Bork, 2021).

*Genomic Functional Potentials Analysis*

The ResFinder 4.0 (Zankari *et al.*, 2012) database was used to predict ARGs in the assembled genomes (>95% identity). The VFGs in G2M6 and G6M1genomes (with 90% nucleotide identity and query coverage) were identified using VFDB v6.0 (Chen *et al.*, 2016) database. The draft genomes were also annotated using the RAST (Rapid Annotation using Subsystem Technology) server, v2.0 (Aziz *et al.*, 2008), to identify metabolic function related genes/pathways under different subsystem categories.

*Statistical Analyses*

Data were entered into Microsoft Excel 2020® (Microsoft Corporation, Redmond, WA, USA) and analyzed using Excel and SPSS version 20 (IBM Corp., Armonk, NY, USA). The Pearson's chi-square test was performed to compare the prevalence of *Streptococcus* spp. in three different sample categories (*e.g.*, milk, feces and soil). The AMR patterns, resistance, intermediate and sensitivity were calculated through the CLSI guideline 2018 using the cut-off as provided in the brochure of the manufacturer (Liofilchem®, Italy). For the test, $P<0.05$ was considered statistically significant.

## Results and Discussion

*Association of Streptococcus spp. in Bovine Clinical Mastitis*

In this study, 22 samples (36.67%), comprising 9 (15.0%) milk samples, 7 (11.67%) feces samples, and 6 (10.0%) soil samples, tested positive for *Streptococcus* spp. through cultural and molecular examination. From these samples (n=22), 42 isolates of *Streptococcus* spp. were screened through selective culture. Ribosomal gene (*16S rRNA*) sequencing and phylogenetic analysis identified 59.52%, 30.95%, 7.14% and 2.38% of the *Streptococcus* isolates as *S. uberis*, *S. agalactiae*, *S. hyovaginalis* and *S. urinalis*, respectively (Table 1). The milk samples had the higher prevalence (42.75%) of *S. uberis* mastitis than feces (36.80%) and soil (20.45%) samples. Likewise, *S. agalactiae* was prevalent in 51.5%, 37.60% and 10.90% milk, feces and soil samples, respectively. *Streptococcus* is one of the bacterial genera that can cause mastitis in dairy cows (Hassan *et al.*, 2023b). *Streptococcus* species frequently linked to mastitis encompass *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* (Calvinho *et al.*, 1998). Our observations align with numerous prior studies that have documented the presence of these pathogens in bovine CM (Hassan *et al.*, 2023b; Zadoks *et al.*, 2001). Recently, Hassan *et al.* (2023a) identified and reported four different species of *Streptococcus*, including *S. agalactiae*, *S. uberis*, *S. hyovaginalis* and *S. urinalis* from bovine CM milk (Hassan *et al.*, 2023b) supporting our present findings. Thus, identification of these species in milk and feces samples collected from bovine clinical mastitis cases implies a potential association with the pathogenesis of mastitis.

**Table 1.** Prevalence of *Streptococcus* spp. through ribosomal gene (*16S rRNA*) sequencing

| ***Streptococcus* spp.** | **Positive isolates (n=42)** | **Prevalence (%)** |
|---|---|---|
| *S. uberis* | 25 | 59.52 |
| *S. agalactiae* | 13 | 30.95 |
| *S. hyovaginalis* | 3 | 7.14 |
| *S. urinalis* | 1 | 2.38 |

The *in vitro* antibiogram profiling of 42 isolates was attempted for 15 commonly used antibiotics from 12 different groups. Among these, 90.48% (38 out of 42) isolates exhibited multidrug resistance (MDR), with resistance to more than three antibiotics in disk diffusion tests. The primary resistance was observed against beta-lactams (ampicillin, oxacillin), aminoglycosides (gentamicin, streptomycin), tetracycline, macrolides (azithromycin), nitrofurans (nitrofurantoin), and fluoroquinolones (nalidixic acid). It was revealed that *Streptococcal* isolates

displayed 85 to 95% resistance against oxacillin, aztreonam, sulfonamide, cefoxitin and nalidixic acid followed by 50 to 80% resistance against gentamicin, streptomycin, tetracycline and azithromycin (Figure 1). However, the isolates showed lower resistance (<20%) against chloramphenicol and nitrofurantoin. Furthermore, these isolates were highly susceptible to nitrofurantoin, ciprofloxacin, doxycycline, and imipenem (80 to 100 %) However, the susceptibility of *Streptococcal* isolates to nalidixic acid, streptomycin, oxacillin, cefoxitin, aztreonam and sulfonamide was significantly lower, indicating that these antibiotics are less effective against these bacteria (Figure 1).

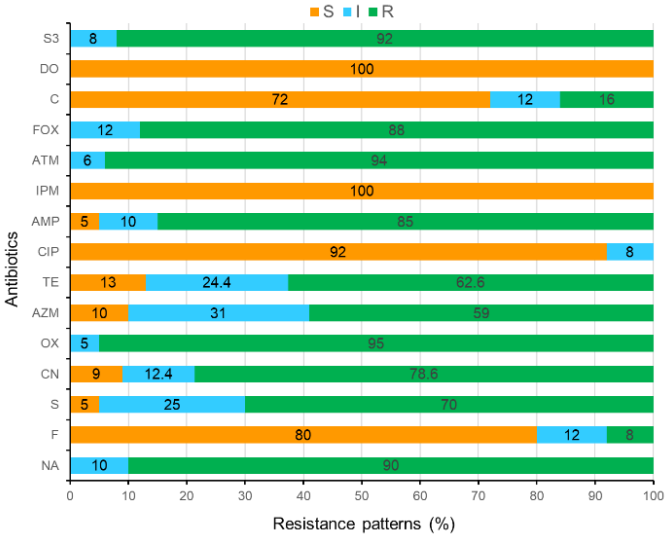


**Figure 1.** Overall antibiotic resistance patterns of *Streptococcal* isolates (n=42).

Legends: AMP = Ampicillin, ATM = Aztreonam, AZM = Azithromycin, FOX = Cefoxitin, C = Chloramphenicol, CIP = Ciprofloxacin, DO = Doxycycline, CN = Gentamicin, IPM = Imipenem, NA = Nalidixic acid, F = Nitrofurantoin, OX = Oxacillin, S = Streptomycin, S3 = Sulfonamide, TE = Tetracycline. Here S, I and R denote susceptible, intermediate and resistant.

The antibiogram profiling showed that 96.0% *S. uberis* and 88.75% *S. agalactiae* isolates were MDR isolates. From these MDR isolates, one *S. uberis* strain (*S. uberis* strain G2M6, isolated from milk) and *S. agalactiae* strain (*S. agalactiae* strain G6M1, isolated from feces) were selected for WGS. Antimicrobial resistance (AMR) poses a significant challenge, complicating the control of mastitis through systemic or intramammary therapies (Hoque *et al.*, 2018; Hoque *et al.*, 2020b). Furthermore, the widespread use of antimicrobials in mastitis control has led to the presence of antimicrobial residues in milk, potentially entering the human body through the food chain. Species specific AMR revealed that more than *S. uberis* and *S. agalactiae* isolates were MDR isolates. These findings of high MDR patterns in murine mastitis associated *Streptococcus* spp. are in line with many of previous studies on bovine and bubaline mastitis (Hassan *et al.*, 2023b; Martins *et al.*, 2021).

*Genome Characteristics of the Streptococcus Strains*

Genomic characteristics of both strains are shown in Table 2. The draft assembly sizes of G2M6 and G6M1 were 1,960,858 base pair (bp) and 2,303,841 bp, respectively (Figure 2). These two isolates were typed as *S. uberis* sequence type 155 (ST155) and *S. agalactiae* sequence type 58 (ST58) according to seven-gene MLST (*adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A and *rec*A) on cgMLST scheme. Phylogenetic analysis revealed that both *S. uberis* strain G2M6 and *S. agalactiae* strain G6M1 were evolutionarily diverse (Figure 3). The RAST FIGfams v.70 annotations revealed that the G2M6 genome contained 367 metabolic features in SEED subsystems with 32% coverage, 4,343 protein coding sequences (CDS) and 68 RNA geneswhile 378 SEED subsystem features with 32% coverage, 4,607 CDS and 76 RNA genes were predicted in the G6M1 genome. Seventy-eight (G2M6=45; G6M1= 41) ARGs conferring resistance to multiple antibiotics and metals, and 276 virulence factors (G2M6=160; G6M1=178) related genes were detected in the draft genomes. One plasmid replicon such as IncY of 4,012 bp, was identified in the G6M1 genome (with 95% identity and 60% coverage) while G2M6 genome harbored none (Table 2). Genome completeness analysis with BUSCO showed the presence of 100% complete BUSCOs in the hybrid assembly of both genomes. The introduction of WGS for bacterial pathogens has established a novel avenue for exploring their molecular epidemiology and assessing their potential for virulence (Coll *et al.*, 2020; Hassan *et al.*, 2023a).

**Table 2.** General genomic features of the *Streptococcal* strains isolated from in this study

| Features(s) | *Streptococcus* strains | |
|---|---|---|
| | ***S. uberis* G2M6** | ***S. agalactiae* G6M1** |
| Genome size (bp) | 1,960,858 | 2,303,841 |
| Genome coverage (x) | 60 | 65.5 |
| GC content (%) | 50.8 | 50.9 |
| Total contigs | 41 | 57 |
| Largest contig (bp) | 404,720 | 204,508 |
| Shortest contig (bp) | 6,753 | 3072 |
| Contig $N_{50}$ (bp) | 340,886 | 184,919 |
| $L_{50}$ | 3 | 19 |
| Total genes | 4,307 | 4,535 |
| CDS | 4,343 | 4,451 |
| Protein coding genes | 4,111 | 4,313 |
| RNA genes | 76 | 84 |
| tRNA genes | 67 | 74 |
| rRNAs | 1 | 1 |
| ncRNAs | 8 | 9 |
| Pseudo genes | 120 | 138 |
| Genes assigned to SEED subsystems | 1,901 | 2,020 |
| Number of subsystems | 367 | 378 |
| CRISPR arrays | 2 | 2 |
| No. of plasmids (% identity) | 0 | 1 (99.08) |
| No. of prophages | 6 | 9 |
| Sequence type (ST) | ST155 | ST58 |
| No. of ARG | 59 | 77 |
| No. of VFG | 162 | 181 |

Here, CDS = coding sequence, ARG = antimicrobial resistance gene, VFG = virulence factor gene.

The draft genomes analyzed in this study exhibited high quality genome features for analysis, with 41 to 57 contigs and $N_{50}$ values ranging from 140 to 184 kb for contigs larger than 1000 bp. While both genomes contained multiple prophage regions with more than 75 gene features, only the G6M1 genome harbored a plasmid replicon, specifically the IncY plasmid, which is commonly associated with beta-lactam resistance in *Streptococcus* (Arcilla *et al.*, 2016). Our investigation of the genomes also revealed the presence of three CRISPR arrays in each genome harboring 12 signature genes. CRISPR arrays, which have been identified in many bacterial pathogens (including *Streptococcus* spp.) causing mastitis, play a significant role in host adaptive immune response and virulence (Alawneh *et al.*, 2020). Our results from core-genome typing (ST155 and ST58) align with various prior studies that have noted the correlation of ST155 and ST58 *Streptococcus* strains with instances of bovine mastitis (Käppeli *et al.*, 2019; Yang *et al.*, 2013).

The phylogenetic positioning of the *Streptococcal* isolates responsible for mastitis corresponds with the core genome phylogeny. The examination revealed distinct clustering of G2M6 and G6M1, indicating a closer relationship to mastitis-causing *Streptococcal* strains identified in Germany (Moawad *et al.*, 2023) and France (Käppeli *et al.*, 2019). We further anticipated several significant genes and proteins in the G2M6 and G6M1 genomes linked to diverse subsystem categories and metabolic functions, reinforcing their potential involvement in mastitis pathogenesis (Hassan *et al.*, 2023a; Ievy *et al.*, 2022; Saha *et al.*, 2021). Prior studies have suggested that bacterial metabolites contribute to the modulation of host immune functions and the pathophysiology of diseases (Hoque *et al.*, 2020c; Hoque *et al.*, 2022b; Zeng *et al.*, 2017). Furthermore, we detected various ARGs and clusters of VFGs in the genome sequences of G2M6 and G6M1. These identified ARGs and virulence factors or genes may potentially play a role in the pathogenesis of mastitis (Hassan *et al.*, 2023b; Hoque *et al.*, 2020b; Käppeli *et al.*, 2019). Therefore, further investigation should be conducted to explore the ARGs and virulence genes produced by *Streptococcus* spp., and their pathogenic properties in mastitis.

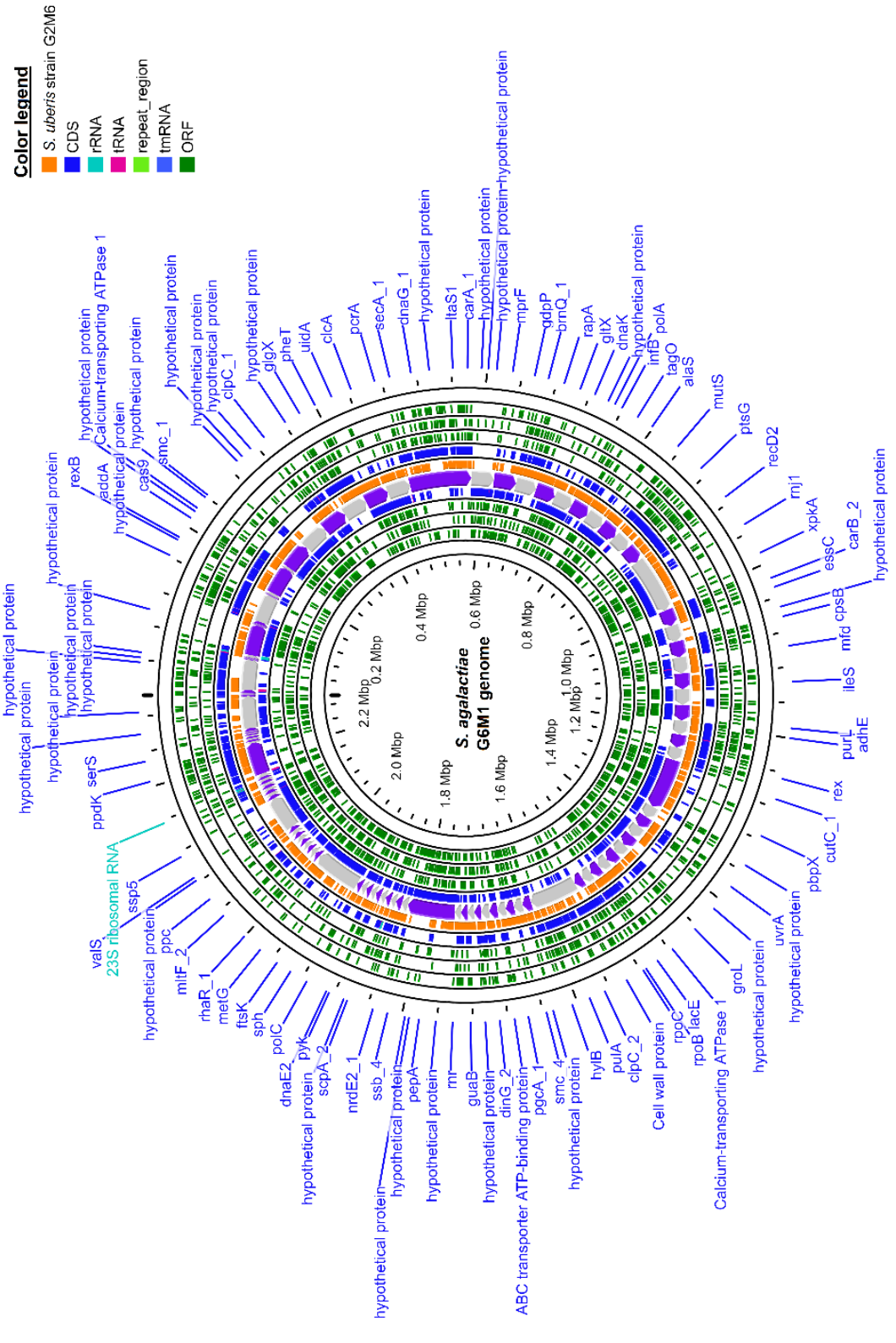


**Figure 2.** Circular representation of genome using CGView Server (http://cgview.ca). Circular genome representation of *S. agalactiae* strain G6M1 compared with *S. uberis* strain G2M6. The six innermost layers in the graphic portray the genome coordinates (mega base pairs—Mbp, purple), open reading frames (ORF, dark green), coding sequences (CDS; blue), backbone of both genomes (purple color). The other colored rings, from the outermost to innermost, depict the nucleotide BLAST alignment of *S. uberis* strain G2M6 (orange) followed by CDS (blue) representing the positions of both genomes. Image created using CGview Server

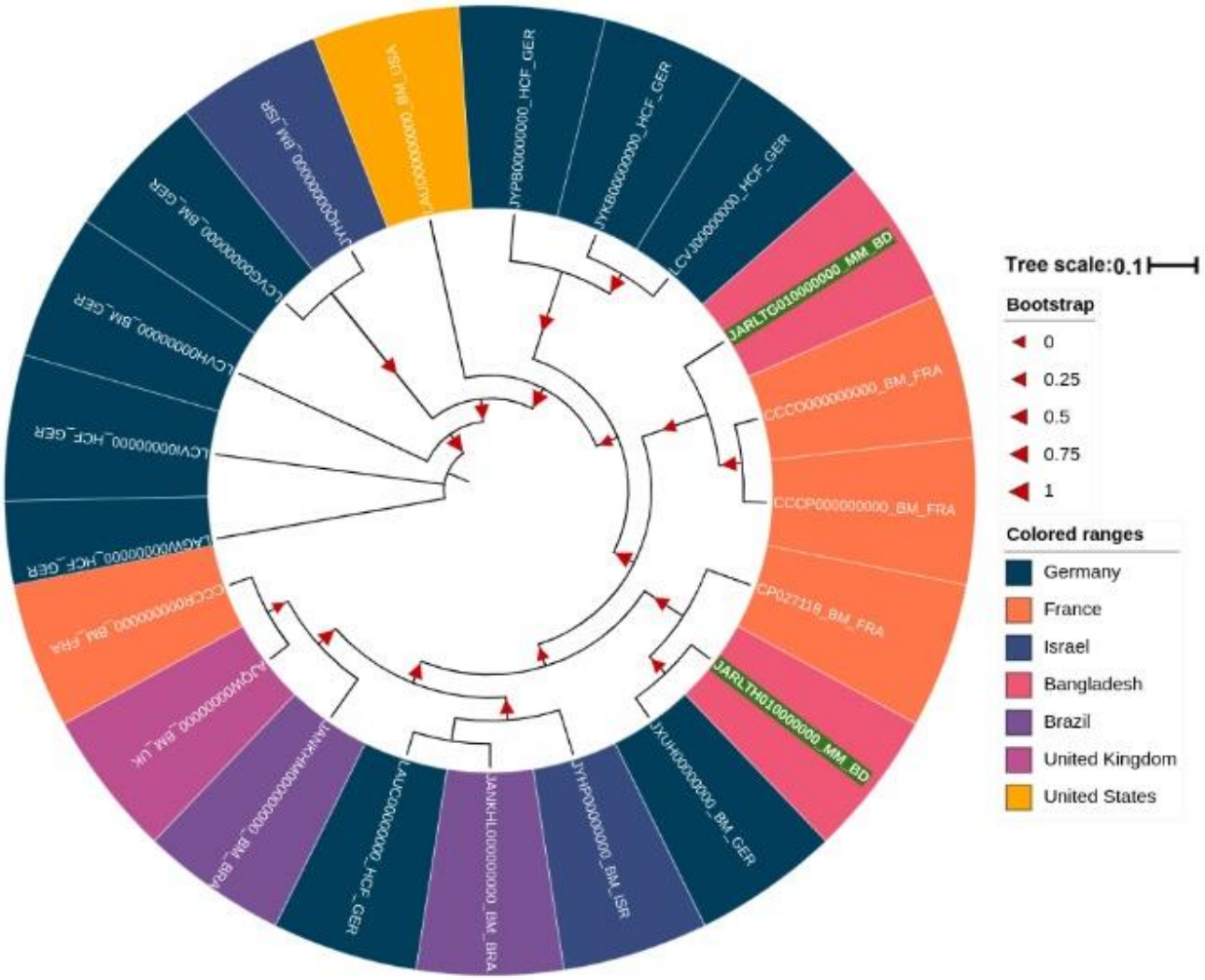


**Figure 3.** The evolutionary relationships of *Streptococcal* genomes sequenced from 7 countries of the world. Whole genome sequences of 21 strains of human and animal origin retrieved from NCBI were used for phylogenetic analysis. The midpoint rooted tree was constructed using the NCBI Tree Viewer (https://www.ncbi.nlm.nih.gov/tools/treeviewer/), and visualized with iTOL (interactive tree of life). The evolutionary relationship was inferred using the maximum-likelihood method. Different colors (*e.g.*, dark blue for Germany, orange for France, pink for Bangladesh, light navy for Israel, purple for Brazil, violet for United Kingdom and yellow for United States) are assigned according to the close evolutionary relatedness (clade) of the genomes. The scale bar is in the unit of the number of substitutions per site. The values on the branches are bootstrap support values based on 1000 replications. All the sequences were indicated by their accession numbers followed by the host and country code. The country codes according to the standard abbreviation are: United States of America (USA), United Kingdom (UK), Germany (GER), Brazil (BRA), Israel (ISR), France (FR) and Bangladesh (BD). The genome of the *S. uberis* strain G2M6 (JARLTH000000000_MM_BD) and *S. agalactiae* strain G6M1 (JARLTG000000000_MM_BD) are highlighted in green color.

## Conclusions

Examining these genomic features alongside functional genomic validation in prevalent mastitis pathogens like *Streptococcus* spp. might enhance our comprehension of molecular pathogenesis. The genomic data from *S. uberis* strain G2M6 and *S. agalactiae* strain G6M1, generated in this study, uncovered two novel STs and potential virulence characteristics. This information might be significantly contributed to the creation of new tools for virulence profiling, ST typing, and the development of vaccines to control mastitis caused by emerging pathogens. Phylogenetic and comparative genome analysis revealed genetic similarities between these two strains are related to bovine mastitis-causing *Streptococcal* strains of diverse geographical regions. Furthermore, the generated sequence data could be utilized to explore the genomic background influencing the evolution of this pathogen and its enhanced environmental fitness over time.

## Acknowledgements

This research was done by the financial support (SRG-221177, 2022–2023) from the Ministry of Science and Technology, Government of the People's Republic of Bangladesh.

## Declaration

The authors declare that the research findings reported in this article do not have any conflicting interest.

## Authors' Contributions

MNH conceptualized, resourced, wrote, and reviewed the manuscript as well as was responsible for funding acquisition, visualization, and supervision; MR, TS, MMR, and NS performed the methodology, investigation, and formal analyses and wrote the original draft; MSI, MMH, ANMAR, and ZCD were responsible for resourcing, writing, reviwing, and editing the manuscript as well as being responsible for project administration.

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