Study of the molecular mechanisms involved in high-level macrolide resistance of Spanish *Campylobacter jejuni* and *Campylobacter coli* strains

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**Objectives:** To investigate the molecular mechanisms involved in the high-level erythromycin resistance of clinical Spanish *Campylobacter jejuni* and *Campylobacter coli* strains.

**Methods:** Overall susceptibilities of 678 *C. jejuni* and 119 *C. coli* strains, collected from 10 Spanish provinces during 2006 and 2007, were determined by Etest. In high-level erythromycin-resistant strains, molecular determinants were studied. The analysis was focused on region V of the 23S rRNA gene, the *rplD* and *rplV* ribosomal genes, and the regulatory region of the CmeABC efflux pump.

**Results:** The global resistance rate to erythromycin was 3.8%. Among the resistant strains, 93% were *C. coli* and 7% were *C. jejuni*. The A2075G mutation in the 23S rRNA gene was detected in all of the resistant strains except for two, which carried the A2074G mutation. None of the ribosomal *rplD* and *rplV* genes harboured the described mutations that confer resistance to macrolides. Different mutations affecting the regulatory region of the CmeABC efflux pump were also found.

**Conclusions:** *C. coli* strains are clearly more resistant to erythromycin than *C. jejuni*. The mutation A2075G in the 23S rRNA gene was responsible for the resistance in most of the strains; A2074G was only found in two strains. Further studies are required to ascertain the effect of mutations in the regulatory region of cmeABC. Our data indicate that the rate of resistance was similar to that of other European countries.

**Keywords:** erythromycin, 23S rRNA, *rplD*, *rplV*, CmeABC

**Introduction**

*Campylobacter* spp. is the leading cause of bacterial acute diarrhoea in humans in developed countries.1 Most of these infections are caused by *Campylobacter jejuni*,2 although *Campylobacter coli* is of increasing importance,3 as evidenced by an increased number of human cases.4,5 The majority of infections are acquired through handling or consuming infected meat, usually undercooked food.6 *Campylobacter*, as a zoonotic disease agent, is commonly found in meat animals (poultry, swine, cattle and sheep). Human campylobacteriosis does not require antimicrobial treatment, being normally self-limiting; however, antimicrobial therapy is recommended in immunocompromised patients, and cases of bacteraemia and severe and prolonged diarrhoea.1,4,5 Fluoroquinolones and macrolides are the treatment of choice for these severe cases.7

Although resistance rates vary among countries,3,6,10 fluoroquinolone resistance rates are usually so high (up to 90%) in many countries, such as Thailand or Spain11,12) that fluoroquinolone use as empirical treatment should be discouraged. For that reason, erythromycin and other macrolides are the best choices in severe cases of campylobacteriosis. Resistance to macrolides has been reported, although the resistance rates differ between species. In the USA, resistance rates reportedly varied during 1997–2003, between 0.3% to 3.0% for *C. jejuni* and between 4.0% to 37.5% for *C. coli*.13 In other countries, the rates also vary, but, generally, human isolates are reported with a low resistance rate.7,9 In contrast, high rates of macrolide resistance have been reported among *Campylobacter* strains from meat animals.7,9,13 It is generally accepted that *C. coli* is more resistant to macrolides than *C. jejuni*.7,14 This is probably caused by the use of macrolides, such as tylosin, as growth promoters during previous years15 in the *C. coli* main animal host (swine).

Macrolides act by binding to the 50S ribosomal subunit of the bacteria, interfering with elongation of nascent proteins. Several
molecular mechanisms have been described as being responsible for macrolide resistance in bacteria. In Campylobacter spp., the most prevalent mechanism among highly resistant strains is a mutation at nucleotide 2075 of domain V of the 23S rRNA gene. This mechanism also appears in many other species, such as Streptococcus pneumoniae,16 Staphylococcus aureus,17 Helicobacter pylori18 and others. Another less frequently reported mechanism is the mutation of nucleotide 2074 of domain V.19 These nucleotides (positions 2074 and 2075) belong to the peptide transference loop and macrolides join specifically to this position. Other mechanisms associated with macrolide resistance are mutations in the rplD and rplV genes. These genes encode the ribosomal proteins L4 and L22, which are probably involved in the conformational switches in the ribosome that occur during the translation process.19,20 Parts of L4 and L22 (extended loops) act along the peptide exit channel controlling the rate of translation.21 Mutations along those extended loops may affect the peptide synthesis. In Campylobacter spp., there are three mutations described for rplD: Gly-74→Ala/Val; and Gly-74→Ala21,22 for rplV, an insertion of three or four amino acids at position 86 or 98 in the L2221 protein has been described as being responsible for macrolide resistance.

Another resistance mechanism consists of a chromosomally encoded resistance–nodulation–cell division-type efflux system,23,24 named the CmeABC efflux pump, which protects Campylobacter against bile salts, helping Campylobacter to colonize the animal intestinal tract.23 It also protects against dyes, detergents and antimicrobials.25 It is well known that changes in expression levels of CmeABC also contribute to macrolide resistance, acting in synergy with the described mutations.15,21,25 CmeABC is transcriptionally repressed by CmeR, which binds to an inverted repeat (IR) region between cmeR and cmeA. It has been shown that mutations in the repressing site lead to an overexpression of the efflux pump and to enhanced resistance to several antibiotics.1,25

The aim of this work was to analyse the molecular mechanisms involved in high-level macrolide resistance in C. jejuni and C. coli. Human strains isolated in Spain, focusing on the analysis of region V of the 23S rRNA gene, the rplD and rplV genes, and the regulatory region of the cmeABC operon (CmeABC efflux pump), after studying the antimicrobial susceptibilities.

Materials and methods

Bacterial strains

Seven hundred and ninety-five human Campylobacter spp. strains, without a known epidemiological link, from 10 Spanish provinces were received at the Campylobacter Laboratory of the Spanish National Center of Microbiology during 2006 and 2007 for identification and susceptibility testing purposes. Campylobacter spp. strains were grown on mCCDA (modified charcoal cefoperazone deoxycholate agar; Oxoid, Madrid, Spain) medium for 48 h at 42 °C. Presumptive colonies were streaked on Mueller–Hinton sheep blood agar (Becton Dickinson, Madrid, Spain) and cultured for 48 h at 42 °C. DNA extraction was carried out as previously described.21 Identification was performed using a previously described multiplex PCR.28 When additional confirmation of the species was required, two other described multiplex PCRs were performed.25,30

Antimicrobial susceptibilities

An antimicrobial susceptibility test for erythromycin was performed and MICs were determined as previously described21 using Etest (AB Biodisk, Solna, Sweden). The MIC breakpoints were those defined by the CLSI: susceptible, ≤8 mg/L; intermediate, 8–32 mg/L; and resistant, ≥32 mg/L.32 C. jejuni strain ATCC 33560 was used as a susceptibility test control, as recommended by the CLSI.32 Strains with MICs >256 mg/L were considered to have high-level resistance to erythromycin.

Analysis of 23S rRNA, rplD and rplV genes, and the cmeR and cmeABC intergenic region

All high-level erythromycin-resistant strains were characterized for four genetic loci potentially responsible for resistance: the 23S rDNA gene; the rplD and rplV ribosomal genes; and the intergenic region between cmeR and cmeABC. The 23S rDNA gene was amplified using the primers 23sunvF′+41783 (5′-GCTCGAGGTAAATGATG-3′) and 23sunvR-42635 (5′-GCTCTGGCAGAACAC-3′), based on the C. jejuni subsp. jejuni 81116 sequence (GenBank accession number CP000814.1A). Amplification was carried out using the Ready-to-Go system (GE Healthcare Life Sciences, UK) with 25 cycles of 94 °C for 30 s, 52 °C for 20 s and 72 °C for 35 s. Ribosomal genes rplD and rplV were amplified using the primers and method previously described.21

The regulatory region of cmeABC was studied by two different amplifications. The entire cmeR gene (950 bp), the cmeR and cmeABC intergenic region (94 bp), and the first part of cmeA (180 bp) were first amplified with a pair of previously described primers.31 For 23 strains with negative amplification, a second PCR with new primers was designed (CmecoliF3, 5′-AATGTTTGAAGCCTATC-3′; and CmecoliR4, 5′-AACACGGTCTACCTTGGG-3′) on the basis of previously sequenced bacteria (GenBank accession number FJ797670–3).13 These primers amplified a fragment of 428 bp that included part of the cmeA gene (108 bp), the cmeR and cmeABC intergenic region (107 bp), and the first 231 bp of the cmeA gene. PCR consisted of 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s.

PCR products were purified using Invitrogen Fragment CleanUp system (Invitrogen, Carlsbad, USA) following the manufacturer’s instructions. Sequences were obtained with ABI PRISM 377 equipment (Perkin-Elmer, Applied Biosystems Division, Foster City, USA) using the BigDye Terminator protocol (Applied Biosystems). The sequences were assembled using Lasergene Seqman II software (DNA Star, Inc., Madison, WI, USA), then they were aligned and amino acids deduced using the CLUSTAL W routine of MegAlign software (v.6.1, DNA Star, Inc.). The GenBank accession numbers of all the sequences obtained in this study are presented in Table S1 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)].

Results

Identification and antimicrobial resistance rates

Six hundred and seventy-eight (85.1%) out of 797 strains received at our laboratory were identified as C. jejuni, while 119 (14.9%) were C. coli. With the CLSI breakpoint of ≥32 mg/L, 30 strains (3.8%) of the received strains were erythromycin resistant. All of these displayed high-level resistance (MIC ≥ 256 mg/L, Etest highest level).

The distribution of high-level resistant strains by Campylobacter species was 93% C. coli and 7% C. jejuni. Among each species, the distribution was: C. jejuni, n=2 strains (0.3%); and C. coli, n=28 (23.5%) (as seen in Table 1). The modal MIC was 2 mg/L for C. coli strains (32.8%) and 1 mg/L for C. jejuni strains (41.3%).

23S rRNA analysis

All but two highly resistant strains had the A2075G mutation. One C. jejuni and one C. coli carried the A2074G mutation instead. In all but one of these mutant strains, it appeared
that the three copies of the 23S rRNA gene were mutated. In one
C. coli
strain (GenBank accession number GU384931), it seems
that the A2075G mutation was present in two of the three
copies of the gene, because the chromatogram showed a
double peak at that position, the peak for guanine being two
times higher than the peak for adenine. In addition to these,
nine
C. coli
strains and one
C. jejuni
strain contained further
mutations, which are summarized in Table 2.

C. coli
strains
CNM20070177, CNM20070178 and CNM20070497 had the
highest number of polymorphisms detected in 23 rRNA, 11 in
addition to A2075G.

rplD and rplV analysis
For the
rplD
gene, none of the high-level erythromycin-resistant
strains harboured the described mutations Gly-57
\rightarrow
Asp/Val and Gly-74
\rightarrow
Asp, which confer resistance to macrolides. However, 11 and 10 non-synonymous mutations were identified
for the
rplD
gene and the
rplV
gene, respectively (Table 3). In addition to this, two
rplV
insertions were detected (ins119 Thr-Thr-Thr-Lys-Ala and ins124 Thr).

cmeR–cmeA intergenic region analysis
With the primers designed by Lin et al.,
11 only two
C. jejuni
and
C. coli
strains had a positive amplification of the cmeR–cmeA inter-
genic region (length of 94 nucleotides). The IR region was located
from −46 to −31 upstream of the cmeA gene (consensus
sequence: TGTAAATTTACTA). With the newly designed pair
of primers, the remaining 23 resistant strains could be analysed.
The intergenic region possessed a length of 107 nucleotides
instead of 94 nucleotides and the IR region was located from
−50 to −35 upstream of the cmeA gene (consensus sequence:
TGTTAAATTTACTA). Between the two intergenic regions, a simi-
larity rate of 78.7% was determined. Polymorphisms and inser-
tions inside of
CmeR–cmeA
intergenic region are represented in
Figure 1.

Discussion
The frequency of macrolide resistance of Spanish
C. jejuni
and
C. coli
human clinical strains from 2006 to 2007 reported here
(3.7%) is similar to frequencies previously reported in our
country in earlier studies: 3.2%–7.3%;
13 3.2% in the period
1987–93,
14 and 2.3% in the period 1988–92.
15 In other
European countries, the resistance rates are variable: 1%–3%
in Finland; 3.5% in France; 7.8%–11.3% in Italy;
11 and 11.3% in
Northern Ireland
11 (all these data refer to human isolates). As
has been observed before, C. coli
strains are more frequently
macrolide resistant than
C. jejuni; in our survey 23.5% of
C. coli
strains but only 0.3% of
C. jejuni
(two strains) had an MIC
\geq
32 mg/L, and all of these had an MIC \geq 256 mg/L. It is

Table 1. Distribution of the erythromycin MICs for clinical
C. jejuni
(\textit{n}=678) and
C. coli
(\textit{n}=119) strains received from various provinces in Spain
during 2006 and 2007

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Erythromycin MICs (mg/L)\textsuperscript{ab}</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>\textit{C. jejuni} (\textit{n}=363)</td>
<td>0.016 0.032 0.064 0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 &gt;256</td>
<td>GU384930 GU384930</td>
</tr>
<tr>
<td>2007</td>
<td>\textit{C. jejuni} (\textit{n}=315)</td>
<td>0.016 0.032 0.064 0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 &gt;256</td>
<td>GU384945 GU384954</td>
</tr>
<tr>
<td>2006</td>
<td>\textit{C. coli} (\textit{n}=65)</td>
<td>0.016 0.032 0.064 0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 &gt;256</td>
<td>GU384939</td>
</tr>
<tr>
<td>2007</td>
<td>\textit{C. coli} (\textit{n}=54)</td>
<td>0.016 0.032 0.064 0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 &gt;256</td>
<td>GU384942</td>
</tr>
</tbody>
</table>

\textsuperscript{a}CLSI breakpoints MICs: susceptible, \leq 8 mg/L; intermediate, 8–32 mg/L; and resistant, \geq 32 mg/L.
\textsuperscript{b}Modal MIC is indicated in italics.
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Table 3. L4 and L22 protein polymorphisms identified in high-level erythromycin-resistant C. jejuni and C. coli strains

<table>
<thead>
<tr>
<th>Polymorphisms and insertions</th>
<th>No. of C. coli strains</th>
<th>No. of C. jejuni strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein L4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala-24→Thr</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Thr-91→Lys</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Asn-95→Ser</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Val-121→Ala</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Ala-140→Thr</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Ile-142→Val</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Val-176→Ile</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Thr-177→Ser</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Val-184→Ile</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Met-192→Ile</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>Ala-196→Val</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Protein L22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile-65→Val</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Ala-103→Val</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Thr-109→Ser</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Thr-109→Ala</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ala-111→Glu</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ala-114→Thr</td>
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<tr>
<td>Thr-119→Ala</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Val-121→Ala</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Val-130→Ala</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Glu-131→Lys</td>
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<td>2</td>
</tr>
<tr>
<td>ins119 Thr-Thr-Thr-Lys-Ala</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ins124 Thr</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

noteworthy that none of the strains possessed an intermediate level of resistance to erythromycin.

With regard to the study of the molecular mechanisms among the erythromycin-resistant strains, we have focused our investigation on well-known mechanisms associated with mutations in region V of the 23S rRNA gene, the rplD and rplV genes, and the regulation region of the cmeABC operon. A2075G and A2074C mutations in region V of the 23S rRNA gene have been associated with high-level macrolide resistance (128–1024 mg/L),15,22,36 by impeding the anchorage of macrolides to the ribosome.37

In our survey, the most frequent mutation detected was A2075G. This mutation, as other authors have shown,15 seems to provide some biologically advantageous features versus another mutations in 23S rRNA, such as A2074G or A2074C. Despite the biological cost of the mutations, Campylobacter strains carrying these mutations seem to be not only stable in culture, but to maintain their ability to colonize their host.22

Despite the conserved nature of the 23S rRNA gene, 24 changes were found (Table 2). Most of the variations (11 out of 24) appeared in three strains. These three strains were isolated at the same hospital but at two different times. With our data, we can not be sure whether they share the same source or the epidemiological relationship between them. The fact that most of the polymorphisms were concentrated in a few strains could be a reflection of the clonal nature of several groups of C. coli strains.38,39

The influence of these polymorphisms in 23S rRNA in the modulation of the final erythromycin MICs for the strains is, at the moment, difficult to ascertain.

L4 and L22 are highly conserved proteins among eubacteria, due to their function in ribosomes. In Campylobacter spp., several mutations in L4 and L22 have been found in the loop region, leading to macrolide resistance. In our survey, no variation was found at amino acids 55–77 (loop region) of the L4 protein. Most of the changes were concentrated in the region at amino acids 91–196, except in two strains that harboured the Ala-24→Thr replacement, which is not associated with macrolide resistance.

All of the L22 protein substitutions, except Ile-65→Thr in a C. coli strain, were focused in the C-terminal region and outside the region between amino acids 78 and 98. Previously, none of them has been associated with macrolide resistance.36 In L22, the deletions/insertions seem to be more important than substitutions.40 The insertions ins86 Ala-Arg-Ala-Arg and ins98 Thr-Ser-His, which give resistance to macrolides, as described by Cagliero et al.,21 were not present in any strain. All the insertions found outside the loop region (ins119 Thr-Thr-Thr-Lys-Ala and ins124 Thr) were identified in susceptible and resistant strains.36 Probably, these insertions do not affect the entry of macrolides to the ribosome. In addition, C. coli strains showed less polymorphism at rplV than C. jejuni strains, possibly due to the clonal nature of C. coli.

The transcriptional regulation of the efflux pump operon cmeABC is carried out by the repressor CmeR, which binds specifically with an IR region upstream of the cmeA gene. The length and sequence of this intergenic regulatory region differs between C. jejuni (94 bp) and C. coli (>107 bp). These differences in the sequence do not seem to affect the function of the repressor, as long as the sequence of the IR region (TGTAAAATACGACGCTCA) is maintained. In our study, five C. coli strains possessed an intergenic region with length and sequence typical of C. jejuni strains. By two additional PCRs,29,30 their species identification as C. coli was confirmed. These C. coli strains could be an example of genetic exchange between these two species.

To our knowledge, two confirmed mutations affecting the regulation have been described in the IR region: a deletion of one of the nucleotides between the two half-sites of the IR region;11 and a C to T transition in the IR sequence. This transition has been previously associated with CmeABC overexpression, increasing the MICs of macrolides and other antimicrobials for an in vitro mutant strain.15 As seen in Figure 1, we have found the C-36→T transition in a C. coli strain. To our knowledge, this is the first time that such a mutation has been described in vivo. Other mutations, Δ→36C and T→48→C transition, theoretically would avoid the binding of the half-sites and consequently would deregulate the expression of the cmeABC operon. The insertion of six nucleotides (TATTAC) inside the IR sequence theoretically does not hamper the formation of the hairpin in which CmeR binds; however, as other authors have pointed out, the two half-sites of the CmeR sequence have to be correctly spaced for the correct binding of the repressors and consequently for correct regulation of the operon.11,14 Therefore, a misregulation of the operon could be expected in this strain.
The real contribution of the rest of the mutations surrounding the IR region is still unknown. More studies are necessary to ascertain the real contribution to the regulation of the cmeABC operon.

In conclusion, our survey showed that human Spanish C. jejuni and C. coli strains had low erythromycin resistance rates. The resistance could be explained through mutations in region V of the 23S rRNA gene, the main described mechanism among campylobacters. It is noteworthy that while rplD and rplV (other mechanisms of resistance) were not affected, we found several mutations in the cmeABC regulation zone, which may lead to an enhanced tolerance of macrolides and other antimicrobials.

Acknowledgements
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Figure 1. Partial multiple alignment of the two different intergenic regions between cmeR and cmeA identified in high-level erythromycin-resistant C. jejuni and C. coli strains. Polymorphisms are shown in black squares. The IR regions are shown inside the open squares and under the arrows the consensus sequence is shown. Repeated sequences are underlined. Positions are indicated on the top of the alignment. Partial sequences are shown. (a) Alignment of the 94 nucleotide intergenic region. Four different sequences are presented: IGR1, includes GenBank accession numbers GU384955–6, GU384960, GU384965 and GU384967; IGR2, GU384950–1, GU384953–4, GU384955–6, GU384960, GU384965 and GU384967; IGR3, GU384957 and GU384958; IGR4, GU384972; IGR5, GU384970; IGR6, GU384959, GU384961–4, GU384966, GU384968–9 and GU384971; IGR7, GU384970; IGR8, GU384957 and GU384958; IGR9, GU384967; IGR10, GU384960, GU384965 and GU384967; IGR11, GU384962. (b) Alignment of the 107 nucleotide intergenic region. Seven different sequences are presented: IGR1, includes GenBank accession numbers GQ868050–1, GQ868053–4, GU384955–6, GU384960, GU384965 and GU384967; IGR2, GU384950–1, GU384953–4, GU384955–6, GU384960, GU384965 and GU384967; IGR3, GU384957 and GU384958; IGR4, GU384972; IGR5, GU384970; IGR6, GU384959, GU384961–4, GU384966, GU384968–9 and GU384971; IGR7, GU384970; IGR8, GU384957 and GU384958; IGR9, GU384972; IGR10, GU384960, GU384965 and GU384967; IGR11, GU384962.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

Transparency declarations
None to declare.