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Identification of the main quinolone resistance determinant in *Campylobacter jejuni* and *Campylobacter coli* by MAMA-DEG PCR

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ABSTRACT

Among zoonotic diseases, campylobacteriosis stands out as the major bacterial infection producing human gastroenteritis. Antimicrobial therapy, only recommended in critical cases, is challenged by resistance mechanisms that should be unambiguously detected for achievement of effective treatments. Quinolone (ciprofloxacin) resistance of *Campylobacter jejuni* and *Campylobacter coli*, the 2 main *Campylobacter* detected in humans, is conferred by the mutation *gyrA* C-257-T, which can be genotyped by several methods that require a previous identification of the pathogen species to circumvent the sequence polymorphism of the gene. A multiplex PCR, based on degenerated oligonucleotides, has been designed for unambiguous identification of the quinolone resistance determinant in *Campylobacter* spp. isolates. The method was verified with 249 *Campylobacter* strains isolated from humans (141 isolates) and from the 3 most important animal sources for this zoonosis: poultry (34 isolates), swine (38 isolates), and cattle (36 isolates). High resistance to ciprofloxacin, MIC above 4 μg/mL, linked to the mutated genotype predicted by MAMA-DEG PCR (mismatch amplification mutation assay PCR with degenerated primers) was found frequently among isolates from the different hosts.

1. Introduction

Campylobacteriosis is the most prevalent zoonotic disease, with infection numbers in humans being more than twice that of salmonellosis in the European Union during 2013 (EFSA, 2015a). Thermophilic *Campylobacter* spp. are the causal agents of this infection, with 2 major species found asymmetrically distributed in animals and humans. *Campylobacter jejuni* is mainly found in poultry and cattle, while *Campylobacter coli* is mainly found in swine. In humans *C. jejuni* is the predominant species, which might indicate that poultry and cattle are the major sources of campylobacteriosis in human patients (Nielsen et al., 1997).

Ciprofloxacin (CIP), a fluoroquinolone, and erythromycin (ERY), a macrolide, had been considered the antibiotics of choice for treating *Campylobacter* infections until very recently, when an increasing trend in CIP resistance has been observed, whereas resistance to ERY and aminoglycosides like gentamycin (GEN) remains very low (Ge et al., 2013). Quinolone resistance in Gram-negative bacteria occurs mostly by point mutations and corresponding amino acid substitutions in the quinolone resistance-determining region (QRDR) of the *gyrA* gene. The genetic polymorphism C-257-T (Thr-86-Ile) is the most frequently observed mutation in *Campylobacter* conferring high-level resistance to all quinolones as nalidixic acid and CIP (Wang et al., 1993).

Amplification refractory mutation system (ARMS) and mismatch amplification mutation assay (MAMA) are similar PCR techniques capable of detecting allele specific mutations by PCR with primers including more than 1 mismatch, at the 3′-end, that confer a high degree of specificity (Cha et al., 1992; Newton et al., 1989). Together with a positive control pair of primers, these techniques enable the detection of the presence of either the mutated or the wild-type allele. Species-specific MAMA-PCR can identify the main mutation conferring quinolone resistance, *gyrA* C-257-T, in *C. jejuni* or *C. coli* (Zirnstein et al., 1999, 2000). Bypassing the polymorphism of *gyrA* between these species, this work describes a MAMA-PCR technique based on degenerated primers, MAMA-DEG PCR (mismatch amplification mutation assay PCR with degenerated primers), to detect wild-type and mutated genotypes of *C. jejuni* and *C. coli* in a single PCR, removing the time-consuming species identification procedures and reducing the number of PCRs required to survey CIP resistance status of *Campylobacter*.

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2. Materials and methods

2.1. Bacteria and antibiotic resistance

A total of 249 *Campylobacter* spp. isolates collected from humans (134 *C. jejuni*, 7 *C. coli*), poultry (16 *C. jejuni*, 18 *C. coli*), swine (38 *C. coli*), and cattle (33 *C. jejuni*, 3 *C. coli*) were collected between 2010 and 2012 and used in this study (Supplementary Table 1). Bacteria from humans were isolated by the clinical units of 3 hospitals located in Middle West Spain: "Campo Arañuelo", Navalmoral de la Mata, Cáceres: 7 *C. jejuni*, 2 *C. coli*; "S. Pedro de Alcántara", Cáceres: 84 *C. jejuni*, 1 *C. coli*; and "Universitario de Salamanca", Salamanca: 43 *C. jejuni*, 4 *C. coli*. Animal isolates were selected randomly from animal farms and abattoirs, located all around Spain, by the Spanish Surveillance Network of Antimicrobial Resistance in Bacteria of Veterinary Origin (VAV Network) (Moreno et al., 2000) and the clinical unit of the Veterinary Faculty of the University of Córdoba. Microorganisms were cultured on blood agar in a microaerophilic atmosphere (CampyGen™; Thermo Scientific, Lenexa, KS, USA) at 42 °C for 24–48 h. Species identification was done with a Vitek-MS MALDI-TOF system (bioMérieux, Marcy l'Etoile, France), and the MIC was determined by agar dilution methods according to CLSI (2010).

2.2. MAMA-DEG PCR

A set of 4 primers designed to amplify the QRDR of *gyrA* from *C. jejuni* and *C. coli* in a MAMA-DEG PCR (Table 1 and Fig. 1) was analyzed in silico for compatibility using software Oligo V6.57 (Molecular Biology Insights, Colorado Springs, CO, USA). PCR was performed with crude DNA extracted by the boiling method. One to 2 fresh colonies, originated from pure culture, were suspended in 250 μL of mili-Q water and heated to 100 °C in a thermobloc for 5 minutes. The heat-treated cell suspension was centrifuged at 10,000 × g for 10 minutes, and the supernatant was used as DNA template. PCR mix contained 0.2 mmol/L dNTPs (Takara-Clontech, Kusatsu, Shiga, Japan), 0.5 μmol/L of each of the 4 primers, 0.025 U/μL Taq polymerase (Biotools, Madrid, Spain), 1× PCR buffer with 1.5 mmol/L $MgCl_2$ (Biotools), and 5 μL of DNA template in a total volume of 20 μL. PCR thermocycle conditions were conducted as follows: 1 initial step of 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes was performed. When indicated, DNA fragments were purified from agarose gels with Speedtools PCR clean-up kit (Biotools), followed by sequencing (STAB service, DNA Sequencing facilities of the Universidad de Extremadura, Spain).

3. Results and discussion

3.1. Antimicrobial resistance of Campylobacter isolated from humans in Middle-West Spain

Campylobacter isolated from humans were analyzed for antimicrobial resistance against the 3 antibiotics of choice for treatment of campylobacteriosis: CIP, ERY, and GEN (Ge et al., 2013). Considering the resistance threshold defined by CLSI (2010), the antibiotics ERY and GEN (breakpoint of 8 μg/mL and 6 μg/mL, respectively) were strongly effective against the 141 *Campylobacter* isolates, with only 3 and 1 isolates in the limits of resistance, respectively (Supplementary Table 1). On the other hand, CIP (breakpoint of 4 μg/mL) was not effective in the inhibition of growth in 85.1% of *Campylobacter* (81.6% in *C. jejuni* and 71.4% in *C. coli*) isolated from human infections, a result that is slightly lower than the previously reported based on the ecological cut-off value of 0.5 μg/mL recommended by the EFSA (2015b), which indicated that Spain was the EU member estate with the highest level of resistance in *C. jejuni* (91.5%) and *C. coli* (94.3%) in 2013.

Campylobacter isolates from humans analyzed in this work presented a diversity of origins, 3 distant hospitals, located in Middle-West Spain, over a 3-year period, including different species, *C. jejuni* and *C. coli*, and variable antimicrobial resistance profiles (Supplementary Table 1). The interest in surveying the trend of CIP resistance in *Campylobacter* justifies the development of a more effective diagnostic tool, like MAMA-DEG PCR, which was validated on a collection of isolates genetically diverse.

Table 1
Nucleotide sequences of primers used in this work.

Primer	Sequence (5′-3′)	PCR Product (bp)	
		258-C	258-T
gJC-F	GAGATGG(T/C)TTAAAGCCTGTTCA	337[a]	337[a]
gJC-R	TGGAA(C/T)AAAATC(T/G)ACCGTATCT		
sWT-F	GGTATCA(C/T)CCACATGG(A/C)GATTC	223[b]	
sMU-R	AC(T/C)AA(A/G)GCATC(G/A)TAAAC(T/A)GCCA		157[c]

[a] Amplified with gJC-F and gJC-R primers.
[b] Amplified with sWT-F and gJC-R primers.
[c] Amplified with sMU-R and gJC-F primers.

3.2. Design of a MAMA-DEG-PCR for genotyping the gyrA C-257-T polymorphism in C. jejuni and C. coli

gyrA sequences from the 2 species are closely related (Fig. 1), although numerous polymorphisms like C-257-T produce quinolone resistance (Wang et al., 1993). To allow amplification of *gyrA* DNA fragments from *C. jejuni* or *C. coli*, regardless of species, the 4 primers designed include degenerated positions (Table 1) covering the polymorphisms found among 174 *C. jejuni* and 71 *C. coli* sequences (BLASTN program available at the NCBI server, NR database last accessed 04/29/2013). The entire QRDR of *gyrA* was amplified using external primers gJC-F and gJC-R, which produce a 337-bp DNA fragment (Table 1) that functions as a positive control in the PCR and allows the possibility to conduct sequence analysis. Detection of the quinolone resistant genotype was performed by 2 MAMA-DEG PCR internal primers (Table 1): sWT-F, a forward primer that together with gJC-R reverse external primer amplify a 223-bp DNA fragment from the CIP susceptible genotype; and sMU-R, a reverse primer that together with the gJC-F forward external primer amplify a 157-bp DNA fragment from the CIP-resistant genotype. The 2 last positions at the 3′-end of MAMA-DEG PCR primers contribute to selectivity, with the last one being the specificity determinant for wild-type (C-257) or mutant (T-257) alleles (Fig. 1). In addition, an obligated mismatch was introduced in the penultimate positions from the 3′-end to reduce the PCR efficiency below detectable levels only when there is a gap in the last nucleotide, according to the predictions performed on the basis of empirical testing of MAMA PCR (Cha et al., 1992).

3.3. Discrimination of gyrA C-257-T genotype by MAMA-DEG-PCR

C. jejuni (HSA12, HCC18) and *C. coli* (HCC43, HSA46) isolates, which present CIP resistance (HCC18, HCC43) and CIP susceptibility (HSA12, HSA46), were selected among human isolates after their identification by MALDI-TOF and resistance phenotype analysis (Supplementary Table 1). Crude DNA samples of the 4 bacterial strains were used to validate the MAMA-DEG PCR designed for genotyping the polymorphic position that determines quinolone resistance in *Campylobacter* (Fig. 2). The DNA fragment of 337 bp, which is produced by external primers gJC-F and gJC-R, was used as an internal control. The other 2 DNA fragments, of 223 bp or 157 bp, were specifically produced by isolates carrying the *gyrA* 257-C or *gyrA* 257-T alleles, respectively, from either *Campylobacter* species, *C. jejuni* or *C. coli* (Fig. 2). The sequencing of the 337-bp band that works as an internal control from the 4 strains confirmed the genotypes identified by MAMA-DEG PCR, corresponding to *gyrA* 257-C or 257-T conferring CIP susceptible or resistance in the isolates, respectively (Fig. 1).