Clonal Dissemination of *Yersinia enterocolitica* Strains with Various Susceptibilities to Nalidixic Acid

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Ten epidemiologically related *Yersinia enterocolitica* clinical isolates were studied. Six isolates were nalidixic acid resistant (MIC > 512 µg/ml), with mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene, suggesting clonal dissemination of a nalidixic acid-susceptible *Y. enterocolitica* strain which has acquired different mutations generating resistance to nalidixic acid.

*Yersinia enterocolitica* is a gram-negative bacillus mainly causing gastrointestinal infection. Antibiotics are usually not required for gastrointestinal disease; however, they are necessary for treating systemic infections in immuno compromised patients (4). Fluoroquinolones show good in vitro activity against this microorganism (3). The main purpose of this study was to investigate the epidemiological relationship and the mechanisms of resistance to quinolones among 10 nalidixic acid-resistant and nalidixic acid-susceptible *Y. enterocolitica* clinical isolates.

From July 2000 to March 2001, 31 *Y. enterocolitica* strains recovered from outpatients from the so-called Sixth Area of Madrid were isolated at the C. E. Argüelles Microbiology Laboratory (Hospital Universitario Puerta de Hierro). This area includes some neighborhoods in Madrid and nearby towns, the farthest being Collado Villalba, 40 km from Madrid. Ten of these 31 strains were studied. Bacterial strains were isolated in ceftuladin-irgasan-novobiocin agar and identified by biochemical techniques. All clinical strains analyzed are shown in Table 1. The control strains 37DV and 66599 used in this study were isolated in the Hospital Clinic, Barcelona, Spain, far from Madrid. Serotyping was performed by the slide agglutination test with commercial antisera (Sanofi Diagnostics Pasteur, Paris, France). The PCR amplification of the *yst* gene was carried out with primers 1a (5′-AAA GAT AGT TTT TGT TCT TGT-3′) and 1b (5′-GCA GCC AGC ACA CGC GGG-3′) under the following conditions: 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and a final extension at 65°C for 16 min. All the strains belonged to serotype O:3, the most prevalent in Spain (9), and presented the chromosomal gene *yst*, which encodes a heat-stable enterotoxin (4).

Antibiotic susceptibility was determined by the microdilution method according to the NCCLS guidelines (12). MICs of nalidixic acid and ciprofloxacin are shown in Table 1.

Epidemiological analysis was performed by two different methods. The first was analysis of chromosomal DNA by digestion with low-frequency restriction enzymes (ApaI and *XhoI*) followed by pulsed-field gel electrophoresis (PFGE) under conditions previously described (7, 9), although changes were introduced in the original protocol to optimize the technique for *Y. enterocolitica*. We added 20 mM thiocyanate to the TE-1 buffer to inhibit the powerful DNase of *Y. enterocolitica*. Electrophoretic conditions were also modified as follows: initial switch time, 5 s; final switch time, 8 s; run time, 20 h; gradient, 6.0 V/cm; temperature, 14°C. The second method was repetitive-extragenic palindrome PCR (REP-PCR) using the conditions previously described by Navia et al. (13). Tenover’s criteria were used to define the relationship among the studied strains, which were analyzed by PFGE (16). When PFGE was performed on the DNA after digestion with ApaI, 10 similar patterns, differing in no more than three bands, were found among all the strains. When *XhoI* was used, 9 of the 10 strains showed the same pattern, whereas for strain 915892 there was a loss of one band. In both cases the two control strains presented totally different patterns. All the patterns resulting from REP-PCR were identical but differed from those for the control strains (Table 1). The epidemiological studies clearly showed a close epidemiological relationship among the analyzed strains. The PFGE method has been extensively used to analyze the epidemiological relationship among strains of *Y. enterocolitica* (1, 6, 8, 11). In our study the same discriminatory power for both REP-PCR and PFGE was observed. Thus, the speed and ease of REP-PCR make it useful for analyzing the epidemiological relationship among *Y. enterocolitica* strains.

Mutations in the *gyrA* and *parC* genes were studied by PCR amplification of their quinolone resistance-determining regions (QRDR). To amplify the *gyrA* gene, consensus primers were designed upon comparison of the *gyrA* gene sequences from *Yersinia pestis* and *Yersinia pseudotuberculosis* (*gyrAY1*, 5′-CGC GTA CTG TTT GCG ATG AA-3′; *gyrAY2*, 5′-CGG AGT CAC CAT CGA CGG AA-3′); to amplify the *parC* gene, the primers parC 1 (5′-CGC GAC GGC CTG AAG CCG GGG-3′) and parC 2 (5′-GCC GTC GCG CGA ACC GAA G-3′) were used. The PCR and DNA sequencing conditions for both reactions have been described elsewhere (18).
Mutations were found in the QRDR of the \textit{gyrA} genes of the six nalidixic acid-resistant strains (Table 1). Four had a substitution at the codon for Ser-83 (AGC), changing it to an Arg codon (two to AGG and two to AGA). The fifth strain exhibited a mutation in the same codon producing a change from Ser-83 to Ile (ATC). The remaining strain presented a mutation in the codon for amino acid Asp-87 which changed it to a Tyr codon (TAC). Mutations in the \textit{parC} genes were not found in any of the studied strains. Our results agree with those for \textit{Escherichia coli} and other \textit{Enterobacteriaceae} in which a mutation at the codon for amino acid Ser-83 is the most frequently found among clinical isolates, being related to a moderate level of resistance to fluoroquinolones but high levels of resistance to nalidixic acid (14, 15, 19). This is the first time these QRDR have been sequenced in \textit{Y. enterocolitica}, and they show a high similarity to those of \textit{Y. pestis} and \textit{Y. pseudotuberculosis}. The percentages of DNA sequence similarity between the \textit{gyrA} and \textit{parC} genes of \textit{Y. enterocolitica} and those of other microorganisms are shown in Fig. 1.

**FIG. 1.** Comparison of the nucleotide sequences of the amplified regions of the \textit{gyrA} (A) and \textit{parC} (B) genes of \textit{Y. enterocolitica} and other bacteria. \textit{P. aeruginosa}, \textit{Pseudomonas aeruginosa}; \textit{C. jejuni}, \textit{Campylobacter jejuni}.

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**TABLE 1.** Characteristics of \textit{Y. enterocolitica} clinical isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date of isolation</th>
<th>Place of isolation</th>
<th>MIC ((\mu)g/ml) of:</th>
<th>PFGE pattern(^a)</th>
<th>REP-PCR pattern</th>
<th>Serotype</th>
<th>GlyA QRDR amino acid at position (d):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CIP(^a) NAL(^b)</td>
<td>XhoI Apal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>892287</td>
<td>July 00</td>
<td>Las Matas</td>
<td>0.5 &gt;512 A B C</td>
<td>O:3</td>
<td>Arg (AGG)</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>854820</td>
<td>Aug. 00</td>
<td>Collado Villalba</td>
<td>0.5 512 A B C</td>
<td>O:3</td>
<td>Arg (AGG)</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>836314</td>
<td>Sep. 00</td>
<td>Aravaca</td>
<td>0.5 512 A B C</td>
<td>O:3</td>
<td>Arg (AGG)</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>842656</td>
<td>Nov. 00</td>
<td>Boadilla del Monte</td>
<td>1 512 A B C</td>
<td>O:3</td>
<td>Arg (AGG)</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>861247</td>
<td>Nov. 00</td>
<td>Collado Villalba</td>
<td>1 512 A B C</td>
<td>O:3</td>
<td>Arg (AGG)</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>828365</td>
<td>Nov. 00</td>
<td>Aniceto Marinés</td>
<td>0.25 512 A B C</td>
<td>O:3</td>
<td>Ser (Tyr)</td>
<td>Tc (TAC)</td>
<td></td>
</tr>
<tr>
<td>915892</td>
<td>Nov. 00</td>
<td>Majadahonda</td>
<td>0.125 16 A B C</td>
<td>O:3</td>
<td>Ser (Tyr)</td>
<td>Tc (TAC)</td>
<td></td>
</tr>
<tr>
<td>881000</td>
<td>Feb. 01</td>
<td>Pozuelo de Alarón</td>
<td>0.125 2 A B C</td>
<td>O:3</td>
<td>Ser (Tyr)</td>
<td>Tc (TAC)</td>
<td></td>
</tr>
<tr>
<td>990372</td>
<td>Feb. 01</td>
<td>Torrelodones</td>
<td>0.125 8 A B C</td>
<td>O:3</td>
<td>Ser (Tyr)</td>
<td>Tc (TAC)</td>
<td></td>
</tr>
<tr>
<td>839104</td>
<td>Mar. 01</td>
<td>Aravaca</td>
<td>0.015 8 A B C</td>
<td>O:3</td>
<td>Ser (Tyr)</td>
<td>Tc (TAC)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) CIP, ciprofloxacin.
\(^b\) NAL, nalidixic acid.
\(^c\) Subscripts indicate subtypes of the major clone.
\(^d\) Codons are in parentheses.
Several articles have reported outbreaks caused by the serotypes O:8, O:9, and O:3 of *Y. enterocolitica* (5, 10, 17). However, no reports have described an epidemiological relationship among clinical isolates with different antimicrobial susceptibilities from a wide geographical area.

The emergence of nalidixic acid-resistant *Y. enterocolitica* around Madrid posed the question of possible clonal dissemination. Susceptible and resistant strains were selected from several towns. All the strains analyzed belonged to the O:3 serotype, showed an epidemiological relationship, and carried the gyrA gene.

Fluoroquinolones have been ranked as the second most widely used antimicrobial agent both in Spanish hospitals and the community (2). This high level of usage, together with the use of antibiotics in animal feed, may explain the increase in the resistance to quinolones in *Y. enterocolitica* clinical isolates. Our results suggest the clonal dissemination of a nalidixic acid-susceptible *Y. enterocolitica* strain which has acquired different mutations that generate resistance to nalidixic acid and which has probably emerged due to the selective pressure exerted by the overuse of fluoroquinolones.

Nucleotide sequence accession numbers. Accession numbers for the gyrA and parC genes of *Y. enterocolitica* in the GenBank are AY064398 and AY064399, respectively.

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**REFERENCES**


