Caught in the Act: *In Vivo* Development of Macrolide Resistance to *Campylobacter jejuni* Infection[▽]

J. C. Lindow,[1][†][*] F. Poly,[2][†] D. R. Tribble,[2,3] P. Guerry,[2] M. P. Carmolli,[1] S. Baqar,[2][§]
C. K. Porter,[2] K. K. Pierce,[1] M. J. Darsley,[4][¶] K. S. Sadigh,[1] E. A. Dill,[1]
the *Campylobacter* Study Team,[1,2,4] and B. D. Kirkpatrick[1]

*University of Vermont (UVM) College of Medicine, Department of Medicine, Unit of Infectious Diseases and Vaccine Testing Center,
Burlington, Vermont[1]; Naval Medical Research Center (NMRC), Department of Enteric Diseases, Silver Spring, Maryland[2];
Uniformed Services University of the Health Sciences, Department of Preventive Medicine and Biometrics, Bethesda,
Maryland[3]; and ACE Biosciences, Hillerød, Denmark[4]*

**We report the first instance of macrolide resistance developing *in vivo* following appropriate antibiotic use
in a healthy volunteer as a part of a controlled human infection with *Campylobacter jejuni*. *In vivo* development
of macrolide resistance may be an important contributor to antibiotic resistance in *C. jejuni*.**

CASE REPORT

As part of a well-controlled inpatient experimental human infection study, a 21-year-old healthy male was dosed with 8.5×10^4 CFU of *Campylobacter jejuni* CG8421 (21). This bacterial stock was prepared and stored under good manufacturing practices (cGMP), lacks ganglioside mimicry, is susceptible to macrolide and fluoroquinolone (FQ) antibiotics, and has been fully characterized (19). At 72 h postinoculation, the subject experienced the onset of diarrhea preceded by a high fever (maximal temperature, 103.1°F). Blood cultures were negative. The total episode of campylobacteriosis consisted of 12 loose/liquid stools (4 stools prior to antibiotics; total volume of all stools, 1,220 ml) without gross blood, accompanied by abdominal cramping and two episodes of vomiting. At 72 h after inoculation but before initiation of antibiotics (80 h), the subject shed *C. jejuni*, which was confirmed to be *C. jejuni* CG8421 ($2.95 \times 10^5 \pm 1.8 \times 10^5$ CFU bacteria/gram of stool). Per protocol, azithromycin (500 mg orally once daily for 5 days) was administered. The subject's symptoms resolved within 48 h; stool cultures became negative and remained consecutively negative until discharge on day 9 postinoculation and at follow-up visits on days 10 and 14 postinoculation. On the day 21 follow-up visit, the subject was asymptomatic, but stool cultures were positive for *C. jejuni*, which was again confirmed to be CG8421 but now determined to be macrolide resistant. The subject was further treated with both ciprofloxacin (500 mg orally twice daily) and azithromycin (500 mg orally once daily, begun before antibiotic sensitivities were known). The subject remained asymptomatic, and all weekly stool cultures taken for 5 weeks post-antibiotic treatment were negative. HIV antibody, serum immunoglobulins and subsets, com-

plement levels, and response to protein vaccination were all negative or normal. The subject mounted robust *C. jejuni*-specific immunologic responses postinfection, including serologic responses (8-, 16-, and 4-fold increases in IgM, IgA, and IgG, respectively), fecal IgA (8-fold rise), antibody-secreting cells (ASC) (maximal response, 129 IgA ASC/10^6 peripheral blood mononuclear cells), and *in vitro C. jejuni*-specific gamma interferon (IFN-γ) (4-fold rise).

After *C. jejuni* inoculation, the first two stools produced each day were cultured using validated standard operating procedures with a level of detection of 1.5 CFU/100 mg stool. Cultures were performed within 4 h of collection using *Campylobacter*-specific medium (Campy CVA or Campy blood agar plates) in chambers containing 5% O_2, 10% CO_2, and 85% N_2 at 42°C for 48 h. Following administration of antibiotics, to maximize the detection of *C. jejuni*, stools were cultured within 2 h of specimen production and plated in sextuplicate.

The day 3 and day 21 *C. jejuni* isolates were confirmed as identical to the challenge strain, CG8421. Both isolates were evaluated by PCR, using previously described primers specific to CG8421 (4). These primers are complementary to genes located in a phage-like element unique to CG8421; *C. jejuni* strain 81-176, which lacks these sequences, was used as a negative control. As anticipated, the day 3, day 21, and CG8421 *C. jejuni* strains generated PCR products, while the negative control, *C. jejuni* 81-176, did not. Pulsed-field gel electrophoresis (PFGE) was also performed on the day 21 strain using a BssHII enzyme digestion and previously described parameters (4). Identical digestion patterns shown in Fig. 1B and C confirmed the PCR results that the day 21 isolate was CG8421.

Antibiotic sensitivity tests for the day 21 isolate and the inoculum strain were performed in parallel by MIC testing (MICs) using Etests (AB Biodisk, Solna, Sweden). New, high-level macrolide resistance (azithromycin and erythromycin) was observed in the day 21 recrudescence strain (Table 1).

The most common mechanism of macrolide resistance in *C. jejuni* is mutation of the 23S ribosomal gene (9, 23, 24). In *C. jejuni*, macrolide resistance is mostly due to a reduction in macrolide binding affinity to the clarithromycin-binding site

* Corresponding author. Mailing address: Vaccine Testing Center, University of Vermont College of Medicine, 95 Carrigan Drive, 110 Stafford Hall, Burlington, VT 05405. Phone: (802) 656-7717. Fax: (802) 656-0881. E-mail: janet.lindow@uvm.edu.
† These authors contributed equally to the study.
§ Current address: National Institutes of Health, Bethesda, MD.
¶ Current address: TD Vaccines A/S, Skørping, Denmark.
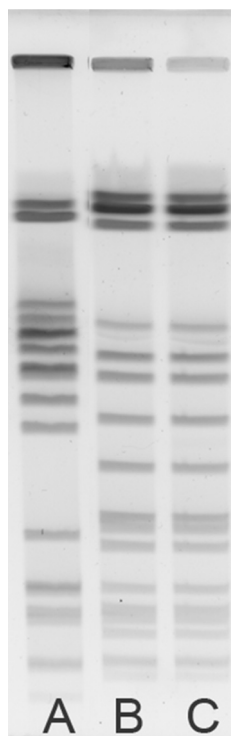▽ Published ahead of print on 16 June 2010.

FIG. 1. Pulsed-field gel electrophoresis. Genomic DNA was digested with BssHII enzyme (8 U; New England Biolabs, Beverly, MA) overnight at 37°C. Samples were run in a 1% agarose gel with a switch time of 2.2 to 17.6 s and a field strength of 6 V/cm for 23 h on a contour-clamped homogeneous electric field (CHEF) apparatus (Bio-Rad). Lane A, *C. jejuni* reference strain 81-176; lane B, inoculum strain *C. jejuni* CG8421; lane C, macrolide-resistant *C. jejuni* CG8421 isolate from day 21.

located on the 23S rRNA genes. Such resistance is, in most cases, associated with a point mutation at position 2075 that replaces an adenine by a guanine residue (A2075G) (8, 23, 24). This mutation must occur in at least two of the three copies of the rRNA genes to confer the resistance phenotype (8, 23). A less frequent point mutation is also observed at position 2074, in which the adenine is replaced by a cytosine residue (A2074C) (8, 23, 24). For the day 3 and day 21 isolates, the 23S genes of *C. jejuni* were amplified by PCR and sequenced using previously described primers (23). A single transition mutation, A2075G, was identified in the *C. jejuni* strain isolated on day 21 compared to the starting challenge strain. The DNA sequence of the amplicons did not indicate any mixed signals at position 2075, which is consistent with mutations at all three sites. Importantly, the day 3 strain, which was shed prior to antibiotic treatment and characterized as CG8421 by PCR, was macrolide sensitive since it did not contain mutations at positions 2074 or 2075, suggesting that macrolide resistance developed *in vivo* following antibiotic use.

The mutation rate of the challenge strain was determined as follows to ensure that there was not a higher mutation rate in CG8421 resulting in antibiotic-resistant bacteria. A cGMP seed lot vial was plated on Mueller-Hinton (MH) agar plates at 42°C overnight as per the protocol for the inoculation. The cells were scraped off the plate and resuspended in phosphate-buffered saline. Aliquots of the suspensions were incubated on MH agar supplemented with 10 μg/ml erythromycin at 37°C for 6 to 7 days, and the total number of cells plated was enumerated on MH plates without selection and incubated for 48 h at 37°C. The mutation rate was compared to that of a second, well-characterized *C. jejuni* strain used in human studies, 81-176. The mutation rate for CG8421 (1.76×10^{-10}) was similar to that of 81-176 (2.1×10^{-10}).

We present the first clinical case of *in vivo*-acquired macrolide resistance causing recrudescent infection in a healthy subject infected with *C. jejuni. Campylobacter* is a leading cause of food-borne enteritis worldwide, causing an estimated 400 million cases of gastroenteritis annually. Although antibiotic use is usually reserved for moderate to severe infection, antibiotic options are limited, and increasing antibiotic resistance is a growing concern (2, 3, 9, 16, 20). Through randomized control trials, macrolide antibiotics have been recognized as efficacious against *Campylobacter* since the 1980s and are currently considered the antibiotic of choice for moderate to severe human infection (10, 15). In the United States, the current rate of macrolide resistance is approximately 2% in human *C. jejuni* isolates and has fluctuated from 0.6% to 5% since 1998 (6, 9). In addition to being used in humans, macrolides (such as tylosin) are widely used in animal husbandry as antibiotics and growth promoters. The contribution of macrolide use in animals to overall macrolide resistance trends is controversial, but the volume of animal use appears to exceed human use (1).

Macrolide resistance in *C. jejuni* strains has remained relatively stable, particularly compared to results with FQ antibiotics, in which *Campylobacter* resistance has rapidly increased in the United States from approximately 15 to 26% over a 10-year period (6). FQ resistance in clinical isolates was not seen on the Asian and African continents before 1991 and has rapidly increased in both regions, reaching over 80% in Thailand and Hong Kong (16). The highest rates of resistance are in Spain, where 99% resistance has been reported (16). The FQs are also heavily used in animal care, and *in*

TABLE 1. Etest MICs demonstrate macrolide resistance

Antibiotic	Etest result[a] for isolate and inoculum				Resistance (μg/ml)[b]
	Day 0		Day 21		
	Test 1	Test 2	Test 1	Test 2	
Ciprofloxacin	0.125	0.094	0.064	0.064	4
Tetracycline[d]	ND[c]	24	64	24	≥16
Levofloxacin	0.094	0.094	0.094	0.094	
Trimethoprim-sulfamethoxazole[d]	>32	>32	>32	>32	
Imipenem	0.125	0.094	0.094	0.094	
Azithromycin	0.125	0.125	>256	>256	≥8
Ampicillin	0.75	1.0	1.0	1.0	
Erythromycin	1.0	1.0	>256	>256	≥32

[a] Etest MIC (μg/ml) for the *C. jejuni* CG8421 day 21 isolate, compared to the inoculum strain (Day 0) (cGMP lot), after 48 h of incubation.
[b] Resistance levels according to 2007 NARMS report (6).
[c] ND, not determined.
[d] CG8421 is known to be resistant to tetracycline and trimethoprim-sulfamethoxazole.

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vivo Campylobacter resistance to FQ has been well described in humans (7).

The microbiological rationale for the differences in prevalence of *C. jejuni* resistance between FQ and macrolides has two likely explanations. *C. jejuni* strains gain macrolide resistance at the cost of biological fitness, whereas FQ resistance causes a gain in *C. jejuni* fitness (11, 12, 17). Second, only one of several mechanisms associated with macrolide resistance (mutations within 23S rRNA) is stably maintained whereas all mutations generating FQ resistance are stably maintained (5, 14, 17).

In this case, the close clinical and bacteriological monitoring of this study permitted the detection of new macrolide resistance that was selected following appropriate antibiotic use. Following infection with a well-characterized, known macrolide-sensitive strain, the subject shed the antibiotic-sensitive strain (day 3) prior to antibiotic use (19, 21). Recrudescence of infection with the newly macrolide-resistant strain occurred after a 15-day period of negative stool cultures. This observation illustrates the risk of antibiotic resistance even with appropriate use of antibiotics and in the most controlled clinical settings. Clinically asymptomatic shedding of macrolide-resistant *Campylobacter* infection is likely to be more common than recognized, particularly since stool cultures are generally not performed in the absence of gastrointestinal symptoms.

Our findings do not appear to be strain specific; no evidence of increased mutation rates was found in our inoculum strain, and no evidence exists to suggest that this strain (*C. jejuni* CG8421) has characteristics of increased clinical virulence. Indeed, compared to another strain used in clinical testing (*C. jejuni* 81-176), strain CG8421 appears clinically less virulent (21, 22). Clinical precautions implemented after recognition of recrudescence of *Campylobacter* in the clinical setting have permitted continued safe use of strain CG8421 in human studies; these include dual use of antibiotics and extended microbiological monitoring of subjects.

The mechanism of that recrudescence remains unknown, but luminal replication of the strain may have resumed as antibiotic levels dropped, permitting microbiologic relapse due to survival and growth of a now-resistant strain. Although *Campylobacter* has survived up to a week in macrophages *in vitro*, no gallbladder, diverticulum, or appendiceal carriage of *Campylobacter* has thus far been clinically identified (13). It is interesting to postulate that other enteric bacterial pathogens might follow the same pattern and to consider the clinical significance (or lack thereof) of asymptomatic shedding (18). Further research is clearly needed to clarify these issues.

Clinical *Campylobacter* model work, which uses very closely monitored subjects dosed with *C. jejuni*, has brought to light the incidence of recrudescent infection in otherwise healthy adults. We have observed recrudescence in ~5% of antibiotic-treated persons, all following a period in which *Campylobacter* cannot be isolated from stool by optimized, standard microbiological techniques (4 and unpublished data). Our data suggest that following antibiotics, microbiologic recrudescence can occur 14 to 28 days after the initial infection, but it is not known if recrudescence is dependent upon antibiotic treatment. Although not seen in this case, recrudescence may occur in the setting of suboptimal immune responses to *C. jejuni* and may or may not be clinically apparent (4). Therefore, there may be

several mechanisms by which recrudescence can occur during *C. jejuni* infections. Given the increasing *Campylobacter* resistance to FQs and the limited antibiotic treatment options for treating this infection, it will be important to monitor trends in resistance to the macrolides. Although the development of *in vivo* macrolide resistance is likely underrecognized, our description of the development of *in vivo* resistance after appropriate use of antibiotics is a reminder of the principles of careful antibiotic stewardship.

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