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Original Article

Detection of Mutation in 23S rRNA in Isolates of *Streptococcus pyogenes* Resistant to Erythromycin

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Abstract

Background: In this study, erythromycin resistance isolates from the students with aged 12-15 yr old were studied and the mutations in the 23S rRNA were identified by sequencing.

Methods: Throat samples of 327 students with 12-15 years old from 3 schools of Tehran were cultured on the selective *Streptococcus pyogenes* medium and 23 strains were primarily selected as *S. pyogenes*.

Results: All strains were susceptible to penicillin and bacitracin (0.04U). Minimum inhibitory of concentrations (MICs) for erythromycin were determined in which 5 strains were found intermediate, 2 strains resistance and the remaining 16 strains susceptible to erythromycin. MIC for the erythromycin-resistance strains, BT_3 and BT_4, were 8.2 and 12.4 µg/ml, respectively. PCR was performed for each six 23S rRNA operons and a fragment with 550 bp was sequenced. For two resistance strains, BT_3 and BT_4 there were a mutation in position 2059 (A to G). Also another mutation in position 2529 (G to A) was detected in BT3.

Conclusion: In addition to mutation at the position 2058 other point mutations in different positions of domain V involve in the resistance to macrolides.

Keywords: Streptococcus pyogenes, *23S rRNA, erythromycin-resistance*

Introduction

Streptococcus pyogenes (group A streptococcus, GAS) is an important pathogen causing pharyngitis in children, scarlet fever, erysipelas, cellulitis, impetigo & necrotizing fasciitis (1- 3). Although penicillin is uniformly active against GAS and first choice for treatment of pharyngial and most of the infections caused by this organism, in the second line drug of choice, erythromycin, which was recommended as an alternative treatment for penicillin-hypersensitive patients (3, 4). Macrolide antibiotics such as erythromycin inhibit protein synthesis in a wide range of pathogenic bacteria. The antibiotics bind to a single site in the large ribosomal subunit located near the entrance to nascent peptide tunnel and are thought to sterically hinder the growth of the polypeptide chain (2, 5). Macrolide binding site is composed primarily of 23S rRNA, in central loop of domain V and loop of hairpin from domain II (5). Mutation in these loops cause the macrolide resistance due to inability in antibiotic binding. Additional mechanisms for macrolide resistance are target modifications by the *erm* (erythromycin ribosome methylation) genes and the macrolide efflux that was performed by increasing the transport of 14-and 15-membered macrolides from cells of *S. pyogenes* (2, 5).

S. pyogenes has six 23S rRNA operons, so mutations in rRNA genes might escape detection because the mutations are recessive or alter only a small fraction of the ribosome. Mutations at the domains V and II of 23S rRNA are known to cause macrolide resistance in *S. pyogenes*. A 2 kb length DNA fragment from the beginning of the 23S rRNA includes these domains. According to some reports mutation in a region around 550 bp (near the position A2058, numbered in *E. coli*) results in resistance to macrolides. This region is macrolide binding site and all mutation in the resistance strains have converting in (2, 5).

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Material and Methods

Bacterial strains and Media

A total of 327 students from ranged 12-15 yr old were studied and their throat samples were cultured on the selective *S. pyogenes* medium (5% sheep blood agar with sulfamethoxazole-trimethoprim added to suppress the growth of other organisms). Identification of the isolated strains were based on the colony morphology, catalase reaction, beta-hemolysis on Colombia agar base (Merck) with 5% sheep blood, as well as resistance to bacitracin (MAST) and positive reaction in pyrrolidonyl arylamidase (PYR) test (MAST).

Susceptibility test for erythromycin

Initially, disk susceptibility testing was performed according to the quid lines in NCCLS document M2-A5.[3 (ref)] Muller-Hinton sheep blood agar was inoculated with a suspension of each strain equivalent to a 0.5 Mac farland turbidity standard. Disks with 15 µg of erythromycin (MAST) were dispensed on the agar and zone diameters were measured after 20 to 24 h of incubation at 35º C. Bacitracin susceptibility was measured by the disk containing 0.04 U of bacitracin in the same method.

Agar dilution method for MIC

MICs were determined by agar dilution method. The antibiotic was added to the Muller-Hinton sheep blood agar in serial concentrations. Then, 10^4 to 10^5 cfu of the strains inoculated on the medium in a 5 µl spots.

PCR of 23S rRNA Selective sequence

Each operon was amplified by two-step PCR reaction. The first PCR was performed using a common forward primer (U_1) and reverse specific primers for each operon (P_1 to P_6). The second was done on resulted PCR (First) product using one pair of common primers ($U_1$and U_2). All primers were selected based on the 23S rRNA gene sequences in Genbank.

The primers were used in these PCRs including:

U_1 5'GAGAGACTCGGTGAAATTTTAG 3'
U_2 5'GTCCTCTCGTACTAGGAGCAG 3'
P_1 5'AAGACGTATTGAAGCTTACTCTA 3'
P_2 5'AGCTACTTCCCGAACTGATGCA 3'
P_3 5'CATAACCGTCTTCTTTCCCCTTTA 3'
P_4 5'CATATTTCTAACACGGGCAGTAG 3'
P_5 5'CACTGCCACGCTATCTAAACGTA 3'
P_6 5'CAATTGAATAGCCTTCACGTTCG 3'

Sequencing of the 23S rRNA

The second PCR products were purified by high pure gel extraction kit (Qiagen) and were sequenced (MWG, Germany). The blast software was used to check the alignment of resulted sequences of the sensitive and erythromycin resistance strains were performed with the blast in NCBI.

Results

Throat samples of 327 students with 12-15 yr old from three schools of Tehran were cultured on the selective *S. pyogenes* medium and 23 strains were primarily selected as *S. pyogenes*. Identification of the strains was studied by several morphological and physiological properties. Typical colonies were observed on the sheep blood agar with beta-hemolysis. In addition, the colonies were catalase negative and PYR positive. All strains were susceptible to penicillin and bacitracin (0.04 U). The strains were tested against erythromycin. MICs for erythromycin were determined in which five strains were found intermediate, two strains resistance and the remaining 16 strains susceptible to erythromycin. MIC for erythromycin-resistance strains, BT_3 and BT_4, were 8.2 and 12.4 µg/ ml (Table.1).

The resistance strains were cultured in BHI medium and the cell masses were harvested and subjected for total DNA extraction procedure. The purified DNA was used as template for PCR. The first PCR resulted fragments around 2 kb sized of each operon (Fig.1). In the second step, the nested PCR using those fragments resulted 550 bp fragments (Fig.2) which were used for sequencing. The sequences were compared with the sequences of susceptible strain. The results showed three mutations in two operons of strain BT_3 corresponding to 2059 (A to G) and 2529 (G to A) in one of the operons and 2059 (A to G) in the other one. For the strain BT_4, three mutations according to 2059 (A to G) in three different operons were demonstrated.

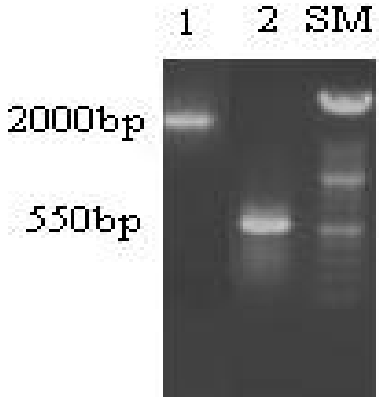

Fig. 1: Lane 1: PCR product for operons (2kb) and Lane 2: Nested PCR product (550bp)

BT3-1
GGACGGA**G**AGACCCCATGGAGCTTTACTGCAGTTTGATATTGAGTATCTGTACCACATGT
ACAGGATAGGTAGGAGCCATTGACTTCGGGACGCCAGTTTCGAATGAGGCGTTGTTGGGA
TACTACCCTTGTGTTATGGCTACTCTAACCCAGATAGGTTATCCCTATCGGAGACAGTGT
CTGACGGGCAGTTTGACTGGGGCGGTCGCCTCCTAAAGAGTAACGGAGGCGCCCAAAGGTT
CCCTCAGATTGGTTGGAAATCAATCGCAGAGTGTAAAGGTATAAGGGAGCTTGACTGCGAG
AGCTACAACTCGAGCAGGGACGAAAGTCGGGCTTAGTGATCCGGTGGTACCGAATGGAAG
GGCCATCGCTCAACGGATAAAAGCTACCCTGGGGATAACAGGCTTATCTCCCCCAAGAGTT
CACATCGACGGGGAGGTTTGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGTA**A**TC
GGTCCCAAGGGTTGGGCTGTTCGCCCATTAAAGCGGCACGCGAGCTGGGTTCAGAACGTC
GTGAGACAGTTCGGTC
BT4-1
GGACGGA**G**AGACCCCATGGAGCTTTACTGCAGTTTGATATTGAGTATCTGTACCACATGT
ACAGGATAGGTAGGAGCCATTGACTTCGGGACGCCAGTTTCGAATGAGGCGTTGTTGGGA
TACTACCCTTGTGTTATGGCTACTCTAACCCAGATAGGTTATCCCTATCGGAGACAGTGT
CTGACGGGCAGTTTGACTGGGGCGGTCGCCTCCTAAAGAGTAACGGAGGCGCCCAAAGGT
TCCCTCAGATTGGTTGGAAATCAATCGCAGAGTGTAAAGGTATAAGGGAGCTTGACTGCG
AGAGCTACAACTCGAGCAGGGACGAAAGTCGGGCTTAGTGATCCGGTGGTACCGAATGGA
AGGGCCATCGCTCAACGGATAAAAGCTACCCTGGGGATAACAGGCTTATCTCCCCCAAGA
GTTCACATCGACGGGGAGGTTTGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGTA
GTCGGTCCCAAGGGTTGGGCTGTTCGCCCATTAAAGCGGCACGCGAGCTGGGTTCAGAAC
GTCGTGAGACAGTTCGGTCCCTATCCGTCGCGGGCGTAGGAAATTTGAGAGGATCTGCTC
CTAGACCAGAGGGACATA
BT4-2
GGACGGA**G**AGACCCCATGGAGCTTTACTGCAGTTTGATATTGAGTATCTGTACCACATGT
ACAGGATAGGTAGGAGCCATTGACTTCGGGACGCCCGTTTCGAATGAGGCGTTGTTGGGA
TACTACCCTTGTGTTATGGCTACTCTAACCCAGATAGGTTATCCCTATCGGAGACAGTGT
CTGACGGGCAGTTTGACTGGGGCGGTCGCCTCCTAAAGAGTAACGGAGGCGCCCAAAGGT
TCCCTCAGATTGGTTGGAAATCAATCGCAGAGTGTAAAGGTATAAGGGAGCTTGACTGCG
AAGCTACAACTCGAGCAGGGACGAAAGTCGGGCTTAGTGATCCGGTGGTACCGAATGGA
AGGGCCATCGCTCAACGGATAAAAGCTACCCTGGGGATAACAGGCTTATCTCCCCCAAGA
GTTCACATCGACGGGGAGGTTTGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGTA
GTCGGTCCCAAGGGTTGGGCTGTTCGCCCATTAAAGCGGCACGCGAGCTGGGTTCAGAAC
GTCGTGAGACAGTTCGGTCCCTATCCGTCGCGGGCGTAGGAAATTTGAGAGGATCTGCTC
CTAGACCAAAAGGGACAAAGCCCGCTCTTTCCCGGAACCACTCCCGCCTGGGGCCCCCCC
ATCCCTAACGCTCGGCCCTCTCCCCTGGGCCTGTCATTTTTGTCTA

Fig. 2: The sequences of mutated operons of BT3 and BT4. The bold letters are showing the mutated ones.

Table 1: MICs (µg/ml) of Erythromycin and Penicillin for *Streptococcus pyogenes* isolates

Isolated strains	Erythromycin	Penicillin
BT1	0.40	0.001
BT2	0.1	0.001
BT3	8.20	0.004
BT4	12.40	0.002
BT5	1.20	0.001
BT6	0.10	0.001
BT7	0.40	0.002
BT8	2.40	0.001
BT9	0.80	0.004
BT10	0.60	0.001
BT11	0.40	0.001
BT12	3.00	0.004
BT13	0.60	0.002
BT14	0.20	0.002
BT15	0.40	0.001
BT16	0.10	0.002
BT17	1.60	0.001
BT18	1.20	0.002
BT19	0.80	0.001
BT20	0.20	0.002
BT21	0.20	0.001
BT22	0.40	0.002
BT23	0.40	0.001

Discussion

Macrolide resistance usually results from over use of these drugs for treatment of pharyngitis. It also could be a result of the unsuccessful treatment of infections with macrolide antibiotics and self-treating without physician prescription (1, 4, 6). Three types of erythromycin resistance mechanisms in *S. pyogenes* have been reported. The first ones that modify the ribosome, which is target of the antibiotic; the second ones modify the antibiotic itself; and the last ones that affect the rate of transport of the antibiotic across the cell membrane. Target modification is performed by methyl transferase enzymes encoded by the *erm* (erythromycin resistance methylase). Other groups of the resistance genes are the genes increase macrolide transportation from cells of *S. pyogenes*. These genes were known as *mef* (macrolide efflux) and believed to encode a membrane protein that pumps macrolides from the interior of the cell (2, 5, 7, 8).

The major component of macrolide binding site on the ribosome is a 2058 and several neighboring positions in the central loop of domain V. Mutations of this part of rRNA confer resistance to erythromycin. These mutations reduce the affinity of macrolides for the ribosome. In addition, several reports were shown the mono-or dimethylation of the A2058 dramatically reduces the affinity of various macrolides (2, 5, 9). However, it should be mentioned that some other mutations have been also reported in domain V of 23S rRNA as a cause of macrolide resistance in S*treptococcus pyogenes* (9- 11).

In this study in young Iranian population, we found a number of point mutations in position 2059 (A to G) of two operons of BT_3 three operons of BT_4, which are in agreement with the previous studies (2, 5).

The other point mutation in position 2529 (G to A) was detected just in one operon in BT_3. It might be involved in erythromycin resistance but is not proved experimentally. Conclusively the data obtained in this study demonstrates different point mutations in addition to the reported positions involve in erythromycin resistance of the strains isolated from Iranian children.

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The authors declare that they have no conflict of interests.

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