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ORIGINAL ARTICLE

Erythromycin Resistance in *Streptococcus pyogenes* causing Pharyngo-tonsillitis in Egyptian Children and its Association with the Presence of Fibronectin Binding Protein F1 (*prtf1*) Gene

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Key words:

Macrolide, Clindamycin, PCR, Virulence, Pediatrics

ABSTRACT

Background: *Erythromycin resistance among Streptococcus pyogenes (S. pyogenes) has been reported with alarming rates throughout the world. Fibronectin-binding proteins, especially F1 (PrtF1) encoded by prtF1 gene have been implicated in mediating internalization of S. pyogenes within epithelial cells.* ***Objectives:*** *The current study aimed to detect erythromycin resistance among S. pyogenes causing acute pharyngo-tonsillitis in Egyptian children and to explore the association between erythromycin resistance and the presence of prtf1 gene.* ***Methodology:*** *Throat swabs were collected from 100 pediatric patients with age ranging from 5 to 12 years and suffering from pharyngo-tonsillitis. S. pyogenes was recovered from samples and identified by conventional methods. In-vitro susceptibility of S. pyogenes isolates to different antimicrobials was done using disk diffusion method. Minimum inhibitory concentration for erythromycin was determined using E test strips. The phenotype of macrolide resistance was determined using the double-disk test. The presence of prtF1 gene was detected by polymerase chain reaction.* ***Results:*** *S. pyogenes were isolated from 45% (45/100) of the patients' throat swabs. Erythromycin resistance was detected in 11 (24.4%) isolates, among which 5 (45.5%) exhibited the M phenotype of macrolide resistance; 4 isolates had the constitutive phenotype of resistance while 2 isolates had the induced phenotype. prtF1 gene was detected in 16/45 isolates (35.6%). There was statistically significant association between prtF1 gene presence and erythromycin resistance (OR: 4.8611; 95% CI: 1.14-20.63, p= 0.032).* ***Conclusion****: The present study provides a preliminary idea about the frequency of macrolide resistance and contribute to a better understanding of the pathogenesis of S. pyogenes causing pharyngo-tonsillitis in Egyptian children.*

INTRODUCTION

Streptococcus pyogenes (S. pyogenes) is a group A Streptococcus (GAS) and is considered an important and common human bacterial pathogen, especially in the pediatric age group [1]. It can cause a wide range of clinical illnesses ranging from pharyngo-tonsillitis and skin infections to more severe manifestations such as bacteremia, toxic shock-like syndrome and necrotizing fasciitis [2–4].

Besides, the immunological sequelae of streptococcal infections in the form of acute rheumatic fever and post-streptococcal acute glomerulonephritis represent a significant streptococcal disease burden in most of the developing countries[5].

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Since the 40s, penicillin has been the drug of choice for GAS infections and in case of severe invasive diseases, high-dose penicillin and clindamycin are recommended [3,6]. Resistance to penicillin has not been described yet[7–9]. However, problems of treatment failures have been reported [10,11]. Macrolides (such as erythromycin, azithromycin, clarithromycin, spiramycin, etc.) and lincosamides (clindamycin) are considered the alternative treatment in patients allergic to β-lactams or in case of failure of β-lactams therapy[9,12]. Unfortunately, resistance has emerged to these efficient agents mainly due to their wide general use. Erythromycin resistance has been noticed since the 90s and has peaked worldwide during the last decade with resistance rates varying between different geographical areas [6,8,13–20].

S. pyogenes expresses resistance to erythromycin by two main mechanisms; the first one is a target site modification, mediated by the *erm* genes which encode production of methylases targeting 23S rRNA. This modification leads to a decreased binding of all macrolides, lincosamides, and streptogramin type B

(MLSB) to their targets on the ribosomal RNA (MLSB phenotype). This type of resistance can either be constitutive (cMLSB phenotype) or induced (iMLSB phenotype) [1,14]. The other resistance mechanism is due to the presence of an active efflux pump, mediated by the *mefA* gene. The efflux pump selectively removes 14 and 15-membered macrolides from the bacterial cell but not 16-membered macrolides or lincosamides leading to M phenotype resistance[6].

Meanwhile, studies have proved that *S. pyogenes* possess the ability to invade epithelial cells and survive for a limited time [2]. More than a dozen *S. pyogenes* surface proteins that are involved in cell adherence and invasion have been named[21]. Fibronectin-binding proteins (FBPs), especially F1 (PrtF1) encoded by *prtF1* gene have been implicated in mediating internalization within epithelial cells[22]. Some studies reported an association between the presence of *prtF1* gene and macrolides resistance [23,24]. Authors thus hypothesized that *S. pyogenes* might be escaping antimicrobials and immune response of the host by invading epithelial cells with the aid of PrtF1. The *prtF1* gene has also been reported to be prevalent among persisting GAS from asymptomatic carriers [2,11].

A noticeable feature present in *S. pyogenes* protein F1 and other high-affinity FBPs is a structure containing tandem repeats found adjacent to the conserved C-terminal domain. PrtF1 in particular has got two fibronectin-binding domains. The one located towards the C-terminus, repeat domain type 2 (RD2), was documented to have a variable number of repeats, ranging from 1 to 5 [2] yet, this feature is not related to their ability of binding fibronectin [25].

Accordingly, the RD2 region of *prtF1* gene is expected to consist of variable number of repeats and a polymerase chain reaction (PCR) analysis of this region should yield products of different sizes ranging from approximately 125 to 570 bp[25].

Owing to the rare local data about erythromycin resistance in Egypt, this study was conducted to detect erythromycin resistance among *S. pyogenes* causing acute pharyngo-tonsillitis in Egyptian children and to explore the association between erythromycin resistance and the presence of *prtf1* gene.

METHODOLOGY

1. Patients:

Throat swabs were collected from 100 pediatric patients with age ranging from 5 to 12 years presenting to the Outpatient Clinic of Ain Shams University (ASU), Children's Hospital with symptoms of pharyngo-tonsillitis during the period from October, 2014 to April, 2015.

All patients in the study were subjected to complete physical examination and full history taking. Patients who had received antimicrobials before sampling or who had used antibiotics within the two weeks' period prior to the current illness were excluded from the study.

All patients have participated in the study after obtaining informed consents from their parents or their legal guardians. The work has been approved by ASU Ethics Committee and in accordance with the ethical guidelines of the Declaration of Helsinki, 1975.

2. Isolation and Identification of *S. pyogenes*:

S. pyogenes isolates were recovered from throat swabs and identified in accordance with standard laboratory procedures[26] at the Medical Microbiology and Immunology department, Faculty of Medicine, ASU. Briefly, throat swabs were immediately inoculated onto 5% defibrinated sheep blood agar, and plates were incubated overnight at 37°C in 5% CO_2. *S. pyogenes* were identified on the basis of colony morphology, β-hemolysis, Gram's stain, catalase test, bacitracin disc test (0.04 U) (Oxoid, UK) and by a commercial identification system (RapID STR system, Remel, UK) according to the manufacturer's instructions. GAS confirmation was performed by agglutination with specific Group-A streptococci antisera (Slidex Strepto-kit; BioMérieux, France).

3. Antimicrobial Susceptibility Testing:

The in-vitro susceptibility pattern of *S. pyogenes* isolates to different categories of antimicrobials was determined using the disk diffusion method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI)[27]. Erythromycin (15ug), azithromycin (15ug), clarithromycin (15ug), clindamycin (2ug), chloramphenicol (30 ug), penicillin (10 U), levofloxacin (5ug), tetracycline (30ug) and vancomycin (30 ug) disks were used (Bioanalyse, Turkey).

E test strips (Liofilchem, Italy) were used to determine the minimum inhibitory concentrations (MIC) for erythromycin and the results were also interpreted according to the CLSI recommendations [27].

4. Phenotypic Determination of Macrolide Resistance:

The phenotype of macrolide resistance was determined by the double-disk test as described previously[28]. Briefly, erythromycin (15 ug) and clindamycin (2 ug) disks were placed 12 mm apart, on Mueller-Hinton agar (Oxoid, UK) supplemented with 5% sheep blood agar that was inoculated with a 0.5 McFarland suspension of the organism. After overnight incubation at 37°C, the absence of a significant zone of inhibition around the two disks indicated constitutive resistance (cMLSB phenotype), blunting of the clindamycin zone of inhibition proximal to the erythromycin disk indicated inducible resistance (iMLSB phenotype), and susceptibility to clindamycin without blunting of the inhibition zone around clindamycin disk indicated the M phenotype of resistance.

5. Detection of *prtF1* gene by PCR:

Total genomic DNA was isolated from *S. pyogenes* isolates with QIAamp DNA Mini Kit (Qiagen, California) according to manufacturer's instructions. DNA primer pairs complementary to the flanking region of RD2 in the *prtf1* gene were used : (5'-TTTTCAGGAAATATGGTTGAGACA and 5'-TCGCCGTTTCACTGAAACCACTCA)[29]. PCR amplification was performed on a DNA thermal cycler (Thermo PxE 0.21, England) as previously described [30]. The PCR tests were done in a final volume of 50 µl reaction mix; 25 µl of a 2x QIAgen HotStar Taq Master Mix (Qiagen, California), 11 µl of RNase-free water, 2µl of each primer, and 10 µl of tested DNA. Negative control (QIAgen RNase-free water) was included in each PCR run. QIAgen Taq DNA polymerase activation was performed by incubation at 95°C for 15 min, followed by 35 cycles at 94°C for 40 seconds, 64°C for 1 minute, and 7°C for 2 minutes. Amplified DNA fragments were separated on 2.0% agarose by gel electrophoresis and then visualized by ethidium bromide staining. The number of RD2 repeats of *prtF1* was determined on the basis of the amplicon size [31] (Figure 1).

6. Statistical analysis

Statistical Package for Social Science (IBM SPSS) version 20 was used for data collection and entry. Qualitative data were presented as number and percentages and compared by using the Chi-square test. The confidence interval was set to 95% and p-value was considered non significant at the level of > 0.05, significant at the level of > 0.05 and highly significant at the level of < 0.01.

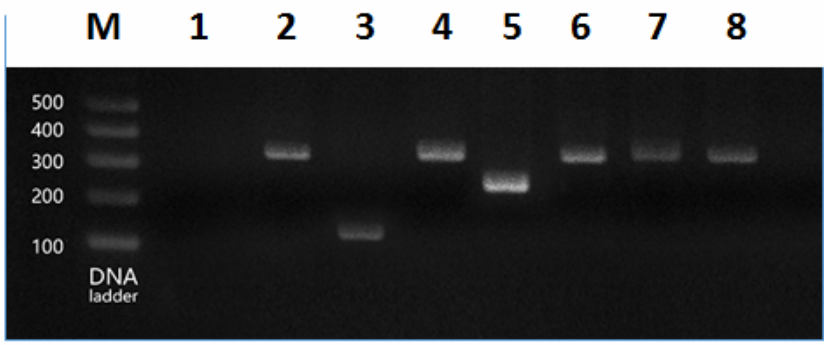

Fig. 1. Size variation of PCR products of *prtF1* gene among some *S. pyogenes* isolates (representative gel)
M: Molecular size marker (ladder)
Lane 1: negative control
Lanes 2, 4, 6, 7 and 8: amplicon size of 349 bp, suggesting the presence of three RD2 repeats
Lane 3, amplicon size of 127 bp, suggesting the presence of a single RD2 repeat
lane 5, amplicon size of 238 bp, suggesting the presence of two RD2 repeats

RESULTS

Of the 100 patients who participated in the study, 46 were males and 56 were females, ranging in age from 5 to 12 years (mean ± SD, 7.32 ± 1.78 years). *S. pyogenes* were isolated from 45% (45/100) of the patients' throat swabs.

Patients who had positive throat swab cultures for *S. pyogenes* were 21 (46.7 %) males and 24 (53.3 %) females.

The results of in-vitro antimicrobial susceptibility testing of 45 *S. pyogenes* isolates are shown in table 1.

Erythromycin resistance was detected in 11 (24.4%) isolates (MIC range; 96->256µg/mL). All erythromycin resistant isolates were resistant to azithromycin and clarithromycin. Four isolates (8.9%) showed resistance to clindamycin.

All *S. pyogenes* isolates (100%) were susceptible to penicillin, levofloxacin and vancomycin.

Among the 11 erythromycin resistant isolates, 5 (45.5%) exhibited the M phenotype of macrolide resistance; 4 isolates had the cMLSB phenotype of resistance while 2 isolates had the iMLSB phenotype (Table 2).

prtF1 gene was detected in 16/45 isolates (35.6%) among which 7 (43.75%) isolates were resistant to erythromycin.

There was statistically significant association between *prtF1* gene presence and erythromycin resistance (OR: 4.8611; 95% CI: 1.14-20.63, *p*= 0.032) (Figure 2).

Patients' sex did not show significant association with either erythromycin resistance or *prtF1* gene presence (data not shown).

Table 1: Susceptibility of *S. pyogenes* isolates (n=45) to nine antimicrobial agents

Antimicrobial agent	*Susceptible*	*Intermediate*	*Resistant*
Erythromycin	34 (75.6%)	-	11 (24.4%)
Clarithromycin	34 (75.6%)	-	11 (24.4%)
Azithromycin	34 (75.6%)	-	11 (24.4%)
Clindamycin	41 (91.1%)	-	4 (8.9%)
Chloramphenicol	34 (75.6%)	3 (6.7%)	8 (17.8%)
Tetracycline	30 (66.7%)	-	15(33.3%)
Penicillin	45 (100%)	-	0 (0.0%)
Levofloxacin	45 (100%)	-	0 (0.0%)
Vancomycin	45 (100%)	-	0 (0.0%)

Data are presented as number (percentage)

Table 2: Phenotypic characteristics of erythromycin resistant isolates (n=11)

Phenotype	*Number*	*Percent*
M phenotype	5	45.5%
cMLSB phenotype	4	36.4%
iMLSB phenotype	2	18.2%

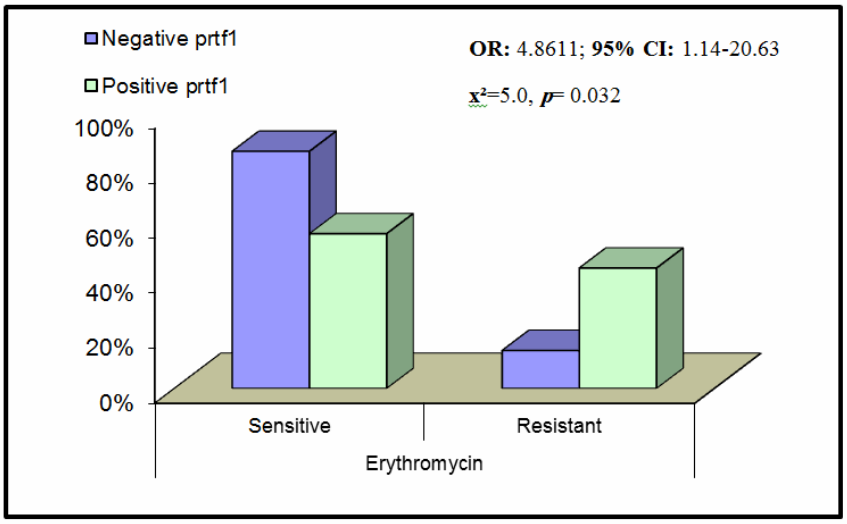

Fig. 2: Association between *prtF1* gene and erythromycin resistance OR, odds ratio, CI, confidence interval

DISCUSSION

This study included 100 children suffering from acute pharyngo-tonsillitis whose age ranged from 5 to 12 years. This age selection owes to the fact that GAS as a cause of pharyngitis is most commonly observed in this age group [32]. *S. pyogenes* could be recovered from 45% (45/100) of the patients' throat swabs. Comparable rates of detection of *S. pyogenes* were reported in other studies that focused on the same age group [33,34].

Erythromycin resistance was detected in 11 (24.4%) of the *S. pyogenes* isolates. We could find one study addressing macrolide resistance among *S. pyogenes* causing pharyngo-tonsillitis in Egyptian children[33], in which, the authors reported 70% macrolide resistance rate which is a much higher rate than ours. This difference could be attributed to that 92.8% of macrolide resistant strains in that study were isolated from children who had a history of recurrent attacks of pharyngio-tonsillitis and had previously received multiple macrolides treatment courses.

Generally, rates of macrolide resistance vary considerably among *S. pyogenes* strains isolated in various geographical areas and over time. Comparable rates of resistance to ours were documented in Korea (23%) 16 and Canada (30%) [35] for instance. On the other hand, our rates far exceeded those reported from Turkey (2.3%)[36] Chile (3.5%)[37], Romania (5%)[38] Lebanon (6%) [19] Serbia (6.8%) [17] and France (7%)[12]. In contrast, much higher rates of erythromycin resistance have been documented in China (97%) [39].

Interestingly, decline in rates of macrolides resistance were reported in some countries after the implementation of antimicrobial stewardship programs.

In France, rates of macrolide resistance decreased from 22.4% between 2002 and 2003 to 3.2% between 2009 and 2011 in children with acute pharyngitis owing to the decrease in macrolide consumption[40]. With a similar strategy, the rate of macrolide resistance in pediatric *S. pyogenes* isolates in Germany fell drastically from 13.6% (1999–2003) to 2.6% (2005–2009) [41]. In Japan, restricting the use of erythromycin contributed to lowering resistance rates from 30 % to 7.4% [42], while in Taiwan, a decline in resistance rates was observed after the national health insurance system denied reimbursement for the costs of antibiotics for the treatment of acute upper respiratory tract infections that had no evidence of bacterial involvement [43].

In the current study, erythromycin-resistant isolates were also resistant to the other two tested macrolides, azithromycin and clarithromycin, as was reported elsewhere[15,16,33,44].

Clindamycin resistance was encountered in 4 isolates (8.9%) that were also resistant to erythromycin. Comparable rates of resistance to clindamycin were also found in Lebanon (5%) [19], Spain (6.5 %) [45] and Taiwan (11 %) [43].

All *S. pyogenes* isolates (100%) in this study retained full susceptibility to penicillin as had been documented over decades by many researchers [8,12,17,24]. The consistency of this finding had led researchers to hypothesize that the reason behind the clinically documented treatment failures with β-lactams might be the reflection of other factors, such as reinfection, lack of patients' compliance, the intracellular persistence of *S. pyogenes* or β-lactamase produced by adjacent bacteria [15].

S. pyogenes isolates showed also full susceptibility to vancomycin and levofloxacin, that also came in accordance with previous studies [8,15,17,33,38,46].

Tetracycline resistance was noticed in 33.3 % of *S. pyogenes* isolates. Similar rates were documented in European countries [24,47] while a 100 % resistance to tetracycline was observed in Senegal [46].

Phenotypic characterization of macrolide resistance also shows considerable variations among different areas. In the current study, the M phenotype of macrolide resistance was the most common phenotype (45.5%) followed by the cMLSB (36.4) and iMLSB (18.2%) phenotypes. M phenotype predominance was noticed in many countries in Europe [15,17,44,48–51], Canada [52] and Latin America [53]. Fortunately, the 16-membered macrolides, potent against strains of the M phenotype, could still yield better treatment results in diseases caused by *S. pyogenes* than 14-and 15-membered macrolides.

The results of the present study showed that the proportion of isolates harboring the *prtF1* gene was (35.6%) and that was comparable to the proportions generally reported by other studies [11,29,54,55], though it was found to be 70% by Musumeci et al. [30].

Our results support those reported in previous studies [24,30,31] showing that there was a statistically significant association between *prtf1* gene possession and erythromycin resistance in *S. pyogenes*. The ability of the bacteria to internalize pharyngeal cells adds to their virulence capabilities by avoiding host defenses and escaping antimicrobials that are confined to the extracellular space. Indeed, a significant correlation between *prtF1* gene presence and GAS eradication failure was detected by Cocuzza et al. [56]. On the other hand, Baldassarri et al. 31 and Hotomi et al. [57] had noticed that the prevalence of the *prtF1 g*ene was significantly higher in isolates recovered from noninvasive infections than those from invasive infections. An interesting finding, but its explanation remains elusive.

A limiting point in our study is that the genetic basis of erythromycin resistance was not elucidated.

In conclusion, the results of this study should provide a preliminary idea about the magnitude of macrolide resistance and contribute to a better understanding of the pathogenesis of *S. pyogenes* causing pharyngo-tonsillitis in Egyptian children.

Further wider scale surveillance studies and the adoption of antimicrobial stewardship programs on a local institutional and national basis are advisable.

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