

Full Length Research Paper

Molecular screening for erythromycin resistance genes in *Streptococcus pyogenes* isolated from Iraqi patients with tonsillo-pharyngites

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Streptococcus pyogenes is the leading cause of uncomplicated bacterial pharyngitis and tonsillitis commonly referred to as strep throat. Erythromycin is administered for patients allergy to penicillin. In this study, 125 throat swab samples were collected from children between 2-12 years old with tonsillo-pharyngitis attended to at the AL-Imammain AL-Kadhimain Medical City-Baghdad-Iraq and Pediatric Caring Hospital-Baghdad-Iraq from February 2014 to February 2015. Only 72 throat swab samples showed bacterial growth. The isolates were identified using Vitek 2 Compact system for Gram-Positive. Antibiotics susceptibility was examined using the BioMérieux Vitek2 compact system AST card. For direct molecular identification of *S. pyogenes*, 16S rRNA and 16S-23S rRNA gene amplification were used. Molecular screening for erythromycin resistance genes *erm(A)*, *erm(B)* and *mef(A)* were done using PCR. The results of identification using Vitek2 GP show that 21 (29.2%) samples were *S. pyogenes*-positive while 51(70.8%) of samples were due to other causes of tonsillo-pharyngitis. The results of molecular identification of *S. pyogenes* strains using 16S rRNA and 16S-23S rRNA amplification showed that only four strains were positive for 16S-23S rRNA, while two strains out of four were also positive for 16S rRNA. According to the results of antibiotic sensitivity, there were seven strains resistant to erythromycin. The results of molecular screening for erythromycin resistant genes showed that all these resistant strains did not contain the resistant genes *erm(A)*, *erm(B)* or *mef(A)*. We conclude that, maybe there was a specific sequence variations in genes used for identification of *S. pyogenes*. Also, resistance to erythromycin could be attributed to causes other than the studied mutations, such as structural modification of erythromycin by phosphorylation, glycosylation or lactone ring cleavage by erythromycin esterase.

Key words: *Streptococcus pyogenes*, molecular identification, erythromycin resistance genes.

INTRODUCTION

Streptococcus pyogenes is the leading cause of uncomplicated bacterial pharyngitis and tonsillitis commonly referred to as strep throat. It causes up to 15 to 30% of cases of acute pharyngitis that occurs in children in the age between 5 to 15 years. Other respiratory infections include sinusitis, otitis, and pneumonia. Also, it causes

skin infections and post-streptococcal sequel, rheumatic fever, glomerulonephritis that may follow streptococcal diseases, and occur in 1 to 3% of untreated infections (Cunningham, 2000; Carapetis et al., 2005; Tart et al., 2007). Accurate diagnosis is essential for appropriate antibiotic selection. Penicillin should be a first choice of

antibiotics in acute tonsillitis while macrolides such as erythromycin is reserved for patients allergic to penicillin. Ketolides such as telithromycin have the activity against *S. pyogenes* which is resistance to erythromycin (Ben Zakour et al., 2012; Shulman et al., 2012). The mechanisms of action of erythromycin involve the inhibition of bacterial protein synthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl-tRNA. The action is bacteriostatic, but can also be bactericidal in high concentrations (Giguere, 2013). Resistance to erythromycin in *S. pyogenes* can be caused by the following main mechanisms: 1) modification of the 23S rRNA by rRNA adenine-N6-methyltransferase encoded by horizontally acquired *erm A* and *erm B* (Jasir et al., 2000; Giovanetti et al., 2003; Albrich et al., 2004; Farrell et al., 2006; Brenciani et al., 2007); 2) active drug efflux via a trans-membrane pump encoded by horizontally acquired *mef*. This mechanism is mediated by a membrane-associated protein that pumps the antibiotic out of the cell, keeping intracellular concentrations low and preventing the binding of antibiotics to the ribosome (Nord et al., 2004; Del Grosso et al., 2011; Giovanetti et al., 2012; Giovanetti et al., 2003) mutations comprising a change in domain V of 23S rRNA as a result of a mutation in all the six copies of *rRNA* gene (Bingen et al., 2002). Additional mechanisms of erythromycin resistance include structural modification of erythromycin by phosphorylation (Davies and Davies, 2010), glycosylation (Hawkey and Jones, 2009), and lactone ring cleavage by erythromycin esterase (Levi and Marshal, 2004).

The 16S rRNA and 23S rRNA are targets for identification of microorganisms at the species, genus or family level. These genes contain both conserved regions and areas of variability sufficient for specific identification of bacteria. The ribosomal intergenic spacer region (ISR), a stretch of DNA that lies between the 16S rRNA and the 23S rRNA subunit genes, proved to be much more variable than the adjacent 16S and 23S ribosomal genes and this region can be used as method of differentiation of many species within genus and as method of identification of certain bacteria (Hassan et al., 2003). In this study, molecular identification of *S. pyogenes* using 16SrRNA and 23SrRNA and screening for erythromycin resistance genes was performed.

MATERIALS AND METHODS

Samples collection

One hundred and twenty five throat swab samples were collected from children between 2 to 12 years old with tonsillo-pharyngitis infections attended to by the AL-Imammain AL-Kadhimain Medical

city-Baghdad-Iraq and Pediatric Caring Hospital-Baghdad-Iraq from February 2014 to February 2015. Throat swab samples were taken according to clinical evaluation recommendation of physicians (Vandepitte et al., 2003). Information from patient parent was taken including age, sex, duration of infection, previous treatment and stage of throat infection (acute or chronic). The study protocol was approved by The Ethical Committee of College of Medicine-Al Nahrain University.

Identification of *S. pyogenes*

Throat swab sample was cultured on blood agar plate that was incubated aerobically with 5 to 10 % CO₂ at 37°C for 18 to 24 h in a candle jar (Vandepitte et al., 2003). The blood agar plates were examined for morphology and cultural characteristic that include appearance of colonies and beta-hemolytic zone around colonies on blood agar plate. For purification, growing beta-hemolytic streptococci was inoculated on sodium azide media, which is consider as a selective agar used for the selective isolation of *S. pyogenes*. Also, catalase test, microscopical examination of Gram stain and Bacitracin sensitivity test were done (Vandepitte et al., 2003). The isolates were identified with Vitek 2 Compact system for Gram-Positive Identification, card 2GP (bioMérieux-France).

Antibiotics susceptibility assay

Minimal inhibitory concentration (MIC) and antibiotics susceptibility were examined using the BioMérieux Vitek2 compact system AST card (bioMérieux-France) according to manufacturer instructions. It is an automated colorimetric method used for identification of bacteria and for detection susceptibility of bacterial isolates against different type of antibiotics. A suspension of overnight pure culture of *S. pyogenes* was prepared by transferring sufficient quantity of bacterial colonies to 3 ml of sterile saline (0.45%NaCl). The turbidity was adjusted to (0.5-0.63) MacFarland turbidity range and measured using a turbidity meter. Then, the suspension was transferred to the apparatus which contain the card that loaded with 9 type of antibiotics included erythromycin, as indicated in Table 1. Measurement of MIC and sensitivity were done using optical system inside the apparatus and the result that was obtained after 18 h of incubation were computerized analyzed which referred to the MIC and whether this isolate had sensitivity, intermediate sensitivity or resistant to each antibiotic found in the card.

Identification of *S. pyogenes* strains using PCR

Genomic DNA was extracted from *S. pyogenes* strains using WIZARD Genomic DNA Extraction Kit (Promega, USA) following manufacture instructions. For direct molecular identification of *S. pyogenes*, 16S rRNA and 16S-23S rRNA were used. Two primer sets were used for molecular identification of *S. pyogenes* (Table 2) (Nandi et al., 2008). Briefly, two PCR master mixes (final volume 25 µl per reaction) were prepared, one for each gene as in the following: (final concentration per one réaction): 1XPCR buffer (Promega, USA), 200 µm dNTPs (Promega, USA), 100 pmol of each forward and reverse primers (Alpha, USA) and 1.25 U/reaction of GoTaq DNA polymerase (Promega, USA). Two microliters (equivalent to 100 ng) of DNA was added for each reaction tube, except the no template control tube (NTC). PCR

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Table 1. Vitek2 GP susceptibility cards contents.

Antibiotic	Concentrations µg/ml	Calling range	
		≤	≥
Ampicillin	0.5,1,4,8	0.25	16
Benzylpenicillin	0.06,0.12,0.5,2	0.06	8
Cefotaxime	0.25,0.5,1,2	0.12	8
Ceftriaxone	0.12,0.25,1,4	0.12	8
Clindamycin	0.12,0.25,0.5	0.25	1
Erythromycin	1,2,4,16	0.12	8
Levofloxacin	1,2,4,16	0.25	16
Tetracycline	0.12,0.25,1,4	0.25	16
Trimethoprim/ Sulphamethaxazole	8/152,16/304,64/1216	10	320

Table 2. Primer sequences and molecular size used in molecular identification of *S. pyogenes*.

Gene	Sequence of forward primer	Sequence of reverse primer	Product size (bp)
16S rRNA (A)	5'AAGAGTTTGATCCTGGCTCAG3'	5'GGTTACCTTGTTACGACTT3'	1500
16-23S rRNA (B)	5'TTGTACACACCGCCCGTCA3'	5'GGTACCTTAGATGTTTCAGTTC3'	800

Table 3. Primer sequences and molecular size used in erythromycin resistance genes.

Gene	Sequence of forward primer (5' - 3')	Sequence of reverse primer (5' - 3')	Product size (bp)
<i>erm (A)</i>	AGAAGGTTATAATGAAACAGA	GGCATGACATAAACCTTCAT	260
<i>erm(B)</i>	GAAAAGGTACTIONCAACCAAATA	AGTAACGGTACTTAAATTGTTTAC	640
<i>mef(A)</i>	AGTATCATTAATCACTAGTGC	TTCTTCTGGTACTAAAAGTGG	350

reaction tubes were transferred into thermal cycler (ependrof, Germany) that was programmed as following: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 49°C for Gene A and 55°C for Gene B for 2 min, 72°C for 2 min. Final extension was done at 72°C for 10 min. The selection of optimum annealing temperature came after multiple optimization experiments. PCR products (10 µl from each) were resolved by 1% agarose gel electrophoresis.

Molecular screening for erythromycin-resistant genes in *S. pyogenes*

The sequence of oligonucleotide primers sets used in PCR reactions to amplify resistance genes *erm(A)*, *erm(B)* and *mef(A)* are shown in Table 3. PCR reaction was done according to Morosini et al. (2003). Optimization for annealing temperature was done at 52°C.

Statistical analysis

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Nominal data were expressed as number and percent. Fischer Exact test was used for comparison of frequency. P-value less than 0.05 were considered significant.

RESULTS

Isolation and identification of *S. pyogenes*

This study was carried out in 125 throat swabs isolated

from throat of children from 2 to 12 years old. Fifty three of total samples were excluded from this study because no bacteria were isolated. The remaining 72 samples were identified using bacteriological test. Also, commercial Vitek2GP identification card was used and the identifications probabilities were ranged from 86 to 99%. The results obtained by using Vitek2 GP identification system showed that 21 (29.2%) samples were *S. pyogenes* positive and 51(70.8%) samples were due to other causes of tonsillo-pharyngitis as shown in Table 4. The results of molecular identification of *S. pyogenes* strains using 16SrRNA and 16S-23SrRNA showed that only four strains were positive for 16S -23SrRNA, while two strains out of four were also positive for 16S rRNA, as seen in Figure 1.

Distribution of streptococci throat infection in disease phases acute and chronic tonsillopharyngitis

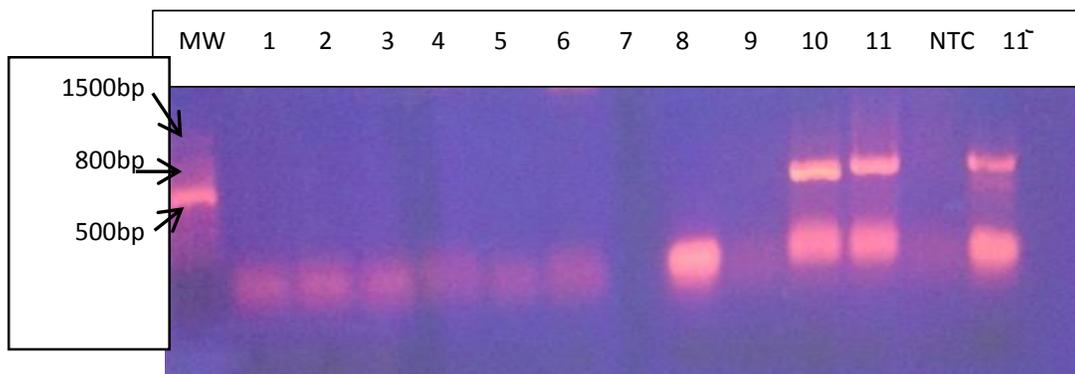
In this study, the number of isolates from acute cases were 5 isolates which represent (23.81%) of total cases while from chronic cases were 16 isolates which represent (76.19%) of total cases.

Antimicrobial susceptibility patterns of *S. pyogenes*

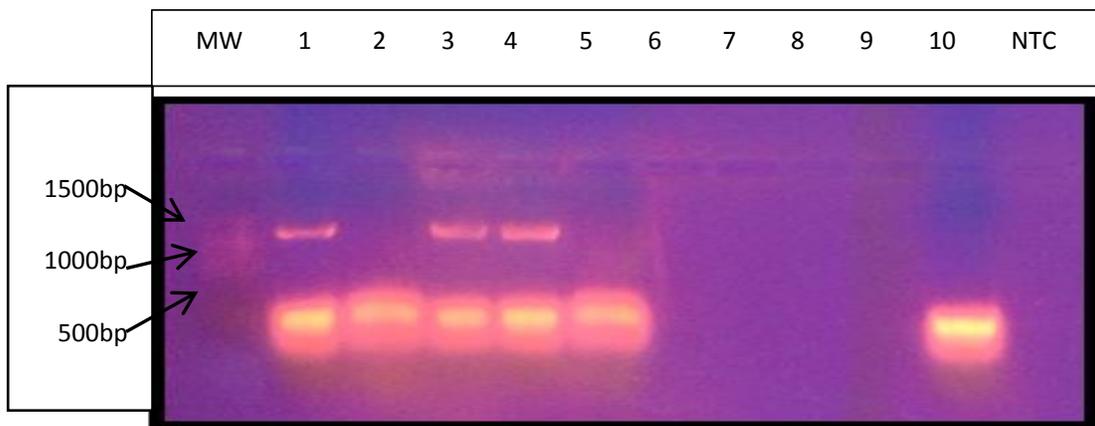
The antimicrobial susceptibility patterns of *S. pyogenes*

Table 4. Type and number of bacterial strains isolated from children with tonsillopharyngitis by using Vitek identification kit.

Throat swab bacteria isolated	Number of strains	Percentage (%)
<i>Streptococcus pyogenes</i>	21	29.2
<i>Streptococcus agalactiae</i>	2	2.8
<i>Streptococcus pneumonia</i>	4	5.6
<i>Streptococcus mitis</i>	2	2.8
<i>Streptococcus parasanguinis</i>	2	2.8
<i>Staphylococcus aureus</i>	41	56.8
Total	72	100



(A)



(B)

Figure 1. Agarose gel electrophoresis of amplified products of identification genes of *S. pyogenes*. **(A)** Agarose gel electrophoresis of amplified products of *16-23SrRNA* of *S. pyogenes*. Lane MW, molecular weight ladder of 100 bp. lane 1, 2, 3 to 9: negative results of *16-23S rRNA* (800 bp); lane 10, 11 and 11' (as double): amplified products of *16-23S rRNA* (800 bp); lane NTC, no template control. **(B)** Agarose gel electrophoresis of amplified products of *16S rRNA* of *S. pyogenes*; lane MW, molecular weight ladder of 100 bp; lane 1, 3, 4: amplified products of *16SrDNA* (1500 bp); lane 2, 5 to 10, negative results of *16SrRNA* (1500 bp); lane NTC: no template control.

Table 5. The percentage of sensitivity patterns of *S. pyogenes* isolates against different types of antibiotics.

Antibiotic	Sensitive		Resistant		Intermediate	
	Number	%	Number	%	Number	%
Benzyl Penicillin	21	100	0	0	0	0
Ampicillin	21	100	0	0	0	0
Cefotaxime	8	38.09	13	61.90	0	0
Ceftriaxone	8	38.09	13	61.90	0	0
Levofloxacin	20	95.23	0	0	1	4.76
Erythromycin	11	52.38	7	33.33	3	14.28
Clindamycin	15	71.43	6	28.57	0	0
Tetracycline	10	47.62	11	52.38	0	0
TMS	21	100	0	0	0	0

strains against various types of antibiotics according to Vitek 2 system were shown in Table 5.

Molecular screening for erythromycin resistant genes in *S. pyogenes*

According to the results of antibiotic sensitivity obtained by using Vitek2 AST system, there were seven strains resistant to erythromycin. The results of molecular screening for erythromycin resistant genes showed that none of these resistant strains have the resistant genes *erm(A)*, *erm(B)* or *mef(A)*.

Telithromycin activity against erythromycin resistant strains of *S. pyogenes*

The result of sensitivity test of telithromycin disc against erythromycin-resistant strains of *S. pyogenes* showed that 6 out of 7 strains have full sensitivity to Telithromycin disc with MIC value of ≤ 0.5 $\mu\text{g/ml}$ while the remaining resistant strain showed intermediate sensitivity against this antibiotic with MIC value range from 1 to 2 $\mu\text{g/ml}$.

DISCUSSION

Identification of *S. pyogenes*

The important cause of the tonsillitis is bacterial and viral causes and about 30 to 40% of bacterial tonsillitis cases are caused by *S. pyogenes* (Abd Al-Kareem et al., 2004). Result of this study showed that from 72 patients with tonsillo-pharyngitis, 21 strains indicated the presence of *S. pyogenes*, 2 strains of *Streptococcus agalactiae*, 4 strain of *Streptococcus pneumoniae*, 2 strains of *Streptococcus mitis*, 2 strains of *Streptococcus parasanguinis* and 41 strain of *Staphylococcus aureus*. Kurien et al. (2000) and Wessels (2011) showed that the

most common bacterial pathogens in the upper respiratory tract infection were *S. pyogenes* and *S. aureus*. In addition to identification by using Vitek2, *S. pyogenes* was identified using 16S rRNA and 16S-23S rRNA. The result showed that 4 strain out of 21 carried 16S-23S rRNA, 2 out of these 4 strains additionally carried 16S rRNA. The absence of identification genes in the remaining *S. pyogenes* strains may be due to the genetic variations. It was referred to that the absence of intra-species genetic variation at 16S rRNA subunit but documented variation in inter-genic 16S-23S spacer region (Clarridge, 2004; Petti et al., 2005; Nandi et al., 2008; Lal et al., 2011).

In this study, the percentage of *S. pyogenes* that caused chronic tonsillopharyngitis was 76.19%; this result was significantly higher than the results of Afaf et al. (2004) in Egypt who found that (18.5%) of the *S. pyogenes* strains were responsible for chronic tonsillopharyngitis. The low incidence of streptococcal tonsillopharyngitis in present study may be due to the relatively small number of the throat samples collected from patient with tonsillo-pharyngitis.

Antibiotic susceptibility patterns of the *S. pyogenes* using Vitek2 AST system

In this study, the susceptibility test of *S. pyogenes* strains using Vitek2 AST showed that all *S. pyogenes* strains were susceptible to penicillin group and penicillin remain the drug of choice for treatment of streptococcal pharyngitis, because the circumstances favorable for the development of resistance have not yet occurred, because this antibiotic is out of use in clinical practice in Iraq nowadays and the preference of the newest antibacterial drugs for prescription, as well as inefficient mechanisms for genetic transfer or barriers to DNA uptake and replication and β -Lactamase may not be expressed or may be potentially toxic to *S. pyogenes* (Malhotra-Kumar et al., 2005; Ramalhinho et al., 2012;

Rubio-Lopez et al., 2012). Also, it could be that PBP_s of *S. pyogenes* contain no lengthy regions of similarity with genes from other streptococci, making it unlikely that the acquisition of penicillin resistance arises by homologous recombination with genes from other species (Ferretti et al., 2001). This study shows that the susceptibility of strains to cefotaxime and ceftriaxone was 38.09% which is due to the extensive and random prescribing of these antibiotics before doing culture and sensitivity test, as well as those antibiotics in Iraq are supplied as over counter medicines in private pharmacies against the regulations. The results obtained by Young et al. (2004) in South Korea, Oliver et al. (2007) in Spain and Huang et al. (2014) in Taiwan, shows that the susceptibility of *S. pyogenes* to cefotaxime and ceftriaxone was 100%.

In patients who are allergic to penicillin, macrolides such erythromycin and lincosamides such as clindamycin are alternative treatment choices (Shulman et al., 2012). In this study, the percentages of resistance of clindamycin and erythromycin for *S. pyogenes* were 28.75 and 33.33%, respectively. Huang et al. (2014) showed that the resistance to clindamycin and erythromycin were 2.1 and 16.4%, respectively. Other studies referred to low percentages of erythromycin resistance such as in America (8.6%), Asia-pacific region (10.9%), Europe (9.7%) and Latin America (2.7%) (Gordon et al., 2002). Shibl (2005) in Saudia Arabia showed that the resistant was only 6.3%, and similar percentages (4 to 10%) have been reported in Germany, UK, Portugal, Greece and Canada. The level of erythromycin resistance among strains was low which may be related to the low consumption of macrolides in these regions, or may be due to the absence of clonal spread of erythromycin-resistant strains (Silva-Costa et al., 2012). The high percentage that is found in the current study could be attributed to high misuse of antibiotic in Iraq.

Screening for erythromycin resistant gene

Increases in macrolide resistance have been reported and the rapidly growing problem of antibiotic resistant *S. pyogenes* is increasing (Ray et al., 2010). In Iraq, information regarding the screening for erythromycin resistant genes of *S. pyogenes* strains was largely loosing. In this study, there were no strains related to *S. pyogenes* carrying the resistant genes. Bingen et al. (2000) referred that the predominance of a particular resistance genotype among macrolide-resistant strains were *mefA* in Spain (97% of 437 strains), Belgium (84% of 131 strains), Germany (56% of 54 strains) and Canada (92% of 72 strains) and *ermB* in France (55% of 93 strains). Richter et al. (2005) refereed to that of the population of macrolide-resistant *S. pyogenes* strains in the United States comprises similar proportions of strains containing *mefA* (43%) and *ermA* (46%), with a smaller fraction of strains having *ermB* (8.5%) and considerable variation among regions. A study by Dundar et al. (2010)

in Turkey show that of 3 of 11 erythromycin resistant strains of *S. pyogenes* did not have *erm(A)*, *erm(B)*, and *mef(A)* and this may be due to the ribosomal mutations.

Effect of telithromycin on erythromycin resistant *S. pyogenes*

In this study, 6 out of the 7 erythromycin resistant strains had high sensitivity to telithromycin with MIC value \leq 0.5 μ g/ml while the remaining strain show intermediate resistance with MIC value range between 1 to 2 μ g/ml. Such results may justify the effectiveness of this antibiotic as alternative of erythromycin in the treatment of streptococcal pharyngitis (Camara et al., 2013). Telithromycin was more active than 14 and 15 membered ring macrolides (azithromycin and clarithromycin) against erythromycin resistant *S. pyogenes* strains (Jalava et al., 2001). Telithromycin show good activity against clinical *S. pyogenes* isolates including erythromycin A-resistant strains harboring the *erm(A)* or *mef(A)* (efflux) genotype.

Conflict of interests

The author(s) did not declare any conflict of interest.

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