Emergence of Fluoroquinolone Resistance in Group B Streptococcal Isolates in Taiwan (Revised Manuscript AAC00350-08)

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Running title: Fluoroquinolone resistance in GBS isolates in Taiwan.
ABSTRACT

Of the 1,994 group B streptococcal isolates collected, 26 (1.3%) of the isolates were resistant to levofloxacin, and cross resistance was observed to other fluoroquinolones. Emergence and prevalence of high level fluoroquinolone resistance in genetically unrelated isolates was linked to the presence of gyrA, parC and parE triple mutations in each isolate.
Resistance to fluoroquinolones has emerged in different *Streptococcus* spp. (4, 5, 8, 15), mainly due to efflux or mutations in the quinolone resistance-determining regions (QRDR) of the genes coding for type II topoisomerase enzymes: DNA gyrase (*gyrA/*gyrB*) and topoisomerase IV (*parC/*parE*) (7). Recently, Biedenbach *et al.* showed that fluoroquinolone resistant GBS (FR-GBS) were identified in the USA in 1997; however in the western Pacific region in Japan it was detected in the year 2002 (1, 8). Although ciprofloxacin has been available since 1990, and levofloxacin was introduced in 2000 in Taiwan, none of the studies reported the presence of fluoroquinolone resistance in GBS isolates from 1990 to 2007 (6). The objective of this study was to: 1) determine the prevalence of fluoroquinolone resistance, 2) to characterize the QRDR mutations, and 3) to determine the clonal relation among resistant isolates by pulsed-field gel electrophoresis (PFGE).

A total of 1,994 GBS isolates were collected from 1993 to 2006 in the Department of Pathology, National Cheng Kung University Hospital in southern Taiwan. Clinical isolates of Gram-positive cocci with beta hemolysis and a positive CAMP test were tested with Lancefield group B antiserum (Streptex; Murex Biotech, UK). Those cocci agglutinating with grouping–specific antiserum were identified as *S. agalactiae*. Clinical isolates were stored in Todd–Hewitt medium (Difco Laboratories, Detroit, MI) with 15% glycerol at -70°C until further testing. Antibiotic susceptibilities were performed by disk diffusion for the following antibiotics: ampicillin, cefazolin, clindamycin, erythromycin, gentamicin, levofloxacin, penicillin and vancomycin (2). Minimum inhibitory concentration (MIC) was determined by agar dilution method for levofloxacin (MIC breakpoint ≥ 8 µg/ml) and other fluoroquinolones, including
moxifloxacin, gatifloxacin, trovafloxacin, garenoxacin, and ciprofloxacin (3). PCR amplification and DNA sequencing of QRDR regions responsible for the fluoroquinolone-resistant phenotype were performed as described previously (1, 14). PFGE of Smal-digested genomic DNA samples was carried out with a contour-clamped homogeneous electric field system (CHEF Mapper XA; Bio-Rad Laboratories, Hercules, CA) according to the instruction manual. PFGE banding patterns were interpreted as described previously (13).

Of the 1,994 GBS isolates, 26 (1.3 %) isolates were resistant to levofloxacin. The MIC of levofloxacin resistant (LR) GBS isolates was found to be $\geq 16 \mu g/ml$. Since mutations in the QRDR leads to cross resistance to fluoroquinolones, we determined the MICs by the agar dilution method and found that all the LR-GBS isolates had elevation in MICs to ciprofloxacin (MIC, 32-64 $\mu g/ml$), garenoxacin (MIC, 2-4 $\mu g/ml$), gatifloxacin (MIC, 4-8 $\mu g/ml$), moxifloxacin (MIC, 2-4 $\mu g/ml$), and trovafloxacin (MIC, 8-16 $\mu g/ml$) (Table 1). The first FR-GBS clinical isolate was isolated in November 2004 from the urogenital tract of a 40 year old female patient. Since 2004, the annual prevalence rate of FR-GBS isolates significantly increased from 0.33% (1 isolate) in 2004 to 3.83% (13) in 2005 and 5.04% in 2006 (12). The antimicrobial susceptibility pattern test showed that all the FR-GBS isolates were totally susceptible to penicillin, ampicillin, vancomycin, and cefazolin. Resistance to gentamicin was found in 100% of isolates, followed by erythromycin (65%) and clindamycin (62%).

$gyrA$, $parC$ and $parE$ triple mutations were identified in all the FR-GBS isolates (Table 1). In $gyrA$, a Ser-81-to-Leu (Ser81Leu) mutation was predominant, while in $gyrB$ mutations were absent. But in $parC$, a Ser79Tyr mutation was present in 16 isolates and...
10 isolates had a Ser79Phe mutation. In \textit{parE}, all the isolates had a unique Ile495Leu mutation. Isolates with the \textit{parE} mutation also had \textit{gyrA} (Ser81Leu) and \textit{parC} (Ser79Phe) mutations predominantly. To determine the clonal relation of isolates resistant to fluoroquinolones, we performed PFGE. Among the 26 resistant isolates, 17 pulsed types (A to Q) were identified (Fig. 1, Table 1).

In Taiwan, the presence of fluoroquinolone resistance in GBS isolates have not been reported before. After isolating the first FR-GBS strain in November 2004, the annual incidence rate, which was 0.33% in 2004, significantly increased to 3.83% in 2005 and 5.04% in 2006. This indicates that FR-GBS isolates not only emerged, but that they are significantly more prevalent since late 2004 in this region. Overall, the FR-GBS prevalence rate in our study was 1.3%, which is similar to prevalence rates reported earlier in Barcelona, Spain (1.16%, 2003-2004) and higher than in the USA (0.7%, 1997-2004) (1, 12).

In GBS, QRDR mutations have been reported previously in \textit{gyrA} and \textit{parC} only. High level resistance could be linked to the presence of a triple mutation (\textit{gyrA-parC-parE}) in each isolate. Mutations in \textit{gyrA} occurred predominantly at amino acid position 81 (Ser-81-to-Leu) and in \textit{parC} at Ser79Tyr and Ser79Phe, which were similar to earlier reports (1, 9, 14). The QRDR of \textit{gyrA} is the primary target which mediates fluoroquinolone resistance in several gram negative bacteria, while in gram positive bacteria \textit{parC} is the primary target, which substantially increases the probability of a second mutation in \textit{gyrA}, resulting in high level fluoroquinolone resistance (4, 7, 10). Based on our data we were unable to determine whether gyrase or topoisomerase IV is the primary target for fluoroquinolones or the other antibiotic(s) associated with the triple
mutations. Further analysis of the minor targets gyrB and parE, revealed that mutations were absent in gyrB in FR-GBS isolates, similar to the previous reports on fluoroquinolone resistance in GBS (1, 8, 14). A unique mutation (Ile495Leu) was present in parE, but since gyrB and parC mutations were also present in the isolates with the parE mutation, its significance is unknown. It can be either a silent mutation or a mutation similar to a S. pneumoniae parE (Ile-460-Val) variant that led to reduced susceptibility to fluoroquinolones (9). Presence of 17 pulsed types and absence of a predominant pulsed type indicates that the fluoroquinolone resistant isolates are genetically unrelated. Spread of FR due to single and multiple clones was observed in other S. pneumoniae and S. pyogenes isolates (5, 11). In GBS, Wehbeh et al., suggested the nosocomial spread of levofloxacin resistant GBS isolates based on two major clusters identified by PFGE (14).

Since all the FR-GBS isolates were totally susceptible to ampicillin, penicillin, cefazolin and vancomycin, these antibiotics remain the preferred choice to treat GBS infections empirically. Since resistance to fluoroquinolones can develop during therapy and cross-resistance to other fluoroquinolones is likely to occur, when prescribing fluoroquinolones for GBS infections susceptibility testing and monitoring during therapy should be done to avoid treatment failure (5, 10). Continuous molecular level surveillance is needed to prevent further dissemination of FR-GBS clones in Taiwan.

In conclusion, our study confirmed the emergence of genetically unrelated FR-GBS isolates in Taiwan and showed a significant increase in prevalence from 2004 to 2006. High level fluoroquinolone resistance in the isolates could be linked to the presence of gyrA-parC-parE triple mutations in each isolate.
This work was partially supported by grants DOH93-DC-1110, DOH94-DC-1008, and DOH95-DC-1038 from the Bureau of Center of Disease Control, Department of Health, Taiwan.
REFERENCES


FIGURE LEGEND

FIGURE 1

PFGE patterns of 26 fluoroquinolone-resistant GBS isolates. The designation of isolates that were represented by each pulsed type are shown below (A) and (B) gels. Lane M contains a lambda ladder (Gibco) that served as a molecular marker.
FIGURE 1.

(A)

(B)
TABLE 1. MICs of fluoroquinolones and mutations in QRDR of gyrase (gyrA/gyrB) and topoisomerase IV (parC/parE) in GBS.

<table>
<thead>
<tr>
<th>No. of GBS isolates</th>
<th>MIC (µg/ml)</th>
<th>QRDR mutation</th>
<th>PFGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cip</td>
<td>Lev</td>
<td>Gar</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>32</td>
<td>2-4</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
<td>16-32</td>
<td>2-4</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>16</td>
<td>2</td>
</tr>
</tbody>
</table>

Cip, ciprofloxacin; Lev, levofloxacin; Gar, garenoxacin; Gat, gatifloxacin; Mox, moxifloxacin; Tro, trovafloxacin