23S rRNA Gene Mutations Contributing to Macrolide Resistance in *Campylobacter jejuni* and *Campylobacter coli*

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Abstract

The genetic basis of macrolide resistance in *Campylobacter coli* (*n* = 17) and *C. jejuni* (*n* = 35) isolates previously subjected to in vivo selective pressure was investigated to determine if the number of copies of 23S rRNA genes with macrolide-associated mutations affects the minimum inhibitory concentration (MIC) of macrolides. Sequence data for domain V of the 23S rRNA gene revealed that two macrolide-resistant *C. coli* isolates had adenine→guanine transitions at position 2059 (A2059G, *Escherichia coli* numbering). One of the two isolates had the A2059G transition in only two of the three gene copies. Among the macrolide-resistant *C. jejuni* isolates (*n* = 9), two different point mutations within domain V were observed. Three macrolide-resistant *C. jejuni* isolates had A2059G transitions. One of these three *C. jejuni* isolates had the A2059G transition in only two of the three gene copies. Six macrolide-resistant *C. jejuni* isolates had an adenine→cytosine transversion at position 2058 (A2058C, *E. coli* numbering) in all three copies of the 23S rRNA gene. *Campylobacter jejuni* isolates with the A2058C transversion had higher erythromycin MICs (>256 μg/mL) compared to *C. jejuni* isolates with A2059G transitions (64–128 μg/mL). In addition, the *C. jejuni* and *C. coli* isolates with only two copies of the 23S rRNA gene having A2059G substitutions had lower macrolide MICs compared to isolates with all three copies of the gene mutated. No isolates were observed having only one copy of the 23S rRNA gene with a mutation. Sequence analysis of ribosomal proteins L4 (*rplD*) and L22 (*rplV*) indicated that ribosomal protein modifications did not contribute to macrolide resistance among the collection of *Campylobacter* examined.

Introduction

*CAMPYLOBACTER* is recognized as a major cause of acute bacterial gastroenteritis in humans worldwide (Friedman *et al*., 2000). Within the United States, an estimated 1.5 million cases of human gastroenteritis are attributed to *Campylobacter* infection annually (Samuel *et al*., 2004). *Campylobacter jejuni* and *C. coli* are the species most frequently isolated from cases of human infection, with *C. jejuni* accounting for over 90% of infections, and *C. coli* being identified in most of the remaining cases (Lastovica and Skirrow, 2000). Human *Campylobacter* infection is generally an acute gastrointestinal illness characterized by diarrhea, abdominal cramping, and fever (Karmali and Fleming, 1979; Blaser *et al*., 1983). The majority of cases are mild or self-limiting and antimicrobial therapy is not required. Nonspecific supportive and symptomatic treatment, as for any other gastrointestinal illness, is usually sufficient (McNulty, 1987). However, prolonged duration of illness or altered immune function in some individuals may warrant antimicrobial therapy (Aarestrup and Engberg, 2001; Allos, 2001). The macrolide

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erythromycin (ERY) is a primary treatment option for *Campylobacter* infections in humans, and as such, the development of macrolide resistance in *Campylobacter* may pose a public health concern.

Macrolides are a class of antimicrobial compounds derived from secondary metabolism products of streptomycetes bacteria (Vester and Douthwaite, 2001). Their structures consist of 14-, 15-, or 16-member lactone rings with two or more amino or neutral sugars attached (Vester and Douthwaite, 2001). These compounds are effective against Gram-positive cocci and bacilli, Gram-negative cocci, and a limited number of Gram-negative bacilli which include *Bordetella pertussis*, *Campylobacter*, *Chlamydia*, *Helicobacter*, and *Legionella* species (Leclercq, 2002).

The basic mechanism of action of macrolides is the inhibition of protein synthesis. During protein synthesis, the macrolide desosamine sugar group forms hydrogen bonds with the polar groups on the 23S rRNA nucleotides 2058 and 2059 (*Escherichia coli* numbering) at the narrowest portion of the 50S subunit tunnel wall. This effectively blocks growth of the nascent peptide chain (Franceschi et al., 2004; Poulsen et al., 2000).

Single base substitutions at positions 2058 and 2059 in domain V of 23S rRNA (*E. coli* numbering) have been shown to confer macrolide resistance in *Campylobacter* and several other bacterial genera (Jensen and Aarestrup, 2001; Vester and Douthwaite, 2001). Bacterial species possessing multiple copies of the 23S rRNA gene may require more than one mutated copy to confer macrolide resistance. A mutation in one of the two 23S rRNA copies of *Helicobacter pylori* has been shown to confer macrolide resistance (Hultén et al., 1997). While transformation studies utilizing *Streptococcus pneumoniae*, which has four copies of 23S rRNA, have shown that susceptibility to ERY decreases as the number of mutated gene copies increases (Tait-Kamradt et al., 2000). Macrolide-associated mutations in a single copy of the three *Campylobacter* 23S rRNA genes have not been reported, nor has there been any correlation of the number of mutated target gene copies with levels of macrolide resistance.

In this study, the genetic basis of macrolide resistance in a collection of *C. coli* and *C. jejuni* isolates previously subjected to *in vivo* selective pressure was investigated to determine if the number of copies of 23S rRNA genes with macrolide-associated mutations affects the minimum inhibitory concentration (MIC) of three macrolides or a lincosamide. The macrolide-susceptible parent strains used in this study were confirmed to have wild-type 23S rRNA genes by sequence analysis of domain V for each of the three gene copies. It was hypothesized that the MICs of derived strains would be proportional to the number of mutated gene copies.

**Materials and Methods**

**Campylobacter isolates**

*Campylobacter* isolates used in this study were obtained from chicken ceca (17 *C. coli* and 35 *C. jejuni*), as part of a previous study in which broilers were challenged with macrolide-susceptible strains of *Campylobacter* (three strains of *C. jejuni* or three strains of *C. coli*) and then administered TYL at subtherapeutic or therapeutic concentrations (Ladely et al., 2007). All isolates had previously been tested for susceptibility to ERY using the agar dilution method (CLSI, 2006). Approximately 79% of the isolates used in this study were susceptible to macrolides, even though they had been recovered from broilers administered TYL. The susceptible isolates were included in the study to increase the probability of identifying strains with less than three mutated copies of the 23S rRNA gene.

**Antimicrobial susceptibility testing**

For each isolate, the MICs to three macrolides (azithromycin [AZM], ERY, and TYL) and a lincosamide (clindamycin [CLI]) were determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Isolates were considered resistant to AZM, ERY, and CLI with MICs $\geq8$, $\geq32$, and $\geq8\mu g/mL$, respectively. Interpretive criteria for TYL susceptibility testing have not been established (CLSI, 2006).
Campylobacter jejuni ATCC 33560 was used as a quality control strain for susceptibility testing.

Polymerase chain reaction (PCR) amplification and DNA sequencing

The primers used for amplification and sequencing of domain V of the 23S rRNA gene and the rplD and rplV genes that encode the L4 and L22 ribosomal proteins, respectively, are listed in Table 1. Genomic DNA for PCR was prepared using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to manufacturer’s directions. Primers flanking each operon were utilized to amplify the three copies of the 23S rRNA gene in all C. coli and C. jejuni isolates. Campylobacter jejuni amplifications were performed in three separate reactions as described by Gibreel et al. (2005), using forward primers FI, FII, or FIII, paired with the conserved reverse primer CJ-copy-R. Similarly, C. coli amplifications were performed using forward primers Fla, Flia, or FlIia, paired with reverse primer CJ-copy-R. Potential macrolide-associated mutations were then identified by sequencing a 508-bp fragment (Vacher et al., 2003) for each copy of the target gene.

To assess the contribution of mutations within L4 and L22 ribosomal protein genes (rplD and rplV, respectively) to macrolide resistance, sequence analysis of L4 and L22 ribosomal protein genes of macrolide-resistant Campylobacter strains (C. coli n = 2, C. jejuni n = 9) and their macrolide-susceptible parent strains were compared. PCR amplifications of rplD and rplV genes were performed as describe by Cagliero et al. (2006) and Corcoran et al. (2006), respectively. PCR products were analyzed by gel electrophoresis, purified using the QIAquick PCR purification system (Qiagen, Valencia, CA) and were sequenced using the BigDye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyzer (Applied Biosystems). Forward and reverse sequence data were assembled and compared using Sequencher version 4.2 (Gene Codes Corporation, Ann Arbor, MI).

Results

Resistance patterns of Campylobacter isolates

The MICs to the three macrolides (AZM, ERY, and TYL) and a lincosamide (CLI) were determined for 17 C. coli (Table 2) and 35 C. jejuni isolates (Table 3) using agar dilution. Eighty-eight percent (15/17) of the C. coli and 74% (26/35) of the C. jejuni isolates were susceptible to AZM, ERY, and CLI. Two C. coli isolates (TPS61 and TSP62) were resistant to AZM and ERY; one (TPS61) was also resistant to CLI. Nine C. jejuni isolates (26%) were resistant to AZM,
ERY, and CLI (Table 3). The TYL MICs for C. coli and C. jejuni isolates ranged from 2 to >256 μg/mL.

**Sequence analysis of 23S rRNA and L4 and L22 ribosomal protein genes**

Operon-specific PCRs were performed to amplify the three copies of the 23S rRNA gene for each isolate. The resulting PCR products were all of the expected sizes. Sequencing data for domain V of the 23S rRNA gene showed that all macrolide-susceptible C. coli and C. jejuni isolates had nonmutated wild-type 23S rRNA. None of the isolates, susceptible or resistant, were found to have only one mutated copy of the 23S rRNA gene.

The two macrolide-resistant C. coli isolates (TPS61 and TPS62) had adenine→guanine transitions at position 2059 (A2059G, E. coli numbering) of the 23S rRNA gene (Table 2). One of the macrolide-resistant C. coli isolates (TPS61) had A2059G transitions in all three target genes, the other (TPS62) had the A2059G transition in only two of the three copies of the 23S rRNA gene.

Among the nine macrolide-resistant C. jejuni isolates, two different point mutations within domain V of the 23S rRNA gene were observed...
Two resistant *C. jejuni* isolates (TPS49 and TPS50) exhibited the A2059G transition in all three 23S rRNA gene copies, while one *C. jejuni* isolate (TPS53) had A2059G transitions in only two of the three copies of the target gene. The remaining six of these nine macrolide-resistant *C. jejuni* isolates (TPS42, TPS43, TPS45, TPS46, TPS47, and TPS48) had an adenine→cytosine transversion at position 2058 (A2058C) in all three copies of the 23S rRNA gene. The six *C. jejuni* isolates (TPS42, TPS43, TPS45, TPS46, TPS47, and TPS48) with the A2058C transversion had higher ERY MICs (>256 μg/mL) compared to the three *C. jejuni* isolates (TPS49, TPS50, and TPS53) with A2059G transitions (64–128 μg/mL). In addition, *C. jejuni* (TPS53) and *C. coli* (TPS62) isolates with only two copies of the 23S rRNA gene having the A2059G substitution had lower ERY, AZM, and TYL MICs compared to isolates in which all three copies of the target gene were mutated (Tables 2 and 3).

Sequence analysis of L4 and L22 ribosomal protein genes of macrolide-resistant *Campylobacter* strains (n = 2 *C. coli*, n = 9 *C. jejuni*) and their macrolide-susceptible parent strains were compared. Complete DNA sequence identity was observed between macrolide-resistant strains and their susceptible parent strains.

**Discussion**

Domain V of each of the three copies of 23S rRNA gene were sequenced for 41 macrolide-susceptible (15 *C. coli* and 26 *C. jejuni*) and 11 macrolide-resistant *Campylobacter* isolates (two *C. coli* and nine *C. jejuni*). Sequencing data showed that all macrolide-susceptible *C. coli* and *C. jejuni* isolates had wild-type 23S rRNA. No isolates recovered from poultry with known exposure to TYL, regardless of whether they were resistant, were found to have only one mutated copy of the 23S rRNA gene. No previous reports in which individual operons were sequenced, have identified *Campylobacter* strains with only one copy of the 23S rRNA gene mutated. Further genetic characterizations of susceptible and low-level macrolide-resistant *Campylobacter* strains may identify strains with only a single mutated copy of the 23S rRNA gene. However, since the ancestral state of the study isolates was known to have all three 23S rRNA genes with wild-type alleles, conversion to the second mutated copy of this gene must be extremely rapid, suggesting selective pressure against strains with just one mutant copy. The resistance profile of the isolates with two mutated copies of the 23S rRNA implies that conversion of the third copy is not necessary for fitness in the presence of AZM but may be needed for TYL and/or ERY. No conclusions on the rate of conversion of the third copy can be made and further studies are warranted.

Mutations were identified at positions 2058 or 2059 (*E. coli* numbering system) in all high-level ERY-resistant isolates, as previously reported (Jensen and Aarestrup, 2001; Payot et al., 2004; Alonso et al., 2005; Corcoran et al., 2006). These mutations also provided resistance to AZM and in most cases conferred cross-resistance to the lincosamide CLI, which is also consistent with earlier studies (Taylor and Chang, 1991; Cagliero et al., 2005; Mamelli et al., 2005). Limited data are available regarding TYL MICs in *Campylobacter* species. Cagliero et al. (2005), reported TYL MICs of 32 μg/mL for wild-type *C. coli* and MICs of ≥2048 μg/mL for *C. coli* isolates with A2059G transitions in all three copies of the 23S rRNA gene. TYL MICs in the present study tended to be lower, in part due to a narrower range of testing (1–256 μg/mL). However, both studies found TYL MICs to be 2–32 fold higher than ERY and AZM MICs among wild-type *C. coli* isolates. Interestingly, one macrolide-resistant *C. coli* isolate (TPS62), with the A2059G mutation in two of the three 23S rRNA gene copies had a TYL MIC similar to wild-type isolates (4 μg/mL).

Five of the eleven *Campylobacter* isolates with point mutations in domain V of the 23S rRNA exhibited the A2059G transition, which has been the most common mutation identified among macrolide-resistant *Campylobacter* isolates (Jensen and Aarestrup, 2001; Alonso et al., 2005; Corcoran et al., 2006). One *C. coli* (TPS53) and one *C. jejuni* isolate (TPS62) were found to have an A2059G transition in only two copies of the 23S rRNA gene, and previous studies have also reported this genotype at a low frequency (Jensen and Aarestrup, 2001; Payot et al., 2004; Gibreel et al., 2005; Vacher et al., 2005). In the present study, the isolates with two copies of the
mutated target gene had lower levels of ERY resistance compared to isolates of the same Campylobacter species having all three copies mutated. It was observed in previous studies (Payot et al., 2004; Gibreel et al., 2007) that Campylobacter isolates with the same point mutation (A2059G) in two copies of the 23S rRNA gene had lower ERY MICs compared to isolates carrying mutations in all three copies of the target gene. Similarly, Vacher et al. (2005), observed one C. jejuni isolate with an A2059T transversion in only two copies of the 23S rRNA gene that had a lower ERY MIC (8 μg/mL) compared to isolates with mutations in all three copies of the target gene. Similar observations have been noted in other bacterial species. Transformation studies utilizing Streptococcus pneumoniae, which has four copies of 23S rRNA, have shown that the level of ERY resistance increases as the number of mutated 23S rRNA gene copies increases (Tait-Kamradt et al., 2000). In contrast, Gibreel et al. (2005), observed no difference in Campylobacter ERY MICs with regard to the number of mutated copies of the target gene.

An A2058C transversion was identified in six C. jejuni isolates in our study. This mutation was recently identified among a collection of Campylobacter isolates by Vacher et al., (2003) at a low frequency (2%). Across bacterial species, transversional substitutions (pyrimidine→purine or purine→pyrimidine) such as the A2058C seen here, generally occur at a much lower frequency than transitional substitutions (pyrimidine→pyrimidine or purine→purine) (Li, 1997) such as the A2059G observed in most 23S rRNA mutations. The higher frequency of A2058C transversions observed in this study may be an artifact of the limited genetic diversity among the Campylobacter strains used (derived from Campylobacter-challenged poultry). Similarly, Lin et al. (2007) observed A2058G transitions in in vivo selected mutants, in contrast to the more commonly observed A2059G mutation. Both studies suggest that genetic features of a given strain may influence the specific point mutation observed.

Campylobacter jejuni isolates with the A2058C transversion had consistently higher ERY MICs compared to those with A2059G transitions. Differences in the level of macrolide resistance have been shown to be dependent on the position of base substitution in other bacterial species. For example, in H. pylori, base substitutions at position 2058 have been shown to confer higher levels of macrolide resistance than similar base substitutions at position 2059 (Wang and Taylor, 1993). Furthermore, in H. pylori A2058C transversions confer similar levels of macrolide resistance to A2058G substitutions (Wang and Taylor, 1993). This may suggest that base substitutions at position 2058 are more effective at disrupting macrolide binding. In contrast, a previously reported A2058T transversion in C. jejuni (Vacher et al., 2005) had a lower ERY MIC than the other mutations detected (A2058C and A2059G). However, in that case the A2058T transversion was only present in two copies of the target gene.

Other resistance mechanisms, in particular efflux systems have previously been shown to provide low-level macrolide-resistance in Campylobacter (Payot et al., 2004; Corcoran et al., 2006). Gibreel et al. (2007), suggest that efflux systems may act synergistically with A2059G mutations, and Cagliero et al. (2006), indicate that modifications in ribosomal proteins L4 and L22 may act synergistically with the CmeABC efflux system in conferring macrolide resistance. We did not evaluate the efflux systems in our isolates, however, previous susceptibility testing showed that the ancestral strains used were all susceptible to ERY (Ladely et al., 2007) and no highly resistant (ERY MICs >32 μg/mL) isolates were found without changes in at least two 23S rRNA genes. Investigation of ribosomal proteins L4 and L22 by sequence analysis of macrolide-resistant strains and their susceptible parent strains indicated that ribosomal protein modifications did not contribute to macrolide resistance among the collection of Campylobacter isolated we examined. Other mechanisms conferring macrolide resistance such as methylation of the drug-binding site and drug inactivation have not yet been observed in Campylobacter (Yan and Taylor, 1991; Gibreel et al., 2005; Corcoran et al., 2006).

In conclusion, these data show that A2058C transversions (E. coli numbering) in the C. jejuni isolates studied exhibit higher ERY MICs compared to C. jejuni isolates with A2059G transitions, indicating that base substitution position may influence macrolide resistance levels in this
pathogen. The number of copies of the 23S rRNA gene carrying mutations may also influence the level of resistance, as A2059G transitions in only two copies of this gene conferred lower macrolide MICs than C. coli and C. jejuni isolates with mutations in all three copies of the target gene. Even though the majority of strains used in this study were derived from Campylobacter-challenged poultry with known exposure to macrolides, no Campylobacter strains were identified with only one copy of the 23S rRNA gene mutated. Conversion to the second mutated copy appears to be extremely rapid, suggesting selective pressure against strains with just one mutated copy.

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References


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