

ANTIMICROBIAL RESISTANCE IN *CAMPYLOBACTER JEJUNI* ISOLATED FROM BROILER CHICKENS IN ESTONIA DURING PERIODS 2002–2003 AND 2005–2006

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SUMMARY

Our study was conducted in 2002–2003 and 2005–2006 to isolate campylobacters from a poultry production chain and determine the prevalence of antimicrobial resistance. All together we studied 167 broiler chicken *Campylobacter jejuni* isolates of Estonian origin. In 2002 and 2003 using a disc diffusion method and by E-test the resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44.4%, 44.4%, 22.2%, 19.4%, and 16.6% among the 36 *Campylobacter jejuni* isolates. We found no simultaneous resistance, of isolated strains, to three or more unrelated antimicrobial agents. Resistance to one or more antimicrobials was detected in 24 isolates (66.7%). None of the chicken isolates were resistant to gentamicin. In 2005 and 2006 a total of 131 *C. jejuni* isolates were collected over a 13-month period and the MICs were determined. Resistance to one or more antimicrobials was detected in 104 isolates (79.4%). A high proportion of the isolates were resistant to enrofloxacin (73.3%) and nalidixic acid (75.6%). Multidrug resistance (to three or more unrelated antimicrobials) was detected in 36 isolates (27.5%), all of which were resistant to enrofloxacin. Our results showed that multidrug resistance was significantly associated with enrofloxacin resistance ($p < 0.01$) and the use of enrofloxacin may select multiresistant strains.

Keywords: broiler chicken, *Campylobacter jejuni*, susceptibility, multiresistance

MATERIALS AND METHODS

Isolates

We studied 36 broiler chicken *Campylobacter* isolates from 396 raw broiler chicken meat samples obtained from retail stores in Estonia between January 2002 and December 2003. In 2005 and 2006 our study included 105 *Campylobacter* strains analysed from a total of 1254 fresh faecal samples at an Estonian chicken farm and from 264 chicken caecal contents at slaughterhouse level. Furthermore, 26 isolates from 340 randomly purchased fresh chicken meat samples at the retail level in Estonia were analysed. All 167 isolates were identified as *C. jejuni*.

One loopful (10 μ l) of faecal material or intestinal contents from the caecum was taken, and the material was transferred into tubes containing 10 ml of Preston enrichment broth (Oxoid; Basingstoke, Hampshire, England). The tubes with enrichment broths were stored at 4°C and transported immediately to the laboratory. Enrichment broths were incubated at $42 \pm 0.5^\circ\text{C}$ for

24 h in microaerobic conditions. Analyses for campylobacters were carried out at the State Veterinary and Food Laboratory, Tartu, Estonia.

Fresh poultry meat samples were analysed for campylobacters using the method of Nordic Committee on Food Analysis (Anonymous, 1990), which includes an enrichment phase in Preston broth. Briefly, 250 ml of Preston enrichment broth (Oxoid; Basingstoke, Hampshire, England) was added to a 25-g of meat sample, and the sample was stomached for 60 s. The enrichment broth was incubated at 42 ± 0.5 °C for 24 h in microaerobic conditions.

After 24 h incubation a loopful of the enrichment broth both from faecal and meat samples was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid; Basingstoke, Hampshire, England) and examined for typical growth after 48 h microaerobic incubation. Organisms growing on mCCDA plates were streaked on Brucella blood agar (Oxoid) and confirmed by Gram staining, motility analysis and oxidase and catalase tests as campylobacters. One randomly chosen colony from each positive sample was analysed for hippurate hydrolysis, and hippurate-positive isolates were regarded as *C. jejuni*. After the original isolation, the strains were stored at -70 °C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

Antimicrobial susceptibility testing

In 2002 and 2003 all *Campylobacter* isolates were tested by disc diffusion method against ampicillin (25 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), and tetracycline (10 µg) (Oxoid), and by the E-test (AB Biodisk, Solna, Sweden) against ampicillin, ciprofloxacin, erythromycin, and tetracycline.

Campylobacter isolates were first grown on blood agar plates and were transferred in 5 ml of Mueller-Hinton (MH) broth (Oxoid) and incubated at 37°C for 24 h in microaerobic conditions. Inoculum from the MH broth was diluted and a turbidity equivalent of a 0.5 McFarland standard was adjusted in physiological peptone-saline water and the growth suspension was spread on the MH blood agar plates (Oxoid, supplemented with 7% horse blood), the disks or E-test strips containing antimicrobial compounds were laid on the plates. The plates were incubated at 37 °C for 24 h in microaerobic conditions. The diameter of the growth inhibition zone was measured according to the CLSI (2004). MIC values were determined by E-test according to the instructions given by the manufacturer (AB Biodisk). *C. jejuni* 143483 was used as control strain in the antimicrobial susceptibility testing.

The following zone diameter (mm) and MIC breakpoints for resistance were applied: ampicillin ≤ 13 mm and MIC ≥ 32 µg/ml, ciprofloxacin ≤ 26 mm and MIC ≥ 4 µg/ml, erythromycin ≤ 26 mm and MIC ≥ 32 µg/ml, gentamicin ≤ 12 mm, nalidixic acid ≤ 26 mm, and tetracycline ≤ 31 mm and MIC ≥ 16 µg/ml (Anonymous, 2004; CLSI, 2004).

In 2005 and 2006 all 131 *C. jejuni* isolates were tested for minimal inhibitory concentration (MIC) by a broth microdilution method (National Veterinary Institute, Uppsala, Sweden) against ampicillin, enrofloxacin, erythromycin, gentamicin, nalidixic acid and oxytetracycline. The MIC-based microdilution was carried out at the laboratory of the Department of Food Science and Hygiene of the Estonian University of Life Sciences, Tartu, Estonia. *Campylobacter* isolates were first cultured on Brucella blood agar (Oxoid, Basingstoke, Hampshire, England) and incubated at 37 °C for 48 h. A loopful (1 µl) of bacterial growth was transferred to 10 ml of cation-adjusted Mueller-Hinton (CAMHB) broth (Oxoid, Basingstoke, Hampshire, England) and then incubated at 37 °C for 24 hours to achieve a level of around 10^8 CFU/ml. The bacterial suspension was diluted to 10^6 CFU/ml. One hundred microliters (µl) of bacterial suspension was inoculated into each well of microtitre plates. The plates were incubated at 37 °C for 40 h in microaerobic

conditions. The MIC was read as the lowest concentration completely inhibiting visible growth of campylobacters in accordance with the instructions given by the test manufacturer (National Veterinary Institute, Uppsala, Sweden). Control of the purity of the bacterial suspension was carried out by plating 10 µl of bacterial suspension on Brucella agar. The density of the bacterial suspension was controlled according to the guidelines of the Estonian Veterinary and Food Laboratory, and colony counts from 50 to 250 per plate were accepted (Anonymous, 2004b and Anonymous 2005b). *C. jejuni* ATCC 33560 was used as a control strain in the antimicrobial susceptibility testing. The following MIC breakpoints for resistance were applied: ampicillin 32 µg/ml, enrofloxacin 1 µg/ml, erythromycin 16 µg/ml, gentamicin 8 µg/ml, nalidixic acid 32 µg/ml and oxytetracycline 4 µg/ml (Anonymous, 2004b and Anonymous 2005b).

Statistical analysis

All individual results were recorded using MS Excel 2003 software (Microsoft Corporation; Redmond, WA, USA), and the statistical analysis was performed with the Statistical Package for Social Sciences 13.0 for Windows (SPSS Inc.; Chicago, IL, USA). Non-parametric Spearman's rank order correlation coefficients with two-tailed p-values and odds ratios (ORs) were calculated for bivariate cross-correlations between resistances to the six antimicrobials analysed as well as between antimicrobials and multiresistance, which was defined as resistance to three or more unrelated antimicrobials simultaneously. Furthermore, a non-parametric Mann-Whitney independent samples test was conducted to compare the level of antimicrobial resistance between multiresistant and non-multiresistant strains.

RESULTS

In 2002 and 2003 by disc diffusion method the resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44.4%, 44.4%, 22.2%, 19.4%, and 16.6% of the isolates ($n = 36$). All isolates were susceptible to gentamicin. Isolates with resistance to ciprofloxacin were also resistant to nalidixic acid. Resistance of isolates to two unrelated antimicrobials was mainly to a combination of ciprofloxacin/nalidixic acid and tetracycline (8/36, 22.2%). Three isolates showed a resistance combination of ampicillin and erythromycin, and two isolates of ampicillin and ciprofloxacin/nalidixic acid. We found no simultaneous resistance, of isolated *Campylobacter jejuni* strains, to three or more unrelated antimicrobial agents in 2002 and 2003. Results of disk diffusion method and the E-test were similar and all isolates resistant or susceptible by the disk diffusion method showed the same results by E-test.

In 2005 and 2006 resistance to one or more antibiotics was detected in 104 isolates (79.4%). Twenty isolates (15.3%) were resistant to three unrelated antimicrobials, thirteen isolates (10%) to four unrelated antimicrobials and three isolates (2.3%) to all tested antimicrobials. Enrofloxacin and nalidixic acid were regarded as one group of antimicrobials. Resistance of isolates to three unrelated antimicrobials was mainly to a combination of enrofloxacin/nalidixic acid, erythromycin and oxytetracycline (4.6%). Resistance of isolates to four unrelated antimicrobials was mainly to a combination of enrofloxacin/nalidixic acid, erythromycin, gentamicin and oxytetracycline (8.4%). Three isolates were resistant to five unrelated antimicrobials, comprising a combination of ampicillin, enrofloxacin/nalidixic acid, erythromycin, gentamicin, and oxytetracycline (2.3%). The highest frequency of resistance was to nalidixic acid and enrofloxacin (75.6% and 73.3%, respectively), followed by oxytetracycline (32.1%), erythromycin (19.8%),

gentamicin (19.1%) and ampicillin (7.6%). Multidrug resistance (to three or more unrelated antimicrobials) was significantly ($p < 0.01$) associated with enrofloxacin and nalidixic acid resistance. The level of antimicrobial resistance was higher for nalidixic acid in multiresistant *C. jejuni* strains than in non-multiresistant strains (Mann-Whitney test, $p = 0.026$), while resistance for other antimicrobials was not statistically different ($p > 0.05$) between multi- and non-multiresistant strains.

DISCUSSION

An important finding of our study in 2002 and 2003 was the recognition of a high number of *Campylobacter* isolates with increased antimicrobial resistance to ciprofloxacin, 44.4% (16 isolates MIC ≥ 32 $\mu\text{g/ml}$). Enrofloxacin and flumequine, both fluoroquinolone group antimicrobials, are accepted for poultry treatment in Estonia (Anonymous, 2005), possibly explaining the high level of resistance detected among Estonian isolates.

Ampicillin is a widely used antimicrobial in veterinary medicine and from this group the amoxicillin is accepted for use in veterinary medicine in Estonia (Anonymous, 2005). Resistance to ampicillin in Estonian broiler isolates was 19.4%.

The resistance of Estonian *Campylobacter* isolates to erythromycin was 16.6%. None of the chicken isolates showed resistance to gentamycin in our study performed in 2002 and 2003.

In the period of 2005 and 2006, an important finding was the high number (79.4%) of antimicrobial-resistant *Campylobacter jejuni* isolates, 36 (27.5%) of which exhibited multiresistance (resistance to three or more unrelated antimicrobials). Resistance was especially high to enrofloxacin (80 isolates MIC ≥ 4 $\mu\text{g/ml}$). In the present study, two different fluoroquinolones were studied. Cross-resistance between the different fluoroquinolones has been previously documented (Rautelin et al., 2003; Griggs et al., 2005), as their modes of action are similar (inhibition of DNA gyrase). Most of the *Campylobacter* strains for which enrofloxacin MICs were high were also not inhibited by low concentrations of nalidixic acid. Gentamicin and erythromycin resistance was rather high among our *C. jejuni* strains 19% and 19.8%, respectively. The reason for this is unknown but could be associated with the veterinary use of latter antimicrobial agents in broiler chicken production. High MICs of both macrolides and fluoroquinolones for isolates pose a problem and because erythromycin is considered as a first-line choice of treatment for human *C. jejuni* infections, this resistance has an important public health impact. We found a high proportion of multidrug-resistant isolates (27.5%); all of these were resistant to enrofloxacin and all except one resistant to nalidixic acid. Our results showed that multidrug resistance was significantly associated with enrofloxacin and nalidixic acid resistance (correlation coefficient 0.372 and 0.310, $p < 0.01$). These findings suggest that the use of fluoroquinolones may select multiresistant strains since resistance to erythromycin, gentamicin or oxytetracycline was exceptional without simultaneous resistance to fluoroquinolones. In conclusion, multidrug resistance in Estonian broiler chicken isolates was one of the highest reported in studies of broiler chicken *Campylobacter* isolates. The widespread emergence of multiresistant isolates poses a threat to humans and limits therapeutic medication. The European ban of the antimicrobial growth promoters (came into force since 01.01.2006 in Estonia) should be strictly followed. It will be important to monitor the trends in resistance after the withdrawal and over time. In Estonia, more restricted use of antimicrobial agents, especially fluoroquinolones, in food animal production should be implemented.

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