RESEARCH NOTE

A POSSIBLE MECHANISM OF MACROLIDE RESISTANCE AMONG MULTIPLE RESISTANT *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ISOLATED FROM THAI CHILDREN DURING 1991-2000

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Abstract. A total of 495 *Campylobacter jejuni* and 122 *C. coli* isolated from Thai children were screened for macrolide (erythromycin and azithromycin) resistance by disk diffusion assay. Minimum inhibitory concentrations for erythromycin, azithromycin, nalidixic acid, ciprofloxacin, tetracycline, streptomycin, gentamicin and chloramphenicol were further determined for these macrolide-resistant *Campylobacter* isolates. Presence of known point mutations resulting in reduced susceptibility to macrolides was investigated by PCR and DNA sequencing. Seventeen percent (23/122) of *C. coli* and 2.4% (12/495) of *C. jejuni* isolates were resistant to macrolides. By sequencing domain V of the 23S ribosomal DNA from all 35 macrolide-resistant isolates, a known point mutation of 23S rRNA associated with reduced susceptibility to macrolides was detected in all isolates except one. Among the macrolide-resistant isolates, all were multiply resistant to nalidixic acid and ciprofloxacin, of which the latter is the preferred antimicrobial used for diarrheal treatment in Thailand. Furthermore, most macrolide-resistant isolates were also resistant to tetracycline and streptomycin. The spread of macrolide and quinolone resistant *Campylobacter* should be monitored closely in Thailand and elsewhere as these antimicrobials are preferred drugs for treatment of diarrhea.

INTRODUCTION

Among *Campylobacter* species, *C. jejuni* and *C. coli* are the most common species associated with gastroenteritis worldwide with the prevalence of *C. jejuni* generally being higher than *C. coli* (Bodhidatta *et al*, 2002). Antimicrobial resistance in *Campylobacter* spp has increased and multiple resistant isolates have emerged as a public health problem. In Thailand, fluoroquinolones have previously been used for treatment of diarrhea and high level fluoroquinolone resistance has led to increased use of macrolides, *eg* erythromycin or azithromycin, for treatment of campylobacteriosis (Murphy *et al*, 1996; Hoge *et al*, 1998). Any emergence and spread of multiple resistant *Campylobacter* spp is of great concern and should be monitored closely.

There exist several mechanisms resulting in reduced susceptibility to macrolides, *eg* efflux pumps, drug inactivation or alteration

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of drug target site. Our study aim was to determine the genetic background for macrolide (both erythromycin and azithromycin) resistance among clinical *C. jejuni* and *C. coli* isolated from Thai children during 1991-2000 and to determine other antimicrobial resistance phenotypes among the macrolide resistant *Campylobacter* spp.

MATERIALS AND METHODS

Bacterial strains

During 1991-2000, a total of 968 *C. jejuni* and 200 *C. coli* isolates from diarrhea etiology studies from different parts in Thailand were identified by standard microbiological procedures at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. Briefly, stool cultures were processed by a modified filtration method (Steele and McDermott, 1984). All *C. jejuni* and *C. coli* were identified by biochemical tests and differentiated by hippurate hydrolysis. Confirmed *C. jejuni* and *C. coli* isolates were kept frozen at -70ºC in glycerol medium for further characterization.

Disk and minimum inhibitory concentration (MIC) susceptibility testing

All 495 *C. jejuni* and 122 *C. coli* isolates were tested as part of routine laboratory analyses for antimicrobial susceptibility by disk diffusion assay as described by Bauer *et al* (1966) with minor modifications. An 18-48 hours subculture of *C. jejuni* and *C. coli* on sheep blood agar plates, isolates were suspended in Mueller Hinton broth (BD-Diagnostic Systems, Sparks, MD, USA) to obtain a turbidity equivalent to a 1.0 McFarland standard, and inoculated onto Mueller Hinton II agar supplemented with 5% sheep blood. Isolates were tested for the following antimicrobials using disk diffusion (BD): erythromycin (15 mg), azithromycin (15 mg), nalidixic acid (30 mg) and ciprofloxacin (5 mg). As no standardized interpretive criteria exist for *Campylobacter*, the inhibition zones were interpreted following the disks manufacturer's instructions. *Campylobacter* spp isolates found resistant to both erythromycin and azithromycin by disk diffusion tests were further tested for MICs by the agar dilution method (National Committee for Clinical Laboratory Standards, 2004a,b) to the following antimicrobials with MICs ranges indicated in brackets: erythromycin (0.2-32 μg/ml), azithromycin (0.25-32 μg/ml), nalidixic acid (1-128 μg/ml), ciprofloxacin (0.03-16 μg/ml), chloramphenicol (1-64 μg/ml), gentamicin (0.25-32 μg/ml), streptomycin (1-64 μg/ml) and tetracycline (0.5-32 μg/ml).

PCR and DNA sequencing

DNA templates were prepared by the boiled culture method. PCR was conducted using the universal primers of 23S rDNA (forward primer 5′-GTA AAC GGC GGC CGT AAC TA 3′ and reverse primer 5′-GAC CGA ACT GTC TCA CGA CG-3′) as described previously (Jensen and Aarestrup, 2001). The 699-bp amplicons were separated and purified by QIAquick PCR purification kit (Qiagen, CA, USA).

An internal amplicon of the domain V of the 23S rDNA of each isolate (12 *C. jejuni* and 23 *C. coli* that were all resistant to erythromycin and azithromycin) were submitted for sequencing. Sequences were edited, aligned and analysed using Sequencher program version 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). All 23S rDNA sequences were compared with 23S rDNA sequences obtained from GenBank (*C. jejuni* ATCC 700819 and *C. coli* U0961) and from erythromycin and azithromycin susceptible *Campylobacter* isolates in this study (3 *C. jejuni* and 1 *C. coli*).

RESULTS

Among the 617 *Campylobacter* spp tested (495 *C. jejuni* and 122 *C. coli* isolates), resistance to both erythromycin and azithromycin were detected in 12 *C. jejuni* and 23 *C. coli* isolates. As compared to erythromycin

Table 1
Minimum inhibitory concentration (MIC) of selected antimicrobials among 35 macrolide resistant *C. jejuni* and *C. coli* isolates.

Antimicrobial drug (MIC range μg/ml)	MIC (μg/ml)	No. of resistant isolates/ total isolates
Nalidixic (1-128)	= 64	9/35
	≥128	26/35
Ciprofloxacin (0.03-16)	= 8	2/35
	≥ 16	33/35
Tetracycline (0.5-32)	= 32	1/35
	≥ 32	32/35
Streptomycin (1-64)	= 16	2/35
	= 32	2/35
	≥ 64	25/35
Gentamicin (0.25-32)	> 32	3/35

and azithromycin susceptible isolates, MICs > 32 μg/ml for both erythromycin and azithromycin was observed in all 35 isolates, while all four susceptible isolates (3 *C. jejuni* : CJ-558-1, CJ 1409 and SPH-2353-1) and one *C. coli* (CJ-558) showed MICs ranging from ≤ 2-32 μg/ml and ≤ 0.25-2 μg/ml for erythromycin and azithromycin, respectively. However, at the time of this study in 2004, there were no MICs ranges for a control *Campylobacter* strain with nalidixic acid, azithromycin, streptomycin and chloramphenicol. Therefore, the break points to these four antimicrobials followed NCCLS standard interpretation of gram-negative and gram-positive bacteria (National Committee for Clinical Laboratory Standards, 2002a). MICs ranges for those isolates tested with nalidixic acid (MIC ≥ 64 μg/ml), ciprofloxacin (MIC ≥ 4 μg/ml), tetracycline (MIC ≥ 16 μg/ml) and streptomycin (MIC ≥16 μg/ml) were considered as resistant. Among the erythromycin and azithromycin resistant isolates (Table 1), 100% (35 /35) showed resistance to both nalidixic acid (MICs ≥ 64 μg/ml) and ciprofloxacin (MICs ≥ 8-16 μg/ml). Moreover, 94% (33/35) and 83% (29/35) of these isolates were also resistant to tetracycline (MICs ≥ 32 μg/ml) and streptomycin (MICs ≥ 16 μg/ml). Resistance to gentamicin (MIC > 32 μg/ml) was low at 8% (3/35) and resistance to chloramphenicol was not detected. The multiple resistance patterns among *C. jejuni* and *C. coli* are shown in Table 2. The most common multiple resistant phenotype was type A, showing resistance to erythromycin, azithromycin, nalidixic acid, ciprofloxacin, tetracycline and streptomycin.

DNA sequences of 35 amplicons of 699 bp-size from the erythromycin and azithromycin resistant *Campylobacter* isolates were determined, analyzed and compared with similar 23S rDNA nucleotide sequences of the control strains *C. coli* U 09691, *C. jejuni* ATCC 700981 as well as of four erythromycin and azithromycin susceptible *C. jejuni* and *C. coli* isolates from this study. All macrolide-resistant isolates, except one (SPH-1977-1) showed a single point mutation of 23S rDNA sequence at the position of 2230 created by a base substitution of A to G compared to *C. coli* U09691(number equivalent to 2059 in *E. coli* J01695) from GenBank. A summary of the 23S rDNA mutations found is shown in Table 2. Three *C. coli* isolates (CJ-1732-1, CJ-1782-1 and CJC 1761-8) showed an additional base substitution at position of 2252 from C to T.

Table 2
Multiple resistance phenotype and base transition of 23S rDNA of 23 *C. jejuni* and 12 *C. coli* isolates from Thailand.

Isolates (Year)	Pathogen	Serotype (Lior's)	Resistance phenotype	Base transition and position [b]
RVB-184-1 (1999)	*C. coli*	Un-typed	A	A2059G
RVB-203-1 (1999)	*C. coli*	Un-typed	A	A2059G
CJ-120-1 (1999)	*C. coli*	Un-typed	B	A2059G
CJ-190-1 (1996)	*C. coli*	Un-typed	A	A2059G
CJ-284-1 (1996)	*C. coli*	Un-typed	A	A2059G
CJ-1113-1 (1996)	*C. coli*	Un-typed	A	A2059G, A2279G, G2305A, T2307G, T2310G, T2325G, A2326C, T2328A, C2354T, C2372T, G2378A, C2388T, A2390G, G2393A
CJ-1172-1 (1997)	*C. coli*	Un-typed	A	A2059G
CJ-1456-2 (1997)	*C. coli*	Un-typed	A	A2059G
CJ-1524-3 (1997)	*C. coli*	Un-typed	A	A2059G
VC-949 (1995)	*C. coli*	Un-typed	B	A2059G
VC-348 (1994)	*C. coli*	Un-typed	A	A2059G
VC-336 (1994)	*C. coli*	110	A	A2059G
CJ-1782-1 (1997)	*C. coli*	20	A	A2059G, C2252T
CJ-1732-1 (1997)	*C. coli*	20	A	A2059G, C2252T
SPH-2353 (1996)	*C. coli*	20	A	A2059G
VC-1324 (1995)	*C. coli*	20	C	A2059G
RVB-128-1 (1995)	*C. coli*	29	C	A2059G
CJ-1566-1 (1997)	*C. coli*	29	D	A2059G
V-1068-1 (1995)	*C. coli*	44	A	A2059G
CJ-243-1 (1996)	*C. coli*	45	A	A2059G
CJC-1761-8 (1997)	*C. coli*	55	B	A2059G, C2252T, C2372T, G2378A, C2388T, A2390G, G2393A
RV-0305D1 (1999)	*C. coli*	8	A	A2059G
CJ-516-6 (1996)	*C. coli*	8	A	A2059G
RV-0304 (1999)	*C. jejuni*	Un-typed	A	A2059G
RV-0323 (1999)	*C. jejuni*	Un-typed	A	A2059G
RVB-0064 (1999)	*C. jejuni*	Un-typed	A	A2059G
RVB-175-1 (1999)	*C. jejuni*	Un-typed	A	A2059G
CJ-1724-2 (1997)	*C. jejuni*	Un-typed	B	A2059G
SPH-1977-1 (1995)	*C. jejuni*	Un-typed	A	No mutation
CJ-874-1 (1996)	*C. jejuni*	Un-typed	C	A2059G
CJ-481-1 (1996)	*C. jejuni*	19	C	A2059G
V-863-1 (1995)	*C. jejuni*	19	A	A2059G
VC-114 (1993)	*C. jejuni*	19	B	A2059G
AX-0247 (1999)	*C. jejuni*	28	D	A2059G
CJ-1839-1(1997)	*C. jejuni*	6	A	A2059G
CJ-558-1[a]	*C. coli*	44	NAL,CIP, TET, STR	No mutation
CJ-558-1[a]	*C. jejuni*	36	NAL, CIP, TET	No mutation
CJ-1409 [a]	*C. jejuni*	Un-typed	No resistance	No mutation
SPH-2353-4[a]	*C. jejuni*	11	No resistance	No mutation
ATCC 700981	*C. jejuni*	Unknown	Not tested	No mutation

A, resistance to erythromycin, azithromycin, nalidixic acid, ciprofloxacin, tetracycline, streptomycin
B, resistance to erythromycin, azithromycin, nalidixic acid, ciprofloxacin, tetracycline, streptomycin, gentamicin
C, resistance to erythromycin, azithromycin, nalidixic acid, ciprofloxacin, tetracycline
D, resistance to erythromycin, azithromycin, nalidixic acid, ciprofloxacin
NAL (nalidixic acid), CIP (ciprofloxacin), TET (tetracycline), STR (streptomycin)
[a], Macrolide susceptible *Campylobacter* isolates used as controls
[b], number of position equivalent to *E. coli* J01695

Additionally, several more point mutations at different positions were detected in two macrolide resistant *C. coli* isolates (CJ-1113-1 and CJC 1761-8).

DISCUSSION

In this study, a possible genetic basis of erythromycin and azithromycin resistance among *C. jejuni* and *C. coli* isolates was by a common point mutation causing a substitution of base A to G at the equivalent position of 2230 compared to 23S rDNA of *C. coli* (equivalent to position 2059 of *E. coli* J01695) (Jensen and Aarestrup, 2001; Vester and Douthwaite, 2001). This mutation causes an alteration of the drug binding target site of domain V in the 23S rRNA. None of the *C. jejuni* and *C. coli* isolates had mutations at position 2058 compared to *E. coli* J01965 as reported previously (Vester and Douthwaite, 2001). Additional sequence mutations in 23S rDNA were detected in four *C. coli* isolates (CJ-1113-1, CJ 1732-1, CJ 1782-1 and CJC-1761-8) in Table 2. In this study, *C. jejuni* isolates were less frequently resistant to macrolides than *C. coli* (2.4% and 17% resistance, respectively). High prevalence of macrolide resistance among *C. coli* isolates from swine has been cited in a previous publication and thus the use of macrolides in animal production may lead to the emergence of macrolide-resistant isolates in human (Gibreel *et al*, 2005). In this study, the first multiple resistant (resistant phenotype B, Table 2) was observed from *C. jejuni* (VC-114) isolated in 1993. In addition, there was no association between multiple resistant isolates and Lior serotypes. The finding of mutation of 23S rRNA in our *Campylobacter* isolates does not rule out other possible mechanisms involved in macrolide resistance (Gibreel *et al*, 2005). MIC testing with both erythromycin and azithromycin was not performed at the concentration >32 μg/ml to observe whether a further increase of MICs might relate to additional point mutations in the two *Campylobacter* isolates CJ-1113-1 and CJC-1761-8.

In summary, point mutation of 23S rRNA similar to those previously reported was associated with reduced susceptibility to macrolides in clinical *C. jejuni* and *C. coli* isolated in Thailand. The high occurrence of multiple resistances among macrolide-resistant isolates is of concern as such strains will not respond to standard therapeutic treatment of campylobacteriosis and their spread should therefore be monitored closely. Although prevalence of macrolide resistance among human *Campylobacter* isolates from Thailand has not reached the level of fluoroquinolone resistance in Thailand and elsewhere (Hoge *et al*, 1998), the switch to antimicrobial usage could have selected for the multiple resistance seen in the tested human *Campylobacter,* leaving very few available treatment options.

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