emm typing, antibiotic resistance and PFGE analysis of *Streptococcus pyogenes* in Lebanon

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One hundred and three *Streptococcus pyogenes* isolates recovered mainly from streptococcal throat infections in Lebanon were characterized by *emm* and PFGE typing. Thirty-three *emm* types and subtypes were detected among the isolates. PFGE was more discriminatory as a typing method. The prevalent *emm* types were *emm1* (12.6%), *emm22* (8.7%), *emm28* (7.7%), *emm88* (7.7%) and *emm4* (6.8%) and all isolates were susceptible to vancomycin and penicillin G. Ten per cent of the isolates were resistant to erythromycin and 3% were resistant to erythromycin and clindamycin, showing the macrolide–lincosamide–streptogramin B phenotype. The *emm* sequences and PFGE pattern database that were generated in this study will serve as a basis for information for long-term evolutionary and epidemiological studies of local *S. pyogenes* recovered not only in Lebanon, but also in neighbouring countries.

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INTRODUCTION

Streptococcus pyogenes is one of the most clinically important Gram-positive pathogens. *S. pyogenes* causes disease states that vary between mild sore throat to the flesh-eating disease necrotizing fasciitis. *S. pyogenes* causes, other than the acute diseases, a set of severe post-disease sequelae such as rheumatic fever and post-streptococcal glomerulonephritis (Yoonim *et al.*, 2005).

The successful nature of *S. pyogenes* as a pathogen relates to its wide array of virulence factors, be they secreted or cell bound. *S. pyogenes* strains express a multitude of genes coding for DNases, antibiotic resistance and the highly virulent superantigens that cause streptococcal toxic shock syndrome in addition to other nonsuppurative sequelae (Aziz & Kotb, 2008). *S. pyogenes* also exhibits C5a peptidase, streptolysin O and streptolysin S activity, which aid in streptococcal cytotoxicity and virulence (Sagar *et al.*, 2008). The *S. pyogenes* M protein is probably the most notorious virulence factor that this micro-organism possesses. It is a fibrillar cell wall protein that aids in adherence to human cells and prevention of opsonophagocytosis. This protein, due to molecular mimicry within protein sequences found in human tissues, is implicated in rheumatic fever (Guilherme *et al.*, 2006).

S. pyogenes emm typing has become one of the best approaches used in molecular typing of this micro-organism. Many methods have been exploited to best type

S. pyogenes, such as 16S rRNA gene sequencing (Stanley *et al.*, 1995), restriction fragment length polymorphism analysis (Desai *et al.*, 1999), random amplified polymorphic DNA analysis (Seppälä *et al.*, 1994) and PFGE (Chiou *et al.*, 2004). However, sequencing-based *emm* typing by the use of oligonucleotides that target the N-terminus of the M-protein coding gene remains the most practical method of *S. pyogenes* typing (Beall *et al.*, 2000). The reason behind this is that the gene coding for the group A *Streptococcus* M protein contains a hypervariable 5' region that is subject to many single nucleotide polymorphisms, which serves as the basis for *emm* typing *S. pyogenes* isolates (Yoonim *et al.*, 2005).

To our knowledge, this study is the first in Lebanon characterizing *S. pyogenes* through PFGE and *emm* typing with the main purpose being to establish *emm* sequences and a PFGE pattern database that can serve as a basis for information for long-term epidemiological and evolutionary studies of local *S. pyogenes* strains.

METHODS

Sample collection. A total of 103 group A *Streptococcus* isolates were recovered from patients between May 2007 and November 2009 at the Clinical Microbiology section of the American University of Beirut Medical Centre in Lebanon and were designated WS1–WS103. In the sample population tested, there was no specific distribution of patients according to gender, ethnicity or age. The majority of the samples resulted from throat ($n=94$), vaginal ($n=1$), pus ($n=4$) and wound ($n=3$) infections. The majority of the samples (80%) were isolated from patients at the emergency room and 20% were from

Abbreviations: CDC, Centers for Disease Control and Prevention; MLS_B, macrolide–lincosamide–streptogramin B.

outpatients. Clinical samples were diagnosed by the American University of Beirut Medical Centre bacteriology department as *S. pyogenes*. This was done by the use of Lancefield grouping (Oxoid) and API 20 Strep strips (bioMérieux).

emm typing. *emm* typing was done according to the guidelines stated by the Centers for Disease Control and Prevention (CDC) and Beall *et al.* (2000) protocol and guidelines. In short, after genomic DNA extraction, the first 500 bp 5' of the *emm* gene was amplified and sequenced. The types and the new subtypes of the sequences were assigned for our isolates by the curators of the CDC database (http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm).

PFGE. PFGE analysis was done on those isolates sharing different *emm* types and subtypes. Bacterial isolates were grown on blood agar plates for 24 h at 37 °C. The cells were harvested, processed into plugs and digested with 5 U *Sma*I according to a modified PulseNet *Listeria* PFGE protocol (Chiou *et al.*, 2004). The DNA fragments were separated on a 1% agarose gel (Bio-Rad Pulsed Field Certified Agarose). The separation was done using a Bio-Rad Chef DR-III system at 14 °C for 20 h in 0.5 × Tris-Borate-EDTA (pH 8.0) at a fixed angle of 120° and a voltage of 6 V cm⁻¹, with time intervals between 4 and 40 s. A difference of three or more bands was set as a criterion to set apart banding patterns (Kao *et al.*, 2005). The genomic DNA of *Salmonella enterica* serovar Braenderup strain H9812 (ATCC BAA-664) digested with *Xba*I was used as the molecular marker for PFGE analysis (Chiou *et al.*, 2004).

Antibiotic susceptibility testing. Antibiotic susceptibility patterns of our isolates were assayed *in vitro* according to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2006). The micro-organisms were plated on Mueller–Hinton agar supplemented with 5% sheep blood and incubated in 5% CO₂ for 24 h at 35 °C. The double disc diffusion method was used to assay the resistance patterns of our isolates against erythromycin (2 µg) and clindamycin (2 µg), and the disc diffusion method was used for penicillin G (10 µg) and vancomycin (30 µg) (Oxoid).

RESULTS

emm typing

emm typing of the 103 isolates showed that the most prevalent *emm* type was *emm1* (12.6%), followed by *emm22* (8.7%), *emm28* (7.7%), *emm89* (7.7%) and *emm4* (6.8%). Twenty-eight different additional *emm* types were also recovered in percentages under 6 for each. Table 1 shows the detected *emm* types and their frequencies. All of our isolates were typable by CDC recommended primers. One strain was denoted as a new subtype, *stC36.0b*, by the curators of the CDC website. This new subtype was recovered from a throat culture and was sensitive to penicillin G, erythromycin, clindamycin and vancomycin.

Antibiotic susceptibility testing

Isolates were tested for resistance against erythromycin, clindamycin, penicillin G and vancomycin. Results obtained revealed that all isolates were susceptible to vancomycin and penicillin G, 10% were resistant to erythromycin and only 3% were resistant to both erythromycin and clindamycin. Two isolates (WS7 and WS38, typed *emm1* and *emm28*,

Table 1. Percentage distribution of *emm* types from our sample collection of 103 isolates

<i>emm</i> type	No. of isolates (%)
<i>emm1</i>	13 (12.62)
<i>emm22</i>	9 (8.73)
<i>emm28</i>	8 (7.76)
<i>emm89.0</i>	8 (7.76)
<i>emm4</i>	7 (6.79)
<i>emm97.1</i>	6 (5.82)
<i>emm12</i>	5 (4.85)
<i>emm110</i>	4 (3.88)
<i>stC5345.1</i>	4 (3.88)
<i>stC6979</i>	4 (3.88)
<i>emm11</i>	3 (2.91)
<i>emm77.0</i>	3 (2.91)
<i>emm9.0</i>	3 (2.91)
<i>emm29.2</i>	2 (1.94)
<i>emm3.19</i>	2 (1.94)
<i>emm75</i>	2 (1.94)
<i>stg245</i>	2 (1.94)
<i>stg5420</i>	2 (1.94)
<i>emm12.1, emm12.23, emm12.48, emm28.4, emm44.0, emm48.1, emm49.0, emm50.0, emm58.0, emm6.4, emm63.2, emm76.0, emm85.0, st1389.1, st1400.0</i>	1 (0.97)*

*One of each indicated type.

respectively) showed the constitutive macrolide–lincosamide–streptogramin B (MLS_B) phenotype, whereas one (WS60, typed as *emm49*) isolate showed inducible MLS_B. It is noteworthy that 50 and 28% of the isolates typed as *emm97.1* and *emm4*, respectively, were resistant to erythromycin, while 7.7% of *emm1* isolates were resistant.

PFGE analysis

PFGE analysis was done on isolates representing each of the types *emm1*, *emm11*, *emm12*, *emm110*, *emm12.0*, *emm22*, *emm28*, *emm3.19*, *emm4*, *emm77*, *emm75*, *emm89*, *emm9*, *emm97.1* and *stC534.1*, along with other isolates representing the remaining *emm* types, including the new subtype. PFGE exhibited higher discriminatory power than *emm* typing for several of the tested *S. pyogenes* isolates. Thirty-two different banding patterns were detected, with isolates such as WS14 and WS15 being typed as *emm11* but exhibiting different PFGE patterns (Fig. 1).

DISCUSSION

In this study, we have collected 103 *S. pyogenes* isolates from the American University of Beirut Medical Centre. To our knowledge, this work was the first attempt at *S. pyogenes* characterization through *emm* typing in Lebanon. Results of this study revealed that the most prevalent *emm* types of *S. pyogenes* in this country were *emm1*, *emm22*,

Fig. 1. PFGE analysis of *S. pyogenes* isolates. Samples WS1, WS2 and WS9 belong to *emm1*; WS14 and WS15 belong to *emm11*; WS17 and WS18 belong to *emm110*; WS29 and WS30 belong to *emm22*; WS38 and WS39 belong to *emm28*. The DNA molecular mass marker is the *Xba*I-digested DNA of *Salmonella enterica* serovar Braenderup ATCC BAA-664.

emm28, *emm89*, *emm4* and *emm97.1*. These *emm* types constituted 49.5% of the *S. pyogenes* isolates. Although Lebanon shares a common geographical location with Israel, *emm* type distribution obtained in this study was different from that previously reported by Nir-Paz *et al.* (2010). *emm* types 81, 14, 5, 6 and 53, the major types in Israel, were not detected in Lebanon. Interestingly, our results were different from those on the CDC website concerning the predominant *S. pyogenes* isolates in the Middle East (http://www.cdc.gov/ncidod/biotech/strep/emmtypes_proportions.htm). We had no untypable isolates, lower percentages of *emm1* and *emm28*, and higher percentages of *emm22*, *emm89* and *emm97.1*. Furthermore, and in contrast to the findings of Steer *et al.* (2009), with respect to the distribution of *emm* types in Africa, Asia and Australia, a lower percentage of *emm12* was detected in Lebanon and higher percentages of *emm4*, *emm28* and *emm110*. However, and similar to our results, Shulman *et al.* (2009) found that within their population of pharyngeal isolates, *emm1*, 12, 28, 4 and 2 were the major types detected, while Ekelund *et al.* (2005) revealed that *emm1* and *emm28* were mainly associated with invasive diseases.

All our tested isolates were sensitive to penicillin and vancomycin, which agrees with the findings of Bessen (2009). This keeps penicillin as a first line of defence against *S. pyogenes* infections in Lebanon. However, resistance to erythromycin was seen in 10% of our samples. The constitutive MLS_B phenotype was seen in two samples showing resistance to erythromycin and clindamycin at the same time and being typed as *emm1* and *emm28*, whereas the induced MLS_B phenotype was seen in one *emm49* isolate. These results were higher than the 5% resistance reported by Luca-Harari *et al.* (2008). The percentage of resistance to macrolide antibiotics in this study was lower than that found by Chen *et al.* (2007) and Lavergne *et al.* (2007), who reported 21.1% and 42.6%

resistance, respectively, but was similar to that described by Michos *et al.* (2009) and Gracia *et al.* (2009) in their studies. Moreover, we found a different distribution of macrolide resistance among *emm* types compared to Metzgar *et al.* (2009) and Chan *et al.* (2009), where isolates typed as *emm97.1* and *emm4* in this study showed 50 and 28% resistance to erythromycin, respectively.

Chiou *et al.* (2004) previously reported that PFGE analysis had a better discriminatory power than *emm* typing of *S. pyogenes* and our results again confirmed this. Only duplicate isolates belonging to types *emm1*, *emm110*, *emm9*, *emm3.19*, *emm29.2*, *stC6979* and *stC5345.1* had identical banding patterns. All duplicates of other types exhibited different PFGE patterns.

This preliminary study, in which *emm* sequences and a PFGE fingerprint database for *S. pyogenes* isolates were generated, will serve as a basis for information for further and more detailed epidemiological studies of this important pathogen in Lebanon.

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