Egypt. J. Vet. Sci., pp. 1-15

Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Genetic Diversity, Antimicrobial Resistance, and Biofilm Formation in *S. agalactiae* and *S. dysgalactiae* Strains Isolated From Milk, Milk Products, and Humans in Sharkia Province, Egypt

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Abstract

STREPTOCOCCUS spp infections pose a significant public health threat worldwide, contributing to a substantial economic burden. These infections are prevalent among both humans and animals, with potential for zoonotic transmission. Understanding the antimicrobial resistance patterns and genetic diversity of Streptococcus strains is crucial for developing effective prevention and control strategies. A total of 31 Streptococcus isolates isolated from milk (12 isolates), milk products (4 isolates), and humans (15 isolates) in Sharkia Province, Egypt were included in this study, with most exhibiting phenotypic resistance to multiple antibiotics. Antimicrobial resistance was widespread, with 96.8% resistance observed for ampicillin, clindamycin, linezolid, ceftriaxone, meropenem, and tetracycline. No significant differences in resistance profiles were found between *S. agalactiae* (18 isolates) *and S. dysgalactiae* (9 isolates). The isolates displayed 13 distinct resistance profiles, with most classified as extensively drug-resistant (80.6%) or pan drug-resistant (9.7%). The *tetO* gene was most prevalent in isolates from cheese (100%), followed by those from milk (80%) and human (76.9%). Additionally, 81.5%, 66.7%, and 70.4% of isolates were positive for *tetO, ermB,* and *Pbp1A*, respectively. Of the isolates, only one (3.2%) was a weak biofilm producer. Phylogenetic analysis revealed a high degree of genetic similarity among the Streptococcus strains, suggesting potential zoonotic transmission. In conclusion, the high prevalence of antimicrobial resistance and the genetic diversity of *Streptococcus* spp in Sharkia Province highlight the urgent need for effective strategies to combat these infections and prevent their further spread.

Keywords: Antimicrobial resistance, Biofilm formation, *Streptococcus spp*., Zoonotic transmission.

Introduction

The economic losses caused by dairy cow mastitis are substantial because of the disease's effects on milk quality, the number of cows that have to be removed from production and the increasing expenses of treatment and feed [1]. Streptococcus bacteria, which are Gram-positive and generally form chains, are a common etiological factor. In humans and other animals, these bacteria can play various roles, including commensals, pathogens, and opportunistic pathogens. There are a number of ecological niches occupied by Streptococcus species that are important in veterinary medicine.

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(Received 02 November 2024, accepted 06 January 2025)
DOI: 10.21608/EJVS.2025.333162.2468

Notably, subclinical and clinical mastitis can be caused by *Streptococcus agalactiae (S. agalactiae), group B Streptococcus (GBS), Streptococcus dysgalactiae (S. dysgalactiae),* and *Streptococcus uberis (S. uberis)* [2].

Dairy farmers suffer from huge financial losses due to the spread of *S. agalactiae*, a highly infectious bacterium that affects dairy cows and causes subclinical and mild to moderate clinical mastitis [3]. In humans, GBS has been associated with conditions that impact both pregnant mothers and newborns. Neonatal gastrointestinal stomatitis virus (GBS) can manifest in two ways: early-onset and late-onset. The former includes bacteraemia, pneumonia, and meningitis, and it is transmitted vertically from contaminated pregnant women [4]. Emerging as a leading cause of streptococcal mastitis or endometritis in domestic animals and skin lesions, meningitis, and bacteraemia in humans, *S. dysgalactiae* is a key player in the mammalian infection landscape [5]. Even though this microbe doesn't perform well in nature, it can live on in the mammary gland permanently and spread to healthy cows through unclean milking practices.

When it comes to phenotypic variability and the identification of bovine Streptococcus species, molecular techniques provide more dependable ways. The *sodA* gene [6], *CAMP* factor gene [7], *Trna* intergenic region [8], *tuf* gene [9], *16S rRNA* gene [7], the intergenic spacer region (IGS) between the *16S* and *23S rRNA* genes [10], and the *23S rRNA* gene [11] are some of the genes that have been amplified in PCR assays designed to detect bovine streptococci.

Reducing the probability of tragic consequences in invasive bacterial infections requires effective treatment with antimicrobial medicines [12]. About sixty to seventy percent of antimicrobial drugs used on dairy farms are devoted solely to mastitis. A major public health concern on a global scale, multidrug-resistant bacteria have emerged as a result of the evolution of germs that are resistant to antimicrobials, which is accelerated by selection pressure and the excessive use of these agents in animal production [13]. Past research has proven that Streptococcus species are resistant to various common antibiotics, including Kanamycin, β-lactams, erythromycin, streptomycin, tetracyclines, and mefE [1].

Biofilm refers to matrix-enclosed microbial aggregates that adhere to both organic and inorganic surfaces. The formation of biofilm is a critical factor in the pathogenesis of several diseases in animals, as it allows bacteria to survive in hostile environments. Moreover, once inside a host, bacteria embedded in biofilm can better evade the host's immune system and become less susceptible to the effects of antibiotics and disinfectants [14, 15].

Research on mastitis to identify its aetiology and assess antimicrobial susceptibility is essential for developing treatment, prevention, and control strategies, as well as mitigating economic losses associated with milk, milk products, and culling. Therefore, the objective of this study was to investigate the genetic relatedness, antibiotic susceptibility patterns, antibiotic resistance genes, and biofilm formation of *S. agalactiae* and *S. dysgalactiae* strains isolated from milk, cheese and humans in Sharkia Province, Egypt.

Material and Methods

Ethical approval

The current study was reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (approval number ZU-IACUC/2/428/2023).

Bacterial isolates

A total of 31 non-duplicated *Streptococcus* spp isolates (20 *Streptococcus agalactiae and* 11 *Streptococcus dysgalactiae*) were included in the present study. These strains were previously isolated from raw milk, cheese and human samples collected from Sharkia Province, Egypt. Milk strains (7 *S. agalactiae and* 5 *S. dysgalactiae*) were recovered from 160 raw milk samples previously collected from retail outlets as well as apparently healthy and mastitic cattle in 4 dairy cattle farms in Sharkia Province. Meanwhile, *Streptococcus* strains from cheese (1 *S. agalactiae and* 3 *S. dysgalactiae*) were recovered from 75 cheese samples collected from farmers' houses in Sharkia province. In addition, human strains (12 *S. agalactiae and* 3 *S. dysgalactiae*) were isolated from 40 hand swabs (collected from dairy farmers), 20 vaginal swabs (collected from pregnant women referred to different laboratories either apparently healthy or suffered from vaginitis) and 40 pharyngeal swabs (collected from children attending private clinics and Zagazig University Paediatric outpatient clinics) in Sharkia Province. The tested strains were previously isolated by culturing on Edward's medium (Biolife, Turkey) and the biochemical identification was

carried out by standard biochemical tests [16]. Serotyping of the isolates was carried out by latex agglutination test using Streptococcal Grouping Test Kit DR0585 (Thermo Scientific Oxoid, Basingstoke, Hampshire, England) as described by the manufacturer`s instructions. *S. agalactiae and S. dysgalactiae* isolates were molecularly confirmed to genus level targeting *tuf* gene as well as to species level targeting *16S rRNA* gene by polymerase chain reaction (PCR) using primer sets provided in Table1.

Sequencing and phylogenetic analysis of S. agalactiaeand S. dysgalactiae16S rRNA gene.

A partial sequencing of the 16S rRNA gene was used to establish the genetic relatedness of Streptococcus strains. This was done for seven representative Streptococcus strains, four of which were *S. agalactiae* strains and three of which were *S. dysgalactiae* strains, which were isolated from animal and human sources. In a nutshell, the amplified PCR products of the 16S rRNA genes of *S. agalactiae* and *S. dysgalactiae* were purified with the help of the QIAquick PCR Product extraction kit (Qiagen, Valencia, California), and then they were sequenced in either the forward or reverse direction with primers that were provided in table 1. A ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, California) was utilized in order to carry out the sequence reaction on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). In the beginning, a BLAST® analysis, which stands for Basic Local Alignment Search Tool, was carried out in order to determine the identity of the sequences to the GenBank accessions. Table 2 contains the results of seven sequences that were evaluated and then uploaded to GenBank with the accession numbers PQ001960-PQ001966. A comparative analysis of sequences was carried out according to the Clustal W technique, with the MegAlign module of the LasergeneDNAStar program Pairwise version 12.1 (Madison, Wisconsin, United States of America) being utilized. Maximum likelihood, neighbor-joining, and maximum parsimony were the three methods that were utilized in the MEGA6 program to create the phylogenetic tree[17].

Antibiotic susceptibility test

All *S. agalactiae* and *S. dysgalactiae*isolates were tested for their susceptibility to a panel of 12 different antibiotics using the disc diffusion method [198]. The tested antimicrobials were penicillin (P, 10 µg), ampicillin (AMP, 10 µg), ceftriaxone (CRO, 30 µg), cefepime (FEB, 75 µg), meropenem (MEM, 10 µg), vancomycin (VA, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), azithromycin (AZM, 15 µg), clindamycin (DA, 2 µg), levofloxacin (LEV, 5 µg) and linezolid (LNZ, 30 µg). Briefly, the bacterial suspension was adjusted to the turbidity of 0.5 McFarland standards (1.5×10^8 CFU) and then streaked on Mueller Hinton Agar plates (Himedia, India). The antibiotic discs were placed on the surface of the Muller Hinton agar plates and the plates were inverted and incubated at 37 °C for 18–24 hours. The inhibition zones were measured for each antibiotic and the zone diameter breakpoints were interpreted according to Clinical and Laboratory Standards Institute [19]. The multidrug-resistant (MDR), extensive drug-resistant (XDR) and pan drug- resistant (PDR) streptococci isolates were categorized according to Magiorakos et al. [20]. The multiple antibiotic resistances (MAR) index was calculated for every isolate by dividing the number of antibiotics to which the isolate exhibited resistance by the total number of antibiotics examined. MAR score greater than 0.2 suggests that this antibiotic was widely used in the isolate's original environment [21].

Phenotypic detection of biofilms in S. agalactiae and S. dysgalactiae isolates.

All *S. agalactiae and S. dysgalactiae* isolates were tested for biofilm formation using microtitre plate assay according to the methods outlined by Ebrahimi et al. [22]. All of the isolates were grown in tryptic soy broth (TSB, Oxoid, Great Britain) for sixteen hours at a temperature of 37 degrees Celsius. After that, the bacterial cells were centrifuged for ten minutes at a force of twenty thousand grams. An absorbance of 1.00 at 595 nm was achieved by removing the supernatants and resuspending the cell pellets in 5 mL of TSB. This was done in order to get the desired result. The BioSpectrometer® (Eppendorf, Hamburg, Germany) was utilized in order to determine the optical densities (ODs) of the bacterial suspensions. The bacterial suspensions were diluted in fresh TSB at a ratio of 1:40. The diluted cell suspension was then applied to each well in a polystyrene microtitre plate with flat bottoms, with eight wells in a row for each strain. The negative control well was only inoculated with sterile PBS, and the plate was then incubated at 37 degrees Celsius for a full 24 hours. After being rinsed three times with sterile phosphate buffered saline (PBS), the plates were allowed in an inverted position for one hour to dry at room temperature to

ensure that they were completely dry. In order to eliminate any excess dye, the plates were washed with sterile PBS three times. After that, the plates were stained with 200 µL of aqueous crystal violet solution (0.2%) for duration of five minutes. For the purpose of removing the crystal violet that was adhered to the biofilms, an ethyl alcohol and acetone mixture with a volume-to-volume ratio of 80:20 was utilized. Additionally, an ELISA plate reader (Sunrise absorbance reader, Tecan, Austria) was utilized in order to take the optical density (OD) reading at 595 nm. There were three separate examples of the experiment. The interpretation of the biofilm production was carried out by utilizing the standards that were discussed by Stepanović et al. [23]. For the purpose of determining the cut-off value (ODc), the mean optical density (OD) of the negative control was combined with three standard deviations of the negative control. Each of the isolates was classified into the following categories: non-biofilm producers, which had an optical density (OD) that was less than or equal to the optical density (ODc), weak biofilm producers, which had an ODc that was less than or equal to 2 × ODc, moderate biofilm producers, which had an ODc that was between 2 × ODc and 4 × ODc, and strong biofilm producers, which had an ODc that was greater than or equal to 4 × ODc.

Molecular surveillance of antibiotic resistance genes in S. agalactiae and S. dysgalactiae isolates from different sources.

Twenty-seven *S. agalactiae* and *S. dysgalactiae* that displayed phenotypic resistance to multiple antibiotics (tetracycline, clindamycin, penicillin, and ampicillin) were tested for the presence of the genes encoding the resistance to tetracycline (*tetO*), clindamycin (*ermB*), penicillin and ampicillin (*Pbp1A*) using PCR. The DNA was extracted from overnight broth cultures of the tested isolates by QIAamp DNA mini kit (Qiagen) with modifications from the manufacturer's recommendations. A uniplex PCR assay was conducted for each target gene using oligonucleotide primers supplied by Biobasic, Canada (Table 1). The PCR reaction volume (25 µl) consisted of a mixture of 12.5 µl of 2x premix Emerald Amp GT PCR mastermix (Takara, Japan), 1 µl of each primer (20 pmol), 6 µl of DNA template and 4.5 µl of PCR grade water. The amplification was conducted in a T3 thermal cycler (Biometra) and the cycling conditions were provided in table 1. The amplified PCR products, 100 bp DNA ladder (Qiagen, USA), positive, and negative controls were loaded to 1.5% agarose gel stained with ethidium bromide and run for 30 min at 1-5 volts / cm. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software.

Statistical analysis

The data was edited using Microsoft Excel version 16 (Microsoft Corporation, Redmond, WA, USA). A Chi- square test (PROC Freq; SAS Institute Inc., Madison, WI, USA, 2012) was performed to investigate the significant differences in the percentages of antibiotic resistance and antibiotic resistance genes. P values < 0.05 were considered significant. Figures were fitted by Graph pad Prism 9 software.

Results

The genetic relatedness between S. agalactiae and S. dysgalactiae isolates.

The phylogenetic tree demonstrated that *16S rRNA* gene sequences of *S. agalactiae* [GenBank accession no. PQ001961, PQ001963, PQ001964 and PQ001966]and *S. dysgalactiae* [GenBank accession no. PQ001960, PQ001962 and PQ001965]strains isolated from raw milk (dairy farms and retail outlets) and human (dairy farmers' hand swabs, pregnant women's vaginal swabs and children's pharyngeal swabs) samples in this study were closely related (98.2%-100% nucleotide identity) and clustered in the same clade (Figure 1). *S. agalactiae* isolates in this study were clustered in the same lineage with other *S. agalactiae* isolated from different countries in the GenBank with 100% genetic similarity [GenBank accession no. KF111279, JQ771298, KF111277 and PP530142, CP021862, CP021866, CP025028 and MK517599].Likewise, *S. dysgalactiae* strains in this study shared 100% identity and clustered in the same lineage with other *S. dysgalactiae* strains isolated from food, animals and humans in the GenBank [GenBank accession no. CP078737, AB002489, AB002510 and CP033391].

Antibiotic susceptibility testing

The result revealed that *Streptococcus* isolates in this study exhibited a high resistance to ampicillin, clindamycin, linezolid, ceftriaxone, meropenem and tetracycline (96.8%, each), followed by penicillin and cefepime (93.5%, each), chloramphenicol (80.6%), azithromycin and vancomycin (77.4%, each) and levofloxacin (45.2%) (Table 3).

Streptococcus isolates of animal origin (milk, cheese) showed a high percentage of resistance to ceftriaxone, meropenem and tetracycline (100%, each), penicillin, ampicillin, clindamycin, linezolid and cefepime (93.8%, each), chloramphenicol (81.3%), vancomycin (75%),

azithromycin (68.8%) compared with levofloxacin (37.5%). On the other hand, human *Streptococcus* isolates exhibited a high resistance to ampicillin, clindamycin and linezolid (100% each), followed by penicillin, cefepime, ceftriaxone, meropenem and tetracycline (93.3%, each), azithromycin (86.7%), vancomycin, chloramphenicol (80%, each) and levofloxacin (53.3%). There were no significant differences (p=0.8929) observed in the antibiotic resistance profiles of *S. agalactiae* and *S. dysgalactiae* isolates from both human and animal sources (Figure 2).

Isolates of Streptococcus were found to exhibit thirteen different resistance profiles, as shown in Table 4. Out of the total number of isolates, three were classified as multidrug-resistant (MDR) (resistant to at least one agent in five to six antimicrobial classes), 25 were classified as XDR (resistant to at least one agent in eight to ten antimicrobial classes), and three were classified as polydrug-resistant (PDR) (resistant to all antibiotics in all antimicrobial classes). The average value of the MAR index was 0.874, and it varied from 0.5 to 1.

Phenotypic characterization of biofilm formation in Streptococcus isolates.

The results of microtitre plate assay clarified that only one *Streptococcus* isolate (3.2%) was weak biofilm producer and the other isolates (n=30, 96.8%) were non-biofilm producers (Table 4). The biofilm producer *S. agalactiae* isolate was isolated from milk and categorized as XDR.

Molecular characterization of antibiotic resistance genes

Twenty-seven *Streptococcus* isolates (18 *S. agalactiae* and 9 *S. dysgalactiae*) that displayed phenotypic resistance to tetracycline, clindamycin, penicillin and ampicillin were screened for the presence of *tetO*, *ermB* and *Pbp1A*. The results clarified that 22 (81.5%), 18 (66.7%) and 19 (70.4%) of the total examined *Streptococcus* isolates were positive for *tetO*, *ermB* and *Pbp1A* genes, respectively (Table 5). The *tetO* gene was predominant in *Streptococcus* isolates from cheese (100%), followed by milk (80%) and human isolates (76.9%). Moreover, *ermB* gene was detected in 70%, 50% and 69.2% of *Streptococcus* isolates from milk, cheese and humans, respectively. There was no significant difference ($P > 0.05$) in the prevalence of *tetO* and *ermB* genes among *Streptococcus* isolates from animal and human sources. However, there was a significant higher prevalence ($P < 0.05$) of *Pbp1A* gene in *Streptococcus* isolates from cheese (100%), followed by milk (80%) and humans (53.8%) (Table 5 and Figure 3).Table 4 showed that 40.7 % (11/27), 37.04 % (10/27) and 22.2% (6/27) of *Streptococcus* isolates harbored 3, 2 and one resistance genes, respectively.

Discussion

S. agalactiae and *S. dysgalactiae* are contagious obligate pathogens of bovine mammary gland and are considered the most common causes of bovine mastitis in dairy herds worldwide[24, 25]. These pathogens are most commonly found in the udder of the cow, and they have the potential to transmit from one cow to another during the milking process. While the presence of organisms that cause mastitis in bulk milk is a powerful signal of the presence of intra-mammary infections in the herd, it also has an impact on the safety and quality of milk and milk products [26]. This subsequently affects the consumers' health especially when the milk is consumed without pasteurization. In this regard, the current work aimed to determine genetic relatedness, antibiotic susceptibility patterns, antibiotic resistance genes, and biofilm formation of *S. agalactiae* and *S. dysgalactiae* strains isolated from milk and humans in Sharkia Province, Egypt.

In the current study, the phylogenetic analysis revealed that *16S rRNA* gene sequences of *S. agalactiae* and *S. dysgalactiae* isolated from raw milk (dairy farm and retail outlet) and human (dairy farmers' hand swabs, pregnant women's vaginal swabs, and children's pharyngeal swabs) samples were closely related and showed strongest homology with other *16S rRNA* gene sequences of *S. agalactiae* and *S. dysgalactiae* in GenBank and isolated from food, animals and humans. This indicated a zoonotic link between these strains and the possibility of transmission between animals and humans through direct contact, exposure of humans to cow faeces, or the consumption of unpasteurized cow milk [27].

At present, antibiotics are the first-line treatment for bovine mastitis. However, the extensive use of antibiotics to eliminate mastitis pathogens has become a public health concern due to the presence of antibiotic residues in milk and the emergence of antibiotic- resistant pathogens [28]. In this study, antibiotic susceptibility profile against 12 antibiotics of 10 different classes was performed on 31 *Streptococcus* isolates. *S. agalactiae* and *S. dysgalactiae* isolates from animal (milk, cheese) and human sources showed a high percentage of resistance toward cephalosporins (cefepime and

ceftriaxone), tetracyclines, penicillins (penicillin and ampicillin), meropenem, clindamycin and linezolid compared with other antibiotics. This could be attributed to the unoptimized use of these antibiotics in treatment of streptococcal infection in veterinary and human medicine in Egypt without prescription. Furthermore, the antibiotic-resistant *S. agalactiae* and *S. dysgalactiae* can be potentially transmitted to humans through unpasteurized milk and posing threats to public health. The high resistance (100%) of *Streptococcus* isolates from animal sources (milk and cheese) to ceftriaxone in this study agreed with Han et al. [29]and contrast the findings of Ismail et al. [14]who recorded that all isolates were sensitive to ceftriaxone. The high resistance of *Streptococcus* isolates from milk and cheese to tetracycline (100%) in this study coincided with previous studies in China [1, 30], and Taiwan [13]. However, a low rate of resistance to tetracycline was previously reported in Turkey [31]. *Streptococcus* isolated from milk and cheese showed higher percentage of resistance to penicillin, ampicillin (93.8%, each). This finding coincided with the results obtained by Han et al. [29], Ismail et al. [14], and Wataradee et al. [32]. On the contrary, Lin et al. [4] and Liu et al. [33] reported no resistance of *Streptococcus* isolates from milk to penicillins. *Streptococcus* isolated from milk and cheese in this study showed a high resistance rate to clindamycin (93.8%) which was nearly similar to Liu et al. [33] and higher than Saed and Ibrahim [2], Lin et al. [4], and Tuzcu et al. [31]. Furthermore, the resistance rates of *Streptococcus* isolated from milk and cheese in this study to linezolid (93.8%), chloramphenicol (81.3%), vancomycin (75%) and levofloxacin (37.5%) were higher than a previous study in Turkey [31]. The inconsistency between these reports and our present findings may reflect differences in the types of antibiotics used in clinical practice across regions.

In the current study, the high resistance of human *Streptococcus* isolates toward ampicillin (100%) and penicillin (93.3%) agreed with the findings from a previous study in Iraqi [34] and higher than other studies reported in different countries [33, 35-39]. Likewise, Ndiaye et al. [38] and Kamińska et al. [39] reported nearly similar resistance rate to tetracycline as in our study. In addition, the resistance rates of human *Streptococcus* isolates in this study toward clindamycin, linezolid, chloramphenicol, vancomycin was higher than other studies [33, 35-37].

A noteworthy observation in this study that all *Streptococcus* isolates recovered from animal and human sources were resistant to multiple antibiotics, 3 isolates (9.7%) were MDR, 25 isolates (80.6%) were XDR, and 3 isolates (9.7%) were PDR. The high prevalence of multiple antibiotic resistant (MAR) *Streptococcus* isolates and the unexpected increase in MAR index (average = 0.874) in this study indicated the exposure to high-risk source contamination and the misuse of antibiotics in dairy farms as well as in human medicine. The high prevalence of MAR*S. agalactiae* strains in bovine milk has been recorded in previous studies [1, 4, 12].

Biofilm formation by *Streptococcus* spp facilitates bacterial persistence in the udder and increases resistance to antimicrobial agents and the host immune system [40]. Surprisingly, only 3.2% of the *S. agalactiae* and *S. dysgalactiae* isolates in this study were weak biofilm producers. Previous studies have reported varying prevalence rates of biofilm formation by *S. agalactiae* and *S. dysgalactiae* isolates, with some studies finding a high prevalence of biofilm producers [14, 41]and others finding a low prevalence[42].Of interest, *tetO* and *Pbp1A* genes play impactful roles in the exceeding resistance of *Streptococcus* species. The *tetO* gene encodes a ribosomal protection protein that binds to ribosomes and prevents tetracycline from binding to its target site, the 30S ribosomal subunit. This prevents the inhibition of protein synthesis by tetracycline[43]. The *Pbp1A* gene encodes a penicillin-binding protein (PBP), an enzyme involved in bacterial cell wall synthesis. Mutations in the *Pbp1A* gene can alter the structure of the PBP, reducing its affinity for beta-lactam antibiotics such as penicillin and ampicillin. This decreased affinity prevents the antibiotics from binding to their target and inhibiting cell wall synthesis [44].

The *tetO* gene was more prevalent in Streptococcus isolates from animal sources (milk and cheese) compared to *ermB* and *Pbp1A*. Conversely, the *tetO* gene was more prevalent in human Streptococcus isolates than *ermB* and *Pbp1A*. These findings suggest that the *tetO* gene is more commonly associated with antimicrobial resistance in Streptococcus isolates from both animal and human sources [2, 13,30, 31]. Previous studies have reported lower prevalence rates of the *tetO* gene in Streptococcus isolates from milk and humans [45, 46].

The *ermB* gene encodes a ribosomal methylase enzyme that modifies the 23S rRNA molecule.

This modification prevents macrolide antibiotics from binding to their target site on the ribosome, rendering the bacteria resistant [46]. It was detected in 66.7% of *S. agalactiae* isolates and 75% of *S. dysgalactiae* isolates from milk. These prevalence rates are higher than those reported in previous studies from Egypt [12, 47], Turkey [31], and China [30], but similar to the prevalence reported in a recent study from Poland [39].

Conclusion

The genetic relatedness of *S. agalactiae* and *S. dysgalactiae* isolates from animal products and humans suggests the potential for zoonotic transmission. The high prevalence of antimicrobial resistance among these isolates, including multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan drug-resistant (PDR) strains, poses a significant threat to public health and animal health. Effective strategies for preventing the spread of these resistant strains are urgently needed, including improved farm hygiene practices and judicious antibiotic use in both human and veterinary medicine.

Acknowledgments

We would like to thank Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt for the technical support.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Zagazig University, Egypt (approval number ZU-IACUC/2/428/2023).

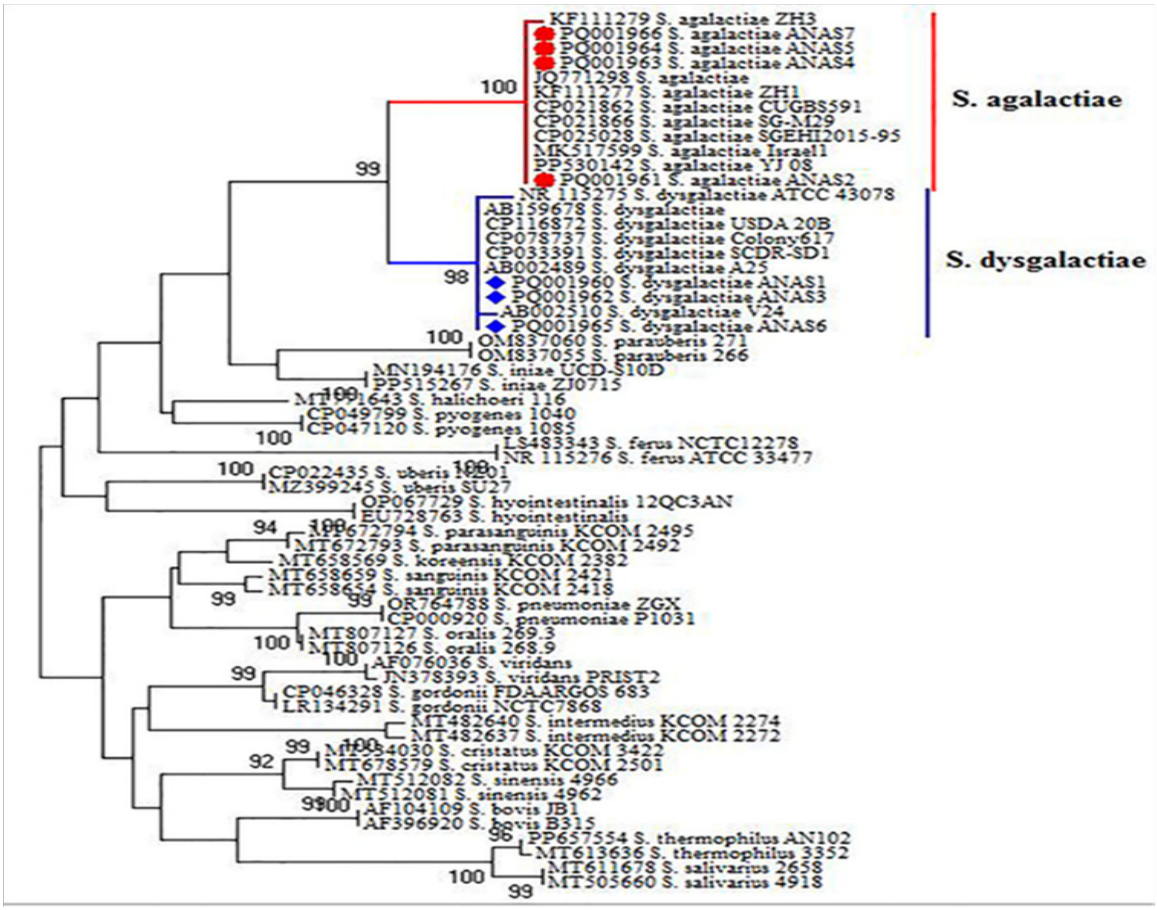

Fig. 1. The phylogenetic tree showing the genetic relatedness between 16S rRNA gene sequences of S. agalactiae and S. dysgalactiae strains isolated from human and milk in this study compared with other sequences retrieved from the GenBank. The tree topology was evaluated by 500 bootstrap analyses.

TABLE 1. Oligonucleotide primers' sequences and PCR cycling conditions used in this study.

Target gene	Primers' sequences 5\-3\	Amplified (product (bp	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Streptococcus spp. *tuf*	GTACAGTTGCTTCAGGACGTATC GCTTCGATTTCATCACGTTG	197	94˚C (5 min)	(C (30 sec˚94	55˚C (40 sec)	72˚C (45 sec)	72˚C (10 min)	Picard et al. [[49
S. agalactiae 16S rRNA	CGCTGAGGTTTGGTGTTTACA CACTCCTACCAACGTTCTTC	405	94˚C (5 min)	94˚C (30 sec)	60˚C (40 sec)	72˚C (45 sec)	C (10˚72 (min	Mashoufet al. [50]
S. dysgalactiae 16S rRNA	GGGAGTGGAAAATCCACCAT AAGGGAAAGCCTATCTCTAGACC	572	(C (5 min˚94	(C (30 sec˚94	60˚C (40 sec)	(C (45 sec˚72	C (10˚72 (min	Shomeet al. [[51
tetO	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	519	(C (5 min˚95	95˚C (1 min)	60˚C (1min)	72˚C (1min)	72˚C (5 min)	Princivalli et al. [52]
ermB	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	95˚C (5 min)	(C (1 min˚95	54˚C (1min)	(C (1 min˚72	(C (5 min˚72	Gygax et al. [53]
Pbp1A	TGGGATGGATGTTTACACAAATG GTCGTACTATTATTTGTGCTTGG	1197	95˚C (5 min)	95˚C (1 min)	60˚C (1 min)	72˚C (1 min)	(C (5 min˚72	Zhanel et al. [54]

TABLE 2. Accession numbers of the sequenced *16S rRNA* genes of *S. agalactiae* and *S. dysgalactiae* isolates.

Sample	Isolate ID	Accession number	Host	Sample type	Location	Date of collection
36	*S. dysgalactiae* ANAS1	PQ001960	Cow	Dairy farm milk	Sharkia	7/2023
127	*S. agalactiae* ANAS2	PQ001961	Human	Dairy farmer's hand swab	Sharkia	8/2023
257	*S. dysgalactiae* ANAS3	PQ001962	Human	Child's pharyngeal swab	Sharkia	10/2023
269	*S. agalactiae* ANAS4	PQ001963	Human	Pregnant woman's vaginal swab	Sharkia	11/2023
213	*S. agalactiae* ANAS5	PQ001964	Cow	Retail outlet milk	Sharkia	8/2023
204	*S. dysgalactiae* ANAS6	PQ001965	Human	Dairy farmer's hand swab	Sharkia	9/2023
245	*S. agalactiae* ANAS7	PQ001966	Human	Child's pharyngeal swab	Sharkia	10/2023

TABLE 3. The resistance of *S. agalactiae* and *S. dysgalactiae*isolates to different antibiotics.

Source/serotype	Antimicrobial class/ Antibiotic											
	Penicillins		Macrolides	Fluroquinolones	Lincosamides	Oxazolidinones	Glycopeptides	Cephalosporins		Phenicols	Carbapenems	Tetracyclines
	P	AM	AZM	LEV	DA	LNZ	VA	FEP	CRO	C	MEM	TE
	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
Milk												
S. agalactiae (7)	6 (85.7)	7 (100)	5 (71.4)	2 (28.6)	7 (100)	7 (100)	4 (57.1)	6 (85.7)	7 (100)	6 (85.7)	7 (100)	7 (100)
S. dysgalactiae (5)	5 (100)	4 (80)	4 (80)	2 (40)	4 (80)	4 (80)	4 (80)	5 (100)	5 (100)	3 (60)	5 (100)	5 (100)
Cheese												
S. agalactiae (1)	1 (100)	1(100)	1 (100)	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
S. dysgalactiae (3)	3 (100)	3 (100)	1 (33.3)	2 (66.7)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
Total (16)	**15 (93.8)**	**15 (93.8)**	**11 (68.8)**	**6 (37.5)**	**15 (93.8)**	**15 (93.8)**	**12 (75)**	**15 (93.8)**	**16 (100)**	**13 (81.3)**	**16 (100)**	**16 (100)**
Human												
S. agalactiae (12)	12 (100)	12 (100)	10 (83.3)	6 (50)	12 (100)	12 (100)	10 (8.3)	11 (91.7)	11 (91.7)	9 (75)	11 (91.7)	11 (91.7)
S. dysgalactiae (3)	2 (66.7)	3 (100)	3 (100)	2 (66.7)	3 (100)	3 (100)	2 (66.7)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
Total (15)	**14 (93.3)**	**15 (100)**	**13 (86.7)**	**8 (53.3)**	**15 (100)**	**15 (100)**	**12 (80)**	**14 (93.3)**	**14 (93.3)**	**12 (80)**	**14 (93.3)**	**14 (93.3)**
Total sensitive	2 (6.5)	1 (3.2)	1 (3.2)	11 (35.5)	0	1 (3.2)	7 (22.6)	2 (6.5)	1 (3.2)	4 (12.9)	1 (3.2)	1 (3.2)
Total intermediate	0	0	6 (19.4)	6 (19.4)	1 (3.2)	0	0	0	0	2 (6.5)	0	0
Total resistant	29 (93.5)	30 (96.8)	24 (77.4)	14 (45.2)	30 (96.8)	30 (96.8)	24 (77.4)	29 (93.5)	30 (96.8)	25 (80.6)	30 (96.8)	30 (96.8)

P: Penicillin, AM: Ampicillin, AZM: Azithromycin, LEV: Levofloxacin, DA: Clindamycin, LNZ: Linezolid, VA: Vancomycin, FEP: Cefepime, CRO: Ceftriaxone, C: Chloramphenicol, MEM: Meropenem, TE: Tetracycline

TABLE 4. Antibiotic resistance phenotypes, antibiotic resistance genes and biofilm grades of *S. agalactiae* and *S. dysgalactiae* in this study.

Isolate	Species (Source)	Antibiotic resistance phenotypes			Antibiotic resistance genes			Biofilm grade(average OD_{595} ± SD)
		Antibiotic resistance profile	Antibiotic resistance category	MAR index	*tetO*	*ermB*	*Pbp1A*	
33	*S. agalactiae* (DFM)	P, AM, DA, LNZ, FEP, CRO, MEM, TE	MDR	0.667	-	-	+	None (0.1060.009±)
38	*S. agalactiae* (DFM)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	-	None (0.0950.010±)
86	*S. agalactiae* (DFM)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	-	+	None (0.0880.006±)
108	*S. dysgalactiae* (DFM)	P, AM, AZM, LEV, DA, LNZ, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.0910.008±)
117	*S. dysgalactiae* (DFM)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.0850.003±)
119	*S. dysgalactiae*(DFM)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	-	+	+	None (0.1060.015±)
122	*S. agalactiae* (DFM)	P, AM, AZM, DA, LNZ, VA, CRO, C, MEM, TE	XDR	0.833	+	+	+	Weak (0.1890.022±)
212	*S. agalactiae* (ROM)	P, AM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.833	+	+	+	None (0.0850.004±)
215	*S. dysgalactiae* (ROM)	P, AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, MEM, TE	XDR	0.917	+	-	-	None (0.0960.012±)
141	*S. dysgalactiae*(Cheese)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.0840.002±)
186	*S. dysgalactiae* (Cheese)	P, AM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	-	+	None (0.0930.01±)
92	*S. agalactiae*(DFHS)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, MEM, TE	XDR	0.833	-	+	+	None (0.120.02±)
129	*S. agalactiae* (DFHS)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	-	-	None (0.0970.005±)
130	*S. agalactiae* (DFHS)	P, AM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.833	+	+	-	None (0.1170.033±)
228	*S. dysgalactiae* (DFHS)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	-	-	+	None (0.1040.004±)
270	*S. agalactiae*(PWVS)	P, AM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.1000.009±)
271	(*S. agalactiae* (PWVS	P, AM, AZM, LEV, DA, LNZ, FEP, CRO, C, MEM, TE	XDR	0.917	+	-	-	None (0.0910.002±)
241	(*S. agalactiae* (CPS	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.833	+	+	-	None (0.0810.001±)
244	*S. agalactiae* (CPS)	P, AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	PDR	1	-	-	+	None (0.1020.004±)
252	*S. agalactiae* (CPS)	P, AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, MEM, TE	XDR	0.917	+	+	-	None (0.0980.023±)
32	(*S. agalactiae* (DFM	AM, AZM, LEV, DA, LNZ, FEP, CRO, C, MEM, TE	XDR	0.833	NE	NE	NE	None (0.0860.004±)
225	*S. agalactiae*(DFHS)	P, AM, AZM, DA, LNZ, VA	MDR	0.5	NE	NE	NE	None (0.0750.002±)
127	*S. agalactiae* (DFHS)	P, AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	PDR	1	+	+	+	None (0.1120.020±)
36	*S. dysgalactiae* (DFM)	P, VA, FEP, CRO, MEM, TE	MDR	0.5	NE	NE	NE	None (0.1020.015±)
257	*S. dysgalactiae* (CPS)	P, AM, AZM, LEV, DA, LNZ, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.0770.005±)
269	*S. agalactiae* (PWVS)	P, AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	PDR	1	+	+	-	None (0.0920.007±)
213	*S. agalactiae* (ROM)	P, AM, AZM, LEV, DA, LNZ, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.1030.003±)
137	*S. agalactiae* (Cheese)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	-	+	None (0.0940.007±)
172	*S. dysgalactiae* (Cheese)	P, AM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.1160.014±)
204	*S. dysgalactiae* (DFHS)	AM, AZM, LEV, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	NE	NE	NE	None (0.0810.009±)
245	*S. agalactiae* (CPS)	P, AM, AZM, DA, LNZ, VA, FEP, CRO, C, MEM, TE	XDR	0.917	+	+	+	None (0.0690.002±)

DFM (Dairy farm milk), ROM (Retail outlet milk), DFHS (Dairy farmer's hand swab), PWVS (Pregnant woman's vaginal swab) and CPS (Child's pharyngeal swab)
MDR (Multidrug-resistant), XDR (Extensive drug-resistant) and PDR (Pan drug-resistant) MAR index: Multiple antibiotic resistance index. NE: not examined. OD (Optical density), SD (Standard deviation).

TABLE 5. Occurrence of antibiotic resistance genes in 27 S. agalactiae and S. dysgalactiae resistant to multiple antibiotics (tetracycline, clindamycin, penicillin and ampicillin).

Source/ Serotype	Antibiotic resistance genes		
	tetO No. positive (%)	*ermB* No. positive (%)	*Pbp1A* No. positive (%)
Milk			
S. agalactiae (n= 6)	5 (83.3%)	4 (66.7%)	5 (83.3%)
S. dysgalactiae (n=4)	3 (75%)	3 (75%)	3 (75%)
Total (n=10)	8 (80%)	7 (70%)	8 (80%)
Cheese			
S. agalactiae (n= 1)	1 (100%)	0	1 (100%)
S. dysgalactiae (n=3)	3 (100%)	2 (66.7%)	3 (100%)
Total (n=4)	4 (100%)	2 (50%)	4 (100%) *
Human			
S. agalactiae (n=11)	9 (81.8%)	8 (72.7%)	5 (45.5%)
S. dysgalactiae ((n=2)	1 (50%)	1 (50%)	2 (100%)
Total (n=13)	10 (76.9%)	9 (69.2%)	7 (53.8%)

*Represent statistically significant difference ($P < 0.05$).

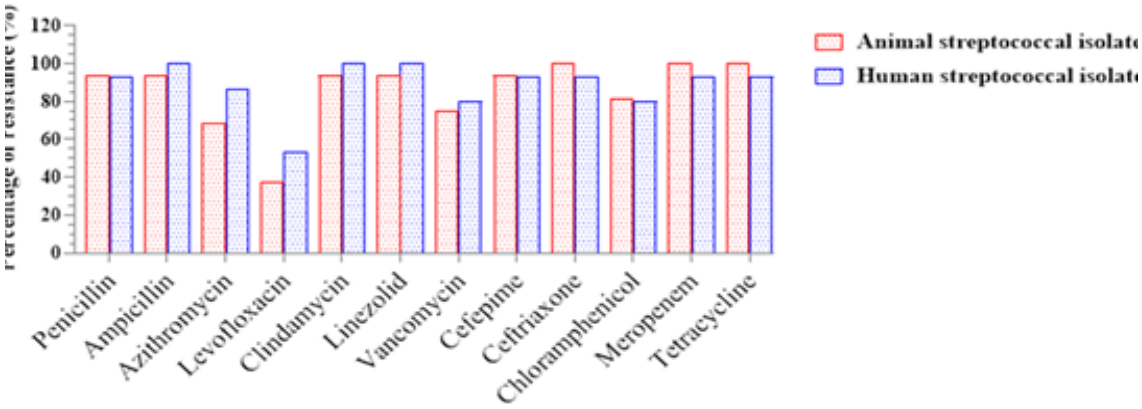

Fig. 2: Percentage of *Streptococcus* isolates resistant to different antibiotics.

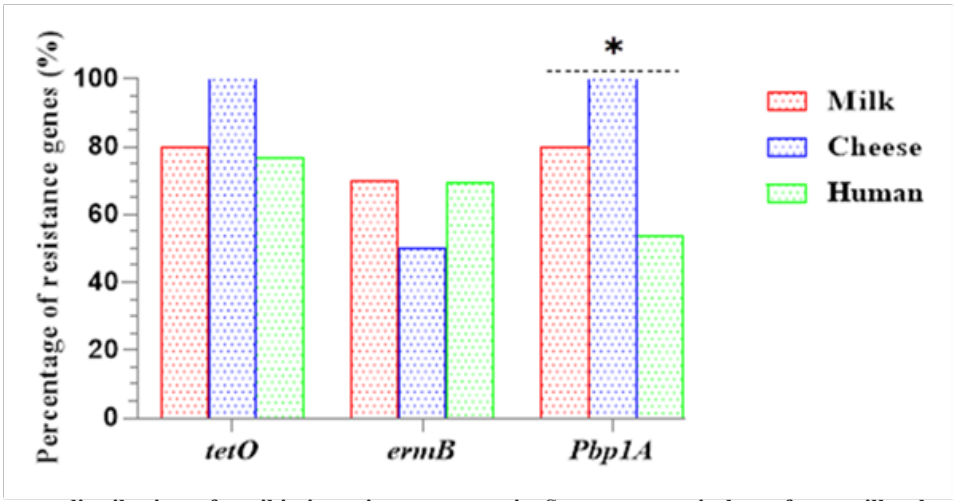

Fig. 3. Frequency distribution of antibiotic resistance genes in Streptococcus isolates from milk, cheese and humans.(*) represents statistically significant difference (P < 0.05).

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التنوع الوراثي ومقاومة مضادات الميكروبات وتكوين الأغشية الحيوية في سلالات الميكروب السبحي اجالاكتيا والميكروب السبحى دساجالاكتيا المعزولة من الحليب ومنتجات الألبان والانسان في محافظة الشرقية، مصر

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الملخص

تشكل عدوى الميكروب السبحى تهديدًا كبيرًا للصحة العامة في محافظة الشرقية بمصر، مما يساهم في عبء اقتصادي كبير. تنتشر هذه العدوى بين البشر والحيوانات، مع إمكانية انتقالها من الحيوانات إلى الإنسان. يعد فهم أنماط مقاومة مضادات الميكروبات والتنوع الجيني لسلالات الميكروب السبحى أمرًا بالغ الأهمية لتطوير استراتيجيات فعالة للوقاية والسيطرة. تضمنت الدراسة ٣١ عزلة من المكورات السبحية، حيث أظهرت معظم العزلات مقاومة ظاهرية للعديد من المضادات الحيوية. كانت مقاومة المضادات الحيوية منتشرة على نطاق واسع، حيث لوحظت مقاومة بنسبة ٩٦,٨٪ للأمبيسلين والكليندامايسين واللينيزوليد والسيفترياكسون والميروبينيم والتتراسيكلين. لم تكن هناك اختلافات كبيرة في المقاومة بين عزلات الميكروب السبحى اجالاكتيا والميكروب السبحى دسجالاكتيا. أظهرت العزلات ١٣ مقاومة ، حيث تم تصنيف معظمها على أنها مقاومة للأدوية على نطاق واسع (٨٠,٦٪) أو مقاومة لجميع الأدوية (٩,٧٪). كان جين المقاومة tetO أكثر انتشارًا في العزلات المأخوذة من الجبن (١٠٠٪)، تليها تلك المأخوذة من الحليب (٨٠٪) والإنسان (٧٦,٩٪). بالإضافة إلى ذلك، كانت ٨١,٥٪ و٦٦,٧٪ و٧٠,٤٪ من العزلات إيجابية لـجينات المقاومة tetO و ermB و Pbp1A على التوالي. ومن بين العزلات، كانت واحدة فقط (٣,٢٪) منتجة للأغشية الحيوية الضعيفة. كشف التحليل الوراثي عن درجة عالية من التشابه الجيني بين سلالات الميكروب السبحى، مما يشير إلى انتقال حيواني محتمل. وانتهت الدراسة الى ارتفاع معدل انتشار مقاومة مضادات الميكروبات والتنوع الجيني لسلالات ستربتوكوكس في محافظة الشرقية مما يسلط الضوء على الحاجة الملحة إلى استراتيجيات فعالة لمكافحة هذه العدوى ومنع انتشارها.

الكلمات الدالة: المقاومة لمضادات الميكروبات , تكوين الغشاء الحيوي , الميكروب السبحي, الامراض المشتركة.