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 Brasilia (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 >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 579A/F mutation in parC that convincingly explains the non-susceptible phenotype.

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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 a MIC ≥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. Materials 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 Brasilia (Brazil) [17]. Patients aged 0–15 years attending three public hospitals in Brazil and one public hospital in 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: ≤0.5/>1 mg/L). Non-susceptibility to ciprofloxacin was defined as a MIC ≥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 antibiotic therapy, age, sex, country of birth, 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 type 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 has 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). 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 (MIC ≥2 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 a MIC ≥4 mg/L and belonged mostly to emm type 6 (one isolate was emm type st1815). The 33 remaining isolates showed a 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.

Table 1 In vitro activities of ciprofloxacin and moxifloxacin against Belgian and Brazilian isolates.

<table>
<thead>
<tr>
<th>City</th>
<th>No. of isolates</th>
<th>Antibiotic</th>
<th>No. (%) of non-susceptible isolates</th>
<th>No. of isolates with MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>≤0.06</td>
</tr>
<tr>
<td>Brussels</td>
<td>204</td>
<td>Ciprofloxacin</td>
<td>46 (22.5)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>0 (0)</td>
<td>7</td>
</tr>
<tr>
<td>Brazil</td>
<td>130</td>
<td>Ciprofloxacin</td>
<td>8 (6)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moxifloxacin</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

MIC, minimal inhibitory concentration.

* The non-susceptible breakpoint for ciprofloxacin is ≥2 mg/L; breakpoints for moxifloxacin are ≤0.5/>1 mg/L for susceptibility and resistance, respectively.

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 dataset. 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, EF031333 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 \((MIC \geq 2 \text{ mg}/\text{L})\). 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 a \(MIC\) of \(2 \text{ mg}/\text{L})\) 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

Fig. 1A and B represents 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
whilst the B clades are composed of E (89% and 92% of the Brazilian and Belgian isolates, respectively), sue tropism. The A clades are mainly composed of emm patterns (A–C, D and E), which serve as an indicator of the tis-
emm types that are distantly related (different Sof status and
within the
lates [26,27]). Based on the organisation of (
locus [25,28], GAS isolates can also be classified into
emm patterns (A–C, D and E), which serve as an indicator of the tis-
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MIC, minimal inhibitory concentration; NT, not tested, since no susceptible isolate was available in our collection.

<table>
<thead>
<tr>
<th>City</th>
<th>emm type</th>
<th>emm subtype</th>
<th>Ciprofloxacin MIC range (mg/L)</th>
<th>No. of strains (% by city)</th>
<th>AA substitution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ParC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ParE</td>
</tr>
<tr>
<td>Brussels 6</td>
<td>6.0</td>
<td>2–8</td>
<td>22 (48)</td>
<td></td>
<td>S79A A378T</td>
</tr>
<tr>
<td></td>
<td>6.14</td>
<td>8</td>
<td></td>
<td></td>
<td>S79A A378T</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>2–4</td>
<td>13 (28)</td>
<td></td>
<td>S79A A378T</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>2–4</td>
<td>4 (9)</td>
<td></td>
<td>S79A A378T</td>
</tr>
<tr>
<td></td>
<td>89</td>
<td>90.0</td>
<td>3 (6.5)</td>
<td></td>
<td>S79F D91N</td>
</tr>
<tr>
<td></td>
<td>st1815</td>
<td>st1815.0</td>
<td>2–4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil 59</td>
<td>59.0</td>
<td>2</td>
<td>2 (25)</td>
<td></td>
<td>S140P</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.0</td>
<td>1 (12.5)</td>
<td></td>
<td>S79A A378T</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>74.0</td>
<td>1 (12.5)</td>
<td></td>
<td>– A471Vb</td>
</tr>
<tr>
<td></td>
<td>44/61</td>
<td>44/61.0</td>
<td>1 (12.5)</td>
<td></td>
<td>– A471Vb</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>94.2</td>
<td>1 (12.5)</td>
<td></td>
<td>– –</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>80.1</td>
<td>1 (12.5)</td>
<td></td>
<td>– –</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>49.3</td>
<td>1 (12.5)</td>
<td></td>
<td>– –</td>
</tr>
</tbody>
</table>

MIC, minimal inhibitory concentration; AA, amino acid.

* New mutation associated with a non-susceptible phenotype.

4. Discussion

Since there are no EUCAST/CLSI established breakpoints for ciprofloxacin resistance in GAS, most studies use a non-susceptibility cut-off (≥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 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).

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. 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.

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], fluorquinolone-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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Ethical approval: This study was approved by the ethical board of all participating hospitals.

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