Journal of Antimicrobial Chemotherapy (2001) **48**, 235–240

JAC

Antimicrobial susceptibilities of *Campylobacter* strains isolated from food animals in Belgium

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***Campylobacter* spp. are a frequent cause of diarrhoea in man, originating mostly from poultry. It has been suggested that the veterinary use of antibiotics is largely responsible for resistance in human isolates, particularly to quinolones. During a 6 month period from June to December 1998, 677 *Campylobacter* isolates were obtained from healthy poultry and pigs. Samples were taken at Belgian slaughterhouses. Species identification was performed by biochemical tests, multiplex PCR and SDS–PAGE of whole-cell proteins. The *in vitro* susceptibility to six antimicrobial drugs was determined by the agar dilution method. *Campylobacter jejuni* was found more often in poultry than *Campylobacter coli* (79% *C. jejuni* versus 21% *C. coli*). In pigs the situation was reversed (6 versus 94%). Erythromycin resistance was significantly higher ($P < 0.05$) in *C. coli*, particularly in *C. coli* isolated from pigs (67.2%). Alarmingly high rates of resistance to ciprofloxacin were also noted, particularly for *C. coli* from broilers (62.1%). The latter indicates that resistance of *Campylobacter* in humans could derive from animals.**

Introduction

Infection with *Campylobacter* spp. has emerged worldwide as one of the leading causes of bacterial diarrhoea.[1] More than 90% of the infections are caused by *Campylobacter jejuni*, with *Campylobacter coli* representing most of the remainder. *Campylobacter lari* comprises <1% of the strains isolated and other species, such as *Campylobacter upsaliensis* and *Campylobacter fetus*, are only occasionally seen in clinical isolates.[2] Usually diarrhoea caused by campylobacters is self-limiting with a duration of 2–5 days but can persist for 2 weeks or longer. Therapy is not required, except in severe cases with prolonged disease and serious symptoms, or in immunocompromised patients.[3,4] Macrolides are the drugs of choice for *Campylobacter* enteritis, but resistance has been reported to be increasing, particularly in *C. coli*.[5,6] Fluoroquinolones have been widely used for the treatment of *Campylobacter* infections and for empirical treatment of patients with gastroenteritis, including traveller's diarrhoea.[7,8] In recent years, however, an increased proportion of *Campylobacter* isolates have been reported to be resistant to these drugs.[9–12]

Campylobacter infection is a mainly food-borne disease because of its widespread commensalism and specific adaptation to the gastrointestinal tracts of domestic and wild animals. The sources most commonly associated with epidemics and sporadic cases have been unpasteurized milk, contaminated drinking water and inadequately cooked meat, particularly poultry.[1,13,14] In the USA, case–control studies have shown that 48–70% of sporadic infec-

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tions are associated with the consumption of *Campylobacter*-contaminated chickens.[13,15] The cross-contamination of other foods during food preparation is also likely to be important.

There is growing scientific evidence that the use of antibiotics in food animals leads to the development of resistant pathogenic bacteria that can reach humans through the food chain. In the present study, resistance in campylobacters isolated from healthy poultry and pigs at slaughter was examined.

Materials and methods

Specimen collection and isolation

From June to December 1998, swabs or samples from healthy pigs (carcass, liver, fresh and minced meat), broilers (carcass, liver and meat), layers (carcass) and turkeys (neck skin) were collected at Belgian slaughterhouses or cutting rooms. Pig carcasses and livers, and carcasses from broilers and layers, were swabbed using a standard cotton swab moistened with *Brucella* broth (Oxoid, Basingstoke, UK). Samples of 100 g of fresh and minced meat from pigs, liver and meat from broilers, and neck skin from turkeys were taken. In the laboratory the swabs or the samples were homogenized into Preston selective broth (Oxoid) and incubated microaerophilically at 42°C for 48 h. The enrichment was streaked on to modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) and incubated at 42°C for 24–120 h.

Identification of isolates

Isolates were identified according to standard criteria including negative Gram's stain, typical morphology, catalase and oxidase reactions, growth at 42°C and hippurate hydrolysis.[16] Further identification was made by the API Campy strip system (bioMérieux SA, Marcy l'Étoile, France) and/or by multiplex PCR.[17] Isolates for which there were contradictory results were additionally examined by SDS–PAGE of whole-cell proteins.[18]

In total, 677 *Campylobacter* isolates were collected during the study period. The number of strains isolated from each animal species were: pigs ($n = 65$), broilers ($n = 351$), layers ($n = 161$) and turkeys ($n = 100$).

Susceptibility testing

MICs were determined by the agar dilution method. Twofold serial dilutions of antibiotics were used at the following concentrations: erythromycin (Sigma, St Louis, MO, USA), 0.03–64 mg/L; ampicillin (Sigma), 0.06–128 mg/L; nalidixic acid (Sigma), 0.03–64 mg/L; ciprofloxacin (Bayer, Brussels, Belgium), 0.03–64 mg/L; tetracycline (Sigma), 0.03–64 mg/L and gentamicin (Sigma), 0.06–128 mg/L. Inocula were prepared in Mueller–Hinton broth (BBL, Becton Dickinson, Cockeysville, MD, USA) at a density adjusted to a 0.5 McFarland turbidity standard, and diluted 1:10. A final inoculum of *c.* 10^4 cfu was delivered on to Mueller–Hinton II agar plates (BBL, Becton Dickinson) with a Steers replicating device. The plates were incubated in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 for 24 h. The MIC was defined as the lowest concentration of an antimicrobial agent that inhibited visible growth completely. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains.

The following NCCLS[19] breakpoints for resistance were used: for ampicillin MIC $\geqslant$ 32 mg/L; for ciprofloxacin MIC $\geqslant$ 4 mg/L; and for tetracycline and gentamicin MIC $\geqslant$ 16 mg/L. Isolates were considered resistant to erythromycin with an MIC $\geqslant$ 8 mg/L[20] and to nalidixic acid with an MIC of $\geqslant$32 mg/L.[21]

Statistical analysis

Data were analysed with Epi Info, version 6 (Centres for Disease Control and Prevention, Atlanta, GA, USA). The χ^2 test and Fisher's exact two-tailed test were used for statistical analysis of the significant difference of resistant rates. An α of 0.05 was used for statistical significance.

Results

The numbers of *C. jejuni* and *C. coli* per animal species were, respectively: pigs, 4/61; broilers, 285/66; layers 105/56; and turkeys, 94/6. *C. jejuni* was the most prevalent species in poultry (79% *C. jejuni* versus 21% *C. coli*), while in pigs *C. coli* predominated (6% *C. jejuni* versus 94% *C. coli*).

Susceptibility testing

For *Campylobacter* spp., no internationally accepted criteria for susceptibility testing including assessments of breakpoints for susceptible versus resistant are available, so these data were analysed with reference to the data available from the NCCLS for aerobic bacteria.[19]

C. jejuni. The results of antimicrobial susceptibility testing for *C. jejuni* strains isolated from broilers, layers and turkeys are shown in Table I. Resistance to erythromycin varied from 6.3% (in broilers) to 8.6% (in layers). A high level of ampicillin resistance was found in *C. jejuni* isolated from turkeys (33.0%). This level was significantly higher ($P < 0.05$) than the levels found in broilers (24.6%) and layers (14.3%). Nalidixic acid resistance was high in broilers (44.2%) and turkeys (44.7%), but for layers the resistance level was lower (29.5%). Similar findings were observed for ciprofloxacin: resistance was found among 44.2, 35.1 and 27.6% of the isolates from broilers, turkeys and layers, respectively. Tetracycline resistance was the

highest in turkeys (37.2%) and broilers (34.4%). All *C. jejuni* isolates were susceptible to gentamicin. *C. jejuni* strains isolated from pigs were not considered because only four strains were isolated.

C. coli. Among *C. coli*, antimicrobial resistance was more common. The results of antimicrobial susceptibility testing for *C. coli* strains are shown in Table II. Erythromycin resistance was significantly higher ($P < 0.05$) in *C. coli*, particularly in *C. coli* isolated from pigs (67.2%). Resistance to ampicillin was found among 18.0, 13.6 and 3.6% of the isolates from pigs, broilers and layers, respectively. Alarmingly high resistance rates to nalidixic acid were also noted, particularly for *C. coli* from broilers (60.6%). Similarly, for ciprofloxacin, 62.1% of *C. coli* isolates from broilers were resistant. For tetracycline the highest level of resistance was found in pigs (62.3%). Isolates from layers showed the lowest level of resistance (28.6%). All *C. coli* isolates from broilers and turkeys (only six isolates, data not shown) were susceptible to gentamicin. Pigs and layers showed low levels of resistance to gentamicin (3.3% and 1.8%, respectively).

Multiresistant isolates

Drug resistance to one or more drugs was detected in over 60% of the strains. Multiresistance, which was defined as resistance to four or more of the drugs tested, was found in 7.6% of the *C. jejuni* strains but was more common in *C. coli* (17%). Multiresistant isolates always remained susceptible to gentamicin (Table III).

Discussion

Alarmingly high resistance rates were observed for erythromycin and ciprofloxacin. Resistance to erythromycin was more prevalent in *C. coli* than in *C. jejuni* ($P < 0.05$). Indeed, erythromycin resistance is more usually associated with *C. coli* than with *C. jejuni*, and several studies have emphasized that *C. coli* isolates are more likely to acquire antibiotic resistance than are *C. jejuni* isolates.[22] In this study, rates of resistance to erythromycin ranged from 6.3% in *C. jejuni* isolated from broilers to 67.2% in *C. coli* isolated from pigs. These findings are similar to those described in DANMAP 98.[23] They found that erythromycin resistance in *C. jejuni* isolated from broilers was 1%, whereas respectively 33% and 68% of the *C. coli* isolated from broilers and pigs were resistant to erythromycin. Macrolides (such as tylosin) have been permitted as growth promotors in pigs (Council Directive 70/524/EEC). Since resistance to erythromycin was the highest for pigs, it seems reasonable to correlate our observation with usage of tylosin in the pig industry. Additionally, tylosin has not been used as a growth promoter in broilers, and this could explain the low level of resistance observed in these strains.

During the past decade, fluoroquinolones have been the

Table I. Antimicrobial susceptibility of *C. jejuni* strains isolated from poultry meat, Belgium, 1998

	Broilers ($n = 285$)				Layers ($n = 105$)				Turkeys ($n = 94$)			
Antibiotic	MIC_{50}[a]	MIC_{90}	range	%R[b]	MIC_{50}	MIC_{90}	range	%R	MIC_{50}	MIC_{90}	range	%R
Erythromycin	2	4	0.06–>64	6.3	2	4	0.06–32	8.6	2	4	1–>64	6.4
Ampicillin	8	32	0.125–>128	24.6	8	32	0.25–>128	14.3	16	64	0.25–>128	33.0
Nalidixic acid	8	>128	0.03–>128	44.2	4	>128	0.06–>128	29.5	8	>128	0.03–>128	44.7
Ciprofloxacin	1	64	0.03–>64	44.2	0.25	64	0.03–>64	27.6	0.5	64	0.06–>64	35.1
Tetracycline	1	>64	0.03–>64	34.4	0.25	64	0.03–>64	20.0	0.5	>64	0.06–>64	37.2
Gentamicin	0.25	0.5	0.06–8	0.0	0.25	0.5	0.06–4	0.0	0.5	0.5	0.125–4	0.0

[a]MIC in mg/L.
[b]%R, percentage resistant.

Table II. Antimicrobial susceptibility of *C. coli* strains isolated from pork and poultry meat, Belgium, 1998

Antibiotic	Pigs (n = 61)				Broilers (n = 66)				Layers (n = 56)			
	MIC_{50}[a]	MIC_{90}	range	%R[b]	MIC_{50}	MIC_{90}	range	%R	MIC_{50}	MIC_{90}	range	%R
Erythromycin	16	>64	0.125–>64	67.2	2	>64	0.06–>64	34.8	2	8	0.06–>64	19.6
Ampicillin	8	64	0.5–>128	18.0	4	32	0.25–128	13.6	8	16	0.125–128	3.6
Nalidixic acid	16	128	0.03–>128	32.8	8	>128	2–>128	60.6	16	>128	0.06–>128	46.4
Ciprofloxacin	0.5	32	0.06–64	27.9	1	64	0.03–>64	62.1	1	16	0.03–>64	41.1
Tetracycline	32	>64	0.03–>64	62.3	0.25	>64	0.03–>64	51.5	1	>64	0.03–>64	28.6
Gentamicin	0.5	1	0.06–>128	3.3	0.25	0.5	0.06–4	0.0	0.25	1	0.06–16	1.8

[a]MIC in mg/L.
[b]%R, percentage resistant.

principal agents in the prophylaxis and treatment of enteric infections. Unfortunately, there has been a rapid emergence of quinolone resistance amongst *Campylobacter* spp. isolates from around the world.[9–12] This rapid emergence of resistance can be attributed to the unique ability of *Campylobacter* to develop high-level resistance to quinolones through a single mutation in the DNA gyrase gene.[24] We also found alarmingly high levels of quinolone resistance, particularly in poultry. For *C. coli* isolated from broilers, 62.1% of the strains were resistant to ciprofloxacin. The percentages of quinolone resistance observed were substantially higher than those described in DANMAP 98.[23] In this Danish study, 3% of the *C. jejuni* and 13% of the *C. coli* isolated from broilers were resistant to nalidixic acid. Further, 13% of the *C. coli* isolates obtained from broilers were resistant to ciprofloxacin. For *C. coli* isolated from pigs, 25% of the strains was resistant to nalidixic acid and 17% to ciprofloxacin.[23]

Poultry constitutes the most important reservoir for human *Campylobacter* infections. Published epidemiological and laboratory data from other countries as well as our study indicate that the use of fluoroquinolones in poultry has a primary role in increasing resistance to quinolones among *Campylobacter* isolates from humans.[9,11,20,21,25,26] In this regard, Endtz *et al.*[9] observed that the emergence of fluoroquinolone-resistant *C. jejuni* infections in humans in The Netherlands coincided with the introduction of enrofloxacin for poultry therapy in early 1987. In Spain, an increase in the percentage of ciprofloxacin-resistant human *Campylobacter* isolates from 0–3% in 1989 to 30–50% in 1991 coincided with the licensing of enrofloxacin for veterinary use in 1990.[5,11,26] Also, in the USA, a significant increase from 1996 to 1998 in quinolone-resistant *C. jejuni* infections that were acquired domestically was temporally associated with the licensing of fluoroquinolones (sarafloxacin in 1995 and enrofloxacin in 1996) for use in poultry.[20] In Belgium, flumequine was licensed for use in poultry in 1982, enrofloxacine in 1988 and difloxacine (hydrochloride) in 1998. It has been described that treatment with enrofloxacin of broiler chickens infected with quinolone-susceptible *C. jejuni* does not eradicate the organism but rather selects for quinolone resistance in *C. jejuni*.[25]

We also found high levels of tetracycline resistance, particularly in *C. coli* strains isolated from pigs (62.3%). A similar observation was described in Spain, where 94.4% of the *C. coli* strains isolated from pigs between 1997 and 1998 were resistant to tetracycline.[27] In contrast, in the DANMAP 98 study,[23] virtually no resistance to tetracycline was found in *Campylobacter* strains isolated from food animals.

In conclusion, alarmingly high resistance rates were observed for erythromycin and ciprofloxacin. These results need to be correlated with antibiotic use in the various animal species before making firm conclusions. However, we consider that the introduction of fluoroquinolones in veterinary medicine might have contributed to the emer-

Table III. Distribution of the *Campylobacter* strains depending on their resistance to erythromycin, ampicillin, nalidixic acid, ciprofloxacin, tetracycline and gentamicin

	No. (%) resistant	
No. antibiotics	*C. jejuni* (n = 488)	*C. coli* (n = 189)
0	151 (30.9)	40 (21.1)
1	119 (24.4)	37 (19.6)
2	119 (24.4)	48 (25.4)
3	62 (12.7)	32 (16.9)
4	31 (6.4)	28 (12.2)
5	6 (1.2)	9 (4.8)

gence of quinolone-resistant *Campylobacter* in man. It is unlikely that the human use of fluoroquinolone alone can be held responsible for the very high resistance rates of human *Campylobacter* to fluoroquinolones observed in Europe and the USA. The detection of multiresistant isolates poses a threat to humans and further limits therapeutic options.

Acknowledgements

The study was part of a study of the surveillance of zoonotic agents in Belgium (directive 92/117/EEC) and was funded by the Belgian Institute for Veterinary Inspection. This work was presented in part at the Thirty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 26–29 September 1999.

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Received 15 January 2001; returned 11 April 2001; revised 30 April 2001; accepted 17 May 2001