# CAMBRIDGE
## UNIVERSITY PRESS

http://www.jstor.org

# Antibiotic susceptibility of campylobacter isolates from sewage and poultry abattoir drain water

P. M. F. J. KOENRAAD[1]*, W. F. JACOBS-REITSMA[2], T. VAN DER LAAN[1], R. R. BEUMER[1] AND F. M. ROMBOUTS[1]

[1] *Wageningen Agricultural University, Department of Food Science, Bomenweg 2, 6703 HD Wageningen, the Netherlands*
[2] *ID-DLO Institute for Animal Science and Health, Research Branch Beekbergen, Spelderholt 9, 7361 DA Beekbergen, the Netherlands*

## SUMMARY

In this study, the *in vitro* susceptibility of 209 campylobacter strains to the quinolones nalidixic acid, flumequine, ciprofloxacin, enrofloxacin, and to ampicillin, tetracycline and erythromycin was tested by the disk diffusion method. The strains were isolated from poultry abattoir effluent (DWA) and two sewage purification plants (SPA and SPB). Sewage purification plant SPA received mixed sewage, including that from a poultry abattoir, whereas SPB did not receive sewage from any meat-processing industry. The quinolone resistance of the DWA isolates ranged from 28% for enrofloxacin to 50% for nalidixic acid. The strains isolated from the sewage purification plants were more susceptible to the quinolones with a range of 11–18% quinolone resistance for SPB isolates to 17–33% quinolone resistance for SPA isolates. The susceptibility criteria as recommended by National Committee Clinical Laboratory Standards (USA) cannot readily be employed for campylobacter isolates. This investigation shows that the resistance of campylobacter bacteria is highest in the plant receiving sewage from a poultry slaughterhouse. Monitoring of antibiotic resistance of aquatic *Campylobacter* spp. is important, as surface waters are recognized as possible sources of infection.

## INTRODUCTION

*Campylobacter jejuni* is recognized as one of the most important causes of acute diarrhoeal disease in humans throughout the world [1]. Four sources appear to account for nearly all cases of campylobacteriosis: poultry meat products, raw milk, untreated surface water, and pets. Most cases of campylobacteriosis are single cases and related to the consumption of (undercooked) poultry meat. However, outbreaks are mostly related to consumption of raw milk or untreated surface water [2–4].

Campylobacter organisms found in surface water most probably originate from wild and domestic animals [5], water run-off from farmland following heavy

---

* Present address: Governmental Food Inspection Service, Department of Microbiology, Rijzertlaan 19, 5223 JS 's-Hertogenbosch, the Netherlands.

rainfall, industrial waste water or effluent from sewage purification plants [6, 7]. In particular, poultry abattoir effluent may contain high numbers of campylobacters and as these organisms can easily pass sewage purification plants, it constitutes an important contamination source for surface water [6–9]. An epidemiological study on the distribution and diversity of campylobacter in a sewage purification plant and poultry abattoir drain water from this plant showed several common phenotypes [10].

Most campylobacter infections are self-limiting. However, if diarrhoea is frequent and/or bloody, or high fever is present, treatment with antimicrobial agents is indicated. Erythromycin is still the drug of choice [11] but nowadays fluoroquinolones are considered to be a good, safe but expensive alternative for the treatment of human campylobacter enteritis [12]. However, Endtz and colleagues [13] reported an increase of quinolone resistance amongst campylobacter strains isolated from human stools and poultry products in the Netherlands between 1982 and 1989. In this country the quinolones enrofloxacin and ciprofloxacin were introduced into veterinary and human medicine in 1987 and 1988, respectively. The prevalence of quinolone-resistant strains isolated from poultry products increased during the period 1982–9 from 0 to 14 % and that of human isolates increased from 0 to 11 % [13]. Most recently, Jacobs-Reitsma and colleagues [14] observed that 29 % of campylobacter isolates from poultry were quinolone-resistant. This rapid increase in quinolone resistance may have implications for the treatment of human diarrhoeal diseases.

Until recently, campylobacter speciation was difficult. Discrimination between the closely related species, *C. jejuni*, *C. coli*, and *C. lari* on the basis of phenotypic characteristics was time consuming and not always correct [15, 16]. A polymerase chain reaction (PCR) technique based on species-specific 23S rRNA fragments has been developed which may be suitable for rapid speciation of campylobacter strains [17].

In the present investigation the prevalence of antibiotic resistance in campylobacter isolates obtained from aquatic environments was determined. Campylobacter strains isolated from surface water, sewage and poultry abattoir drain water, were speciated by the PCR technique [17] and their *in vitro* susceptibility to the quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin, and to ampicillin, tetracycline, and erythromycin was tested by the disk diffusion method [18]. Ampicillin and oxytetracycline are frequently used in Dutch broiler production. The performance standards for antimicrobial disk susceptibility tests, as recommended by the National Committee Clinical Laboratory Standards [19], which is a communication forum in the USA providing international standards, were evaluated. The evaluation was performed by calculating and plotting the population distributions of the measured inhibition diameters for the various antimicrobial agents.

## MATERIALS AND METHODS

### Bacterial strains

Isolates from influx and efflux of two municipal sewage purification plants, namely an activated sludge plant (SPA) and a trickling filter plant (SPB) were tested. The activated sludge system, with a capacity of 60000 citizen equivalents,

receives sewage from households and from various small industries, including a poultry abattoir. The trickling filter system, with a capacity of 130000 citizen equivalents, receives both domestic and industrial waste water, but no waste water from any meat-processing industry. Campylobacter strains isolated from the drain water of the poultry abattoir (DWA), connected with the activated sludge plant, were included in the present study. This poultry abattoir is located at approximately 4 km from the activated sludge plant.

The samples were analysed under micro-aerobic conditions by selective enrichment (42 °C) in Charcoal Cefoperazone Deoxycholate (CCD) Broth, after a pre-enrichment step (37 °C). Campylobacter strains were isolated on selective CCD plates [7]. Confirmation of identity of suspected campylobacter isolates was based on microscopic appearance, resistance to cephalothin (KF Oxoid, Basingstoke, UK, 30 $\mu$g), growth under micro-aerobic conditions in Brain Heart Infusion Broth (BHI, 0037-01-6 Difco Laboratories, Detroit, USA) at 42 °C ($+$) and 25 °C ($-$), presence of oxidase ($+$) and catalase ($+$).

The isolates were stored at $-80$ °C in BHI containing 20% (v/v) glycerol. For DNA preparations and antibiotic susceptibility testing, isolates were pre-cultured in BHI for 2 days at 42 °C under micro-aerobic conditions.

*Antibiotic susceptibility testing by disk diffusion*

Antibiotic susceptibility was determined by the disk diffusion method described by Bauer and colleagues [18]. The method was adjusted to the characteristics of campylobacter by Jacobs-Reitsma and colleagues [14]. The BHI suspension was subcultured onto Mueller-Hinton (CM337 Oxoid) plates supplemented with 5% defibrinated, lysed horse blood (MH). Susceptibility to the following agents was tested: nalidixic acid (NA; Oxoid, 30 $\mu$g), flumequine (UB; Oxoid, 30 $\mu$g), ciprofloxacin (CIP; Oxoid, 5 $\mu$g), enrofloxacin (ENRO; Bayer Diagnostics, München, Germany, 5 $\mu$g), ampicillin (AMP; Oxoid, 25 $\mu$g), tetracycline (TE; Oxoid, 30 $\mu$g), and erythromycin (E; Oxoid, 15 $\mu$g). Per MH plate, four disks were placed on each MH plate and after incubation for 2 days (37 °C, micro-aerobic conditions) the diameter of the inhibition zone (including disk diameter = 6 mm) was measured with callipers. As recommended by the National Committee Clinical Laboratory Standards (NCCLS, USA), Subcommittee on Antimicrobial Susceptibility Testing [19], *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as control microorganisms. The applied susceptibility criteria as recommended by the NCCLS are given in Table 1.

*DNA preparation and speciation*

Cultures grown in BHI were subcultured onto Columbia Agar Base plates (CM 331 Oxoid) with 5% (v/v) defibrinated, lysed horse blood (CAB) and incubated for 2 days at 42 °C under micro-aerobic conditions. Bacteria were washed in physiological salt solution containing 1 g l$^{-1}$ peptone (L34 Oxoid) and harvested by centrifugation. The nucleic acids were extracted by the IsoQuick® Nucleic Acid kit (MXT 020-100, MicroProbe Corporation, Washington, USA) according to the manufacturer's instructions. The species were identified by PCR, based on the 23S rRNA gene [17]. The PCR was performed as described in Koenraad and colleagues [10].

Table 1. *Susceptibility criteria for inhibition zone diameters for the antimicrobial agents nalidixic acid, flumequine, ciprofloxacin, enrofloxacin, ampicillin, tetracycline and erythromycin*

| Antimicrobial agent | Indication | Disk content ($\mu$g) | Criteria* (mm) |
|---|---|---|---|
| Nalidixic acid | NA | 30 | $\geqslant 19$ |
| Flumequine | UB | 30 | $\geqslant 18$† |
| Ciprofloxacin | CIP | 5 | $\geqslant 21$ |
| Enrofloxacin | ENRO | 5 | $\geqslant 18$† |
| Ampicillin | AMP | 25 | $\geqslant 17$ |
| Tetracycline | TE | 30 | $\geqslant 19$ |
| Erythromycin | E | 15 | $\geqslant 23$ |

* Criteria for nalidixic acid, ciprofloxacin, ampicillin, tetracycline and erythromycin according to National Committee for Clinical Laboratory Standards [19].
† The standards for flumequine and enrofloxacin are deduced from the observations of Endtz and colleagues [13].

Table 2. *Antibiotic resistance of 209 campylobacter aquatic isolates*

| | Resistant campylobacter isolates | | | |
|---|---|---|---|---|
| Antimicrobial agent | DWA* ($n = 99$) | SPA* ($n = 66$) | SPB* ($n = 44$) | $P$† |
| Nalidixic acid | 49 (50%) | 22 (33%) | 8 (18%) | $< 0.01$ |
| Flumequine | 30 (30%) | 13 (20%) | 6 (14%) | $< 0.02$ |
| Ciprofloxacin | 29 (29%) | 12 (18%) | 5 (11%) | $< 0.02$ |
| Enrofloxacin | 28 (28%) | 11 (17%) | 6 (14%) | NS‡ |
| Ampicillin | 33 (33%) | 15 (23%) | 7 (16%) | NS |
| Tetracycline | 34 (34%) | 16 (24%) | 11 (25%) | NS |
| Erythromycin | 15 (15%) | 5 (8%) | 8 (18%) | NS |
| All quinolones§ | 28 (28%) | 11 (17%) | 5 (11%) | $< 0.05$ |
| Multiple resistance‖ | 29 (29%) | 23 (35%) | 10 (23%) | NS |

* DWA, poultry abattoir drain water, which is drained on sewage purification plant SPA; SPA, sewage purification plant A; SPB, sewage purification plant B, without meat-processing industries in its drainage area.
† Two-tailed $\chi^2$ test.
‡ NS, not significant.
§ Resistant to the quinolones tested.
‖ Resistant to more than one antimicrobial agent, considering the quinolones as one component.

*Statistical analysis*

Hypotheses about the frequency of antibiotic resistance in the various categories of campylobacter isolates were tested by the two-tailed $\chi^2$ test, considering $P \leqslant 0.05$ to be significant.

### RESULTS

The antibiotic susceptibility of 209 campylobacter strains isolated from sewage purification plants (SPA, SPB) and poultry abattoir drain water (DWA) is shown in Tables 1 and 2. For 60 isolates selected at random, duplicate culturing and determination of susceptibility gave reproducible inhibition diameters (data not

Table 3. *Antibiotic resistance of 121* C. jejuni *isolates and 32* C. coli *isolates*

| Antimicrobial agent | Resistant *C. jejuni* isolates ($n = 121$) | Resistant *C. coli* isolates ($n = 32$) | $P*$ |
|---|---|---|---|
| All quinolones† | 26 (21%) | 5 (16%) | NS‡ |
| Ampicillin | 28 (23%) | 3 (9%) | NS |
| Tetracycline | 23 (19%) | 16 (50%) | < 0·001 |
| Erythromycin | 11 (9%) | 8 (25%) | < 0·02 |

* Two-tailed $\chi^2$ test.
† Cross-resistant to nalidixic acid, flumequine, ciprofloxacin and enrofloxacin.
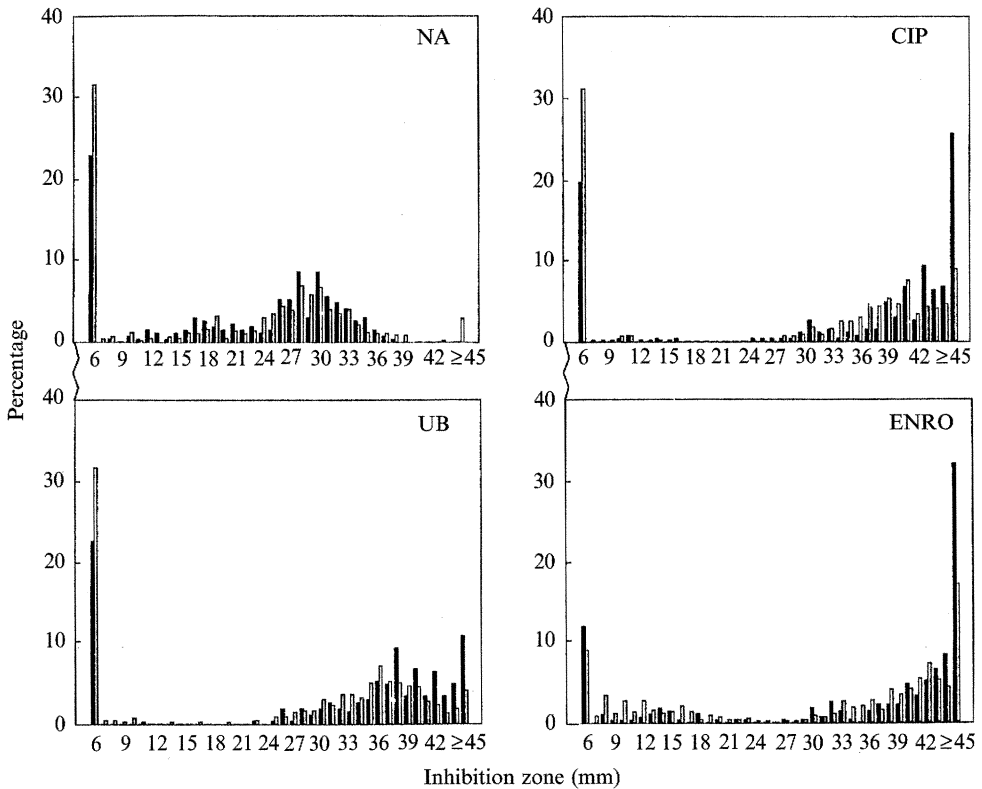‡ NS, not significant.



Fig. 1. Population distributions of antibiotic susceptibility (as measured in inhibition zone diameters) of 617 poultry and 209 aquatic campylobacter isolates to nalidixic acid (NA), flumequine (UB), ciprofloxacin (CIP), and enrofloxacin (ENRO). ■, aquatic isolates; ▨, poultry isolates.

shown). Resistance to nalidixic acid, flumequine, ciprofloxacin and enrofloxacin was significantly lower among SPB isolates. Quinolones have a similar chemical structure and therefore, cross-resistance against these agents is often observed [14]. In this study resistance to all quinolones was lower among the aquatic isolates from SPA and SPB than among DWA isolates. Susceptibility to ampicillin, tetracycline, and erythromycin did not differ between the isolates from the various sources.
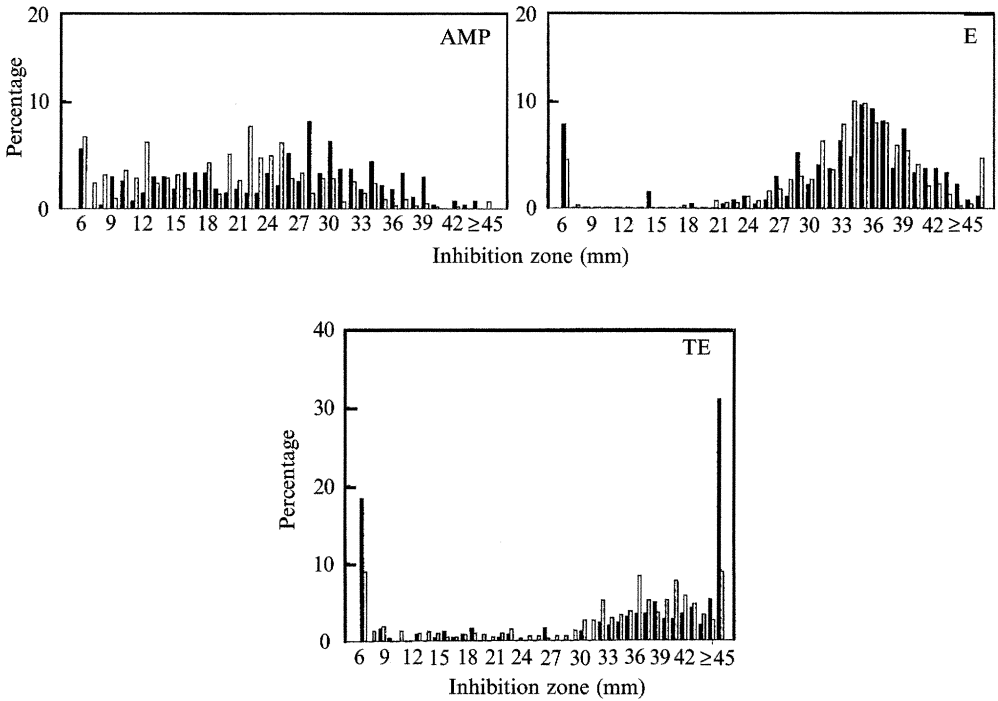
Fig. 2. Population distributions of antibiotic susceptibility (as measured in inhibition zone diameters) of 617 poultry and 209 aquatic campylobacter isolates to ampicillin (AMP), tetracycline (TE) and erythromycin (E). ■, aquatic isolates; ▨, poultry isolates.

One hundred and sixty-nine campylobacter isolates were speciated by PCR. For 16 (9%) isolates originating from all three sources, no PCR product could be observed, which cannot be explained from the results in this investigation. In total, 121 strains (72%) were identified as *C. jejuni* and 32 (19%) belonged to the *C. coli* species (Table 3). *C. coli* isolates were more resistant to tetracycline ($P <$ 0·001) and erythromycin ($P < 0·02$). Resistance to the quinolones and to ampicillin did not differ between the species. The *C. jejuni* and *C. coli* isolates showed a similar extent of multi-resistance, which was defined as resistance to more than one agent treating the quinolones as a single agent.

For the various antimicrobial agents, the population distribution of the inhibition diameters of these campylobacter strains isolated from aquatic environments and poultry [14] were calculated and are shown in Fig. 1 and 2. The population distributions were similar for both strain collections. All population distributions showed a variation in frequency at different inhibition diameters, with the exception of the rather flat distribution for ampicillin inhibition diameters.

## DISCUSSION

In the present study, the prevalence of antibiotic resistance among aquatic campylobacter isolates was studied. Strains isolated from sewage (SPA, SPB) and

poultry abattoir drain water (DWA) were tested for susceptibility to the quinolones nalidixic acid, flumequine, ciprofloxacin, enrofloxacin, and to ampicillin, tetracycline and erythromycin by the disk diffusion method.

Among the isolates from the poultry abattoir drain water (DWA) percentages of strains resistant to the quinolones, ampicillin and tetracycline were similar to those of the poultry isolates obtained in the investigation of Jacobs-Reitsma and colleagues [14]. The lower prevalence of resistance among strains from the sewage purification plant SPA may be due to dilution with household and other waste water, in which campylobacter isolates will have a lower degree of resistance. Quinolones, ampicillin and tetracycline are frequently used in veterinary medicine. Resistance to nalidixic acid, flumequine, and ciprofloxacin was significantly lower among the isolates from sewage purification plant SPB. This municipal plant does not receive drain water from meat-processing industries, supporting the above hypothesis. Resistance to enrofloxacin did not differ significantly between the three sources, though a trend towards a lower enrofloxacin resistance among SPB campylobacter isolates was observed. This was in contrast with the almost complete quinolone cross resistance reported by Jacobs-Reitsma and colleagues [14]. Ampicillin and tetracycline resistance did not differ significantly among the aquatic campylobacter isolates tested. Erythromycin resistance was significantly higher among the SPB and DWA isolates in comparison with the previously documented erythromycin resistance among campylobacter poultry isolates [14] and the SPA campylobacter isolates. These erythromycin-resistant campylobacter isolates might originate from human patients [20], but this fact cannot explain high erythromycin resistance among DWA isolates.

The resistance to quinolones and ampicillin did not differ among the aquatic *C. jejuni* and *C. coli* isolates, which is at variance with the observations of several investigators [13, 14]. The aquatic *C. coli* isolates were more resistant to erythromycin and tetracycline. This agrees with the observations of Navarro and colleagues, Rautelin and colleagues and Reina and colleagues [21–23].

The disk diffusion method described by Bauer and colleagues [18] is easy to perform and the reproducibility is high (data not shown). For the various antimicrobial agents, the population distributions of the inhibition diameters were not dependent on the source of the isolates. The cut-off values (susceptibility criteria) recommended by the National Committee Clinical Laboratory Standards [19] for the inhibition diameters to discriminate between susceptible and resistant strains (Table 1) are mainly based on results obtained with Staphylococci and *Pseudomonas* spp. However, as shown in Figs. 1 and 2 they cannot readily be employed for *Campylobacter* spp. for which separate cut-off values should be established.

This investigation suggests that the resistance of the campylobacter isolates from the two sewage purification plants reflects the extent of veterinary antibiotic usage in the drainage area of the plants. Resistance to quinolones can be induced readily by enrofloxacin treatment of campylobacter colonized broiler chicks [24]. Campylobacters can survive the purification process in the plants [7], so the effluent can act as a vehicle for the spread of resistant campylobacter organisms in the environment. As surface waters are recognized as sources of human campylobacter infections, it is important to continue surveillance of resistance in

campylobacter strains isolated from surface waters and other aquatic environments.

## ACKNOWLEDGEMENTS

## REFERENCES

1. Taylor DN, Blaser MJ. *Campylobacter* infections. In: Evans AS, Brachman PS, eds. Bacterial infections in humans. New York: Plenum Publishing Corp, 1991; 151–72.
2. Aho M, Kurki M, Rautelin H, Kosunen TU. Water-borne outbreak of campylobacter enteritis after outdoors infantry drill in Utti, Finland. Epidemiol Infect 1989; **103**: 133–41.
3. Hudson SJ, Lightfoot NF, Coulson JC, Russell K, Sisson PR, Sabo AO. Jackdaws and magpies as vectors of milkborne human campylobacter infection. Epidemiol Infect 1991; **107**: 363–72.
4. Riordan T, Humphrey TJ, Fowles A. A point source outbreak of campylobacter infection related to bird-pecked milk. Epidemiol Infect 1993; **110**: 261–5.
5. Park RWA, Griffiths PL, Moreno GS. Sources and survival of campylobacters: relevance to enteritis and the food industry. J Appl Bacteriol 1991; Symposium Supplement **70**: 97S–106S.
6. Jones K, Betaieb M, Telford DR. Correlation between environmental monitoring of thermophilic campylobacters in sewage effluent and the incidence of *Campylobacter* infection in the community. J Appl Bacteriol 1990; **69**: 235–40.
7. Koenraad PMFJ, Hazeleger WC, Van Der Laan T, Beumer RR, Rombouts FM. Survey of *Campylobacter* spp. in sewage plants in the Netherlands. Food Microbiol 1994; **11**: 65–73.
8. Brennhovd O, Kapperud G, Langeland G. Survey of thermotolerant *Campylobacter* spp. and *Yersinia* spp. in three surface water sources in Norway. Int J Food Microbiol 1992; **15**: 327–38.
9. Höller C. Long-term study of occurrence, distribution and reduction of *Campylobacter* spp. in the sewage system and waste water treatment plant of a big town. Wat Sci Tech 1988; **20**: 529–31.
10. Koenraad PMFJ, Ayling R, Hazeleger WC, Rombouts FM, Newell DG. The speciation and subtyping of campylobacter isolates from sewage plants and waste water from a connected poultry abattoir using molecular techniques. Epidemiol Infect 1995; **115**: 485–94.
11. Skirrow MB, Blaser MJ. Clinical and epidemiological considerations. In: Nachamkin I, Blaser MJ, Tompkins LS, eds. *Campylobacter jejuni*: current status and future trends. Washington: American Society for Microbiology, 1992; 3–8.
12. Mattila L, Peltola H, Siitonen A, Kyrönseppä H, Simula I, Kataja M. Short-term treatment of traveller's diarrhea with norfloxacin: a double-blind, placebo-controlled study during two seasons. Clin Infect Dis 1993; **17**: 779–82.
13. Endtz HPh, Ruijs GJ, Van Klingeren B, Jansen WH, Van Der Reijden T, Mouton RP. Quinolone resistance in campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother 1991; **27**: 199–208.
14. Jacobs-Reitsma WF, Koenraad PMFJ, Bolder NM, Mulder RWAW. *In vitro* susceptibility of campylobacter and salmonella isolates from broilers to quinolones, ampicillin, tetracycline, and erythromycin. Vet Quart 1994; **16**: 206–8.
15. Penner JL. The genus *Campylobacter*: a decade of progress. Clin Microbiol Rev 1988; **1**: 157–72.
16. Totten PA, Patton CM, Tenover FC, et al. Prevalence and characterization of hippurate-negative *Campylobacter jejuni* in King County, Washington. J Clin Microbiol 1987; **25**: 1747–52.

17. Eyers M, Chapelle S, Van Camp G, Goossens H, De Wachter R. Discrimination among thermophilic *Campylobacter* species by polymerase chain reaction amplification of 23S rRNA fragments. J Clin Microbiol 1993; **31**: 3340–3.
18. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1970; **53**: 149–58.
19. National Committee for Clinical Laboratory Standards (NCCLS) Performance standards for antimicrobic disk susceptibility tests. NCCLS Document M2-A4 1990; **10**: no. 7, 4th edn.
29. Sjögren E, Kaijser B, Werner M. Antimicrobial susceptibilities of *Campylobacter jejuni* and *Campylobacter coli* isolated in Sweden: a 10-year follow-up report. Antimicrob Agents Chemother 1992; **36**: 2847–9.
21. Navarro F, Miró E, Mirelis B, Prats G. *Campylobacter* spp. antibiotic susceptibility. J Antimicrob Chemother 1993; **32**: 906–7.
22. Rautelin H, Renkonen O-V, Kosunen TU. Emergence of fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli* in subjects from Finland. Antimicrob Agents Chemother 1991; **35**: 2065–9.
23. Reina J, Borrell N, Serra A. Emergence of resistance of erythromycin and fluoroquinolones in thermotolerant *Campylobacter* strains isolated from faeces 1987–1991. Eur J Clin Microbiol Infect Dis 1992; **11**: 1163–6.
24. Jacobs-Reitsma WF, Kan CA, Bolder NM. The induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment. Lett Appl Microbiol 1994; **19**: 228–31.