Phenotypes and genotypes of macrolide-resistant *Streptococcus pyogenes* isolated in Seoul, Korea

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The mechanisms of resistance to macrolides in 51 erythromycin-resistant clinical isolates of *Streptococcus pyogenes* collected from 1997 through 2003 in Seoul, Korea were evaluated. They were characterized by their antimicrobial susceptibility, phenotype (using triple-disc and induction tests), resistance genotype, *emm* genotyping (M typing) and phylogenetic analysis. Erythromycin resistance was observed in 23 % of isolates. Inducible phenotype was the most common (iMLS, 51 %, 26 strains), followed by the constitutive phenotype (cMLS, 31 %, 16 strains) and the M phenotype (18 %, 9 strains). Eight of twenty-six iMLS isolates exhibited the iMLS-C phenotype. The remaining 18 isolates gave small inhibition zones (< 12 mm) around all three discs, and mild blunting of the spiramycin and clindamycin zones of inhibition proximal to the erythromycin disc. They showed remarkable inducibility in erythromycin and clindamycin resistance. The MIC₉₀ of erythromycin and clindamycin rose from 8 to > 128 µg ml⁻¹ and from 0.5 to > 128 µg ml⁻¹, respectively. Their resistance characteristics did not fit into any known iMLS subtype reported so far in the literature. So, it was named as an iMLS-D, new subtype. All of these iMLS-D strains harboured the *erm(B)* gene, demonstrated the *emm12* genotype, except one, and formed a tight cluster in a phylogenetic tree, with 89.2 to 100 % sequence homology, suggesting that they are closely related. Nine of sixteen cMLS strains had the *emm28* genotype, which had been reported to be associated with multiple drug resistance.

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INTRODUCTION

Streptococcus pyogenes causes a wide array of infections in humans (Bisno, 1991). Macrolides have been effective in the treatment of individuals with *S. pyogenes* infection and a good alternative in patients who are allergic to other agents (e.g. penicillin). Unfortunately, because of the general use of these agents, macrolide-resistant *S. pyogenes* have been isolated in many countries. Resistance is caused by two different mechanisms: target-site modification (Weisblum, 1995) and active drug efflux (Sutcliffe *et al.*, 1996). In these organisms, target-site modification is mediated by a methylase enzyme that reduces the binding for macrolides, lincosamides and streptogramin B (MLS) antibiotics with their target site in the bacterial ribosome. MLS resistance can be expressed either constitutively (cMLS phenotype) or inducibly (iMLS phenotype) in Gram-positive cocci. Three subtypes of the iMLS macrolide resistance phenotype have been distinguished: iMLS-A, iMLS-B and iMLS-C (Giovanetti *et al.*, 2002). Active efflux is associated with the M phenotype. The *emm* gene of *S. pyogenes* encodes the

cell-surface M virulence protein that is putatively responsible for at least 100 known M serospecificities of *S. pyogenes* (Beall *et al.*, 1998, 2000; Jones & Fischetti, 1988; Whatmore *et al.*, 1994). A new typing system, which is based on a sequence analysis of the portion of the *emm* gene, which encodes M serospecificity, was introduced to overcome many problems with the traditional M protein serotyping system, such as limited availability of M typing sera, newly encountered M types and difficulty in interpretation. Several studies have shown that increased macrolide consumption is associated with the occurrence of macrolide resistance (Granizo *et al.*, 2000; Seppala *et al.*, 1992). Various resistance rates have been reported up to approximately 50 %, depending on the country and geographical region (Beall *et al.*, 1998; Bisno, 1991; De Azavedo *et al.*, 1999; Sungho *et al.*, 2001; Giovanetti *et al.*, 2002). However, data on macrolide resistance of *S. pyogenes* in Korea are relatively scarce. The prevalence of erythromycin resistance in Korea increased rapidly from only 2.0 % in 1994 to 44.8 % in 2002 (Sungho *et al.*, 2001; Kim & Lee, 2004). The aim of this study was to determine the antimicrobial resistance, phenotypic and genetic characteristics of erythromycin-resistant *S. pyogenes* in Seoul, Korea.

Abbreviation: MLS, macrolides lincosamides and streptogramin B.

METHODS

Bacterial strains. A total of 51 erythromycin-resistant isolates were evaluated among 222 *S. pyogenes* isolates from clinical specimens from January 1997 to December 2003 in Seoul, Korea. The specimens were collected from two university hospitals and were from the culture of 26 throat swabs, 16 pus samples, 5 sputa samples and 4 blood samples. Of these, 25 isolates were recovered from children (1–16 years old, median age 5 years) and 26 isolates from adults. Multiple isolates from the same patient were avoided. Isolates were identified as *S. pyogenes* by colony morphology, β -haemolysis on sheep blood agar, and Lancefield grouping by using a commercially available agglutination technique (Seroiden Strepto kit) and bacitracin disc test (0.04 U, Taxo A, BBL Microbiology system). The strains were maintained in glycerol at -70°C until all of the isolates were collected and then subcultured twice on blood agar before undergoing susceptibility testing.

Antimicrobial susceptibility testing. Penicillin, erythromycin, spiramycin, clindamycin, tetracycline, chloramphenicol, azithromycin and clarithromycin were obtained in the form of standard reference powders of known potency from Sigma or from their respective manufacturers. The antibiotics were incorporated into the medium (Mueller–Hinton agar medium with 5% sheep blood) in a $\log_2$ dilution series of 0.03 to $128\ \mu\text{g ml}^{-1}$. Inocula were prepared by diluting bacterial suspensions equivalent in turbidity to a McFarland 0.5 standard, resulting in 10^4 c.f.u. in each spot when applied by a Steer's replicator (Craft Machine). The plates were incubated overnight at 35°C in an atmosphere containing 5% carbon dioxide. The interpretative categories for each antibiotic were those recommended by Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 2005). MIC breakpoints suggested by the CLSI were used for erythromycin, clarithromycin and clindamycin (susceptible at $\leq 0.25\ \mu\text{g ml}^{-1}$, resistant at $\geq 1\ \mu\text{g ml}^{-1}$), azithromycin (susceptible at $\leq 0.5\ \mu\text{g ml}^{-1}$, resistant at $\geq 2\ \mu\text{g ml}^{-1}$), tetracycline (susceptible at $\leq 2\ \mu\text{g ml}^{-1}$, resistant at $\geq 8\ \mu\text{g ml}^{-1}$), chloramphenicol (susceptible at $\leq 4\ \mu\text{g ml}^{-1}$, resistant at $\geq 16\ \mu\text{g ml}^{-1}$) and penicillin (susceptible at $\leq 0.12\ \mu\text{g ml}^{-1}$). Given the lack of CLSI recommendations for spiramycin, we used the Comité de l'Antibiogramme de la Société Française de Microbiologie recommendation of a susceptible MIC of $\leq 1\ \mu\text{g ml}^{-1}$ and a resistant MIC of $\geq 4\ \mu\text{g ml}^{-1}$ (Soussy *et al.*, 2000). *Streptococcus pneumoniae* ATCC 49619 was used for quality control.

Triple-disc test for the phenotypes of macrolide resistance.

A triple-disc test was set up by adding spiramycin (a 16-ring macrolide) to the erythromycin and clindamycin double-discs, thereby enabling us to distinguish the subtypes of inducible-resistance phenotypes. Commercial discs (Oxoid) of erythromycin (15 μg), clindamycin (2 μg) and spiramycin (100 μg) were used. Three discs were placed 15 to 20 mm apart from each other in a triangular formation. Three different patterns (clindamycin-susceptible, erythromycin-resistant; clindamycin-inducible, erythromycin-resistant; clindamycin-resistant, erythromycin-resistant) were recognized as being related to the well-recognized phenotypes (M, inducible, constitutive) of MLS resistance. Subtypes of the inducible macrolide-resistance phenotype were determined as described by Giovanetti *et al.* (1999).

Induction of MLS resistance. Induction of MLS resistance was evaluated under pre-growth conditions (3 h at 35°C) in erythromycin at a subinhibitory concentration ($0.05\ \mu\text{g ml}^{-1}$), as described by Giovanetti *et al.* (1999). The culture was then washed, and the cells were used to prepare the inoculum for MIC testing using the same agar dilution method. The post-induction MICs for those antibiotics were evaluated.

Detection of erythromycin-resistance gene. The *erm(A)*, *erm(B)* and *mef(A)* genes were detected by PCR, using previously published primers (Sutcliffe *et al.*, 1996; Claire *et al.*, 2003). DNA preparation, amplification and detection, as well as visualization of the product, were carried out using established procedures (Sutcliffe *et al.*, 1996). The expected sizes were 459 bp for *erm(A)*, 638 bp for *erm(B)* and 348 bp for *mef(A)*; their specificity was confirmed with an appropriate restriction enzyme (*XmnI*, *XmnI* and *PvuII*, respectively).

emm genotyping (M typing) and phylogenetic analysis. For *emm* genotyping, the 5' terminal ends of *emm* gene were amplified with M1 (5'-ATAAGGAGCATAAAAATGGCT-3') and M2 (5'-AGCTTAATTTCTTCTTTGCG-3') (Saunders *et al.*, 1997) and were partially sequenced (129 bp). The *emm* sequences analysis of all erythromycin-resistant *S. pyogenes* was performed with BioEdit v5.0.9 (Hall, 1999). Multiple sequence alignment was conducted with CLUSTAL X, version 1.81 (Thompson *et al.*, 1997), with a gap-opening penalty of 15 and a gap extension penalty of 6.66. The phylogenetic tree was prepared in MEGA 3 (Kumar *et al.*, 2004) using the Kimura two-parameter neighbour-joining method. Nodal confidence values indicate the results of bootstrap resampling ($n=1000$).

RESULTS AND DISCUSSION

In this study, a total of 222 *S. pyogenes* isolates from clinical specimens from patients referred to the university hospitals in Seoul, Korea over 6-year period, from 1997 through 2003, were included. The resistance rates of erythromycin during the study period were 20.0% in 1997, 27.8% in 1998, 24.3% in 1999, 17.9% in 2000, 27.3% in 2001, 22.2% in 2002 and 21.5% in 2003. Table 1 shows the results of susceptibility testing of 51 erythromycin-resistant isolates *in vitro*, as well as the MIC ranges, and the calculated MIC₅₀ and MIC₉₀ values of antibiotics tested. Erythromycin-resistant strains were resistant to the other macrolides, clarithromycin and azithromycin, as was reported elsewhere (Hsueh *et al.*, 2002; Kim & Lee, 2004). The azithromycin MIC₅₀ was higher than that of clarithromycin, because isolates with the iMLS-D phenotype had high azithromycin MICs ($>128\ \mu\text{g ml}^{-1}$). Fourteen isolates with a high level of erythromycin resistance (MIC $>128\ \mu\text{g ml}^{-1}$) were all resistant to clindamycin and spiramycin.

The patterns of susceptibility to MLS antibiotics according to the phenotypes of macrolide resistance are presented in Table 2. Out of a total of 51 erythromycin-resistant strains, 26 (51%) had the iMLS phenotype, 16 (31%) had the cMLS phenotype and 9 (18%) expressed the M phenotype. These features differed from those observed in European countries and Canada, where the M phenotype was most common (De Azavedo *et al.*, 1999; Giovanetti *et al.*, 1999; Grivea *et al.*, 2006; Kataja *et al.*, 2000). Among the inducible phenotypes, neither iMLS-A nor iMLS-B was found, but the iMLS-C and iMLS-D phenotypes were identified on the basis of the diameter of the inhibition zone. The iMLS-C phenotype had the same pattern as that described in another report (Giovanetti *et al.*, 1999), with blunted spiramycin and clindamycin zones of inhibition proximal to the erythromycin disc, and a visible zone of inhibition around the erythromycin disc. All except one had the *erm(A)* resistance gene. The iMLS-D phenotype had smaller inhibition zones

Table 1. *In vitro* susceptibilities to 9 antimicrobial agents of 51 erythromycin-resistant isolates of *S. pyogenes*

Antimicrobial agent	Erythromycin-resistant isolates (51)			
	Range ($\mu\text{g ml}^{-1}$)	MIC ₅₀ * ($\mu\text{g ml}^{-1}$)	MIC ₉₀ * ($\mu\text{g ml}^{-1}$)	Resistance (%)
Erythromycin	1–> 128	8	> 128	100
Clarithromycin	1–> 128	4	> 128	100
Azithromycin	1–> 128	> 128	> 128	98
Spiramycin	< 0.03–> 128	8	> 128	67
Clindamycin	< 0.03–> 128	0.25	128	35
Tetracycline	0.25–16	16	16	80
Chloramphenicol	2–4	4	4	0
Penicillin G	< 0.03	< 0.03	< 0.03	0

*MIC₅₀ and MIC₉₀, concentration of drug that inhibited 50 and 90% of strains tested, respectively.

(< 12 mm) around each disc and a lesser degree of blunting in the spiramycin and clindamycin discs proximal to the erythromycin disc than iMLS-C (Fig. 1). The ability to induce MLS resistance was confirmed by the increase in MIC after incubation with erythromycin. The MICs of erythromycin before induction were in range of 1–8 $\mu\text{g ml}^{-1}$, which was compatible with known iMLS-C, and were

> 128 $\mu\text{g ml}^{-1}$ after induction, which was compatible with known iMLS-A or iMLS-B. This remarkable inducibility of erythromycin resistance was characteristic of iMLS-D. The MICs of clindamycin and spiramycin were closer to those for known iMLS-A isolates while the MICs of clarithromycin were closer to those for known iMLS-C isolates (Giovanetti *et al.*, 1999). All of these iMLS-D strains had

Table 2. MIC ($\mu\text{g ml}^{-1}$) ranges of 48 isolates of among 51 erythromycin-resistant *S. pyogenes* to MLS antibiotics according to phenotypes of macrolide resistance

Phenotype (no. of strains)	Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$)					
		Pre-induction			Post-induction		
		Range	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀
cMLS (13)*	Erythromycin	> 128	> 128	> 128			
	Clarithromycin	> 128	> 128	> 128			
	Azithromycin	128–> 128	> 128	> 128			
	Spiramycin	> 128	> 128	> 128			
	Clindamycin	> 128	8	128			
iMLS-C (8)	Erythromycin	1–8	1	2	4–32†	8	32
	Clarithromycin	1–4	1	2	2–32†	8	32
	Azithromycin	1–32	16	16	8–> 128	128	> 128
	Spiramycin	0.25–16	0.5	16	4–32	16	32
	Clindamycin	< 0.03–0.5	0.06	0.25	> 128	> 128	> 128
iMLS-D (18)	Erythromycin	1–8	4	8	> 128	> 128	> 128
	Clarithromycin	1–8	4	4	16–> 128	> 128	> 128
	Azithromycin	16–> 128	> 128	> 128	> 128	> 128	> 128
	Spiramycin	0.25–16†	8	16	> 128	> 128	> 128
	Clindamycin	0.25–0.5	0.25	0.5	64–> 128	128	> 128
M (9)	Erythromycin	8–16	8	16			
	Clarithromycin	8–32	8	32			
	Azithromycin	4–16	8	16			
	Spiramycin	0.25–0.5	0.25	0.5			
	Clindamycin	< 0.03–0.06	< 0.03	0.06			

*Three strains with atypical cMLS phenotypes were not included.

†One isolate showed erythromycin, clarithromycin and spiramycin MIC $\geq$ 128 $\mu\text{g ml}^{-1}$.

Fig. 1. Phenotypes of erythromycin-resistant *S. pyogenes* isolates determined by the triple-disc test: (A) cMLS phenotype, (B) iMLS phenotype (subtype iMLS-C), (C) iMLS phenotype (subtype iMLS-D), (D) M phenotype. Clockwise from left on each plate are the erythromycin disc, the spiramycin disc and the clindamycin disc.

the *erm(B)* gene. These strains did not fit into any known subtype reported in the literature so far.

Conventionally, the inducible clindamycin resistance has been detected using a double-disc test with erythromycin and clindamycin discs. The triple-disc test was set up by adding a spiramycin disc to the double-disc test and it was useful to classify heterogeneous MLS resistance phenotypes, especially when the discs were arranged in triangular formation. The inducible nature of iMLS-D phenotype was distinct by the triple-disc test while it was not by the double-disc test.

The prevalence of the recognized phenotypes of erythromycin-resistant *S. pyogenes* seemed to vary considerably from area to area. Empirical erythromycin administration to patients with infection by the iMLS-D phenotype *S. pyogenes* might result in the induction of a high level of erythromycin and clindamycin resistance and, consequently, lead to treatment failure of erythromycin-resistant and clindamycin-susceptible isolates. There was a case report describing clindamycin treatment failure in a patient with meticillin-resistant, erythromycin-resistant and clindamycin-susceptible *Staphylococcus aureus* (Siberry *et al.*, 2003). More specific information about resistance phenotype using the

triple-disc test could help physicians choose appropriate antibiotics for treating infections by *S. pyogenes*.

Most of the cMLS phenotypes (13 out of 16) showed a high degree of resistance ($\text{MIC}_{90} > 128 \mu\text{g ml}^{-1}$) to MLS antibiotics (erythromycin, clarithromycin, azithromycin, spiramycin and clindamycin). They had no inhibition zone around the triple discs with the *erm(B)* gene. There were three isolates showing atypical subtypes of cMLS strains, having a small inhibition zone around the erythromycin and spiramycin discs and no inhibition zone around the clindamycin disc. Their MIC_{50} and MIC_{90} of erythromycin were 32 and $> 128 \mu\text{g ml}^{-1}$, respectively, while the MIC_{50} and MIC_{90} of clarithromycin, azithromycin, spiramycin and clindamycin were all $> 128 \mu\text{g ml}^{-1}$. Their resistance genes were *erm(A)*, *erm(B)* and *erm(A)* plus *erm(B)*, respectively.

Nine strains of the M phenotype were all susceptible to tetracycline, spiramycin and clindamycin. The MIC_{90} values of erythromycin and clarithromycin were higher than those for the isolates with the iMLS phenotype (8–16 and 8–32 $\mu\text{g ml}^{-1}$ vs 1–8 and 1–8 $\mu\text{g ml}^{-1}$). Macrolide resistance induction was not observed in any of the M phenotype strains, all of which had the *mef(A)* gene (Table 3).

Table 3. Distribution of erythromycin-resistant *S. pyogenes* isolates according to their phenotype, genotype and *emm* genotype

<i>emm</i> genotype	cMLS			iMLS-C		iMLS-D	M
	<i>erm(A)</i>	<i>erm(B)</i>	<i>erm(A+B)</i>	<i>erm(A)</i>	<i>erm(A+B)</i>	<i>erm(B)</i>	<i>mef(A)</i>
<i>emm12</i>		1*				17	2
<i>emm28</i>		9					
<i>emm75</i>			1*				3
<i>emm2</i>				4	1		
<i>emm1</i>		2					
<i>emm4</i>				2			1
<i>emm6</i>							1
<i>emm10</i>						1	
<i>emm27</i>	1*						
<i>emm18.1</i>							2
<i>emm22</i>		1					
<i>emm58</i>		1					
NT†				1			
Total		16 (31 %)		8 (16 %)		18 (35 %)	9 (18 %)

NT, Nontypable.

*Strains showing atypical cMLS phenotype.

Fig. 2. Phylogenetic tree of *emm* sequences of 50 erythromycin-resistant *S. pyogenes* isolates using MEGA 3 with the Kimura two-parameter neighbour-joining method. Nodal confidence values indicate the results of bootstrap resampling ($n=1000$). Strain number, phenotype, *emm* genotype and year of isolation are presented. aCMLS, atypical cMLS phenotype.

Two isolates had two resistance genes at the same time – *erm(A)* and *erm(B)*. However, their phenotypic characteristics – e.g. their MICs or inducibility – were similar to those of other isolates within each group.

In this study, *emm* genotyping was used instead of M protein serotyping for epidemiological purposes. PFGE and multi-locus sequence typing were not available. The hypervariable

portion (129 bp) of the 5' terminal end of the *emm* gene in all erythromycin-resistant strains (except one) was sequenced and the phylogenetic tree was analysed. The *emm12*, 28 and 2 genotypes were the most common, comprising 39, 18 and 10 %, respectively, of all genotypes. Seventeen out of twenty *emm12* strains had the iMLS-D phenotype and *erm(B)* gene. There were some reports of an association between the *emm* type and the pattern of

antibiotic resistance (Alberti *et al.*, 2003; Grivea *et al.*, 2006). The *emm28* genotype has been reported to be associated with multiple drug resistance (Mihaila-Amrouche *et al.*, 2004; Perez-Trallero *et al.*, 1999). Our nine *emm28* genotype strains had the cMLS phenotype with multiple resistances, which is consistent with the findings in previous reports. All of the *emm2* genotype strains expressed the iMLS-C phenotype (Table 3).

The cMLS, M and iMLS-C phenotype strains were clustered relatively loosely in the phylogenetic tree based on *emm* sequences (Fig. 2), the sequence homologies were 58.9–100%, 56.1–99.2% and 76.1–98.4%, respectively. The iMLS-D phenotype strains formed tighter clusters than did strains with other phenotypes, with a nucleotide sequence identity of 89.2–100%. These findings suggest that they were genetically closely related.

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