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Determine the bacterial resistance of Streptococcus sobrinus to antibiotics

Hanan Saleh Abdulhussain[1] , Mithal K.A. Al-Hassani[2]
[1,2]University of Al-Qadisiyah / College of Education / Department of Life Sciences
Corresponding author: Edu.bio.posta75@qu.edu.iq
ORCID ID: http://orcid.org/0009-0005-4974-8305

Abstract:

Antibiotics are chemical compound produced by microorganism that kill, or inhibits the growth of the other microorganism. Antibiotics are divided into two types based on their action: bacteriostatic antibiotics, such as tetracycline, and bactericidal antibiotics, such as penicillin. The study aimed was to determine bacterial resistance of *S. sobrinus* to antibiotics and detect resistance genes. The results for *S. sobri*

nus showed that 41.6% of isolates formed biofilms, and 58.3% were non-biofilm-forming. The results show a clear variation in resistance and sensitivity to antibiotics. *S. sobrinus* isolates showed 100% complete resistance to amoxicillin. These isolates also showed high resistance to tetracycline and ampicillin (75%), and 66.67% resistance to ciprofloxacin. The isolates showed sensitivity to s (91.67%), erythromycin (50%), gentamycin (41%), cls (66.67%), and hls (75%).

Keywords: bacterial resistance, Streptococcus sobrinus, antibiotics, resistance genes

Introduction:

Streptococcus sobrinus is a Gram-positive, facultative anaerobic bacterium that naturally inhabits the oral cavity (Chaffanel et al., 2015). These bacteria are generally spherical (coccus) in shape, forming chains during growth. They are non-motile and do not form spores, and are approximately 1-2 µm in diameter. These bacteria grow optimally at temperatures between 18-40°C and in a pH environment between 5.2-7. (Igarashi et al., 2000). It is one of the main genera causing dental carie, and is usually associated with other microorganism such as, *Candida albicans*, *S. sobrinus* possesses multiple pathogenic, and carcinogenic mechanisms. Through its adhesion to a solid surface, cariogenic bacteria can colonizes tooth enamel, and bacterial biofilms, on dental plaque (William et al., 2012). In addition, to their ability to produce organic acids by fermenting carbohydrates (acidophiles) and survive under acidic condition, presence of these bacteria in dental plaque leads to localized decrease in pH on the tooth surface (Oho et al., 2000).

This acidity removes the calcium, and phosphate minerals present in hydroxyapatites crystals of tooth enamels, leading to formation of cavitie known as, dental caries (Abou et al., 2016). The biofilm structures of the

dental plaque enables bacteria to become protected from antibacterial agents and environmental stresses (Haenni et al., 2018). Streptococci require a rich medium for growth, most notably blood agar, and grow at 37°C under anaerobic conditions. After 24 hours of incubation, clear, round, slightly convex colonies appear, fermenting sugars and producing lactic acid without gas. Alpha hemolytic bacteria produce colonies on blood agar surrounded by a small clear zone due to the partial lysis of red blood cells. The colony changes color to green due to the reduction of hemoglobin, similar to *S. Sobrinus* (Loyola-Rodriguez et al., 2018).

These compounds are also specialized secondary metabolites and are effective against many types of microorganisms. Antibiotics are divided into two types based on their action: bacteriostatic antibiotics, such as tetracycline, and bactericidal antibiotics, such as penicillin (Jacob, 2015). They can also be classified according to their spectrum of action; narrow-spectrum antibiotics, such as penicillin, target a specific group of microorganisms, while broad-spectrum antibiotics, such as gentamicin, affect both Gram-positive and Gram-negative bacteria (Tenover et al., 2019). Antibiotic resistance is one of the major determinants of health worldwide. By altering bacterial genes in response to the overuse of drugs or antibiotics, antibiotic resistance occurs. Eventually, resistance develops. These resistant bacteria are more likely to infect humans and cause infections that are more difficult to treat than susceptible bacteria. Antibiotic resistance is rapidly emerging worldwide at a very high level (Chen & LeBlanc, 1992) Therefore, the study aimed was to determine bacterial resistance of *S. sobrinus* to antibiotics and detect resistance genes.

Materials and Methods:

Preparations of culture media:

These media were prepared in laboratory, according to manufacturers' instruction. The media was adjusted to a pH of 7.2 ± 0.2, then boiled in water bath to ensure all component were completely dissolved. Media were sterilized by autoclave at 121°C, 15 psi/in², for 15 mins. Plates, and tubes containing different media were incubated at the 37°C to ensuring no contaminations, and stored at 4°C until using.

Blood agar medium:

Blood agars were prepared according to manufacturer's instruction. Dissolve 40 g of blood agars medium in 1000 ml of dry water. Sterilize at 121°C for 15 minutes and cool to 50°C. Five percent fresh human blood was then added, mixed well until homogeneous, and poured into sterile Petri dish. Finally, cool at 37°C, and allow solidify at room temperatures. This medium used to culture bacterial isolate, and determine the ability of bacteria to the hemolysis blood cell (Cotar et al., 2010).

Mueller-Hinton Agar Medium:

This medium prepared according to company's instruction by dissolve 3881 grams of Mueller-Hinton medium in 1000 ml2 of the distilled water. It was sterilized in an autoclave at 121°C and 15 psi for 15 mins, then poured into plates and allowed to solidify. This medium was used to test bacterial susceptibility to antibiotics using the ready-made tablet method (Cotar et al., 2010).

Brain Heart Infusion Broths:

This medium was prepared according to the manufacturer's instructions and poured into sterile tubes. It is used as a nutrient medium for growing pure bacteria. It preserves the biological properties of the bacteria, and samples are stored for long periods and ready for activation when needed (Hamid et al., 2017).

Nutrient Broth Medium:

This medium was used for growing bacteria and for short-term storage. It prepared according to the manufacturer's instructions (Pillai et al., 2012).

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing was performed using the agar disc diffusion method, as described in CLSI 2024:

Bacterial Sample Preparation:

3-5 well-isolated colonies suspended in the 4-5 ml of brain heart solutions. The bacterial broth incubated for 8 hours at 370°C. Turbidity in the newly grown broth adjusted using steriles broth to achieve a turbidity visually similar to that of a 0.5 McFarland standard tube, which corresponds to a growth concentration (1.5 x 10^8 cells/ml).

Layout of Test Plates:

1. To remove excess inoculums from the swabs, a sterile swab was dipped in the adjusted solution and passed vigorously several times along the inside wall of tube above liquid level.
2. The coated swab used to draw lines across the entire surface of the Mueller Hinton agar plate. To achieve a uniform distribution of bacteria, lines were drawn two more times, rotating the plate 60° each time. The edge of the agar was wiped as a final step.
3. Before applying antibiotic discs, the Petri dishes allowed to dry 15–20 mins at room temperatures.

Use of Antibiotic Discs:

1. Antimicrobial placed on the surface of the inoculated agar plate, and each disc was carefully pressed onto the surface. The agar surface was inverted to achieve optimal contact.
2. The plates were inverted and incubated at 37°C for 18 hours.
3. Using a transparent ruler, the diameter of the growth inhibition zone was measured after incubation.
4. The results were compared with the CLSI (2024) inhibition assay.

Detection of Biofilm Formation:

Congo Red Agar Test:

Streptococcal strains incubated at 24 to 42 hours for 37°C in Brain Heart Infusion (BHI) broth supplement with 5% (w/v) sucrose, and 0.08% (w/v) Congo Red. Strains that formed red colony with a dry, crystalline texture considered to be producing exopolysaccharide, while white or pink colony indicated poor exopolysaccharide production.

Detection of Virulence Factors:

Congo Red Acara:

Tested streptococcal strains incubated at 24–42 hours for 37°C in Brain Heart Infusion broth supplement with 5% (w/v) sucrose, and 0–8% (w/v) Congo Red. Strains forming red colonies with a dry, crystalline texture considered to producing exopolysaccharides, while white-pink colony reflected greater exopolysaccharide productions. Genetic Detection of Virulence Genes

Extraction of Genomic DNA from Bacterial Media:

Genomic DNA extracted from streptococcal isolates using Genomic DNA Purification Kit from Turkey (Geneaid) according to the manufacturer's instructions. Bacterial cultures were seeded in 1 ml

nutrient broth and cultured overnight at 37°C in an incubator. Transfer up to 1 x 109 bacterial cells to a 1.5 m microcentrifuge tube and centrifuge for 1 min at 14-16,000 × g, then discard the top liquid.

1. A total of 180 µL of GT Buffer was added, then the cells were suspended using a pipette. Proteinase K was added, ensuring ddH2O was added. The tube was then inverted every 3 min while incubating at 60°C for at least 10 min.
2. 200 µL of GB Buffer was added, pipetted for 1 second, and incubated for at least 10 min at 70°C to ensure the sample was free of contamination. The tube was inverted every 3 min during the incubation period. At this point, the required elutions solutions (200 µL per sample) was heated to 7°C in preparation for the next step (DNA elution).
3. 200 µL of 100% ethanol was added and mixed vigorously immediately. If a precipitate formed, the shaking was stopped as much as possible using a pipette, and the mixture (including any insoluble precipitate) transferred to a GD columns in a 2 mL collection tube, and centrifuge at 14–16,000 × g for 2 mins. The flow-through 2 mL collection tube discarded, and GD column placed in a new 2 mL collection tubes.
4. 400 µL of W1 solution added to GD column, and centrifuge for 30 seconds at 14–16,000 × g; the flow-through discarded by returning the GD column to 2 mL collection tube.
5. 600 µL of wash buffer (with ethanol) was added to the GD column, which was then centrifuged for 30 seconds at 14–16,000 × g. The eluent discarded, and GD column reintroduced into a 2-mL collection tube, and vortexed for 3 minutes at 14–16,000 × g to dry the column matrix.
6. The GD column transferred to clean 1.5-mL micro centrifuge tube, and 1 µL of pre-warmed elution buffer added to center of the column matrixs. The eluate allowed to sit for at least 3 minutes after centrifugation at 14–16,000 × g for 30 secs to extract pure DNA. The DNA then stored at 2–8°C.

Results and Discussion:

Biofilm Formation:

Streptococcal strains were incubated for 24 to 42 hours at 37°C in Brain Heart Infusion (BHI) broth supplement with the 5% (w/v) sucrose, and 0.08% (w/v) the Congo Red dye (Lobos et al., 2009). Strains that formed red colony with a dry, crystallines texture considered to be exopolysaccharide producers, but white, or pink colony indicated poor exopolysaccharide production. The results for *S. sobrinus* showed that 41.6% of isolates formed biofilms, and 58.3% were non-biofilm-forming.

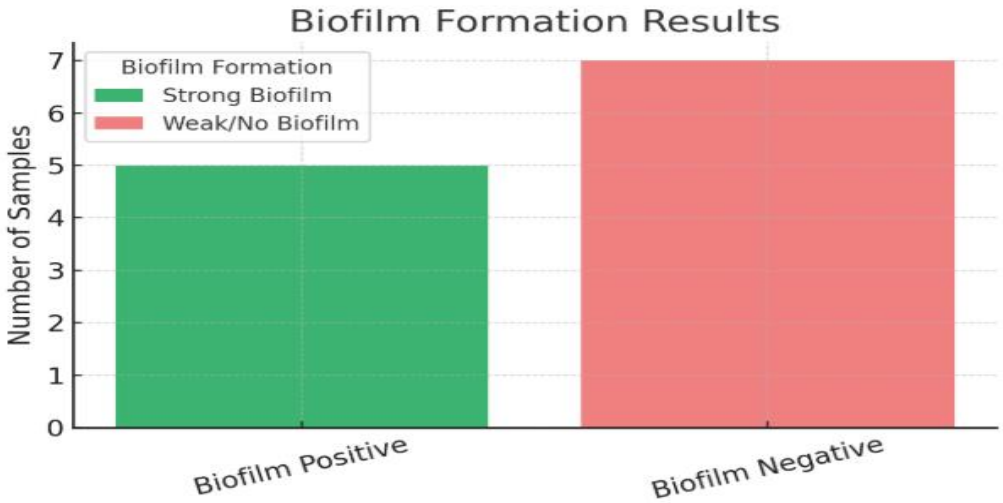

Figure (1) shows the biofilm results of bacterial isolates

Antibiotic Susceptibility Test:

The sensitivity and resistance of isolated bacteria to a number of common antibiotics were tested. The sensitivity of bacterial species to antibiotics was measured using the disk diffusion method, by calculating the diameter of growth inhibition zones around the antibiotic disk used, and comparing it with the results reported in NCLIS 2024.

The results show a clear variation in resistance and sensitivity to antibiotics. *S. sobrinus* isolates showed 100% complete resistance to amoxicillin. These isolates also showed high resistance to tetracycline and ampicillin (75%), and 66.67% resistance to ciprofloxacin. The isolates showed sensitivity to s (91.67%), erythromycin (50%), gentamycin (41%), cls (66.67%), and hls (75%). These results are consistent with what the researcher (Al-Majmai 2024) found in his study, where *S. sobrinus* isolates showed 100% resistance to amoxicillin. These results were close to what the researcher (Okada et al., 2005) found, as *S. sobrinus* isolates showed sensitivity to Gentamycin at a rate of 41.67%, and showed high sensitivity to Streptomycin at a rate of 91.67%. The results are consistent with what the researcher (Maasi et al., 2022) found, as *S. sobrinus* isolate showed high resistances to the Amoxicillin, and Ampicillin.

The biochemical, and genetic basis of the resistance is paramounts importance for designings strategy to limit emergence, and spread of resistance, and for develop innovative therapeutic approach against multidrug resistant organism (MDU). This phenomenon is known as multidrug-resistant organisms (Järvinen et al., 1995). The emergence of resistance among major bacterial pathogen is major public healths threat affecting human worldwid. Multidrug-resistant organism emerged not only in the hospital settings but is now identified in the community setting. Bacterial response to antibiotic "attack" is the first and most sophisticated example of bacterial adaptation (Wu et al., 1995). The emergence of highly resistant bacteria to beta-lactam antibiotics has become a global health problem. (Buckley et al., 1995) confirmed that resistance to ampicillin compounds includes not only the production of beta-lactamase enzymes such as penicillinase and beta-lactamases, but also extend to productions of penicillin-binding protein (PBPS) located in cytoplasmic membrane, and associated with the cell wall, such as carboxypeptidases and transpeptidases. These proteins possess enzymatic activity, such as the target of both ampenicillins and cephalosporins. These proteins alter the target of beta-lactam antibiotics, thus resulting in bacterial resistance to these antibiotics.

Another mechanism of ampicillin resistance is that one of the main enzymes, that inactivates antibiotic, such as almost all beta-lactamase, hydrolyzes those containing an ester, and amide bond, including penicillins, cephalosporin, monobactam, and carbapenem. The presence of this enzyme directly breaks down the beta-lactam ring, leading to reduced antibiotic effectiveness. The bacterial species containing this ring (Al-Mazini, 2020). Most of resistance shown sobrinus isolate is plasmid-mediated, as plasmid plays role in productions of beta-lactam enzyme, and are responsible for encoding some penicillin-binding proteins (PBPS) (Fuda). Streptomycin was discovered as the first antibiotic in this class. Subsequently, series of the aminoglycoside antibiotic discovered, including neomycins (1949), gentamicins (1963), tobramycins (1967), cisomycins (1970), amikacins (1972), and bilobasomycins (2006). All of these antibiotics have demonstrated excellent antibacterial activity not only against Gram-negative bacteria, but also against some Gram-positive bacteria.

Table (1) the antibiotic susceptibility test for *S. Sobrinus* bacteria

antibiotic	Resistant isolates (R)		Moderately resistant isolates (L)	Susceptible isolates (S)	
	No.	Percentage		No.	Percentage
Amoxicillin	12	100%	0	0	0
Tetracycline	9	75%	3	0	0
Erythromycin	1	8.33%	5	6	50%
Levofloxacin	0	0	4	8	66.67%
Streptomycin	0	0	1	11	91.67%
Ciprofloxacin	8	66.67%	4	0	0
Ampicillin	9	75%	2	0	0
Gentamycin	0	0	7	5	41.67%
cls	0	0	4	8	66.67%
Hls	0	0	3	9	75%

The various resistance mechanisms to aminoglycoside antibiotics (AGAs), aminoglycoside modifying enzymes (AMEs) are most commons (MITRAKUL et al., 2020). AMEs divided to three families of the aminoglycoside-modifyings enzymes: phosphotransferase (APHs), acetyltransferase (AACs), and adenyltransferase (ANTs). These coenzymes use enzymes such as acetyl-coenzyme A or ATP to modify the NH3 or OH groups on aminoglycoside molecules, rendering them ineffective. The genes responsible for the production, which promote transfer of aminoglycoside resistance between bacteria. Clinical strains resistant to aminoglycosides typically contain multiple AME genes, and it has been shown that AME gene is often located on same plasmid as the 16S ribosomal methyltransferase gene. The three types of AMEs include several subtypes, each specificity for resistance to different types of aminoglycosides (Gharajalar & Hassanzade, 2017).

Conclusions:

The study concludes a clear variation in resistance and sensitivity to antibiotics. S. sobrinus isolates showed 100% complete resistance to amoxicillin. These isolates also showed high resistance to tetracycline and ampicillin (75%), and 66.67% resistance to ciprofloxacin. The isolates showed sensitivity to s (91.67%), erythromycin (50%), gentamycin (41%), cls (66.67%), and hls (75%). The mechanism of ampicillin resistance is that one of the main enzyme that inactivate antibiotic, such as almost all beta-lactamase, hydrolyzes those containing an ester and amide bond, such as penicillins, cephalosporin, monobactam, and carbapenem.

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Conflicts of Interest Statement

Manuscript title:

Determine the bacterial resistance of *Streptococcus sobrinus* to antibiotics

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Author names:

Hanan Saleh Abdulhussain and Mithal Karim Abbas
Department of Life Sciences
College of Education
University of Al-Qadisiyah

The authors whose names are listed immediately below report the following details of affiliation or involvement in an organization or entity with a financial or non-financial interest in the subject matter or materials discussed in this manuscript. Please specify the nature of the conflict on a separate sheet of paper if the space below is inadequate.

Author names:

Hanan Saleh Abdulhussain and Mithal Karim Abbas
Department of Life Sciences
College of Education
University of Al-Qadisiyah

This statement is signed by all the authors to indicate agreement that the above information is true and correct (a photocopy of this form may be used if there are more than 10 authors):

Author's name (typed)	Author's signature	Date
Hanan Saleh Abdulhussain		**2025/5/17**
Mithal Karim Abbas		**2025/5/17**