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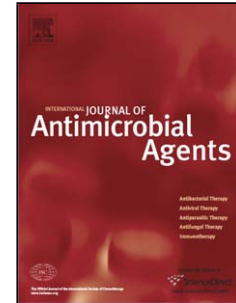
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## Emerging fluoroquinolone-non-susceptible group A streptococci in two different paediatric populations

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**Abstract**

Clonal emergence of group A streptococci (GAS) with reduced susceptibility to fluoroquinolones (FQs) has been increasingly reported. Non-susceptibility is associated with various point mutations in the target-encoding genes and has only been described in a few *emm* types. We used a well characterised GAS clinical paediatric collection from Brussels (Belgium) and Brasília (Brazil) to analyse the molecular basis of FQ non-susceptibility. GAS strains were tested for ciprofloxacin susceptibility and were screened for mutations in DNA gyrase- and topoisomerase IV-encoding genes. Genetic relationships between the different *emm* types were assessed by phylogenetic analysis of the whole surface-exposed part of the M protein. A high proportion (22.5%) of ciprofloxacin-non-susceptible isolates (minimal inhibitory concentration  $\geq 2$  mg/L) was found among the Belgian strains. They belonged mostly to *emm* type 6 (87%). In Brazil, 6% of the isolates, belonging to seven distantly related *emm* types, were non-susceptible. Our phylogenetic analysis showed that non-susceptibility may arise in various genetic backgrounds. Sequence comparison of the quinolone resistance-determining regions (QRDRs) of the ParC- and ParE-encoding genes from susceptible and non-susceptible isolates revealed that most of the mutations were found in both classes of isolates, indicating an *emm* type-linked polymorphism. In conclusion, we observed a clonal spreading of non-susceptible *emm* type 6 GAS strains in Brussels and a polyclonal distribution of non-susceptible isolates in Brazil. All the Brazilian and Belgian *emm* type 6 strains displayed a S79A/F mutation in *parC* that convincingly explains the non-susceptible phenotype.

## 1. Introduction

The use of fluoroquinolones (FQs) is approved in adults for a wide range of community-acquired infections. In 2003, Belgium ranked third among European countries with the highest consumption of FQs for outpatient treatment [1]. No data on Brazilian FQ consumption are available in the literature. Paediatric use of FQs is very limited because of potential joint toxicity observed in young animal studies. Although this was not demonstrated in reports on systemic use of FQs in children [2,3], these drugs are not licensed for general paediatric usage. There is, however, a trend towards increased use in this age group [2,3].

FQ antibiotics target two essential bacterial type II topoisomerases: DNA gyrase, composed of four subunits encoded by the *gyrA* and *gyrB* genes; and topoisomerase IV, formed of four subunits encoded by the *parC* and *parE* genes. In Gram-positive bacteria, ciprofloxacin and levofloxacin preferentially target topoisomerase IV, whilst moxifloxacin and gatifloxacin show the converse specificity [4]. FQ resistance is mainly caused by point mutations in the target-encoding genes (*gyrA*, *gyrB*, *parC* and *parE*). Mutations tend to cluster in a defined region of these genes called the quinolone resistance-determining region (QRDR). FQ resistance appears to occur stepwise, with moderate levels of resistance arising from a single mutation in the primary target of the drug (topoisomerase IV, *parC* gene). A higher level of resistance is reached by the accumulation of additional mutations in the secondary target (DNA gyrase, *gyrA* gene) [4]. Efflux of the FQ via membrane-associated pumps also contributes to resistance [5,6].

Group A streptococci (GAS) are one of the most common paediatric pathogens. They are responsible for a wide spectrum of diseases, ranging from trivial pharyngitis and impetigo to lethal toxic shock syndrome and necrotizing fasciitis. The first description of a GAS clinical isolate resistant to ciprofloxacin [minimal inhibitory concentration (MIC) >32 mg/L] was reported in 2000 [7]. So far, seven cases of highly resistant strains have been described [7–11]. At the same time, GAS with reduced susceptibility to FQs have been increasingly reported worldwide [4,12,13]. Reduced susceptibility to ciprofloxacin was defined for GAS isolates exhibiting an MIC  $\geq$  2 mg/L [12]. The prevalence of such isolates was 3.5% in 1998–1999 in Spain, 5.4% in 1999–2002 in Belgium and 10.9% in 2002–2003 in the USA [4,12,13]. The clinical impact of this reduced susceptibility is unclear. Reduced susceptibility could represent a first step towards a higher level of resistance, hence the emergence of FQ-resistant GAS.

The epidemiology of FQ-non-susceptible GAS was reported to be clonal in developed countries [4,12,14–16], with a predominance of *emm* type 6 isolates [4]. We recently showed that the *emm* type diversity of GAS isolates was much higher in Brazil than in Belgium [17]. The aim of the present study was to compare the FQ non-susceptibility of GAS from two different clinical and microbiological settings.

## **2. Material and methods**

### *2.1. Materials*

GAS clinical isolates analysed in this study were prospectively collected from 1 February to 31 October 2004 from symptomatic children simultaneously in Brussels

(Belgium) and Brasília (Brazil) [17]. Patients aged 0–15 years attending three public hospitals in Brazil and one public hospital of Brussels were included in the study. Isolates were mostly recovered from pharyngitis in Brussels and from cutaneous infections and pharyngitis in Brazil. Twenty distinct *emm* types were identified among 200 Belgian isolates and 48 *emm* types among 128 Brazilian isolates [17,18]. All the isolates were tested for quinolone susceptibility.

## 2.2. Antibiotic susceptibility testing

MICs for ciprofloxacin and moxifloxacin were determined by a reference broth microdilution method (Merlin-Diagnostika, Bornheim-Hersel, Germany) in cation-adjusted Muller–Hinton broth (Difco Laboratories, Detroit, MI) supplemented with 4.6% lysed horse blood as recommended by the Clinical and Laboratory Standards Institute (CLSI) [19]. *Streptococcus pneumoniae* ATCC 49619 was used as a control strain. GAS breakpoints for moxifloxacin were defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (susceptibility/resistance in mg/L:  $\leq 0.5 / > 1$ ). Non-susceptibility to ciprofloxacin was defined as an MIC  $\geq 2$  mg/L [12], as neither EUCAST nor CLSI breakpoints exist. The MICs for ciprofloxacin and moxifloxacin of FQ-non-susceptible isolates were determined in the presence of reserpine (an efflux pump inhibitor) at a final concentration of 25 mg/L [12].

## 2.3. Epidemiological characterisation

The relative risk of several clinical and epidemiological factors (previous antibiotherapy, crowding, sex, age, geographical location and pathology) being

associated with the ciprofloxacin-non-susceptible phenotype was assessed separately for the Belgian and Brazilian isolates.

#### 2.4. Polymerase chain reaction (PCR) amplification and DNA sequencing of the quinolone resistance-determining regions

The QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes of the ciprofloxacin-non-susceptible isolates were sequenced on the two DNA strands (3730 DNA Analyser; Applied Biosystems, Foster City, CA). The QRDRs of *parC* and/or *parE* of a subset of ciprofloxacin-susceptible isolates (30 strains) belonging to the same *emm* types as the non-susceptible isolates were also sequenced. The sequence of the primers used for PCR amplification of the QRDRs of the four target genes have been described previously [6,7]. PCR conditions were as follows: a denaturation step at 94 °C for 4 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 50 °C and 30 s at 72 °C. The QRDRs sequences were compared with the *gyrA* (AF220945), *gyrB* (AE006524), *parC* (AF220946) and *parE* (AE006540) DNA sequences of the *emm* type 1 GAS strain (ATCC 700294).

#### 2.5. Phylogenetic analysis of M proteins

The genetic relatedness of the whole surface-exposed part of the M protein was analysed as described previously [20]. Briefly, the portion of the *emm* gene encoding the whole surface-exposed part of M (including hypervariable and conserved parts of the protein) was sequenced, translated into amino acid sequence and a multiple alignment was performed using ClustalW [21]. In the present study, the relatedness of the Belgian and Brazilian M sequences was analysed separately (19 and 46



Belgian and Brazilian M sequences, respectively) to obtain a phylogenetic tree for each country. The sequence alignments were loaded in MEGA version 4 [22] to generate a bootstrapped tree using the neighbour-joining algorithm [23]. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The variation rate among sites was modelled with a gamma distribution (shape parameter = 0.5) [20]. The frequency of isolates of each *emm* type is proportional to the surface of the circle in Fig. 1.

### 3. Results

#### 3.1. Phenotypic and epidemiological characterisation of ciprofloxacin-non-susceptible isolates

In 2004, 334 GAS isolates were collected in children presenting with GAS-associated pathologies in Brussels ( $n = 204$ ) and Brazil ( $n = 130$ ) [17]; 22.5% (46/204) of the Belgian isolates were non-susceptible to ciprofloxacin ( $\text{MIC} \geq 2\text{mg/L}$ ), whilst 6% (8/130) of the Brazilian isolates were non-susceptible (Table 1). Among the 54 non-susceptible isolates from both countries, 21 presented an  $\text{MIC} \geq 4\text{ mg/L}$  and belonged mostly to *emm* type 6 (one isolate was *emm* type st1815). The 33 remaining isolates showed an  $\text{MIC}$  of 2 mg/L and belonged to nine different *emm* types (*emm* 6, 89, st1815, 59, 74, 44/61, 94, 80 and 49). All the isolates were susceptible to moxifloxacin.

From an epidemiological standpoint, the relative risk (RR) of previous antibiotherapy, crowding, sex, age or geographical location were not statistically associated with

non-susceptibility, indicating that these factors did not constitute a risk for ciprofloxacin-non-susceptible GAS isolation in our data set. However, in Brussels, children with acute otitis media ( $n = 15$ ) had a trend towards an increased rate of infection with non-susceptible GAS (RR = 1.9;  $P = 0.09$ ).

### 3.2. Genotypic characterisation of ciprofloxacin-non-susceptible isolates

Newly identified sequences were submitted to GenBank (accession nos. EF031328, EF031329, EF031330, EF031331, EF031332, EF03133 and EF529735). The 46 non-susceptible Belgian isolates belonged to three different *emm* types (6, 89 and st1815), among which *emm* type 6 was predominant (87%) (Table 2). All the isolates carried at least one mutation in *parC*. The most common mutation was serine79→alanine (S79A), which was present in all *emm* type 6 isolates. It is noteworthy that all isolates of *emm* type 6 recovered were non-susceptible (MIC  $\geq$  2mg/L). Several other mutations were detected both in *parC* and *parE*. Interestingly, most of the mutations were identical for a given *emm* type and varied among different *emm* types (Table 2).

In contrast to the *emm* type clonality observed for the Belgian isolates, the eight non-susceptible Brazilian isolates belonged to seven different *emm* types (59, 6, 74, 44/61, 94, 80 and 49). Only three of these isolates (two *emm* type 59 and one *emm* type 6 isolates) had mutations in the *parC* gene, whilst four isolates carried a mutation in *parE*. Surprisingly, isolates of *emm* types 94 and 49 (both presenting an MIC of 2 mg/L) did not carry any mutation in *parC* or *parE*. The *gyrA* and *gyrB* QRDRs sequences were wild-type for all the non-susceptible isolates and none of

them carried an efflux resistance mechanism, as reserpine did not affect ciprofloxacin non-susceptibility.

### 3.3. Genotypic characterisation of ciprofloxacin-susceptible isolates

To determine the contribution of the mutations described above to ciprofloxacin non-susceptibility, the QRDR sequences of the *parC* and *parE* genes from non-susceptible isolates were compared with those from susceptible isolates belonging to the same *emm* types. Isolates from a given *emm* type within a given geographic location are usually associated with a specific clonal type as defined by multilocus sequence typing (MLST). However, three of the nine non-susceptible *emm* types were associated with more than one clonal type (*emm* 89, 44/61 and 49) [24,25].

Thirty strains from our collection belonging to the same *emm* types as the non-susceptible isolates were checked for the presence of mutations in the *parC* and *parE* QRDRs (Table 3). In contrast to the st1815 non-susceptible isolates, none of the four susceptible st1815 isolates carried a S79F mutation in *parC*. In contrast, most of the other *parC* and *parE* mutations detected in the non-susceptible isolates were present in the corresponding susceptible isolates. The A395T and A471V mutations in *parE* were only observed in a fraction of the susceptible isolates, indicating a certain level of polymorphism in the *parE* QRDR of isolates within *emm* types 80, 74 and 44/61 that were isolated in Brazil. Note that the 44/61 *emm* type has been shown to be associated with more than one MLST clonal type [24,25]. The two Brazilian *emm* type 94 and 49 non-susceptible isolates that did not harbour mutations shared the same sequence as their susceptible counterparts. The S79A mutation in

*parC* and the A378T mutation in *parE* were only found in *emm* type 6 for which no susceptible isolate was available in our collection.

### 3.4. Phylogenetic analysis

Figures 1A and 1B represent the genetic relationships of the whole surface-exposed part of the M protein of Belgian and Brazilian isolates, respectively. The two main clades (A and B) of both trees correlate with the presence or absence of the serum opacity factor (*sof*) (which is one of the classification characteristics of GAS isolates [26,27]). Based on the organisation of *emm* and *emm*-like genes within the *mga* locus [25,28], GAS isolates can also be classified into *emm* patterns (A–C, D and E), which serve as an indicator of the tissue tropism. The A clades are mainly composed of *emm* pattern E (89% and 92% of the Brazilian and Belgian isolates, respectively), whilst the B clades are composed of *emm* patterns A–C and D. Fig. 1A shows that FQ-non-susceptible Belgian isolates are found in *emm* types that are distantly related (different Sof status and *emm* patterns). Similarly, Brazilian non-susceptible *emm* types are scattered along the tree showing that the emergence of non-susceptibility is independent of the *emm* type, the *emm* pattern and the *sof* status (Fig. 1B).

## 4. Discussion

Since there are no EUCAST/CLSI established breakpoints for ciprofloxacin resistance in GAS, most studies use a non-susceptibility cut-off ( $\geq 2$  mg/L). Using this cut-off for screening clinical isolates, as many as 14 mutations have been described in the QRDRs of *parC*, *parE*, *gyrA* and *gyrB* [4,7–10,12,14–16,29] and 4 new mutations were encountered in the present study. The QRDR sequence comparison

of susceptible and non-susceptible isolates of the same *emm* types revealed that the S79F in *parC* is a relevant mutation for ciprofloxacin non-susceptibility. This is in agreement with recent experimental data showing that mutations of S81 in *gyrA* and S79 in *parC* conferred FQ non-susceptibility and resistance [30]. These mutations were also found in the seven ‘fully’ resistant clinical isolates (ciprofloxacin MIC  $\geq$  32 mg/L) described so far [7–11]. Our data indicate that the other mutations in *parC/E* and *gyrA/B* QRDRs are likely to represent *emm* type-linked polymorphism, although we cannot exclude that these mutations could be indirectly associated with FQ non-susceptibility. These mutations were determined by comparison of the QRDR sequences of non-susceptible isolates with that of the M1 GAS reference strain (ATCC 700294). Use of this strain as a reference might not always be appropriate since *emm* type-linked polymorphism appears to exist (as well as some level of polymorphism inside particular *emm* types). To discriminate between clone-specific wild-type and mutant alleles conferring ciprofloxacin non-susceptibility, the sequence of the QRDRs of each ‘wild-type’ clone (belonging to an *emm* type and/or clonal type) should be established and made available in databases.

Because FQ resistance in Gram-positive bacteria is mediated by the accumulation of point mutations, it is intuitive to expect that resistance would emerge in different GAS genetic backgrounds. However, the molecular epidemiology of FQ resistance in a number of bacterial pathogens suggests that the persistence and spread of resistance is associated with a small number of highly successful clones [31]. The epidemiological studies of non-susceptible GAS, all of which were performed in Western countries, underlined this clonal aspect [4,12,14–16]. The common non-susceptible *emm* types described are 6, 75, 89, 22, 28, 1, 5 and 12; *emm* type 6

accounted for 60–100% of the non-susceptible isolates [4,12,14–16]. Our data regarding the Belgian isolates are in agreement with the published data, with only three *emm* types involved in non-susceptibility and a large predominance of *emm* type 6 (87%). By contrast, the eight non-susceptible Brazilian isolates belonged to seven different *emm* types. These *emm* types (59, 6, 74, 44/61, 94, 80 and 49) were different from those described in Western countries and are distantly related [20]. Thus, our data and the in vitro selection of FQ resistance [30] suggest that non-susceptibility to FQs may emerge in a diverse array of genetic backgrounds. The apparent clonality observed in Western countries might thus be due to the reduced number of circulating *emm* types rather than the non-susceptible status as was proposed for FQ-resistant *S. pneumoniae* [32–34].

The 22.5% of ciprofloxacin-non-susceptible GAS observed in Brussels is from far the highest rate ever described in epidemiological prospective studies, particularly in a paediatric population in which FQs are not supposed to be routinely used. We propose that this very high percentage is attributable to an epidemic wave of an *emm* type 6 clone during the spring period (data not shown). A rapid shift in predominance of GAS *emm* types in the community has been demonstrated [35] and, since *emm* type 6 isolates harbour an intrinsic reduced susceptibility to ciprofloxacin [4], it is possible that *emm* type 6 has been selected, notably by the use of FQs. This hypothesis is difficult to prove. However, it is reported that Belgian doctors are prompt in prescribing quinolones [1,36] and regularly prescribe topical treatment for ear infections (often containing quinolones). Topical FQ prescriptions (ear or eye drops) have been estimated to exceed 15 000 per year in Belgium [12]. As children serve as one of the primary community reservoirs for both GAS and *S. pneumoniae*,

topical use of FQs might contribute to the selection of FQ-non-susceptible clones and might constitute an important resistance driver.

In conclusion, we observed a clonal spreading of non-susceptible *emm* type 6 GAS strains in Brussels and a polyclonal distribution of non-susceptible isolates in Brazil. All the Brazilian and Belgian *emm* type 6 strains displayed an S79A/F mutation in *parC* that convincingly explains the non-susceptible phenotype. By contrast, the other non-susceptible isolates presented mutations that likely represent *emm* type-linked polymorphism. The molecular mechanism that confers non-susceptibility to these strains remains to be determined. It could include the plasmid-encoded quinolone resistance gene (*qnr*) [37], fluoroquinolone-modifying enzymes [38] and a variety of drug efflux pumps [39]. To our knowledge, these mechanisms have not or only rarely been described in GAS but may represent an interesting explanation for the wide variety of non-susceptible phenotypes linked to one given QRDR genotype.

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## References

- [1] Ferech M, Coenen S, Malhotra-Kumar S, Dvorakova K, Hendrickx E, Suetens C, et al. European Surveillance of Antimicrobial Consumption (ESAC): outpatient quinolone use in Europe. *J Antimicrob Chemother* 2006;58:423–7.
- [2] Gendrel D, Chalumeau M, Moulin F, Raymond J. Fluoroquinolones in paediatrics: a risk for the patient or for the community? *Lancet Infect Dis* 2003;3:537–46.
- [3] Committee on Infectious Diseases. The use of systemic fluoroquinolones. *Pediatrics* 2006;118:1287–92.
- [4] Orscheln RC, Johnson DR, Olson SM, Presti RM, Martin JM, Kaplan EL, et al. Intrinsic reduced susceptibility of serotype 6 *Streptococcus pyogenes* to fluoroquinolone antibiotics. *J Infect Dis* 2005;191:1272–9.
- [5] Boos M, Mayer S, Fischer A, Kohrer K, Scheuring S, Heisig P, et al. In vitro development of resistance to six quinolones in *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2001;45:938–42.
- [6] Jones HE, Brenwald NP, Owen KA, Gill MJ. A multidrug efflux phenotype mutant of *Streptococcus pyogenes*. *J Antimicrob Chemother* 2003;51:707–10.
- [7] Yan SS, Fox ML, Holland SM, Stock F, Gill VJ, Fedorko DP. Resistance to multiple fluoroquinolones in a clinical isolate of *Streptococcus pyogenes*: identification of *gyrA* and *parC* and specification of point mutations associated with resistance. *Antimicrob Agents Chemother* 2000;44:3196–8.
- [8] Richter SS, Diekema DJ, Heilmann KP, Almer LS, Shortridge VD, Zeitler R, et al. Fluoroquinolone resistance in *Streptococcus pyogenes*. *Clin Infect Dis* 2003;36:380–3.

- [9] Reinert RR, Lutticken R, Al-Lahham A. High-level fluoroquinolone resistance in a clinical *Streptococcus pyogenes* isolate in Germany. Clin Microbiol Infect 2004;10:659–62.
- [10] Rivera A, Rebollo M, Sanchez F, Navarro F, Miro E, Mirelis B, et al. Characterisation of fluoroquinolone-resistant clinical isolates of *Streptococcus pyogenes* in Barcelona, Spain. Clin Microbiol Infect 2005;11:759–61.
- [11] Alonso R, Mateo E, Ezpeleta G, Cisterna R. Characterisation of levofloxacin-resistant clinical isolates of *Streptococcus pyogenes* in Bilbao, Spain. Int J Antimicrob Agents 2007;30:183–5.
- [12] Malhotra-Kumar S, Lammens C, Chapelle S, Mallentjer C, Weyler J, Goossens H. Clonal spread of fluoroquinolone non-susceptible *Streptococcus pyogenes*. J Antimicrob Chemother 2005;55:320–5.
- [13] Perez-Trallero E, Fernandez-Mazarrasa C, Garcia-Rey C, Bouza E, Aguilar L, Garcia-de-Lomas J, et al. Antimicrobial susceptibilities of 1,684 *Streptococcus pneumoniae* and 2,039 *Streptococcus pyogenes* isolates and their ecological relationships: results of a 1-year (1998–1999) multicenter surveillance study in Spain. Antimicrob Agents Chemother 2001;45:3334–40.
- [14] Alberti S, Cortes G, Garcia-Rey C, Rubio C, Baquero F, Garcia-Rodriguez JA, et al. *Streptococcus pyogenes* pharyngeal isolates with reduced susceptibility to ciprofloxacin in Spain: mechanisms of resistance and clonal diversity. Antimicrob Agents Chemother 2005;49:418–20.
- [15] Alonso R, Mateo E, Galimand M, Garaizar J, Courvalin P, Cisterna R. Clonal spread of pediatric isolates of ciprofloxacin-resistant, *emm* type 6 *Streptococcus pyogenes*. J Clin Microbiol 2005;43:2492–3.

- [16] Powis J, McGeer A, Duncan C, Goren R, de Azavedo JC, Bast DJ, et al. Prevalence and characterization of invasive isolates of *Streptococcus pyogenes* with reduced susceptibility to fluoroquinolones. *Antimicrob Agents Chemother* 2005;49:2130–2.
- [17] Smeesters PR, Vergison A, Campos D, de Aguiar E, Deyi VY, Van Melder L. Differences between Belgian and Brazilian group A *Streptococcus* epidemiologic landscape. *PLoS ONE* 2006;1:e10.
- [18] Smeesters PR, Campos D Jr, Van Melder L, de Aguiar E, Vanderpas J, Vergison A. Pharyngitis in low-resources settings: a pragmatic clinical approach to reduce unnecessary antibiotic use. *Pediatrics* 2006;118:e1607–11.
- [19] National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. Twelfth informational supplement. Document M100-S12. Wayne, PA: NCCLS; 2002.
- [20] Smeesters PR, Mardulyn P, Vergison A, Leplae R, Van Melder L. Genetic diversity of group A *Streptococcus* M protein: implications for typing and vaccine development. *Vaccine* 2008;26:5835–42.
- [21] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–80.
- [22] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Mol Biol Evol* 2007;24:1596–9.
- [23] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–25.

- [24] Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. *Infect Immun* 2001;69:2416–27.
- [25] McGregor KF, Spratt BG, Kalia A, Bennett A, Bilek N, Beall B, et al. Multilocus sequence typing of *Streptococcus pyogenes* representing most known *emm* types and distinctions among subpopulation genetic structures. *J Bacteriol* 2004;186:4285–94.
- [26] Espinosa LE, Li Z, Gomez Barreto D, Calderon Jaimes E, Rodriguez RS, Sakota V, et al. M protein gene type distribution among group A streptococcal clinical isolates recovered in Mexico City, Mexico, from 1991 to 2000, and Durango, Mexico, from 1998 to 1999: overlap with type distribution within the United States. *J Clin Microbiol* 2003;41:373–8.
- [27] Beall B, Gherardi G, Lovgren M, Facklam RR, Forwick BA, Tyrrell GJ. *emm* and *sof* gene sequence variation in relation to serological typing of opacity-factor-positive group A streptococci. *Microbiology* 2000;146:1195–209.
- [28] Bessen DE, Carapetis JR, Beall B, Katz R, Hibble M, Currie BJ, et al. Contrasting molecular epidemiology of group A streptococci causing tropical and nontropical infections of the skin and throat. *J Infect Dis* 2000;182:1109–16.
- [29] Alonso R, Galimand M, Courvalin P. *parC* mutation conferring ciprofloxacin resistance in *Streptococcus pyogenes* BM4513. *Antimicrob Agents Chemother* 2002;46:3686–7.
- [30] Billal DS, Fedorko DP, Yan SS, Hotomi M, Fujihara K, Nelson N, et al. In vitro induction and selection of fluoroquinolone-resistant mutants of *Streptococcus pyogenes* strains with multiple *emm* types. *J Antimicrob Chemother* 2007;59:28–34.

- [31] Klugman KP. The role of clonality in the global spread of fluoroquinolone-resistant bacteria. *Clin Infect Dis* 2003;36:783–5.
- [32] Davies TA, Goldschmidt R, Pflieger S, Loeloff M, Bush K, Sahm DF, et al. Cross-resistance, relatedness and allele analysis of fluoroquinolone-resistant US clinical isolates of *Streptococcus pneumoniae* (1998–2000). *J Antimicrob Chemother* 2003;52:168–75.
- [33] Johnson CN, Benjamin Jr WH, Moser SA, Hollingshead SK, Zheng X, Crain MJ, et al. Genetic relatedness of levofloxacin-nonsusceptible *Streptococcus pneumoniae* isolates from North America. *J Clin Microbiol* 2003;41:2458–64.
- [34] Nichol KA, Zhanel GG, Hoban DJ. Molecular epidemiology of penicillin-resistant and ciprofloxacin-resistant *Streptococcus pneumoniae* in Canada. *Antimicrob Agents Chemother* 2003;47:804–8.
- [35] Kaplan EL, Wotton JT, Johnson DR. Dynamic epidemiology of group A streptococcal serotypes associated with pharyngitis. *Lancet* 2001;358:1334–7.
- [36] Goossens H, Ferech M, Vander Stichele R, Elseviers M. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 2005;365:579–87.
- [37] Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, et al. *qnrB*, another plasmid-mediated gene for quinolone resistance. *Antimicrob Agents Chemother* 2006;50:1178–82.
- [38] Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, et al. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* 2006;12:83–8.
- [39] Jacoby GA. Mechanisms of resistance to quinolones. *Clin Infect Dis* 2005;41(Suppl 2):S120–6.

Fig. 1. Ciprofloxacin non-susceptibility arises in distantly related *emm* types. The genetic relationships between the surface-exposed region of the M protein of (A) 18 Belgian Group A streptococci (GAS) and (B) 46 Brazilian GAS are represented. The *emm* types and *emm* patterns are indicated. The surface of the circle is proportional to the relative frequency of each *emm* type. The proportion of susceptible and non-susceptible isolates within a given *emm* type is represented in white and black, respectively. The two main clades (A and B) correlate with the Sof status. The evolutionary history was inferred using the neighbour-joining method [23]. The tree is drawn to scale, with branch lengths proportional to the evolutionary distances. Bootstrap value >70% are shown next to the branches (500 replicates).

Table 1

In vitro activities of ciprofloxacin and moxifloxacin against Belgian and Brazilian isolates

City	No. of isolates	Antibiotic	No. (%) non-susceptible isolates	No. of isolates with MIC (mg/L) <sup>a</sup>										
				≤0.06	0.12	0.25	0.5	1	2	4	8	16		
Brussels	204	Ciprofloxacin	46 (22.5)	1	1	20	98	38	25	19	2			
		Moxifloxacin	0 (0)	7	94	62	38	3						
Brazil	130	Ciprofloxacin	8 (6)			6	70	46	8					
		Moxifloxacin	0 (0)	4	76	41	9							

MIC, minimal inhibitory concentration.

<sup>a</sup> The non-susceptible breakpoint for ciprofloxacin is  $\geq 2$  mg/L; breakpoints for moxifloxacin are  $\leq 0.5$ / $>1$  mg/L for susceptibility and resistance, respectively.

Table 2

Amino acid substitutions in topoisomerase IV subunits of ciprofloxacin non-susceptible isolates

City	<i>emm</i> type	<i>emm</i> subtype	Ciprofloxacin MIC range (mg/L)	No. of strains (% by city)	AA substitution(s)	
					ParC	ParE
Brussels	6	6.0	2–8	22 (48)	S79A	A378T
		6.14	8	1 (2)	S79A	A378T
		6.4	2–4	13 (28)	S79A	A378T
		6.5	2–4	4 (9)	S79A	A378T
	89	89.0	2	3 (6.5)	D91N, S140P	E360D, D493N <sup>a</sup>
		st1815	st1815.0	2–4	3 (6.5)	S79F, D91N
Brazil	59	59.0	2	2 (25)	S140P	–
	6	6.0	2	1 (12.5)	S79A	A378T
	74	74.0	2	1 (12.5)	–	A471V <sup>a</sup>
	44/61	44/61.0	2	1 (12.5)	–	A471V <sup>a</sup>
	94	94.2	2	1 (12.5)	–	–
	80	80.1	2	1 (12.5)	–	A395T <sup>a</sup>
	49	49.3	2	1 (12.5)	–	–

MIC, minimal inhibitory concentration; AA, amino acid.

<sup>a</sup> New mutation associated with a non-susceptible phenotype.



Table 3

Distribution of *parC* and *parE* mutations according to ciprofloxacin susceptibility and *emm* types <sup>a</sup>

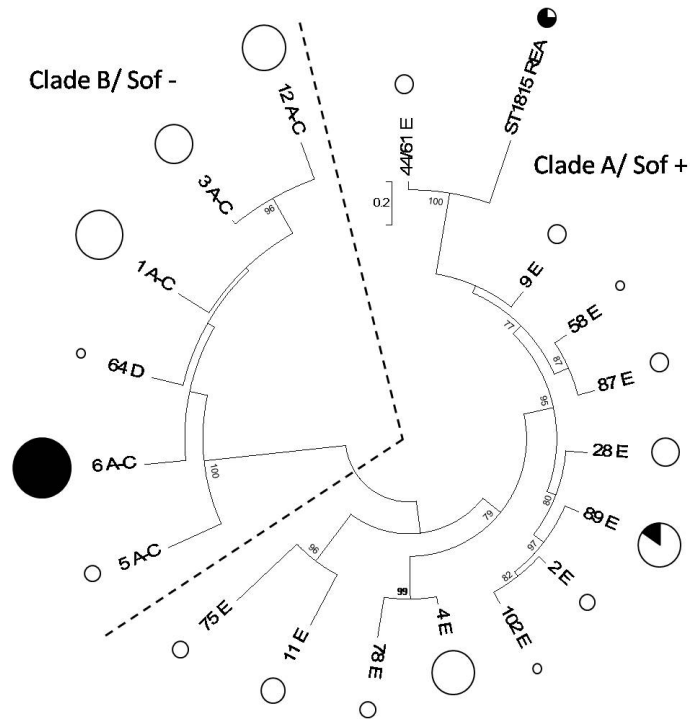
	<i>parC</i>			<i>parE</i>					
	S79A	S79F	D91N	S140P	E360D	A378T	A395T <sup>b</sup>	A471V <sup>b</sup>	D493N <sup>b</sup>
<i>emm</i> types	6	st1815	st1815, 89	89, 59	89	6	80	74, 44/61	89
No. with mutations and MIC $\geq$ 2 mg/L	41	3	6	5	3	41	1	2	3
No. with mutations and MIC < 2 mg/L	NT	0	9	8	2	NT	1	1	2
No. without mutations and MIC $\geq$ 2 mg/L	0	0	0	0	0	0	0	0	0
No. without mutations and MIC < 2 mg/L	NT	4	0	0	0	NT	1	5	0
No. of strains analysed	41	7	15	13	5	41	3	8	5

MIC, minimal inhibitory concentration; NT, not tested, since no susceptible isolate was available in our collection.

<sup>a</sup> Mutations are shown for susceptible (MIC < 2 mg/L) and non-susceptible (MIC  $\geq$  2 mg/L) isolates belonging to the same *emm* types.

<sup>b</sup> New mutations found in this study.

(A)



(B)

