Recovery of cephalosporin-resistant *Escherichia coli* and *Salmonella* from pork, beef and chicken marketed in Nova Scotia

Kevin R Forward MD FRCP1,2, Katherine M Matheson2, Margot Hiltz MSc2, Heather Musgrave MSc2, Cornelius Poppe DVM PhD3

BACKGROUND: Antimicrobial use in farm animals is a potentially important contributor to the emergence of antimicrobial resistance. Resistant *Salmonella* may lead to serious human infections and resistant *Escherichia coli* may transfer plasmid-encoded resistance genes to other pathogens.

OBJECTIVE: To determine the prevalence of *E coli* and *Salmonella* species resistant to the third generation of cephalosporins in retail meat products in Halifax, Nova Scotia in 2002.

METHODS: Ground beef, ground pork and chicken wings were tested for *E coli* and *Salmonella*. *E coli* were selected on ceftriaxone-containing media. Beta-lactamases were characterised by isoelectric focusing, polymerase chain reaction and sequencing. Pulsed field gel electrophoresis was performed to determine the relationship of strains. The transferability of plasmids and location of resistance genes was also determined.

RESULTS: Forty-three of 75 packages of chicken wings contained ceftriaxone-resistant *E coli*; 42 of these contained beta-lactamases with isoelectric points at approximately 8.7. Six of seven CMY primer amplicons that were sequenced contained plasmid-mediated *Citrobacter freundii*-derived blaCMY-2; the other contained a CMY-2-like beta-lactamase. Pulsed field gel electrophoresis patterns demonstrated that strains were not clonal in nature. Four chicken samples contained *Salmonella*, one of which contained blaCMY-2-mediated resistance and an *E coli* bearing the same gene, but on different plasmids. Four of 102 beef samples contained *Klebsiella pneumoniae* resistant *E coli*, none contained *Salmonella*. Two of 75 pork samples contained ceftriaxone resistant *E coli*, one of which encoded for CMY-2. One susceptible *Salmonella* strain was recovered from pork.

CONCLUSIONS: Chicken from retail outlets located in Halifax, Nova Scotia, commonly contained blaCMY-2-bearing *E coli*. The relationship antibiotics used in food-producing animals and its effect on resistance of commensals and pathogens needs to be determined.

Key Words: Cephalosporins; *Escherichia coli*; Resistance; Retail meats; *Salmonella*

There is increasing concern that the use of antimicrobial agents in farm animals is an important contributor to the emergence of antimicrobial-resistant bacteria (1,2). There is also evidence to suggest that resistant organisms contaminate animal products and lead to serious human infections (3,4). Antimicrobial-resistant *Campylobacter jejuni* and *Salmonella* serovars are the most frequently cited examples (5,6). *Salmonella* also develops antibiotic resistance when farm...
animals are exposed to antibiotics (7). Zoonotic Escherichia coli infections are infrequently recognized (with the exception of E coli O157), so there has been less focus on resistance in E coli in packaged meats. However, resistant E coli in animal food products may cause human infection or serve as a source for resistance-bearing plasmids (8). E coli resistant to third-generation cephalosporins and cefamycins are being recovered with increasing frequency from both human and animal sources (7). Ceftiofur, a third-generation parenterally administered cephalosporin increasingly used in food animals, may promote the spread and increase the prevalence of these strains. In the United States, ceftiofur is used in day-old poults (10). We collected packaged ground beef, ground pork and chicken wings from Halifax, Nova Scotia area retail outlets over a three-month period to determine the prevalence of cephalosporin-resistant E coli and Salmonella and to characterize the beta-lactam resistance mechanisms encountered.

MATERIALS AND METHODS
Collection of samples and isolation and identification of E coli and Salmonella
One hundred packages of ground beef, 75 packages of chicken wings and 75 packages of ground pork products from a total of 10 retail outlets in the Halifax area were collected, up to twice weekly, during a period of two months (June to August, 2002). Products were from farms in Ontario, Quebec and Nova Scotia. Retail outlets did not share the same sources of meat and information on the original source of the chicks was not available. Only one of each product was purchased per visit and outlets were visited not more than twice weekly. Samples were double-bagged at the source, refrigerated until delivery to the laboratory and then handled in such a manner as to prevent cross-contamination. Then, 25 g of each ground meat sample was placed in a bag containing 225 mL of lactose broth and macerated using a stomacher. Three chicken wings were placed into a bag containing 110 mL of lactose broth culture was placed each into 10 mL selenite cysteine broth and tetrathionate broth with brilliant green dye. The lactose broth culture was placed each into 10 mL selenite cysteine broth containing 1 mg/L ceftriaxone to select for cephalosporin-resistant E coli. Plates were incubated aerobically at 35°C for 24 h. One millilitre of the ground meat-lactose broth homogenate or chicken-lactose broth culture was placed each into 10 mL selenite cystine broth and tetrazionate broth with brilliant green dye. The tetrazionate and selenite broths were incubated at 42°C and 35°C, respectively. Both were subcultured for 12 h to 16 h and plated onto a xylose lysine deoxycholate plate and incubated at 35°C for 24 h to screen for Salmonella. Suspect colonies were further identified using conventional means. Salmonella isolates were typed serologically.

Determination and characterization of antimicrobial susceptibility
Initial susceptibility testing was performed using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (11). Strains with reduced susceptibilities to cefoxitin or ceftriaxone were further tested using the NCCLS microbroth technique (12). Isoelectric focusing was performed using a method adopted from Matthew and Harris (13).

Amplification and sequencing of blaCMY-2
Polymerase chain reaction (PCR) was used to screen strains for the presence of the Citrobacter freundii-derived AmpC bla gene. Primers described by MFZali et al (14) were used to amplify 631 bp of the blaAmpC gene, and the forward blaTEM-A and reverse blaTEM-E primers of Speldoorren et al (15) to amplify 659 bp of the TEM gene. DNA sequences were determined using the ABI PRISM dye terminator cycle sequencing ready reaction kit and an ABI 377 automated DNA sequencer (Applied Biosystems, USA).

Examination of isolates for plasmid-encoded blaCMY-2
Plasmid DNA for Southern hybridization was extracted from the isolates using an alkaline lysis procedure and electrophoresed on a 1.2% agarose gel (16). The gel was stained with ethidium bromide, visualized under ultraviolet light and subjected to Southern blot analysis as previously described (17). Probing for blaCMY-2 was performed using a blaCMY-2 digoxigenin (DIG) labelled probe, which was prepared using the PCR DIG Probe Synthesis Kit (Roche Diagnostics Canada). Detection was performed using the DIG DNA Labelling and Detection Kit (Roche Diagnostics Canada).

Conjugation and transformation of strains
The donor E coli and Salmonella strains and the recipient E coli C600N (Lac– and nalidixic acid resistant) strain were grown at 37°C to a density of approximately 2×108 cells/mL. The recipient strain was grown for 15 min to 20 min at 45°C before mating. The donor (1 mL) and recipient cells (9 mL) were conjugated for 60 min, and the mating mixture was transferred to Luria-Bertani (LB) broth containing 50 µg/mL of nalidixic acid, incubated at 37°C for 30 min, and thereafter plated onto LB agar containing 50 µg/mL of each of cefoxitin and nalidixic acid (18). Plasmid DNA was used to transform E coli DH10B (Invitrogen, Canada) by electroporation (19). Transformants were selected on LB agar plates containing 50 µg/mL cefoxitin. Plasmids were electrophoresed and Southern blots were hybridized as described above.

Analysis of genetic relatedness by pulsed field gel electrophoresis of total DNA and restriction fragment length polymorphism of blaCMY-2 encoding plasmids
Pulsed field gel electrophoresis (PFGE) and plasmid profiles were compared. Whole-cell DNA for determination of PFGE patterns was prepared as described previously by the Centers for Disease Control (20). PFGE patterns were determined as described by Liebisch and Schwarz (21). To determine the relatedness of the plasmids carrying blaCMY-2, the plasmids were analyzed by restriction fragment length polymorphism (RFLP). Plasmid DNA was isolated from the cefoxitin-resistant E coli DH10B transformants of the E coli MPS57 and S Heidelberg MPS59 isolates using the Clontech BAC Maxi kit (BD Biosciences, Canada), and then digested with BglI, BglII and PstI. The resulting fragments were separated by electrophoresis.

RESULTS
Antimicrobial resistance
Each of the 43 E coli strains that had been isolated from the 75 samples of chicken wings grew in the presence of 1 µg/mL of cefoxitin and were resistant to ampicillin (minimum
TABLE 1

Summary of beta-lactam resistant Escherichia coli and Salmonella recovered from retail meat products (chicken wings) in Nova Scotia

<table>
<thead>
<tr>
<th>Retail outlet</th>
<th>Number of samples</th>
<th>Cefoxitin-resistant</th>
<th>Beta-lactamase</th>
<th>PFGE types</th>
<th>Salmonella</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CMY-2</td>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td>1-6</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>7-8, 9 a-e*</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>1-13</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>14-16, 17 a-f</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>18-19</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>20 a-b, 21 a-b</td>
</tr>
<tr>
<td>G</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>22, 23 a-e</td>
</tr>
<tr>
<td>H-J</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>43</td>
<td>39</td>
<td>41</td>
<td>41</td>
</tr>
</tbody>
</table>

1Within pulsed field gel electrophoresis (PFGE) type 9 there were five subtypes, denoted as a-e; 2Susceptible to cefoxitin; 3Three of the Escherichia coli isolates produced beta-lactamases with a pI of 8.7 but no blaCMY-2 amplicon, whereas the fourth Escherichia coli produced a beta-lactamase with a pI of 5.4, but no amplicon with either of the blaCMY-2 and the blaTEM primers.

Inhibitory concentrations (MICs) more than 32 mg/L, cephalothin (MICs more than 32 mg/L) and cefoxitin (MICs more than 16 mg/L). All but one of the strains had a markedly reduced susceptibility to ceftriaxone and were resistant to cefoxitin and cefotaxime. Only one strain had an MIC less than 4 mg/L. Strain MP3 had an MIC of 0.5 mg/L to ceftriaxone and of 1 mg/L to cefotaxime. This strain produced a beta-lactamase with an isoelectric point of 8.9 and no amplicon was produced using the blaCMY-2 primer set. Of the 43 Escherichia coli isolates from chicken samples, 25 were also resistant to tetracycline, 22 were resistant to trimethoprim/sulfamethoxazole and 10 were resistant to chloramphenicol. Aminoglycoside resistance was uncommon, only six strains were resistant to gentamicin and none were resistant to ciprofloxacin.

In all, Salmonella species were recovered from four (5.3%) of the poultry samples. One S. Heidelberg and one Salmonella oranienburg were fully susceptible to the beta-lactam antibiotics tested, even though one produced a beta-lactamase with an isoelectric point of 5.4. Only four of 100 ground beef samples contained blaCMY-2-bearing Escherichia coli; none contained Salmonella. Only two of 75 pork samples contained Escherichia coli, one of which was encoded CMY-2. One susceptible Salmonella derby strain was recovered from pork.

**Isoelectric focusing, PCR, sequencing of amplicons and PFGE**

Using isoelectric focusing, it was determined that 42 Escherichia coli produced beta-lactamases with isoelectric points at or near 8.7. This isoelectric point is characteristic of ampC-derived beta-lactamases, including blaCMY-2. One strain bore a beta-lactamase with a pI of 5.4. PCR using the CMY and TEM primers were both negative for this strain. Three of 42 strains producing beta-lactamases with an isoelectric point of 8.7 were negative for CMY-like beta-lactamases by PCR. Seven of the amplicons generated by the CMY primers were sequenced; six were entirely homologous with blaCMY-2 and the other was blaCMY-2-like. PFGE examination of strains from each of the retail outlets demonstrated that they were not of a clonal nature (Table 1). However, strains from any one outlet were often related (five of seven from outlet B, six of nine from outlet D, four of six from outlet E and five of seven from outlet G).

**Plasmid profiles, conjugation and transformation of plasmids, and RFLP of plasmid DNA**

One of the chicken samples contained Escherichia coli and S. Heidelberg, both of which produced the 631 bp amplicon by PCR using the blaCMY-2 primers (Figure 1). Both strains contained a 72 Mda (108 Kb) plasmid that was self-transmissible. The transconjugants and transformants harboured plasmids of 72 Mda that hybridized with the labelled blaCMY-2 probe (Figure 2). However, both plasmids displayed dissimilar restriction fragment length polymorphisms upon digestion with BglI, BglII and PvuII.

**DISCUSSION**

Fifty-seven per cent of chicken wings sold in the Halifax area contained Escherichia coli strains resistant to cephalosporins and cephemycins. The resistance was mostly mediated by CMY-2 beta-lactamases that are plasmid-borne. Such plasmids often carry other resistance genes, further limiting the antimicrobial
Cephalosporin-resistant *E. coli* from retail meats

CONCLUSION

The present study found that cephalosporin-resistant *E. coli* occurred commonly in poultry products in Halifax, Nova Scotia. This resistance is predominantly mediated by plasmids bearing blaCMY-2. This observation is troubling and cannot be readily explained. The chicken samples were obtained from retail outlets that purchased poultry products from slaughtering plants in several Canadian provinces. Chickens, mainly broilers, are raised in large numbers at poultry farms. Particularly when only a few days old, small chicks become very readily colonized with commensals like *E. coli* and pathogens like *Salmonella*. The feed they consume often contains antimicrobials such as penicillin or tetracyclines, administered for growth promotion purposes (27). Because many of the *Enterobacteriaceae* are constitutively resistant to penetration by penicillin (28), the effect of consumption of feed containing penicillin may not explain the presence of third-generation *E. coli* on poultry carcasses and parts is the off-label use of ceftiofur. However, we are unaware of a study that examines the occurrence and extent to which Canadian veterinarians and poultry producers may have adopted the practice of prophylactically or therapeutically administering ceftiofur to broiler breeder hens or broilers for indications such as the prevention and treatment of *E. coli* septicemia as in turkey pouls. Broiler chickens may have become colonized with resistant *E. coli* from environmental and other sources at the farm and from other animal species that had been treated with ceftiofur. When they are approximately six to seven weeks of age, they are trucked to, slaughtered and processed at poultry plants. There are many points during this process in the food chain, such as cross contamination during trucking from the shipping cages, during the evisceration

choices available for the treatment of these organisms when they cause infection. *blaCMY-2*-bearing *E. coli* are of concern because they, themselves, may colonize and subsequently cause infection, or they may also transfer their resistance determinants to other pathogens. If *CMY-2*-bearing *E. coli* becomes part of the endogenous flora as a result of consumption of improperly cooked chicken, these strains may result in a variety of infections in which endogenous *E. coli* are deemed important; for example, urinary tract infections. Because *CMY-2*-bearing plasmids may be exchanged between species, other, perhaps more virulent, pathogens may acquire the same resistance determinants, especially when individuals are exposed to the selective pressure of antibiotics.

Others have noted that *E. coli* derived from farm animals often carry beta-lactamase genes. Winokur et al (22) recovered cefhaminic-resistant *E. coli* from 59 of 377 (15.6%) cattle and swine samples and six of 1017 (0.6%) isolates of human *E. coli* from the same geographic region of Iowa. An *ampC* gene could be amplified with *blaCMY-2* primers in 33% of human and 94.8% of animal isolates. There was little clonal relatedness among the animal and human *E. coli* isolates harbouring the *CMY-2* gene. However, they did find significant similarities between plasmids found in *E. coli* and *Salmonella*, suggesting that the molecular environment surrounding the *CMY-2* genes were similar. This suggests, but does not prove, that *CMY-2* plasmids have been transmitted between these two genera of bacteria.

Ground meat products infrequently contained cephalosporin-resistant *E. coli*. Two pork samples contained resistant *E. coli*. We do not know the extent of ceftiofur use in cattle or pigs in Canada. Because this represents only a point prevalence study, we do not know how long these strains have been in ground beef and pork and whether the frequency of their occurrence is increasing. There is clearly a need to follow the spread of resistance plasmids and the bacteria that harbour them on a longitudinal basis.

*Salmonella* strains were recovered from only one of the 75 ground pork samples, none of the beef samples and from four of the packages of chicken wings. White et al (23) examined 200 meat samples collected from supermarkets in the Washington DC, USA area. Forty-one (20%) contained *Salmonella*, of which 16% were resistant to ceftriaxone. Of interest, six of the seven strains containing the *blaCMY-2* were from either turkeys or chickens. Winokur et al (22) also found that *blaCMY-2* was frequently linked to other resistance determinants. Fey et al (24) showed that there was a probable link between antibiotic use on the farm and infection in humans with antibiotic-resistant *Salmonella* species bearing the *blaCMY-2*. Nova Scotian ground pork and beef infrequently contained *Salmonella*. Other studies have also shown a lower *Salmonella* contamination rate of pork and beef than of chicken (25). The explanation may lie in the high susceptibility of young chicks to colonization and infection with *Salmonella* (26) and in the fact that broilers, but not pigs and cattle, are slaughtered at an age of six to seven weeks old. We collected samples from a number of different sources, so the low recovery rate should not reflect the level of hygiene in one particular facility. To date, there has been no conclusive evidence to support the transmission of *blaCMY-2* bearing plasmids between *E. coli* and *Salmonella*.

(CHART)

Figure 2) Plasmid profiles (left) and Southern blot of plasmid DNA hybridized with the *blaCMY-2* probe (right)
process in the slaughtering plant, during chilling and when the chickens are cut in portions and put on trays, that may have resulted in contamination of the chicken wings with antimicrobial-resistant E coli and Salmonella (29,30). Thus, although it is surmised that antimicrobial resistant bacteria recovered from meat products can be traced back to the farm, contamination occurring further down the processing chain may also have contributed to the isolation of cetifiur-resistant E coli from the chicken wings (29,30). The consequences of the treatment of humans with extended-spectrum cephalosporins and its contribution to contamination of sewage, the environment, including rivers, streams and surface waters, and colonization and subsequent infection of animals with antimicrobial resistant E coli and Salmonella are not known (31,32). We need to determine if antimicrobial resistance of E coli and Salmonella isolated from food products is increasing, and at what rate. We also need to study the potential impact on human health. The extent to which the transfer of plasmid-mediated resistance to clinically important antibiotics contributes to adverse patient outcomes is not known. We believe that the present findings should serve as a strong stimulus for veterinarians and poultry farmers to develop alternative strategies to the regular use of antibiotics, and for the poultry processors to minimize cross-contamination of poultry products.

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REFERENCES