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Investigation of Antibiotic Resistance Genes of Beta-Hemolytic and Non-Hemolytic Streptococci With Molecular Methods

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ABSTRACT

Infectious diseases have been one of the most important problems affecting all societies since the existence of mankind. Streptococci, one of the causes of these diseases, can be found in the normal flora of the mouth, nose, throat, skin, digestion and genital system in humans. Streptococci are an important cause of diseases in humans such as meningitis, acute otitis media, rhinosinusitis and community-acquired pneumonia.

In this study, various clinical samples belonging to the disease were taken between January and September 2015. All necessary permission documents and the ethics committee approval numbered 2 and dated 13.11.2014 for clinical investigations were obtained. The collected samples were brought to the Microbiology Laboratory of Van Yüzüncü Yıl University Medical Faculty. Analyzes of determined and identified antibiotic resistance genes of isolated and identified streptococci strains were carried out in the Microbiology laboratory of Yüzüncü Yıl University Faculty of Pharmacy by PCR. The detection of pbp1a for penicillin G antibiotic, pbp2x for cefotaxime antibiotic, and gyrA resistance genes for quinolone antibiotics were targeted. In this study, the primers used in Table-2 were taken as reference.

A total of 30 streptococcus strains were isolated and identified from various clinical samples including 8 sputum, 6 urine, 5 BOS, 4 blood, 4 nasopharyngeal swab, 2 ear effusion and 1 abscess. As a result of the PCR analysis, the resistance of pbp1a was found in 6 of the streptococcal isolates, pbp2x in 8, and resistance in the gyrA gene region in 5. In our study, no three resistance genes were found in 22 isolates and the presence of all three (pbp1a, pbp2x and gyrA) resistance genes in 5 isolates was determined. It is expected that the data obtained from this research will contribute to national and international knowledge accumulation.

Key Words: Streptococcus, pbp1A, pbp2X, gyrA

Introduction

Streptococcus species continue to exist as important human pathogens. They produce and release virulence factors such as several resistance genes, toxins and adhesion molecules. The most common infections caused by them include acute otitis media, pharyngitis, respiratory tract infections (sinusitis), impetigo, erysipelas, endocarditis, meningitis, puerperal sepsis, arthritis, osteomyelitis, post-streptococcal glomerulonephritis and rheumatic fever (1,2). In addition to these infections, they are the pathogens causing severe neonatal infections characterized by maternal infections. They cause osteomyelitis, skin and soft tissue infections, infections associated with endocarditis and bacteremia (3,4). Every year, 4 million of people experience community-based pneumonia in the United States. Community-based pneumonia was reported to be caused by Streptococcus pneumonia in 40-60% and by other bacteria in 3-10% of people (5).

The antibiotics used for the treatment of Streptococcus showed differences over time (4,5-6). In the late twentieth century, vancomycin and cephalosporin were used as the experimental treatment combination against the streptococcal infections causing meningitis (7). At the beginning of the twenty-first century, penicillin was being used traditionally for the treatment of a large number of pneumococcal infections. However, the increase in the incidence of penicillin-resistant S. pneumonia jeopardized the use of β-lactam antibiotics (8). Penicillin, ampicillin and cephalosporin are preferred for the treatment of group B streptococcal infections. Vancomycin, macrolides (erythromycin, azithromycin,

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Table 1. Clinical specimens from which the cultures used in the study were isolated

Clinical specimens	N	(%)
Sputum	8	26.6
Urine	6	20
CSF	5	16.6.
Blood	4	13.3
Nasopharyngeal swab	4	13.3
Ear effusion	2	6.6
Abscess	1	3.3
Total	30	100

Table 2. Oligonucleotide primer sequences used in pcr

Gene Region/Antibiotic	Primary Sequence	Base Wavelength	Reference
Pbp1a (for Penicillin G)	F: 5'-AGCATGCATTATGCAAAC-3' R: 5'-TACGAATTCTCCATTTCTGTAGAG-3'	569 bp	7
Pbp2x (for Cefotaxime)	F: 5'-CGCGGATCCTATGGAACTCATGTATA-3' R: 5'-GCGAATTCTTCTTAGTCTCCTAAAGT-3'	683 bp	9
GyrA (for Quinolone)	F: 5'-GACAAGTGAAATGAAAACGAG-3' R: 5'-CGCTCCATTGACTAATAAATTAGG-3'	474 bp	3

clarithromycin) and lincosamides (clindamycin) can be used as alternative drugs in patients with penicillin or cephalosporin allergy. Among the causes of Streptococcus infections, vancomycin-resistant bacteria were not reported (4).

The targets for β-lactam antibiotics are cell wall synthesis enzymes known as penicillin binding proteins (PBPs). PBP is a membrane-associated serine peptidase. This catalyzes the polymerization and transpeptidation of glycan branches at the final step of the peptidoglycan biosynthesis (8,9). Pneumococci produce five high molecular weight PBPs (1A,1B,2A,2B, and 2X) and a low molecular weight PBP3. The penicillin resistance occurs with the change in PBPs molecule which has four high molecular weight PBP molecules including PBP 1A, 2A, 2B and 2X. The change in pbp2X gene was observed to cause penicillin resistance at low rates. Penicillin resistance at high levels was found to be the result of changes in the Ser-370-Thr-Met-Lys and Ser-428-Arg-Asn patterned regions of pBP1A (7). Among the closely related species, "Mosaic PBPs" occur with recombinational events including the horizontal transfer of PBP genes (8). Although the strains with mosaic PBPs have cross-resistance between penicillin and cephalosporin at different rates, they can be determined (9).

Quinolones were clinically used in the mid-1980s for the treatment of infections. However, in the mid-1980s, quinolone-resistant Streptococcus pneumonia and Streptococcus pyogenes were reported. In the studies conducted, the gyrA and parC regions determining Quinolone resistance were identified to occur as a result of two-point mutation (4,10). In the mutation of the gyrA gene, Serine-81 was transformed into Leucine. As a result of the mutation in the parC gene, Serine-79 was transformed into Phenylalanine. It was reported that the genes causing quinolone resistance could be transferred among Streptococcus agalactia and other Streptococcus species (3).

In the present study, the carriage frequency of pbp1A, pbp2X and gyrA genes in β-hemolytic and non-hemolytic Streptococcus were aimed to be determined in the various specimens collected in 2015.

Materials and methods

Bacterial Isolation and Identification: As the study material, a total of 30 various clinical specimens collected from the patients admitted to Van Yüzüncü Yil University Training and Research Hospital in Van between January-September 2015 were used (Table 1). All necessary permission documents and the ethics committee approval numbered 2 and dated 13.11.2014 for clinical investigations were obtained. The clinical

Table 3. The antibiogram results of Streptococcus isolates

ANTIBIOTICS	Streptococcus (n=30)		
	R (%)	I (%)	S (%)
Penicillin G	5 (%16.6)	11 (36.7%)	14 (46.7%)
Cefotaxime	3 (%10)	10 (33.3%)	17 (56.7%)
Vancomycin	4 (13.3%)	8 (26.6%)	18 (60.1%)
Tetracycline	2 (6.7%)	4 (13.4%)	24 (79.9%)
Meropenem	3 (%10)	5 (16.7%)	22 (73.3%)
Erythromycin	2 (6.7%)	4 (13.4%)	24 (79.9%)
Clindamycin	3 (%10)	4 (13.3%)	23 (76.7%)

*R: Resistant, I: Intermediate Resistant, S: Susceptible

Table 4. Results of the presence of Streptococcus resistance genes (%)

Gene Regions	N	Resistance Gene Is Present	Resistance Gene Is Absent
pbp1A(Penicillin G)	30	6 (20%)	24 (80%)
pbp2X(Cefotaxime)	30	8 (26.7%)	22 (73.3%)
gyrA(Quinolone)	30	5 (16.7%)	25 (%83.3)
pbp1A/pbp2X	30	1 (3.3%)	29 (96.7%)
Pbp1A/pbp2X/GyrA	30	5 (16.7%)	25 (%83.3)

Tablo 5. Pearson chi-square values of antibiotic resistance status of Streptococcus

	Penicillin G				Cefotaxime				Vancomycin			
	N		%	Pearson chi-square	n		%	Pearson chi-square	n		%	Pearson chi-square
	S	R			S	R			S	R		
GB-pbp1a Direnç gene (Positive)	4	2	12.5	0.272	3	3	23.07	0.713	4	2	16.6	0.709
GB-pbp2x Direnç gene (Positive)	5	3	18.75	0.295	5	3	23.7	0.697	5	3	25	0.866
GB-GyrA Direnç gene (Positive)	4	1	6.25	0.102	3	2	15.38	0.869	3	2	16.6	0.1

specimens collected from the patients were brought to Van Yüzüncü Yil University Faculty of Medicine Microbiology Laboratory using BBL Culture Swab Stuart transport media. The isolation of Streptococcus strains was carried out with incubation for 24-48 hours in 5% sheep blood agar (Besimik, blood agar base) at 35°C in a medium containing 5% CO2. The colonies were purified for colony identification according to the results of colony morphology, gram-staining and catalase test. The identification of Streptococcus species was performed in gram-positive panels using BD Phonenix automated microbiology systems (Becton Dickinson, USA) in Van Yüzüncü Yil University Faculty of Medicine Microbiology Laboratory.

Antibiotic susceptibility test: The antibiotic resistance/susceptibility status of the isolated and identified Streptococcus strains was performed according to the Kirby-Bauer disc diffusion method recommended by CLSI (M100-S23; 2013). For this test, penicillin G (P, 10U), cefotaxime (CTX, 30µg), vancomycin (VA, 30µg), tetracycline (TE, 30µg), meropenem (MEM, 10µg), erythromycin (E, 15µg) and clindamycin (DA, 10µg) standard antibiotic discs were used.

DNA Extraction and Molecular Analysis: A column-based ready-to-use kit (DNA Mini kit, Qiagen, Hilden, Germany) was used for the genomic DNA extraction of the isolates. The DNAs obtained were diluted with 50 µl of dilution buffer and stored at -20 ° C until they were analyzed.

The molecular characterization of Streptococcus species was carried out with Polymerase Chain Reaction (PCR). Primers were used in the PCR were shown in (Table 2).

In order to replicate the target gene region, a ready-to-use amplification mixture (TopTaq Master-Mix, Qiagen, Hilden, Germany) was used in PCR. PCR amplification conditions included initial denaturation for 10 minutes at 94°C, afterwards denaturation for 30 cycles and 45 seconds at 94°C, binding at 52°C for 45 seconds and elongation steps at 72°C for 40 seconds, and followed by the last elongation steps at 72°C for five minutes. The PCR products were visualized on a gel imaging device after 1.5% agarose gel electrophoresis.

Statistical Analysis: The descriptive statistics for the investigated characteristics of Streptococcus strains were expressed as numbers and percentages. In respect to these characteristics, the chi-square test was used to determine the association between the strains which developed antibiotic resistance and strains with antibiotic resistance gene. IBM SPSS Statistics (Ver-21.0) statistical package program was used for the calculations (11).

Results

Isolation and Identification Results: Thirty Streptococcus species were isolated from the specimens taken from the humans. The identification revealed that ten strains were S. pyogenes, eight strains were S. agalactiae, five strains were S. mitis, four strains were S. pneumoniae, two strains were S. salivarius, and one strain was S. sanguis.

Antibiogram test results: The antibiotic susceptibility/resistance results of 30 isolated and identified Streptococcus strains were given in (Table 3).

As a result of phenotypic assessment, a Penicillin G resistance at the rate of 16.6% was detected. The lowest resistance rate was found to be for erythromycin (6.7%) and tetracycline (6.7%).

Molecular Analysis Results: The mutation of pbp1A for penicillin G antibiotic, pbp2X mutation for cefotaxime antibiotic and gyrA gene mutation for quinolone antibiotic were examined in respect to antibiotic resistance genes using the PCR method. The wavelength of 569 bp for the pbp1A gene region, 683 bp for the pbp2x gene region and 474 bp for the gyrA gene region were accepted as positive. The pbp1A resistance gene was identified in 20% (6/30) of the streptococcal isolates. The pbp2x resistance gene was found in eight isolates (26.7%) while the gyrA resistance gene was detected in 16.7% of the isolates (5/30). Both pbp1a and pbp2x resistance genes were identified in one of the streptococcal isolates. The pbp1A, pbp2X, and gyrA resistance genes carriages were determined in five isolates (16.7%). Neither of three resistance genes carriages were encountered in 22 (73.3%) isolates in our study. The resistance gene rates in the isolates were shown in (Table 4).

Statistical Analysis Results: Statistically frequency and complete Pearson values of the antibiotic resistance and resistance broad-spectrum status of streptococci are given in (Table 5).

Discussion

Streptococcus strains play role in infections as important human pathogens. Their ability to carry resistance genes and virulence factors contribute to the formation of diseases. In humans, Streptococcus (β-hemolytic and Non-Hemolytic Streptococci) causes pharyngitis, respiratory tract infection, impetigo, endocarditis, meningitis, arthritis, osteomyelitis and rheumatic fever infections (1-12).

Isolation and identification results were determined to be different in the studies conducted in Turkey and in the world (13,14). One hundred twenty β-hemolytic Streptococcus were isolated from the specimens collected in a study conducted in Istanbul between 2004 and 2007. The highest rate of isolation was reported to be from throat culture with the rate of 39.2% (13).Wang et al. (4) isolated 322 S. agalactiae from various specimens between 2007 and 2008. Of these, 73.9% were isolated from urine specimens. In a study conducted in America, 57 β-Hemolytic Streptococcus strains were isolated. Thirty-one of 57 isolates were reported to be obtained from blood cultures (1). In the study by Nagano et al. (14) in 2012, they isolated ten groups of B groups Streptococci from the pharyngeal swap specimens (5), tracheal aspirate specimens (4), pus specimen (1) of eight male and three female patients. In our study, a total of 30 Streptococcus strains were

isolated from a variety of clinical specimens. The highest rate of isolation was determined to be from sputum specimens with the rate of 26.6%. Whereas the lowest isolation ratio was observed to be from abscess specimens with the rate of 3.3%. The differences in isolation are considered to be closely associated with the bacterial flora exposure to humans in their living areas.

Traditionally, penicillin is used for the treatment of streptococcal infections. Recently, in a study conducted in Canada, 21.2% of the streptococci isolated from the respiratory system were reported to be resistant to Penicillin (8). Wang et al. (4) stated that all of 322 S. agalactiae bacteria which were isolated and identified by them were susceptible to penicillin and ceftriaxone. Benzylpenicillin resistance rates of 177 S. pneumonia isolates were determined to increase over time gradually starting from 1987 to 2001 in a study conducted in Japan (15). Of 30 S. pneumonia isolates isolated in America, 15 were resistant, 8 were intermediate resistant and 7 were reported to be susceptible to penicillin (5). No penicillin resistance was encountered in any of 283 group A Streptococcus isolates from 125 different hospitals between 2010-2012. The presence of fluoroquinolone-resistance was revealed in only three isolates (16). In our study, the highest antibiotic resistance was determined to be against penicillin G with the rate of 16.6%. The lowest antibiotic resistance rate was found to be against erythromycin and tetracycline with the rate of 3.3%. Antibiotic use policies are considered to have an impact on the development of resistance.

Penicillin-resistant Streptococcus species carrying the pbp1A and pbp2X genes have been reported in the studies conducted worldwide (7, 8). The authors of the study conducted in South Africa reported that 6 (3.3%) of 183 S. pneumonia isolates showed resistance to high-level penicillin. As a result of the PCR test, it was shown that the isolates carried the pbp1A gene (7). Hiramatsu et al. (15) isolated 177 S. pneumonia from the respiratory system specimens in their study between 1987 and 2001. They reported that 52 of these isolates carried one of pbp1A, pbp2X or pbp2b genes. The simultaneous presence of pbp1A and pbp2X gene mutations was revealed in six isolates. In the study by Sifaoui et al. (9) in France, S. pneumonia isolates carrying pbp2X were determined to show high resistance to cefpodoxime and susceptibility to amoxicillin. Of 10 penicillin resistant group B Streptococcus isolates, five were reported to carry the pbp2X gene in a study conducted in Japan (14). DNA gyrase is known as the target site of fluoroquinolones within the cell. Gyrase is composed of two subunits, namely, A (gyrA) and B (gyrB) subunits. These subunits are encoded by the gyrA and gyrB genes (17). In the study by Kawamura et al. (3) in Japan in 2003, three Streptococcus agalactia isolates were shown to have high-level fluoroquinolone resistance. As a result of the PCR analysis performed, the gyrA gene carriage was found to be positive. Thirty-four fluoroquinolone-resistant S. pneumonia strains were determined in a study conducted in Canada between 1997-2000. Twenty-four isolates showed a low-level fluoroquinolone resistance and 21% of them were revealed to carry the gyrA gene. Whereas 10 isolates were identified to show a high-level fluoroquinolone resistance and 90% of them were carrying the gyrA gene (18). In the study by Bidenbach et al. (1) in 2006, 23 of 57 β-hemolytic Streptococcus isolates were found to be positive for the gyrA gene carriage. Lee et al. (19),2015 reported that 14 S. agalactia strains isolated and identified by them were carrying the gyrA and parC genes. In our study, the highest rate of gene carriage was detected for the pbp2X gene with the rate of 26.7%. While six isolates were revealed to carry more than one resistance genes. Proportionally, the difference in resistance genes was considered to occur as a result of the endogenous and exogenous bacterial flora exposure to humans.

In conclusion, the present study demonstrated that the carriage of resistant streptococci species in various clinical specimens were at remarkable levels. The isolates were shown to have different carriage rates for the pBP1A, pBP2X and gyrA resistance genes. The streptococci with antibiotic resistance genes were predicted to pose a major risk in terms of human health. In order to preclude the risk factors caused by the bacteria developing resistance to antibiotics, epidemiological studies should be conducted frequently in hospitals.

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