Erythromycin-resistant *Campylobacter coli* from slaughtered animals as a potential public health risk

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**ABSTRACT:** Erythromycin-resistant *Campylobacter* were isolated from pig, cattle, and poultry carcasses slaughtered in Poland between 2008 and 2011. A total of 1335 strains were examined and among them 20 (1.5%) showed a high level of erythromycin resistance (≥ 32 mg/l) as determined by the microbroth dilution method. All these isolates were *C. coli* and mainly originated from poultry (15 strains). PCR amplification or DNA sequencing identified the mutation A2075G in the 23S rRNA gene in all strains tested. The vast majority of such *C. coli* were also resistant to quinolones, tetracyclines, and streptomycin whereas none of them revealed resistance to gentamycin. Furthermore, several isolates (14; 70.0%) displayed multi-resistance pattern against quinolones, aminoglycosides, and tetracyclines. PCR analysis identified several putative virulence genes such as cadF, flaA, and iam (present in all erythromycin resistant isolates) as well as the cdtA and filhA markers (19 and 16 strains, respectively) among *C. coli* tested. On the other hand, only two out of 20 isolates were positive for the ciaB and docA genes. Furthermore, none of the analysed strains had the virB11 and wlaN markers. A molecular relationship determination of the erythromycin-resistant *C. coli* performed by pulsed field gel electrophoresis (PFGE) revealed 17 different types. This reflects the high genetic diversity among the examined isolates. The results obtained suggest that erythromycin-resistant *C. coli* from food-producing animals may represent an underestimated potential health risk for consumers.

**Keywords:** *Campylobacter*; carcasses; erythromycin resistance; molecular characteristics; public health

*Campylobacter* is recognised as a major cause of bacterial gastroenteritis in humans worldwide and has been isolated from a diverse range of domestic and wild animals, especially poultry (Scallan et al. 2011; Anonymous 2012a). Macrolides, such as erythromycin, are regarded as drugs of choice for the treatment of severe human intestinal infections or immuno-compromised patients (Gibreel and Taylor 2006). Resistance of *Campylobacter* clinical isolates to this antimicrobial group is relatively rare as compared to other antimicrobials and it is mainly found among *C. jejuni*; however, some strains, especially *C. coli* originating from food, show a high resistance rate (Anonymous 2012b). Several molecular mechanisms have been described as being responsible for macrolide resistance in *Campylobacter* and among them the point mutations in domain V of the 23S rRNA target gene at positions 2074 and 2075 are the most common ones. It has also been shown that the mutation A2075G is usually responsible for a high level of resistance to erythromycin (Alonso et al. 2005; Gibreel and Taylor 2006).

The increasing resistance among *Campylobacter* to macrolides, especially combined with resistance to quinolones, is recognised as an emerging public health problem. Moreover, it was shown that human infections caused by macrolide or quinolone resistant *Campylobacter* are associated with increased risk of adverse events or development of the invasive form of the disease compared to infections with susceptible isolates (Gibreel and Taylor 2006).

Several putative virulence markers have been described in *Campylobacter*; however, the pathogenesis of infection is still not well defined (Tam et al. 2003). Virulence properties such as flagella-mediated motility, adherence to intestinal epithelial...
cells, invasion and survival in the host cells as well as the ability to produce toxins are important in the development of human campylobacteriosis (Tam et al. 2003).

The aim of the present study was to investigate the prevalence of erythromycin-resistant *Campylobacter* among strains isolated from food producing animals. Furthermore, the molecular background of such resistance and genetic relationship of the strains were also investigated. Moreover, the potential risk for public health of such isolates was characterised by investigating antibiotic resistance profiles and the presence of genes putatively associated with virulence.

**MATERIAL AND METHODS**

Sample collection. A total of 1802 swabs were collected from poultry carcasses after cooling at the slaughter level all over Poland between 2008 and 2011 and immediately transported to the laboratory in Amies transport medium with charcoal (Medlab, Szczecin, Poland). The swabs were put into 5 ml of Bolton broth plus 5% leaked horse blood and modified Bolton broth-selective supplement containing the following antimicrobials: vancomycin, cefoperazone, trimethoprim, and amphotericin B to prevent non-target microbials (Oxoid, Basingstoke, UK) and then incubated for 48 h at 41.5 °C under microaerobic conditions using the CampyGen kit (Oxoid). *Campylobacter* isolation and identification were performed according to the ISO 10272-1:2006 standard. From each sample one isolate classified as potentially *Campylobacter* was further identified using PCR methods as described earlier (Wieczorek 2010).

The cattle (*n* = 624) and pig (*n* = 187) carcasses tested in the same period as poultry samples were swabbed at the brisket area using sterile sponges. To each swab, 200 ml of maximum recovery dilution (Oxoid) was added followed by stomaching for 3 min. After centrifugation at 1000 × g for 15 min, pellets were re-suspended in 100 ml of selective enrichment Bolton broth. The enrichment cultures were grown for 48 h at 41.5 °C under microaerobic conditions and then plated onto Karmali agar (Oxoid) and *Campylobacter* blood free agar (Oxoid) with CCDA-selective supplement (Oxoid) followed by re-incubation under the previously described conditions for 48 h. The bacterial isolates were stored at −80 °C until further analysis.

**PCR assays.** One bacterial isolate from each positive sample was tested using PCR. A bacterial colony was suspended in 1 ml of sterile water and centrifuged at 13 000 × g for 1 min. DNA was extracted using the Genomic-Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s instruction. *Campylobacter* species were identified using multiplex PCR (m-PCR) with three sets of primers specific for the simultaneous detection of the *C. jejuni* (the mapA gene target), *C. coli* (ceuE gene), and *Campylobacter*-specific 16S rRNA gene as described previously (Wieczorek 2010). Furthermore, in cases of doubtful results, a second m-PCR was applied to identify the species-specific hipO and 23S rRNA (*C. jejuni*), glyA (*C. coli*, *C. lari*, and *C. upsaliensis*), and sapB2 genes (*C. fetus* subspp. *fetus*), respectively (Wang et al. 2002).

*Campylobacter* isolates were also tested for the presence of the most often described putative virulence genes: flaA, flhA, cadF, docA, cdtA, cdtB, cdtC, ciaB, iam, wlaN, and virB11. The PCR conditions for all amplification reactions were exactly the same as previously described (Wieczorek 2010).

**Antimicrobial susceptibility.** A microbroth dilution method using the Sensititre® custom susceptibility plates, EUCAMP (Trek Diagnostics, East Grinstead, UK) was applied to establish the

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### Table 1. Antimicrobials, dilution ranges and cut-off values used for MIC

<table>
<thead>
<tr>
<th>Antimicrobial class</th>
<th>Antimicrobials</th>
<th>Dilution range (mg/l)</th>
<th>Cut off values (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>gentamycin (GEN)</td>
<td>0.12–16</td>
<td><em>C. jejuni</em> 1</td>
</tr>
<tr>
<td></td>
<td>streptomycin (STR)</td>
<td>1–16</td>
<td><em>C. jejuni</em> 2</td>
</tr>
<tr>
<td>Macrolides</td>
<td>erythromycin (ERY)</td>
<td>0.5–32</td>
<td><em>C. jejuni</em> 4</td>
</tr>
<tr>
<td>Quinolones and Fluoroquinolones</td>
<td>ciprofloxacin (CIP)</td>
<td>0.06–4</td>
<td><em>C. jejuni</em> 1</td>
</tr>
<tr>
<td></td>
<td>nalidixic acid (NAL)</td>
<td>2–64</td>
<td><em>C. jejuni</em> 16</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>tetracycline (TET)</td>
<td>0.25–16</td>
<td><em>C. jejuni</em> 2</td>
</tr>
</tbody>
</table>
minimum inhibitory concentrations (MICs) of six antimicrobial agents for Campylobacter isolates. Antimicrobials, dilution ranges and cut-off values used for MIC determination are described in Table 1. The strains were sub-cultured twice on Columbia agar (Oxoid) at 41.5 °C for 48 h under microaerobic conditions. The MIC of the antimicrobial agents was determined using Mueller-Hinton Broth (Oxoid) supplemented with 2–2.5% horse blood (Trek). The plates were incubated at 37 °C for 48 h under microaerophilic conditions and read using the Vision® system (Trek). The antimicrobials and cut-off values used for the interpretation of the MIC results were in accordance with the values provided by EUCAST (www.eucast.org) and the European Union Reference Laboratory for Antimicrobial Resistance.

PFGE analysis. All Campylobacter isolates were typed by pulsed field gel electrophoresis (PFGE) using the standard operating procedure of PulseNet (Ribot et al. 2001). Briefly, the plugs were prepared from 400 µl of bacterial suspensions to which 20 µl proteinase K (20 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and 400 µl of Seakem Gold Agarose (Lonza, Allendale, NJ, USA) in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) were added. Bacterial cell lysis was performed with 50mM Tris, 50mM EDTA, pH 8.0 + 1% Sarcosyl and 0.1 mg proteinase K per ml. The DNA was digested with SmaI enzyme (Fermentas, Vilnius, EU), 40 IU at 25 °C for 4 h. Salmonella Braenderup H9812 was used as the molecular weight standard. PFGE was performed using the CHEF DR II System (Bio-Rad, Hercules, CA, USA) with the following parameters: initial switch time of 6.8 s, final switch time of 35.4 s for 18 h at 6 V and 14 °C in 0.5 x TBE buffer (Sigma). The gels were stained with ethidium bromide (5 µg/ml) for 15–20 min and the DNA banding pattern was captured with the Gel Doc 2000 system (Bio-Rad).

RESULTS

A total of 2613 swabs from poultry, cattle, and pig carcasses were screened for the presence of Campylobacter. Altogether, 1335 samples (51.1%) were positive for Campylobacter, 716 for C. jejuni and 619 for C. coli (Table 2). It was found that 20 (1.5%) strains, all of them C. coli, were resistant to erythromycin as determined by the microbroth dilution method. The isolates were mainly recovered from poultry carcasses (15 strains, 75.0%) followed by pigs (four isolates) and cattle (one strain). All isolates displayed a high level of resistance, i.e., the MIC value was ≥32 mg/l. PCR amplification identified the mutation A2075G in the 23S rRNA gene in all but one erythromycin-resistant Campylobacter tested. However, the PCR-negative isolate was also confirmed to possess this mutation by DNA sequencing.

The vast majority of C. coli strains examined (19 out of 20 isolates; 95.0%) were also resistant to quinolones (ciprofloxacin and nalidixic acid), tetracyclines (17 strains; 85.0%), and streptomycin (15 isolates; 75.0%) whereas none of these strains revealed resistance to gentamycin (Table 3). The most common (14 isolates; 70.0%) multi-resistance

Reference strains. The following reference strains were included in the study: C. jejuni ATCC 33560, C. coli ATCC 43478, and S. Braenderup H9812 ATCC BAA-664.

Data analysis. BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of PFGE fingerprinting profiles. Dendrograms were generated based on the Dice correlation co-efficient for similarity and the unweighted-pair group method with arithmetic means (UPGMA) was employed for cluster analysis.

Table 2. Erythromycin-resistant Campylobacter isolates identified in the study

<table>
<thead>
<tr>
<th>Source*</th>
<th>Number of strains tested</th>
<th>Number (%) of strains resistant to erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>Poultry</td>
<td>623</td>
<td>519</td>
</tr>
<tr>
<td>Cattle</td>
<td>83</td>
<td>61</td>
</tr>
<tr>
<td>Pig</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>716</td>
<td>619</td>
</tr>
<tr>
<td>Altogether</td>
<td>1335</td>
<td></td>
</tr>
</tbody>
</table>

*the isolates were recovered from carcasses during the slaughter process

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The pattern observed was against quinolones, streptomycin, and tetracycline (Table 3).

The PCR analysis revealed that all erythromycin-resistant *Campylobacter* strains had the *cadF*, *flaA*, and *iam* markers. Most of them also possessed the *cdtA*, *cdtB*, and *flhA* genes. On the other hand, only two out of 20 isolates were positive for the *ciaB* and *docA* genes. Furthermore, none of these strains had the *virB11* and *wlaN* genes (Table 3). It was also shown that the most common virulence gene pattern among erythromycin-resistant *C. coli* was: *cadF*, *cdtA*, *cdtB*, *cdtC*, *ciaB*, *docA*, *flaA*, *flhA*, *iam*, *virB11*, *wlaN*, identified in seven (35.0%) of the isolates (Table 3).

Determination of the molecular relationship of the erythromycin-resistant *C. coli* performed by PFGE with *Sma*I identified 17 different clonal types (with 95% similarity). This reflects the high genetic diversity among the examined isolates. Only three PFGE pulsotypes (numbers 1, 2, and 3) appeared more than once (Table 3). The strains with the common genetic profiles were isolated from the same sources (pig or poultry carcasses) and had the same resistance profile but slightly different virulence gene patterns (Table 3).

**DISCUSSION**

Several reports have documented the isolation and genetic characterisation of campylobacters from humans and food-producing animals (Van Deun et al. 2007; Wang et al. 2011; Egger et al. 2012; Mattheus et al. 2012). However, limited information is available on the drug resistance profiles and genotypic characterisations of erythromycin-resistant *Campylobacter* isolates. Such studies are important because drug-resistant strains from...
food-producing animals may be a source of antibiotic resistance genes to other campylobacters, also those involved in human infections. The results of the present study demonstrated that only 1.5% of the 1335 Campylobacter isolates tested were resistant to erythromycin and the resistance mechanism was due to the point mutation in the domain V of the 23S rRNA gene (Alonso et al. 2005; Gibreel and Taylor 2006). As described in a recent EFSA report, between 0.2% and 2% of C. jejuni and as many as 12% to 25% of C. coli isolates were resistant to erythromycin (Anonymous 2012b). The highest level of resistance to this antimicrobial was in C. coli from pigs, whereas only a small number of erythromycin-resistant C. jejuni of bovine origin were detected (Anonymous 2012b). Similar findings were in the present study where only C. coli were resistant to erythromycin and a higher percentage of the resistant isolates were of porcine origin (10.2%) as compared to cattle (1.6%) and poultry (2.9%) (Table 2). Other studies also confirmed that more C. coli were resistant to macrolides, including erythromycin, as compared to C. jejuni, especially those recovered from pigs (Smole Mozina et al. 2011).

Recently, Quintana-Hayashi and Thakur (2012) performed a longitudinal study on the persistence of antimicrobial-resistant Campylobacter in pigs and found that as much as 20.9% of the isolates recovered from porcine carcasses were resistant to erythromycin. However, the bacterial species were not identified. Qin et al. (2011) analysed 190 C. coli of porcine origin and found that from 37.9% to 54.7% of the isolates were erythromycin-resistant. A similar resistance rate (46.5%) of C. coli recovered from porcine carcasses was identified in Korea (Shin and Lee 2007). In Europe, Pezzotti et al. (2003) isolated 45% out of 47 C. coli from pig carcasses that were resistant to erythromycin. Egger et al. (2012) identified 28 (10.9%) strains with the point mutation A2075G in the 23S rRNA gene responsible for macrolide resistance. However, two of these isolates were sensitive to erythromycin when tested using the MIC method. As shown in the present study all 20 C. coli possessing the A2075G mutation were also phenotypically resistant in the MIC test. Moreover, further characterisation of erythromycin-resistant C. coli strains indicated that most of them (14 out of 20 isolates; 70.0%) displayed a multidrug resistant pattern, i.e., resistance to other antimicrobial classes – quinolones, aminoglycosides and tetracyclines. Other authors have also isolated several multidrug-resistant Campylobacter strains which confirms that such isolates, mainly C. coli, are common, especially among pigs but also poultry and cattle (Wang et al. 2011; Sanad et al. 2011; Schweitzer et al. 2011; Anonymous 2012b; Mattheus et al. 2012). Such data are important from a public health point of view since antimicrobial-resistant Campylobacter in food-producing animals may act as a reservoir of resistance genes that may be disseminated to human strains.

The Campylobacter strains isolated in the present study were characterised for the presence of virulence and toxin genes. Despite the large amount of available data in this area it is difficult to compare the results due to the different genes examined, PCR primers used, number and origin of the samples. Generally, the prevalence of putative virulence genes among erythromycin-resistant C. coli identified in the present study was similar to other data. All strains tested possessed the cadF, flaA, and iam markers, which are involved in the adherence, motility, and invasiveness processes of Campylobacter infection, respectively. Furthermore, most isolates were also positive for the cdtA and cdtB markers responsible for toxin production as well as for the flhA gene involved in the motility of bacterial cells (Tam et al. 2003). On the other hand, none of the isolates possessed the virB11 and wlaN markers that are possibly responsible for the invasion process of the host cells (Tam et al. 2003). These results are in agreement with several other studies in which virulence markers were identified in Campylobacter isolates of poultry, pig, cattle or human origin (Tam et al. 2003; Ripabelli et al. 2010; Wieczorek 2010; Egger et al. 2012; Quintana-Hayashi and Thakur 2012). It should be mentioned that the presence of particular virulence gene factors is not direct evidence that such strains are pathogenic for humans; however, it strongly suggests that they may be potentially able to induce disease.

The PFGE results from these studies showed that the Smal PFGE patterns of erythromycin-resistant C. coli were the same only in the case of three strains, whereas the remaining 17 isolates displayed unique molecular profiles. A good correlation between PFGE patterns and virulence as well as antimicrobial resistance profiles was observed; i.e., C. coli with the same macrorestriction profile had identical drug resistance patterns and very similar virulence gene profiles (Table 3). On the other hand, such correlation among Campylobacter strains of different origin was less pronounced in
the studies of other authors (Thakur and Gebreyes 2005; Van Deun et al. 2007; Wang et al. 2011). It should be underlined that in the present study a limited number of isolated was tested. Since the PFGE technique has a high discriminatory power it may be less valuable for epidemiological studies among Campylobacter isolates when used alone. Thus, a combination of macrorestriction, antimicrobial resistant and virulence gene profiles as performed in the present study may be a valuable tool for determination of the origin and subsequent spread of Campylobacter isolates as well as their antimicrobial resistance genes.

In conclusion, a low percentage (1.5%) of Campylobacter among the strains recovered from slaughtered animals was resistant to erythromycin. However, the majority of these isolates revealed multi-antimicrobial resistance properties and possessed several virulence and toxin genes. Molecular analysis revealed that the mutation A2075G in the 23S rRNA gene was responsible for erythromycin resistance in all isolates tested. Moreover, comparisons of the PFGE and virulence marker profiles of the strains reflected a high genetic diversity among the tested Campylobacter. All these findings suggest that C. coli from food-producing animals may represent an underestimated potential health risk for consumers.

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