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

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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 *bla*CMY-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 *bla*CMY-2-mediated resistance and an *E coli* bearing the same gene, but on different plasmids. Four of 100 beef samples contained *bla*CMY-2-bearing *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 *bla*CMY-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

Présence de bactéries *E coli* et *Salmonella* résistantes aux céphalosporines dans la viande de porc, de bœuf et de poulet des marchés néo-écossais

HISTORIQUE : L'administration d'antibiotiques aux animaux de boucherie contribue potentiellement à l'augmentation de la résistance aux antibiotiques. Les souches résistantes de *Salmonella* pourraient entraîner de graves infections chez l'être humain et celles de *E coli* seraient propices au transfert de gènes de résistance encodés au niveau plasmidique à d'autres agents pathogènes.

OBJECTIF : Déterminer la prévalence des souches de *E coli* et de *Salmonella* résistantes aux céphalosporines de troisième génération dans les viandes vendues au détail à Halifax, en Nouvelle-Écosse en 2003.

MÉTHODE : Le bœuf haché, le porc haché et les ailes de poulets ont fait l'objet de tests de dépistage de *E coli* et de *Salmonella*. Les souches de *E coli* ont été sélectionnées sur un milieu renfermant du ceftriaxone. Les bêtalactamases ont été caractérisées par focalisation isoélectrique, réaction en chaîne de la polymérase et séquençage. L'électrophorèse sur gel en champ pulsé a servi à déterminer le rapport entre les souches. La transférabilité plasmidique et la localisation des gènes de la résistance ont aussi été déterminées.

RÉSULTATS : Quarante-trois paquets d'ailes de poulets sur soixante-quinze renfermaient des souches de *E coli* résistantes au ceftriaxone. Quarante-deux de ces paquets renfermaient des bêtalactamases présentant des points isoélectriques d'environ 8,7. Six amplicons CMY d'amorce sur sept qui ont été séquencés renfermaient le gène *bla*CMY-2 à médiation plasmidique dérivé de *Citrobacter freundii*, l'autre renfermait une bêtalactamase de type CMY-2. L'électrophorèse sur gel en champ pulsé a révélé la présence de souches non clonales. Quatre échantillons de poulet étaient contaminés par *Salmonella*; l'un présentait la souche résistante associée au gène *bla*CMY-2 et une souche de *E coli* porteuse du même gène, mais sur des plasmides différents. Quatre échantillons de bœuf sur cent renfermaient la souche de *E coli* porteuse du *bla*CMY-2. Aucun ne renfermait de *Salmonella*. Deux échantillons de porc sur soixante-quinze renfermaient une souche de *E coli* résistante au ceftriaxone, dont l'une encodée pour le CMY-2. Une souche de *Salmonella* sensible a été isolée à partir de la viande de porc.

CONCLUSION : Le poulet provenant des établissements de détail renfermait souvent la souche de *E coli* porteuse du *bla*CMY-2. Il faut travailler à déterminer quel est le lien entre les antibiotiques utilisés chez les animaux de boucherie et leurs effets sur la résistance des agents pathogènes et commensaux.

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). Antibiotic-resistant *Campylobacter jejuni* and *Salmonella* serovars are the most frequently cited examples (5,6). *Salmonella* also develops antibiotic resistance when farm

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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 cephamycins 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 development and spread of these strains. In the United States, ceftiofur is used in day-old chicks to prevent the development of *E coli* omphalitis and *E coli* septicaemia causing early chick mortality (9). It has not been approved for this indication in Canada. Other uses in both countries include the treatment of bacterial respiratory infections, fever and pododermatitis in cattle, respiratory tract infection in pigs and lambs, and the control of colibacillosis 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 were 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. All meat samples were incubated aerobically at 35°C overnight. The lactose broth was swabbed onto MacConkey agar 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 cysteine broth and tetrathionate broth with brilliant green dye. The tetrathionate 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*. Suspicious 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 *bla*CMY-2

Polymerase chain reaction (PCR) was used to screen strains for the presence of the *Citrobacter freundii*-derived AmpC *bla* gene. Primers described by M'Zali et al (14) were used to amplify 631 bp of the *bla*AmpC gene, and the forward *bla*TEM-A and reverse *bla*TEM-E primers of Speldooren 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 *bla*CMY-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 *bla*CMY-2 was performed using a *bla*CMY-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 2×10^8 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 *bla*CMY-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 *bla*CMY-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 Clonetech BAC Maxi kit (BD Biosciences, Canada), and then digested with *Bgl*I, *Bgl*II and *Pvu*II. 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 mg/mL of ceftriaxone and were resistant to ampicillin (minimum

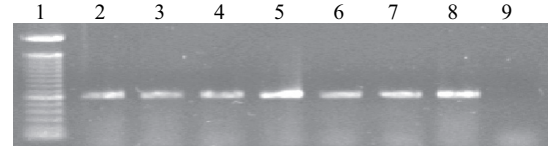
TABLE 1
Summary of beta-lactam resistant *Escherichia coli* and *Salmonella* recovered from retail meat products (chicken wings) in Nova Scotia

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

**Within pulsed field gel electrophoresis (PFGE) type 9 there were five subtypes, denoted as a-e; †Susceptible to cefoxitin; ‡Three of the* E coli *isolates produced beta-lactamases with a pI of 8.7 but no* bla*CMY-2 amplicon, whereas the fourth* E coli *produced a beta-lactamase with a pI of 5.4, but no amplicon with either of the* bla*CMY-2 and the* bla*TEM 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 ceftiofur. 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 ceftiofur. This strain produced a beta-lactamase with an isoelectric point of 8.9 and no amplicon was produced using the *bla*CMY-2 primer set. Of the 43 *E 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 *bla*CMY-2-bearing *E coli*; none contained *Salmonella*. Only two of 75 pork samples contained *E coli*, one of which was encoded CMY-2. One susceptible *Salmonella derby* strain was recovered from pork.



Lane 1 100 base pair ladder
Lane 2 *E. coli* MPS57
Lane 3 *S.* Heidelberg PT3 MPS59
Lane 4 MPS 57 conjugated with *E. coli* C600N
Lane 5 MPS 59 conjugated with *E. coli* C600N
Lane 6 *E. coli* DH10B transformed with 72 Mda from MPS57
Lane 7 *E. coli* DH10B transformed with 72 Mda from MPS59
Lane 8 Positive control
Lane 9 Negative control

Figure 1) *Polymerase chain reaction using* blaCMY-2 *primers produced 631 bp amplicons*

Isoelectric focusing, PCR, sequencing of amplicons and PFGE

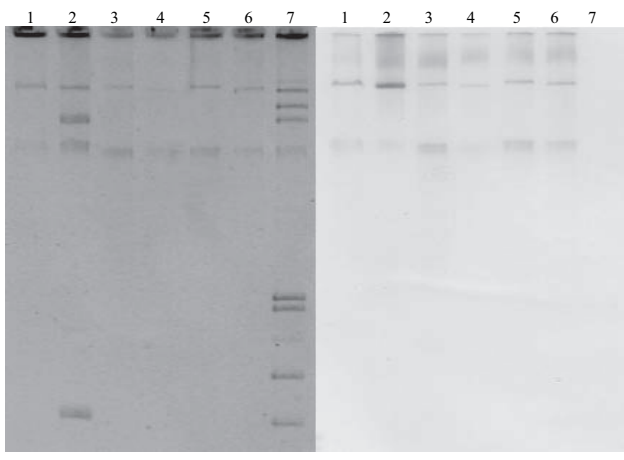
Using isoelectric focusing, it was determined that 42 *E coli* produced beta-lactamases with isoelectric points at or near 8.7. This isoelectric point is characteristic of *amp*C-derived beta-lactamases, including *bla*CMY-2. One strain bore a beta-lactamase with a pI of 5.4. PCR using the CMY and TEM 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 *bla*CMY-2 and the other was *bla*CMY-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 *E coli* and S Heidelberg, both of which produced the 631 bp amplicon by PCR using the *bla*CMY-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 *bla*CMY-2 probe (Figure 2). However, both plasmids displayed dissimilar restriction fragment length polymorphisms upon digestion with *Bgl*I, *Bgl*II and *Pvu*II.

DISCUSSION

Fifty-seven per cent of chicken wings sold in the Halifax area contained *E coli* strains resistant to cephalosporins and cephamycins. 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



Lane 1	*E. coli* MPS57, 2 plasmids of 72 and 66 Mda
Lane 2	*S.* Heidelberg PT3, MPS 59, 3 plasmids of 72, 24 and 1.5 Mda
Lane 3	MPS 57 conjugated with *E. coli* C600N, 72 Mda plasmid
Lane 4	MPS 59 conjugated with *E. coli* C600N, 72 Mda plasmid
Lane 5	*E. coli* DH10B transformed with 72 Mda from MPS 57
Lane 6	*E. coli* DH10B transformed with 72 Mda from MPS 59
Lane 7	Marker plasmids of 96, 62, 36, 23, 3.7, 3.4, 2.8, 2.0 and 1.4 Mda

Figure 2) *Plasmid profiles (left) and Southern blot of plasmid DNA hybridized with the* blaCMY-2 *probe (right)*

choices available for the treatment of these organisms when they cause infection. *bla*CMY-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 cephamycin-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 *amp*C gene could be amplified with *bla*CMY-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 *bla*CMY-2 were from either turkeys or chickens. Winokur et al (22) also found that *bla*CMY-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 *bla*CMY-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 *bla*CMY-2 bearing plasmids between *E coli* and *Salmonella*.

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 *bla*CMY-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 select for cephalosporin-resistant organisms. Data regarding antimicrobial use on farms are often unavailable. A possible explanation for the presence of third-generation cephalosporin-resistant *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 poults. 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

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 ceftiofur-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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