Detection of Resistance to Macrolides in Thermotolerant Campylobacter Species by Fluorescence In Situ Hybridization

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The resistance of enteritis-causing Campylobacter strains to erythromycin is an emerging problem. We therefore evaluated fluorescence in situ hybridization (FISH) for the rapid detection of resistance using 74 campylobacter isolates. FISH showed specificity and sensitivity of 100% for the detection of high-level resistance.

Thermotolerant Campylobacter spp. (C. jejuni, C. coli, C. lari, and C. upsaliensis) are leading causes of bacterial diarrhea (14). Even though most infections are self-limiting, antimicrobial therapy decreases the duration and severity of symptoms and is mandatory in the case of severe illness. Because resistance to quinolones is increasing (5, 14), macrolides are currently the recommended first-line treatment. However, resistance to macrolides is an emerging problem (2, 11). In human isolates, the rate of resistance is about 5%, but rates vary considerably, reaching up to 80% in animal isolates of C. coli (2, 5, 11). Macrolides inhibit protein synthesis in the bacterial cell by binding to the 50S subunit of the ribosome (5). In Campylobacter, high-level resistance to macrolides is conferred by mutations of the 23S strand of the rRNA gene at the binding site of the macrolide (1, 6, 23). Similar mutations are responsible for resistance in Helicobacter pylori (20). While different mutations occur at comparable frequencies in Helicobacter, the change of A to G at position 2059 (Escherichia coli equivalent; corresponds to position 2075 of Campylobacter) dominates macrolide resistance in Campylobacter (1, 4, 5, 20, 23). Rarely, efflux pumps are responsible for a low level of resistance that is of limited clinical relevance at standard antibiotic doses (3, 5, 12).

The identification of drug resistance by phenotypic methods takes at least 1 day. Except for broth dilution and to some extent disk diffusion, these methods are not well standardized (5, 9, 10, 14). Several molecular methods such as PCR and sequencing approaches have been developed as alternative methods (5, 7, 17). For Helicobacter pylori, fluorescence in situ hybridization (FISH) using fluorescently labeled oligonucleotide probes complementary to the rRNA binding site of the macrolides is evidently useful for the detection of macrolide resistance (20, 22, 24). The FISH technique is a robust and technically easy method that does not require sophisticated equipment except for a fluorescence microscope. FISH has also been evaluated for the identification of Campylobacter (15, 16, 19, 21) but not yet for the detection of corresponding macrolide resistance.

We therefore established a corresponding FISH assay using well-characterized Campylobacter strains and then evaluated it using a collection of 70 Campylobacter isolates from humans and animals, including 33 sensitive isolates, 33 isolates with high-level resistance, and 4 isolates with intermediate resistance (Table 1). The Campylobacter strains were obtained from the Laboratory of Bacteriology, Hospital Pellegrin, Bordeaux, France; the Institute of Risk Assessment, Berlin, Germany; the Animal Sciences Group, Wageningen-Lelystad, The Netherlands; and the Institute of Medical Microbiology and Hygiene, University of Ulm, Ulm, Germany. The Campylobacter strains were phenotypically characterized, and resistance to erythromycin was determined by Etest or broth dilution as described previously (8, 10, 11, 19). MICs below 2 µg/liter were judged to be sensitive, those above 8 µg/ml were judged to be high-level resistant, and those between 2 and 8 µg/ml were determined to be intermediately resistant (8, 10, 11). Fresh subcultures of bacteria were fixed on glass slides for 30 min in 2% paraformaldehyde as previously described (19).

For FISH, we used probes that were described previously for the detection of resistance to macrolides in H. pylori (20, 22). The theoretical applicability of these probes for Campylobacter was checked by consulting previous publications (1, 4, 5, 6, 7, 12, 17, 23) and by using the publicly available ARB program (www.ARB-home.de). The wild-type probe (C wt 23S [CGG GGT CTC TCC TT]) was labeled with 6-carboxyfluorescein (6-FAM) and used in combination with a Cy3-labeled resistance probe (C res 23S 2059A G [CGG GGT CTC TCC TT]) at a formamide concentration of 40% in the hybridization buffer and corresponding salt concentrations in the wash buffer as previously described for H. pylori (Thermo Hybaid, Ulm, Germany) (20, 22, 23).

First, FISH probes were tested with one well-characterized erythromycin-susceptible isolate and one resistant isolate of C. coli and C. jejuni each. The A-to-G mutation at position 2059 was previously determined to be responsible for resistance in the corresponding isolates by real-time fluorescence resonance energy transfer PCR (13). In these resistant isolates, FISH showed a strong red fluorescence due to binding of the resistance probe C res 23S 2059A G, while no green fluorescence...
was seen (Fig. 1). In both sensitive isolates, the corresponding 6-FAM-labeled wild-type probe, probe C wt 23S, gave a strong green signal, while the resistance probe C res 23S 2059A/G produced no signal (Fig. 1).

In a second step, FISH was applied to a collection of 70 isolates for which resistance was determined in parallel with phenotypic methods. The FISH assay correctly categorized all 33 sensitive and all 33 highly resistant strains (Table 1). The four strains with intermediate resistance were assigned to the group of sensitive strains by FISH in accordance with the rRNA-independent mechanism of resistance (3, 5, 12). This intermediate resistance is of limited clinical relevance because infections with these strains are likely to be cured with erythromycin in standard dosages (3, 12). According to a recent consensus conference from the Clinical and Laboratory Standards Institute, these isolates may therefore be considered to be sensitive (9).

The FISH approach proved to be a reliable method for the detection of resistance to macrolides. The short time of less than 2 h needed to achieve a result compared to 1 or 2 days for conventional methods may avoid inefficient initial therapy with macrolides and corresponding clinical failure. Compared to other molecular methods, FISH is cost-effective and easy to perform. FISH even allows the identification of resistant bacteria in a mixed population also containing sensitive organisms. An additional advantage of the FISH assay described here is the possibility to use it in conjunction with FISH probes for the identification of thermotolerant Campylobacter spp. (19).

In addition, the assay probably allows the detection of macrolide resistance in close relatives of thermotolerant Campylobacter spp. such as C. fetus and other Helicobacter spp. Due to the limited availability of data or resistant strains, this hypothesis cannot yet be challenged. FISH may also be useful for the detection of macrolide resistance in other bacteria in which macrolide resistance is caused by ribosomal mutations, such as mycobacteria or haemophili. For this purpose, however, the

<table>
<thead>
<tr>
<th>Organism (MIC)</th>
<th>No. of isolates</th>
<th>No. of isolates positive by wild-type probe C</th>
<th>No. of isolates positive by resistance probe C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant (&gt;8 μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. jejuni</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>C. coli</td>
<td>27</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Intermediately resistant (2–8 μg/ml)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C. coli</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Sensitive (&lt;2 μg/ml)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C. jejuni</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>C. coli</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>C. lari</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>C. upsaliensis</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

FIG. 1. Example of FISH for the determination of macrolide resistance. The probe complementary to the wild type (6-FAM labeled) (green) and the probe for the detection of the mutation leading to resistance (Cy3 labeled) (red) were implemented simultaneously, leading to green staining of the sensitive strain (top row) and red staining of the resistant strain (bottom row).
probes need to be adapted to the different sequences flanking the point of mutation.

FISH has been employed for direct investigation of clinical samples including stool samples. In our preliminary experiments using spiked stool samples, however, analysis was complicated by unspecific background fluorescence. This application therefore cannot be recommended as a routine diagnostic procedure.

In summary, FISH is a reliable and useful tool for the rapid detection of macrolide resistance in thermotolerant Campylobacter spp. from culture and may thus serve to speed up the identification of the appropriate therapy for Campylobacter infections.

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REFERENCES
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