ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2011, p. 5939–5941 Vol. 55, No. 12
0066-4804/11/$12.00 doi:10.1128/AAC.00314-11

Ribosomal Mutations as the Main Cause of Macrolide Resistance in *Campylobacter jejuni* and *Campylobacter coli*[▽]

Mirva Lehtopolku,[1,2]* Pirkko Kotilainen,[1,2] Marjo Haanperä-Heikkinen,[1] Ulla-Maija Nakari,[3] Marja-Liisa Hänninen,[4] Pentti Huovinen,[1,5] Anja Siitonen,[3] Erkki Eerola,[5] Jari Jalava,[1] and Antti J. Hakanen[1,2]

Antimicrobial Resistance Unit[1] and Bacteriology Unit,[3] National Institute for Health and Welfare (THL), Turku and Helsinki, Finland; Department of Medicine, Turku University Hospital and University of Turku, Turku, Finland[2]; Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland[4]; and Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland[5]

Received 8 March 2011/Returned for modification 13 April 2011/Accepted 31 August 2011

The aim of this study was to examine macrolide resistance mutations in *Campylobacter* species. In 76 strains studied, point mutation A to G at position 2059 of the 23S rRNA gene was detected in 30 of the 33 erythromycin-resistant strains. An amino acid insertion in the ribosomal protein L22 was found in one resistant strain without a 23S rRNA mutation. The A2059G mutation is the main cause of macrolide resistance in *Campylobacter* species.

Campylobacters are recognized as a leading cause of bacterial gastroenteritis worldwide (1). The most important macrolide resistance mechanisms in *Campylobacter* are due to modifications of the ribosomal target sites. High-level resistance is mainly caused by mutations at positions 2058 and 2059 (*Escherichia coli* numbering) of the 23S rRNA gene (6, 7, 17). Campylobacters contain three copies of this gene (8). Several modifications in the ribosomal proteins L4 and L22, associated with macrolide resistance in other bacteria (5, 13, 15), have been reported in *Campylobacter* (3, 4, 6, 19). We used pyrosequencing to examine the role of 23S rRNA mutations as a cause of macrolide resistance in a large collection of *Campylobacter* strains. We also sequenced the L4 and L22 ribosomal protein genes as well as evaluating the effect of the efflux pump inhibitors on the MIC values.

(This work was presented in part at the 14th International Workshop on Campylobacter, Helicobacter and Related Organisms, Rotterdam, The Netherlands, 2007 [P351].)

The study collection consisted of 76 *Campylobacter* strains isolated from stool specimens of Finnish patients between 2003 and 2008. Of these strains, 53 were from an earlier study collection (14), and 23 strains were collected locally in the area of the Turku University Hospital. The cultivation of the stool samples, the preliminary identification of the isolates, and MIC determinations were done as described previously (14). The MIC breakpoint for erythromycin was ≥16 μg/ml (11). The hippurate hydrolysis test was used for final species identification (16). *Campylobacter jejuni* DSM 4688 type strain and *Campylobacter coli* DSM 4689 type strain (both erythromycin susceptible with wild-type 23S rRNA) were used as control strains. Mutations at positions 2058 and 2059 (*E. coli* numbering) of the 23S rRNA gene were analyzed by pyrosequencing as described by Haanperä et al. (10). L4 and L22 gene sequences of all 33 erythromycin-resistant and 20 erythromycin-susceptible *Campylobacter* strains were determined using standard Sanger sequencing using primers 5′-GAATTTGCTCCA ACACGC-3′ and 5′-ACCATCTTGATTCCCAGTTTC-3′ for L22 protein and primers 5′-GTAGTTAAAGGTGCAGTACC A-3′ and 5′-GCGAAGTTTGAATAACTACG-3′ for L4 protein. The DNA was prepared as described earlier, the suspension was further diluted 1 to 10 in sterile water, and 5 μl was used as a template in PCR (9). The effect of efflux on erythromycin MICs was evaluated in 31 *Campylobacter* strains. The MICs were determined as described above in the presence or absence of efflux pump inhibitor phenylarginyl-β-naphthylamide (PAβN; Sigma, Helsinki, Finland) or 1-(1-naphthylmethyl)-piperazine (NMP; Chess, Mannheim, Germany) at concentrations of 50 μg/ml and 100 μg/ml, respectively (2, 12).

In 76 *Campylobacter* strains evaluated, point mutation A2059G of the 23S rRNA gene was detected by pyroseqencing in 30 (91%) of the 33 erythromycin-resistant (MIC, ≥16 μg/ml) strains but in none of the 43 erythromycin-susceptible strains. Based on pyrosequencing, 28 (93%) of these 30 strains had all three alleles of the 23S rRNA gene mutated. The remaining two strains (one of *C. jejuni* and one of *C. coli*) had two mutated 23S rRNA copies (Tables 1 and 2). Three erythromycin-resistant strains (two of *C. jejuni* and one of *C. coli*) had a wild-type 23S rRNA sequence. None of the studied strains had a point mutation at position 2058 of the 23S rRNA gene (Tables 1 and 2). The comparison of the ribosomal protein L22 and L4 amino acid sequences of the *C. jejuni* and *C. coli* strains to type strains revealed several different amino acid substitutions and their combinations (Tables 1 and 2). Several strains possessed deletions in the ribosomal protein L22. One highly erythromycin-resistant *C. jejuni* strain without a mutation in the 23S rRNA gene (strain 1, Table 1) exhibited a five-amino-acid insertion (GlyAlaValLeuThr) between posi-

* Corresponding author. Mailing address: Antimicrobial Resistance Unit, National Institute for Health and Welfare (THL), Kiinamyllynkatu 13, 20520 Turku, Finland. Phone: 358 20 610 6631. Fax: 358 20 610 6699. E-mail: mipleht@utu.fi.

▽ Published ahead of print on 12 September 2011.

TABLE 1. Ribosomal mutations and erythromycin susceptibility in *Campylobacter jejuni* strains compared to *C. jejuni* DSM 4688 type strain[e]

Strain(s)	Erythromycin MIC (μg/ml)[d]	23S rRNA gene mutation[a]	L4 mutation(s)	L22 mutation(s)	L22 insertion
DSM 4688[b]	0.5				
1	>128	wt	A121V, I192M	A74G, A109T, E111A, T114A, A130T, A132V, A141V, K144E	73-GAVLT-74
2–6	>128	A2059G	A121V, I192M	A74G, A109T, E111A, T114A, A130T, A132V, A141V, K144E	
7	>128	A2059G	wt	A74G, A103V, A141V, K144E	
8[c]	>128	A2059G	wt	A74G, A103V, A141V	
9, 10	>128	A2059G	A121V, I192M, A196V	V65I, A109S, A143V	
11	>128	A2059G	A121V, I192M	Q24R, V65I, A143V	
12	>128	A2059G	A121V, I192M	V65I, A103V	
13	>128	A2059G	A121V, I192M	G86E	
14	128	A2059G	A121V, I192M	A74G, A109T, E111A, T114A, A130T, A132V, A141V, K144E	
15	128	A2059G	wt	V65I, A103V	
16	64	wt	T177S	I65V, D72N	
17, 18	8	wt	A121V, I192M	Q24R, V65I, A143V	
19	8	wt	A121V, I192M	V65I, A109S, A143V	
20, 22	4–8	wt	A121V, I192M	V65I, A103V	
21	4	wt	N95S, A121V, I192M	Q24R, V65I, A143V	
23	2	wr	A129T, T201I	wt	
24, 25	1	wt	A121V, I192M	V65I, A109S, A143V	
26, 30, 31	0.5–1	wt	A121V, I192M, A196V	V65I, A109S, A143V	
27	1	wt	wt	V65I, A103V	
28, 29	1	wt	T177S	V65I	
32	0.5	wt	V80I, A121V, I192M	V65I, A103V	
33	0.5	wt	S132N, T177S	V65I	
34	0.5	wt	A140T	wt	

[a] Position according to *Escherichia coli* numbering.
[b] Reference strain for the ribosomal proteins L4 and L22.
[c] Only two mutated copies of A2059G.
[d] From each MIC, a number of strains were selected for the efflux pump inhibitor (NMP and PAβN) testing, and no decrease in MIC was observed.
[e] Abbreviations: A, Ala; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; wt, wild type, based on corresponding sequences of *C. jejuni* DSM 4688 type strain.

tions 73 and 74 in the ribosomal protein L22. This insertion was not detected in any other strain. Another erythromycin-resistant *C. jejuni* strain (strain 16, Table 1) without mutation in the 23S rRNA gene showed an Asp72Asn substitution in the ribosomal protein L22, which was not found in any other strain. The erythromycin MICs of both susceptible and resistant *Campylobacter* strains remained similar in the presence and in the absence of efflux pump inhibitors PAβN and NMP, except for one *C. coli* strain (strain 51, Table 2). This strain with the A2059G mutation exhibited a 2-fold decrease in the MIC value (512 μg/ml → 128 μg/ml) when NMP was used for inhibiting the efflux pumps. PAβN did not change the MIC value.

In this study, point mutation A2059G of the 23S rRNA gene was found to be the main mechanism behind macrolide resistance among *Campylobacter* strains, as 91% of all erythromycin-resistant strains had this mutation, confirming previous findings (4, 8). For example, while investigating a collection of 23 macrolide-resistant *Campylobacter* isolates, Gibreel and Taylor (8) found that about 78% of their isolates exhibited point mutation A2059G of the 23S rRNA gene and about 13% of the isolates had a mutation at position 2058. Vacher et al. also found position 2058 mutations in two of the 22 macrolide-resistant *Campylobacter* isolates (18). Mutations at position 2058 were not found in our strains, which is also in line with some other reports (6, 17), implying that position 2058 mutations do not have as important a role as do position 2059 mutations in *Campylobacter* species.

Sequencing of the L4 and L22 genes revealed one highly resistant *C. jejuni* strain (strain 1, Table 1) not exhibiting any specific point mutation of the 23S rRNA with insertion between positions 73 and 74 in the ribosomal protein L22. The efflux pump inhibitor PAβN or NMP did not change the MIC values of this strain. We propose that the insertion might be associated with macrolide resistance in *C. jejuni*. According to our knowledge, this has not been previously described.

A number of previous studies have reported modifications in the ribosomal proteins L4 and L22 (3, 4, 6, 19). Several amino acid substitutions in the ribosomal protein L22 were observed also in the present study. They appeared to be more common in the erythromycin-resistant *C. jejuni* strains. In contrast, only a few amino acid substitutions were observed in the ribosomal protein L4, with no obvious difference between the erythromycin-resistant and -susceptible strains. So far, the significance of the amino acid substitutions or deletions found in the ribosomal proteins L4 and L22 remains unknown and warrants further evaluation.

In conclusion, point mutation A2059G of the 23S rRNA gene was found to be the main mechanism behind macrolide resistance among *Campylobacter* species. A five-amino-acid insertion between positions 73 and 74 in the ribosomal protein

TABLE 2. Ribosomal mutations and erythromycin susceptibility in *Campylobacter coli* strains compared to *C. coli* DSM 4689 type strain[e]

Strain(s)	Erythromycin MIC (μg/ml)	23S rRNA gene mutation[a]	L4 mutation(s)	L22 mutation(s)	L22 insertions
DSM 4689[b]	0.5	wt			
36	>128	A2059G	S28P, T176S	wt	
41,[c] 37–40, 42–44	>128	A2059G	S28P	wt	
45	>128	A2059G	S28P, V121A, M192I	wt	
46, 47	>128	A2059G	S28P, V121A, V176I, T177S, V184I, M192I	A103V	
48	>128	A2059G	S28P	G58S	
49	>128	A2059G	S28P, M192I	T95I, A103V, T109A, A111E, A114T, V121A	119-TTTKA-120, 124-T-125
50	>128	A2059G	S28P, M192I	A103V, T109A, A111E, A114T, V121A	119-TTTKA-120, 124-T-125
51[d]	128	A2059G	S28P, V121A, M192I	A103V, T109A, A111E, A114T, V121A	119-TTTKA-120, 124-T-125
52	16	wt	S28P	wt	
53	8	wt	S28P, V121A, V176I, T177S, V184I, M192I	A103V	
54	8	wt	S28P, M192I	A103V, T109A, A111E, A114T, V121A	119-TTTKA-120, 124-T-125

[a] Position according to *Escherichia coli* numbering.
[b] Reference strain for the ribosomal proteins L4 and L22.
[c] Only two mutated copies of A2059G.
[d] NMP caused 2-fold decrease in MIC.
[e] Abbreviations: A, Ala; E, Glu; G, Gly; I, Ile; K, Lys; M, Met; P, Pro; S, Ser; T, Thr; V, Val; wt, wild type, based on corresponding sequences of *C. coli* DSM 4689 type strain. One strain from each row was selected for the efflux pump inhibitor (NMP and PAβN) testing, and only one strain exhibited a 2-fold decrease in MIC.

L22 of one highly resistant *C. jejuni* strain without mutation in the 23S rRNA gene was also detected. Further studies are needed to assess whether this insertion might contribute to *Campylobacter* macrolide resistance.

We thank Tuula Randell and Minna Lamppu for laboratory assistance.

This study was supported by grants from the Maud Kuistila Memorial Foundation, the Finnish Cultural foundation, and the Turku University Central Hospital Research Fund.

There are no conflicts of interest to declare.

REFERENCES

1. **Allos, B. M.** 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. Clin. Infect. Dis. **32:**1201–1206.
2. **Bohnert, J. A., and W. V. Kern.** 2005. Selected arylpiperazines are capable of reversing multidrug resistance in *Escherichia coli* overexpressing RND efflux pumps. Antimicrob. Agents Chemother. **49:**849–852.
3. **Cagliero, C., C. Mouline, A. Cloeckaert, and S. Payot.** 2006. Synergy between efflux pump CmeABC and modifications in ribosomal proteins L4 and L22 in conferring macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. **50:**3893–3896.
4. **Caldwell, D. B., Y. Wang, and J. Lin.** 2008. Development, stability, and molecular mechanisms of macrolide resistance in *Campylobacter jejuni*. Antimicrob. Agents Chemother. **52:**3947–3954.
5. **Chittum, H. S., and W. S. Champney.** 1994. Ribosomal protein gene sequence changes in erythromycin-resistant mutants of *Escherichia coli*. J. Bacteriol. **176:**6192–6198.
6. **Corcoran, D., T. Quinn, L. Cotter, and S. Fanning.** 2006. An investigation of the molecular mechanisms contributing to high-level erythromycin resistance in *Campylobacter*. Int. J. Antimicrob. Agents **27:**40–45.
7. **Gibreel, A., et al.** 2005. Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*: molecular mechanism and stability of the resistance phenotype. Antimicrob. Agents Chemother. **49:**2753–2759.
8. **Gibreel, A., and D. E. Taylor.** 2006. Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. J. Antimicrob. Chemother. **58:**243–255.
9. **Gunell, M., et al.** 2010. In vitro activity of azithromycin against nontyphoidal Salmonella enterica. Antimicrob. Agents Chemother. **54:**3498–3501.
10. **Haanperä, M., P. Huovinen, and J. Jalava.** 2005. Detection and quantification of macrolide resistance mutations at positions 2058 and 2059 of the 23S rRNA gene by pyrosequencing. Antimicrob. Agents Chemother. **49:**457–460.
11. **Hakanen, A., M. Lehtopolku, A. Siitonen, P. Huovinen, and P. Kotilainen.** 2003. Multidrug resistance in Campylobacter jejuni strains collected from Finnish patients during 1995–2000. J. Antimicrob. Chemother. **52:**1035–1039.
12. **Hannula, M., and M. L. Hanninen.** 2008. Effect of putative efflux pump inhibitors and inducers on the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli*. J. Med. Microbiol. **57:**851–855.
13. **Jones, R. N., D. J. Farrell, and I. Morrissey.** 2003. Quinupristin-dalfopristin resistance in *Streptococcus pneumoniae*: novel L22 ribosomal protein mutation in two clinical isolates from the SENTRY antimicrobial surveillance program. Antimicrob. Agents Chemother. **47:**2696–2698.
14. **Lehtopolku, M., et al.** 2010. Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: in vitro activities of 20 antimicrobial agents. Antimicrob. Agents Chemother. **54:**1232–1236.
15. **Malbruny, B., et al.** 2002. Resistance to quinupristin-dalfopristin due to mutation of L22 ribosomal protein in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **46:**2200–2207.
16. **Nakari, U. M., A. Puhakka, and A. Siitonen.** 2008. Correct identification and discrimination between *Campylobacter jejuni* and *C. coli* by a standardized hippurate test and species-specific polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **27:**513–518.
17. **Payot, S., et al.** 2004. Relative contribution of target gene mutation and efflux to fluoroquinolone and erythromycin resistance, in French poultry and pig isolates of *Campylobacter coli*. Int. J. Antimicrob. Agents **23:**468–472.
18. **Vacher, S., A. Menard, E. Bernard, and F. Megraud.** 2003. PCR-restriction fragment length polymorphism analysis for detection of point mutations associated with macrolide resistance in *Campylobacter* spp. Antimicrob. Agents Chemother. **47:**1125–1128.
19. **Zaman, S., M. Fitzpatrick, L. Lindahl, and J. Zengel.** 2007. Novel mutations in ribosomal proteins L4 and L22 that confer erythromycin resistance in Escherichia coli. Mol. Microbiol. **66:**1039–1050.