Of 901 group B streptococcus strains analyzed, 13 (1.4%) were resistant to levofloxacin (MICs of >32 µg/ml for seven isolates, 2 µg/ml for one isolate, and 1.5 µg/ml for four isolates). Mutations in the quinolone resistance-determining regions (QRDRs) of gyrA and topoisomerase IV were identified. A double mutation involving the Ser-81 change to Leu for gyrA and the Ser-79 change to Phe or to Tyr for parC was linked to a high level of fluoroquinolone resistance. In addition, two other mutational positions in parC were observed, resulting in an Asp-83-to-Tyr substitution and an Asp-83-to-Asn substitution. Different mutations were also observed in gyrB, with unknown significance. Most levofloxacin-resistant GBS strains were of serotype Ib and belonged to sequence type 19 (ST19) and clonal complex 19 (CC-19). Most of them exhibited the epsilon gene.

**Streptococcus agalactiae** (group B streptococcus [GBS]) is an important agent of neonatal sepsis and meningitis (1). It is also responsible for different infections in nonpregnant individuals, in particular in elderly people and those with underlying diseases (2). Penicillin is the first-line antibiotic for treatment of GBS infection, even though strains with reduced susceptibility to this drug were recently identified (3, 4). Macrolides are the recommended second-line drugs, moreover, in cases of β-lactam allergy. Nevertheless, considering increasing resistance to erythromycin and clindamycin (5, 6), fluoroquinolones are important alternatives. However, fluoroquinolone resistance has also been reported (3, 7, 8). The purposes of this study were (i) to determine the prevalence of fluoroquinolone resistance, (ii) to characterize the mutations in the quinolone resistance-determining region (QRDR), and (iii) to determine the clonal relationship by multilocus sequence typing (MLST). These strains were further characterized for surface proteins and capsular type, which represent important virulence factors of GBS.

A total of 901 GBS isolates were collected between January 2013 and June 2014. All isolates were from consecutive outpatients who had attended Brescia’s hospital for gynecologic health care control, for normal routine screening during pregnancy, or for the presence of symptoms of urinary infections. Bacteria were isolated by streak plating 1 to 10 µl of transport medium on ChromID Strepto B agar plates (bioMérieux, Florence, Italy). GBS were identified by means of the Vitek system (bioMérieux). Clinical isolates were stored in Todd-Hewitt medium (Difco Laboratories) with 15% glycerol at −70°C until further testing.

Levofloxacin was tested using an automated microdilution broth method (Vitek2; bioMérieux). The concentrations ranged from 0.25 to 8 µg/ml. Breakpoint interpretation was done according to EUCAST guidelines (9), i.e., ≤1 and >2 µg/ml were considered susceptible and resistant, respectively, for levofloxacin.

Initial screening by the automated microdilution broth method (Vitek2) identified 27 isolates that were not susceptible to levofloxacin (2.9%) and 7 isolates resistant to moxifloxacin (0.8%) (Table 1). After suspecting a possible error in levofloxacin susceptibility testing by AST (antibiotic susceptibility testing), we determined the susceptibilities of these isolates by a manual method (Etest; bioMérieux). The test was performed according to the manufacturer’s instructions. Antibiotic concentrations ranged from 0.002 to 32 µg/ml. S. pneumoniae ATCC 49619 was used as a quality control.

The MICs obtained by Etest were not in full agreement with the results of Vitek2. Inaccuracies with AST have already been reported in the literature, particularly for nonfermenters (10, 11). Discrepancies were observed for 14 isolates that had an MIC of 2 µg/ml by Vitek2: among these, four had an MIC of 1 µg/ml by Etest, six had an MIC of 0.75 µg/ml by Etest, and four had an MIC of 0.5 µg/ml by Etest (all susceptible by the EUCAST criteria). The results obtained by Etest were more reliable than the genetic results and were therefore considered definitive for the interpretation of data in this work. A total of 13 isolates (1.4%) were resistant to levofloxacin, including MICs of >32 µg/ml for seven isolates and 2 µg/ml for two isolates. Four isolates expressed intermediate resistance to this antibiotic (MICs of 1.5 µg/ml).

These results highlight important Vitek2 limitations in levofloxacin susceptibility testing. The Vitek2 test produced results with higher MICs, resulting in false resistance findings.

All levofloxacin-resistant isolates were susceptible to penicillin, cefuroxime, cefadroxil, and ceftriaxone. Resistance to tetracycline, erythromycin, and clindamycin was found in 10 (76.9%), 7 (53.8%), and 5 (38.4%) isolates, respectively.

So far, our data show that the overall rate of levofloxacin resistance in GBS isolates was 1.4%. This rate was lower than rates reported in China (37.7%) (12) and in Japan (18.4% resistance) (13) but similar to prevalence rates reported earlier in Spain (11.6%) (14) and higher than those in the United States (0.7%)...
TABLE 1 Serotypes, alp genes, and genetic characteristics of levofloxacin-resistant GBS isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Specimen type</th>
<th>MIC (µg/ml)†</th>
<th>Broth microdilution</th>
<th>Topoisomerase mutation(s)‡</th>
<th>MLST result</th>
<th>Serotype</th>
<th>alp gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Etest (LVX)</td>
<td>LXY</td>
<td>MXF</td>
<td>gyrA</td>
<td>gyrB</td>
<td>parC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(LVX)</td>
<td>LXY</td>
<td>MXF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
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</tr>
<tr>
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<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;8</td>
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<td>Y500Q, F501K</td>
<td>S79F</td>
</tr>
<tr>
<td>3</td>
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<td>WT</td>
<td>ST389 (CC-19)</td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
<td>ST137 (CC-17)</td>
</tr>
<tr>
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<td>Vaginal</td>
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<td>2</td>
<td>0.5</td>
<td>WT</td>
<td>WT</td>
<td>ST10 (CC-1)</td>
</tr>
<tr>
<td>6</td>
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<td>2</td>
<td>&lt;0.25</td>
<td>WT</td>
<td>V186S, I187L</td>
<td>S79F</td>
</tr>
<tr>
<td>7</td>
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<td>&gt;8</td>
<td>4</td>
<td>S81L</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
<td>8</td>
<td>Urine</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>L206C</td>
<td>K310R</td>
<td>WT</td>
</tr>
<tr>
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<td>Vaginal</td>
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<td>2</td>
<td>0.5</td>
<td>WT</td>
<td>V186S, I187L</td>
<td>ST19 (CC-19)</td>
</tr>
<tr>
<td>10</td>
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<td>2</td>
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<td>P211L</td>
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<td>WT</td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
<td>ST1 (CC-1)</td>
</tr>
<tr>
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<td>WT</td>
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</tr>
<tr>
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<td>2</td>
<td>&lt;0.25</td>
<td>WT</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
<td>14</td>
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<td>&gt;8</td>
<td>4</td>
<td>S81L</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
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<td>0.5</td>
<td>WT</td>
<td>D83Y</td>
<td>ST1 (CC-1)</td>
</tr>
<tr>
<td>16</td>
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<td>0.5</td>
<td>WT</td>
<td>V498A</td>
<td>WT</td>
</tr>
<tr>
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<td>2</td>
<td>0.5</td>
<td>WT</td>
<td>WT</td>
<td>ST1 (CC-1)</td>
</tr>
<tr>
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<td>0.5</td>
<td>WT</td>
<td>I461N</td>
<td>ST79F</td>
</tr>
<tr>
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<td>2</td>
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<td>WT</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
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<td>0.5</td>
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<td>WT</td>
<td>S79F</td>
</tr>
<tr>
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<td>WT</td>
<td>S496L</td>
<td>V498C</td>
</tr>
<tr>
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<td>&gt;8</td>
<td>4</td>
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<td>WT</td>
<td>S79F</td>
</tr>
<tr>
<td>23</td>
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<td>&gt;8</td>
<td>4</td>
<td>S81L</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
<td>24</td>
<td>Urine</td>
<td>&gt;32</td>
<td>&gt;8</td>
<td>4</td>
<td>S81L; K193Q; L194F</td>
<td>S79F</td>
<td>ST19 (CC-19)</td>
</tr>
<tr>
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<td>Vaginal</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
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<td>Vaginal</td>
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<td>&gt;8</td>
<td>4</td>
<td>S81L</td>
<td>WT</td>
<td>S79F</td>
</tr>
<tr>
<td>27</td>
<td>Vaginal</td>
<td>0.75</td>
<td>2</td>
<td>&lt;0.25</td>
<td>WT</td>
<td>WT</td>
<td>S79F</td>
</tr>
</tbody>
</table>

†LVX, levofloxacin; MXF, moxifloxacin.
‡WT, wild type.
§Sequence type not associated with any clonal complex and designated as a singleton.

Differences in antibiotic use might be the major factor contributing to geographic differences in fluoroquinolone resistance. PCR amplification and DNA sequencing of the gyrA, gyrB, and parC genes, which include the QRDRs responsible for the fluoroquinolone resistance phenotype, were performed as previously described (3). Streptococcus agalactiae strain 2603V/R (ATCC BAA-611; GenBank accession number NC_004116.1) was used as a reference strain for comparative analysis.

All the resistant isolates had amino acid changes within the QRDRs of gyrA, gyrB, and parC compared to the reference sequence (Table 1). These quinolone-resistant S. agalactiae isolates carried mostly double point mutations of DNA with inferred amino acid substitutions including the change of Ser-81 to Leu in the product of gyrA and Ser-79 to Phe or to Tyr in the product of parC. The level of levofloxacin resistance achieved was dependent on the combinations of gyrA- and parC-encoded amino acid changes present in each isolate. In fact, these double mutations invariably resulted in an MIC of >32 µg/ml. In contrast to these double mutations, a single mutation in parC resulted in intermediate resistance (MIC of 1.5 µg/ml). Two other mutations in the parC protein were observed: an Asp-83-to-Tyr substitution was found in an isolate with an MIC of 2 µg/ml, and an Asp-83-to-Asn substitution was found in another with an MIC of 1.5 µg/ml. There were numerous silent nucleotide base substitutions, especially in parC: Val-186 to Ser and Ile-187 to Leu were both present in two isolates, and Arg-95 to His, Gly-128 to Asp, and Ile-25 to Leu were all present in one isolate together with the Ser-79-to-Tyr substitution. The silent mutations observed in the gyrA protein were Leu-206 to Cys and Pro-211 to Leu. One isolate exhibited a double mutation in addition to the Ser-81 change to Leu: Lys-193 to Gln and Leu-194 to Ile. Six isolates had nucleotide base substitutions in gyrB (Table 1), but since gyrA and/or parC mutations were also present in most of the isolates with the gyrB mutation, their significance is unknown. They could be either silent mutations or mutation variants that contributed to reduced susceptibility to levofloxacin. Only one isolate that had an intermediate resistance to levofloxacin with an MIC of 1.5 µg/ml exhibited a single nucleotide base substitution exclusively in gyrB, resulting in a Val-498 change to Ala.

The main mutation pattern observed in this study was Ser79Phe and Ser79Tyr in the parC gene and Ser81Leu in the gyrA gene, as previously reported (8, 16). Recovery of double mutants...
was associated with a high breakpoint of resistance (MIC > 32 μg/ml). This level is notably higher than the serum level reported during treatment (3 to 5 μg/ml), although tissue concentrations may be higher. From our data, we were unable to determine whether gyrA or topoisomerase IV is the primary target for fluoroquinolones resistance associated with the double mutations. To our knowledge for the first time two novel mutations, Asp-83 to Asn, were found to be encoded in the parC region of *S. agalactiae*, similar to those found in *Streptococcus pneumoniae* that led to reduced susceptibility to fluoroquinolones (17) and in this study were associated with one resistant and one intermediate resistant strain. Other mutations found in ParC were previously reported as silent mutations, such as Arg-95 to His in *Streptococcus pneumoniae* (17) and Gly-128 to Asp in *Streptococcus agalactiae*. This last substitution was found in one resistant isolate, but it was demonstrated that the residue Gly128 was located far from levofloxacin (ca. 26.5 Å) and there was no possible interaction between drug and amino acid (18).

The capsular genotype (Ia, Ib, or II to IX) of *S. agalactiae* was identified by a multiplex PCR assay as previously described (19). DNA was extracted from each isolate by the NucliSENS easyMAG system (bioMérieux) according to the manufacturer’s instructions.

Overall, the most prevalent serotype isolated among the levofloxacin-resistant isolates was serotype Ib (6/13; 46%), followed by serotype V (3/13; 23%). Surface proteins of GBS are likely to play an important role in the pathogenesis of *S. agalactiae* infection, and in order to establish a possible association between *alp* genes, serotypes, and levofloxacin resistance, they were evaluated by using a multiplex PCR as previously described (20).

MLST analysis of all isolates was performed as described elsewhere (21). The sequence types were grouped with the eBURST program ([http://eburst.mlst.net/v3/enter_data/single/](http://eburst.mlst.net/v3/enter_data/single/)) into clonal complexes (CCs) whose members shared at least five of the seven MLST loci (22); otherwise, an ST was considered a singleton.

The χ² test was used to evaluate the differences in distributions of surface proteins, serotypes, and clonal clusters. A P value of <0.05 was considered significant, and a P value of <0.01 was considered highly significant.

An high statistically significant association was found between the Ib serotype and *alpC* (P < 0.01) in resistant isolates. Different associations of *alp* genes were observed in a single isolate (Table 1).

MLST analysis revealed 15 STs, inclusively among isolates expressing the same serotype. All these STs were grouped into three clonal complexes (CCs); 5 singleton STs were identified that were not a part of a cluster. The groups obtained by eBURST analysis were CC-1 (including ST1, ST2, ST413, ST321, and ST293; n = 10 [37%]), CC-19 (including ST19, ST389, and ST28; n = 9 [33%]), CC-17 (including ST17 and ST137; n = 2 [7.4%]) and singletons (ST461, ST23, ST480, ST4, and ST10; n = 6 [22%]).

Of the 13 isolates with resistance to levofloxacin, 6 (46%) belonged to CC-19. This clonal complex has a statistically significant association with serotype Ib (P < 0.05), and most of these isolates had the *epsilon* gene (5/9; 55%). Among the levofloxacin-susceptible isolates, CC-1 was the most predominant clonal complex (7/14; 50%).

Therefore, ST19–CC-19/serotype Ib/epsilon was the most predominant type in levofloxacin-resistant isolates, underscoring the idea that the spread of fluoroquinolone resistance is due to the emergence of this particular clone. Recently, it was demonstrated that the clonal expansion of multidrug-resistant ST19/serotype III was the primary cause of the high prevalence of fluoroquinolones resistance in China (12). The association of ST19 with serotype Ib is not strange, because it was reported in Mediterranean region together with association with serotypes II, III, and IV, which may be due to the horizontal transfer of capsular genes among different clones (12).

In conclusion, our data highlight the emergence of genetically related levofloxacin-resistant GBS strains in Italy and emphasize the need for careful epidemiologic investigation. Furthermore, this study underlines the importance of monitoring antibiotic susceptibility to quinolones. In fact, a clinical concern is the inadvertent consequences of the use of this class of antibiotics, in particular to treat urinary tract infections, which may contribute to the selection of resistant strains, especially considering that GBS is a frequent uropathogen.

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