Role of the plasmid-encoded tet(O) gene in tetracycline-resistant clinical isolates of *Campylobacter jejuni* and *Campylobacter coli*

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The prevalence of tetracycline resistance, tetracycline MICs and tet(O) gene localization were investigated in 83 *Campylobacter* isolates from patients suffering from acute gastroenteritis in Germany. Combined biochemical and molecular markers identified 74 isolates (89%) as *Campylobacter jejuni*, including seven atypical isolates that failed to hydrolyse hippurate, and nine isolates (11%) as *Campylobacter coli*. Tetracycline resistance was detected in six out of nine *Campylobacter coli* isolates (67%) and 13 out of 74 *C. jejuni* isolates (18%). Low-level tetracycline resistance was observed for *C. coli* (MIC 16 μg ml⁻¹ for all strains), whereas *C. jejuni* showed high-level resistance (MIC >256 μg ml⁻¹ for all strains). Both low- and high-level tetracycline resistance was associated with the presence of the tet(O) gene. In *C. jejuni*, tet(O) was plasmid-encoded in 54% of tetracycline-resistant isolates, whereas in *C. coli*, tet(O) appeared to be located on the chromosome.

INTRODUCTION

Over the last few decades, *Campylobacter jejuni* and *Campylobacter coli* have emerged as important food-borne pathogens and are of major public health concern. Both species cause gastrointestinal infections as well as post-infection manifestations, such as Guillain–Barré syndrome and reactive arthritis (Schmidt-Ott et al., 2006). Annually, approximately 60 000 cases of *Campylobacter* enteritis are reported in Germany (RKI, 2006). *Campylobacter* enteritis is a zoonotic disease, and poultry, cattle and pigs can be the source of infection (Mead et al., 1999). Antibiotic supplementation in animal feed constitutes more than half of the total antimicrobial use worldwide (Wegener et al., 1999). Transmission of antimicrobial resistance via the food chain can occur from food animals to humans (Pezzotti et al., 2003; Putnam et al., 2003). In the past few years, increased antibiotic resistance has been reported in *C. jejuni*, particularly tetracycline resistance (Gibreel et al., 2004). In *Campylobacter* species, high-level tetracycline resistance is usually associated with the tet(O) gene carried on transmissible plasmids (Taylor & Courvalin, 1988). The Tet(O) protein belongs to the class of ribosomal protection proteins that confer resistance by dislodging tetracycline from its primary binding site on the ribosome (Connell et al., 2003). Previously, it has been shown that 38.5% of *C. jejuni* isolates in Germany are resistant to tetracycline (Wagner et al., 2003). However, the role of the plasmid-encoded tet(O) gene in tetracycline-resistant clinical isolates of *Campylobacter* in Germany has not been well described. Precise species differentiation is an important prerequisite for such investigations. We therefore combined biochemical and molecular markers for precise differentiation of clinical isolates of *Campylobacter* to determine their prevalence, with a focus on plasmid-encoded tet(O)-mediated tetracycline resistance in German clinical isolates.

METHODS

*Campylobacter strain collection*. Eighty-three isolates of *Campylobacter* were collected from patients suffering from gastroenteritis in the university hospital of Göttingen. Faecal specimens from these 83 patients were examined for the presence of common faecal pathogens. During sample collection, no outbreak of campylobacteriosis was reported in the area.

*Media and growth conditions*. Prior to tetracycline MIC determination, *Campylobacter* isolates were cultured on Columbia agar base (Merck) supplemented with 5% sheep blood, polymyxin B (2.5 IU ml⁻¹), trimethoprim (5 μg ml⁻¹) and vancomycin (10 μg ml⁻¹), and incubated at 42 °C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) for 48 h. *Campylobacter* isolates were biochemically differentiated at the species level by Gram staining, oxidase and catalase activities, hippurate hydrolysis, hydrogen sulfide production and susceptibility to nalidixic acid using a commercially available species differentiation kit (API Campy; bioMérieux).
**Determination of antibiotic resistance and tetracycline MICs.** Campylobacter isolates were initially tested for resistance to ampicillin, ciprofloxacin, erythromycin, gentamicin and tetracycline using a disc-diffusion method (Gaudreau & Gilbert, 1997). The MIC of tetracycline was subsequently determined by an agar dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (NCCLS, 1997; CLSI, 2006). Mueller–Hinton blood agar supplemented with tetracycline concentrations of 4, 8, 16, 32, 64, 128 and 256 mg ml⁻¹ was inoculated with 1 μl brain–heart infusion broth containing 1 × 10⁻¹–2 × 10² bacteria and incubated at 37 °C under microaerophilic conditions for 40–48 h. Two Campylobacter jejuni tetracycline-resistant strains and one C. jejuni strain known to be tetracycline susceptible (MIC 4 mg ml⁻¹) were used as controls. Each experiment was conducted in triplicate. The lowest concentration of the antimicrobial agent that produced no visible growth was considered to be the MIC for the relative isolate. The CLSI has previously described testing conditions for Campylobacter spp., but did not define interpretive breakpoints (CLSI, 2006). Therefore, in agreement with a previously published study (Gaudreau & Gilbert, 1997), we considered isolates having a tetracycline MIC of ≤ 4 mg ml⁻¹ to be sensitive to tetracycline.

**Genomic DNA preparation.** Genomic DNA was prepared by a previously described CTAB (hexadechltrimethyl ammonium bromide) genomic DNA isolation method (Colegio et al., 2001). A lawn culture of Campylobacter grown overnight on Columbia agar base was flooded with physiological 0.9 % NaCl and pelleted at room temperature. The pellet was resuspended in 564 μl TE buffer (pH 7.4), with 30 μl 10 % SDS and 6 μl proteinase K (10 mg ml⁻¹; Qiagen) and incubated for 1 h at 37 °C. Subsequently, 100 μl 5 M NaCl and 80 μl CTAB/NaCl solution were added, followed by a 1 h incubation at 65 °C. Genomic DNA was extracted with chloroform : isoamyl alcohol (1 : 24), ethanol precipitated, resuspended in 50 μl sterile water and stored at −20 °C.

**PCR and Southern blot analysis of the hipO gene.** A previously established PCR method was used to detect the hipO gene (Linton et al., 1997). The presence of the hipO gene in isolates lacking biochemical hippurate hydrolyase activity was confirmed by Southern blot analysis. For Southern blot analysis of the hipO gene, a PCR product labelled with digoxigenin-11-UTP (Roche Diagnostics) was used as a probe and hybridized with the BglII-digested genomic DNA of Campylobacter isolates. The DNA was blotted on a nitrocellulose membrane (Optitran BA-S 85; Schleicher & Schuell). Solutions and conditions were used according to a standard protocol (Sambrook & Russell, 2001). Hybridization was performed at 42 °C for 18 h. Washing of membranes was carried out twice at 37 °C in 2 × SSC, 0.5 % SDS for 15 min and twice at 65 °C in 0.1 × SSC, 0.5 % SDS for 30 min. Digoxigenin was detected with specific peroxidase-labelled antibodies using an enhanced chemiluminescence analysis system (Amersham Pharmacia Biotech) according to the recommendations of the supplier. Hippurate hydrolyase-negative isolates were confirmed as Campylobacter coli by PCR. Genomic DNA was isolated by the CTAB method and a previously reported primer pair (CC18F, 5′-GGTATGATTTC-CTACAAAAGCGA-3′; CC519R, 5′-ATAAAAGACTTGCGGCTG-3′) was used to amplify the expected 583 bp fragment of the aspartokinase (aspA) gene (Linton et al., 1997).

**Plasmid preparation from Campylobacter coli isolates.** Plasmid DNA from Campylobacter coli isolates was purified from an overnight culture on blood agar using mini Qiagen columns as recommended by the manufacturer. The plasmids were designated p(Cj) or p(Cc) for Campylobacter jejuni or Campylobacter coli, respectively, following species differentiation. Restriction digestion of plasmid DNA was performed using HindIII, BglII, PstI and AccI (New England Biolabs) and analysed on 1.2 % agarose gels with TAE buffer (0.04 M Tris/acetate, 0.001 M EDTA).

**Detection and localization of the tet(O) gene in Campylobacter coli isolates.** The presence of the tet(O) gene in tetracycline-resistant Campylobacter coli isolates was confirmed by a tet(O)-specific PCR (Schmidt-Ott et al., 2005). Southern blot analysis was performed to determine the localization of the tet(O) gene. DNA probes were generated and labelled with digoxigenin-11-dUTP by PCR using the above-mentioned primer pair. Plasmid pCjA13 carrying the tet(O) gene was used as a template for this PCR (Schmidt-Ott et al., 2005). The generated probes were hybridized as described above with the HindIII-digested Campylobacter coli plasmids. DNA was then blotted onto Optitran BA-S 85 nitrocellulose membranes and detection of the tet(O) gene was performed with a specific peroxidase-labelled antibody using an enhanced chemiluminescence analysis system.

**RESULTS AND DISCUSSION**

**Species identification of thermophilic Campylobacter strains.**

Precise species identification of Campylobacter is an important prerequisite for epidemiological and resistance studies. Eighty-three thermophilic Campylobacter isolates were identified and tested for the presence of the hipO gene using phenotypic and molecular methods. On the basis of their hippurate hydrolyase activity, 67 isolates (81 %) were identified as Campylobacter jejuni. Sixteen Campylobacter coli isolates (19 %) did not show any hippurate hydrolyase activity. However, in seven of these hippurate hydrolyase-negative isolates, the hipO gene was detected by probing genomic DNA with a digoxigenin-11-dUTP-labelled hipO probe (Fig. 1a).

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**Fig. 1.** Genotypic species differentiation of hippurate hydrolyase-negative thermophilic Campylobacter strains. (a) BglII-digested genomic DNA probed with a digoxigenin-labelled hipO probe. (b) Campylobacter coli-specific PCR amplification of genomic DNA.
isolates were identified as atypical isolates of \textit{C. jejuni} and the remaining nine hippurate hydrolase-negative isolates were confirmed as \textit{C. coli} (Fig. 1b) using a specific PCR (Linton \textit{et al.}, 1997).

**Antibiotic resistance in \textit{C. jejuni} and \textit{C. coli}**

Tetracyclines have been used as an alternative choice in the treatment of \textit{C. jejuni} and \textit{C. coli} enteritis. Large geographical variation in the susceptibility patterns of \textit{C. jejuni} and \textit{C. coli} to tetracycline has been observed. The rate of resistance in Denmark ranges from 0 to 11\% (Aarestrup \textit{et al.}, 1997), in Spain it is 25\% (Gomez-Garces \textit{et al.}, 1995) and in the USA it is 48\% (Nachamkin, 1994). After precise identification at the species level, tetracycline MICs were determined for 19 \textit{Campylobacter} isolates that were identified as tetracycline resistant by the disc-diffusion test. Tetracycline MICs ranged from 16 to >256 mg ml\(^{-1}\). High-level tetracycline resistance was found in \textit{C. jejuni}, whereas in \textit{C. coli} isolates, tetracycline resistance was significantly lower. For the 13 \textit{C. jejuni} strains, the MIC was determined as >256 mg ml\(^{-1}\), whilst the six \textit{C. coli} isolates had an MIC of 16 mg ml\(^{-1}\). The frequency of tetracycline resistance was significantly higher (\(P<0.001\), \(\chi^2\) test) in \textit{C. coli} (67\%) than in \textit{C. jejuni} (18\%).

\textit{C. coli} is frequently found in pigs (Moore \& Madden, 1998), and it is known that the regular use of antimicrobial agents for therapeutic purposes and growth promotion can play a role in the prevalence of antimicrobial-resistant strains of \textit{C. coli} in pigs (Harvey \textit{et al.}, 1999; Payot \textit{et al.}, 2001). Therefore, a higher frequency of tetracycline resistance in clinical isolates of \textit{C. coli} might be linked to the use of related antibiotics in the food chain.

**Plasmid prevalence and tetracycline resistance**

Bacterial resistance to tetracycline commonly arises through one of four identified mechanisms: efflux of tetracycline, modification of tetracycline, ribosomal protection or mutation of the 16S rRNA (Burden, 1991; Ross \textit{et al.}, 1998; Schnappinger \& Hillen, 1996). However, plasmid-mediated \textit{tet(O)}-encoded tetracycline resistance is reported quite frequently in \textit{Campylobacter} spp. (Manavathu \textit{et al.}, 1988; Lee \textit{et al.}, 1994). Plasmids bearing the \textit{tet(O)} determinant have also been isolated from other bacteria, such as \textit{Enterococcus faecalis} and \textit{Streptococcus} spp., and the plasmids were shown to have similar sizes and restriction profiles to those isolated from \textit{C. jejuni} and \textit{C. coli} (Zilhao \textit{et al.}, 1988). Previously, the isolation rate of plasmids from \textit{Campylobacter} species has been reported to be quite variable, ranging from 44 to 91\% for clinical and poultry isolates (Gaudreau \& Gilbert, 1998). In this study, approximately 23\% (\(n=19\)) of \textit{Campylobacter} isolates harboured plasmids, ranging in size from 5 to 66 kb. Significant differences among plasmids were detected in both species of \textit{Campylobacter}: 19\% (\(n=14\)) of the \textit{C. jejuni}

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**Table 1. Characterization of tetracycline-resistant \textit{Campylobacter} isolates**

The method applied in this study allowed the detection of plasmids of 1–66 kb.

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>(\text{hipO})</th>
<th>(\text{aspA})*</th>
<th>Detectable plasmids</th>
<th>\textit{Tet}(^R) (MIC, mg ml(^{-1}))</th>
<th>\textit{tet(O)}</th>
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<tr>
<td>CjA5</td>
<td>+</td>
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<td>+</td>
<td>–</td>
<td>R (16)</td>
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</table>

*\textit{Cj}, \textit{C. jejuni}; \textit{Cc}, \textit{C. coli}.

*Aspartokinase gene of \textit{C. coli}; ND, data not available.*
isolates and 56% (n=5) of C. coli isolates harboured plasmids. Instead of the 33–66 kb plasmids found in C. jejuni, C. coli isolates harboured plasmids of 5–9 kb. Fifty percent (n=7) of plasmid-harbouring C. jejuni and 60% (n=3) of the plasmid-positive C. coli isolates were resistant to tetracycline (Table 1). To determine the localization of the tet(O) gene, plasmid DNA from C. jejuni and C. coli isolates was probed with digoxigenin-11-dUTP-labelled tet(O) (Fig. 2). Our results revealed that 54% (n=7) of the tetracycline-resistant C. jejuni isolates carried the tet(O) gene on their plasmids. Surprisingly, in C. coli none of the plasmids carried the tet(O) gene. Amplification of the tet(O) gene from genomic DNA of tetracycline-resistant C. coli isolates indicated a chromosomal localization of the tet(O) gene. However, considering the limitation of the alkaline lysis method for plasmid isolation, the presence of low-copy-number plasmids larger than 70 kb cannot be totally excluded. It has been suggested previously that recombination events between plasmids and the chromosome, or integration of a plasmid, might occur, which could explain chromosomally mediated tetracycline resistance in these isolates (Boosinger et al., 1990). It is also known that illegitimate recombination can cause integration of a heterologous plasmid in C. coli (Richardson & Park, 1997) and this would ultimately lead to a higher frequency of chromosomally mediated tetracycline resistance in C. coli. We previously confirmed conjugation in two isolates having plasmids of 40.5 kb (pCjA9) and 41.9 kb (pCjA13) (Schmidt-Ott et al., 2005). In this study, Southern blot analysis showed that tetracycline resistance in these isolates was tet(O)-encoded and plasmid-mediated, which ultimately confirmed conjugation transfer of tet(O) in these C. jejuni isolates.

In conclusion, resistance against tetracycline in C. jejuni and C. coli isolates was associated with the tet(O) gene in all cases and there was a strong correlation between tetracycline resistance and plasmid carriage in C. jejuni isolates. Although all plasmid-containing isolates of C. coli were resistant to tetracycline, none of the C. coli isolates carried the tet(O) gene on their plasmid. Instead, the tet(O) gene appeared to be chromosomally encoded in all tetracycline-resistant C. coli isolates.

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**REFERENCES**


