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ORIGINAL ARTICLE

# Genotyping of *Campylobacter coli* isolated from humans and retail meats using multilocus sequence typing and pulsed-field gel electrophoresis

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## Abstract

**Aims:** To determine the antimicrobial resistant profiles and clonality of *Campylobacter coli* isolated from clinically ill humans and retail meats.

**Methods and Results:** A total of 98 *C. coli* isolates (20 from humans and 78 from retail meats) were phenotypically characterized. Antimicrobial susceptibility testing was done using agar dilution method for ciprofloxacin, gentamicin, erythromycin and doxycycline. Seventy *C. coli* isolates including humans ($n = 20$) and retail meats ($n = 50$) were genotyped by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Resistance to ciprofloxacin was found in 29% and 15% of isolates from retail meats and humans. We observed 61 PFGE profiles using two enzymes (*Sma*I, *Kpn*I) with an Index of discrimination of 0·99, whereas MLST generated 37 sequence types. Two clonal complexes were identified with 58 (82%) *C. coli* isolates clustered in the ST-828 complex.

**Conclusions:** Resistance to ciprofloxacin and erythromycin was identified in *C. coli* obtained from retail meats and ill humans. PFGE typing of *C. coli* isolates was more discriminatory than MLST. Grouping of *C. coli* isolates (82%) by MLST in ST-828 clonal complex indicates a common ancestry.

**Significance and Impact of the Study:** A high frequency of resistance found to ciprofloxacin and erythromycin is concerning from food safety perspective. PFGE using single or double restriction enzymes was found to be more discriminatory than MLST for genotyping *C. coli*. Overall, the *C. coli* populations recovered from humans and retail meats were genotypically diverse.

## Introduction

*Campylobacter* is an important bacterial foodborne pathogen, responsible for causing the largest number of foodborne-related infections after *Salmonella* in the US. The overall incidence of campylobacteriosis in the US was 12·79 per 100 000 people in 2007 (Center for Disease Control and Prevention, 2008). However, because of under-reporting, the estimated number of cases is estimated to be 38-fold higher than reported (Mead *et al.* 1999). *Campylobacter jejuni* is responsible for 90–95% of the infections in humans with the remaining because of *Campylobacter coli* [National Antimicrobial Resistance Monitoring System (NARMS), 2008]. A recent population

based surveillance study conducted in the UK concluded that *Campylobacter* caused the greatest impact on the healthcare sector with 16 946 hospitalizations (Adak *et al.* 2002). Consumption of contaminated food, particularly retail meat products originating from poultry, cattle and pigs, milk and water have been implicated as the major source of *Campylobacter* infection (Sacks *et al.* 1986; Zhao *et al.* 2001; Thakur *et al.* 2006; Lévesque *et al.* 2007; Litrup *et al.* 2007; Kwan *et al.* 2008).

Illness as a result of *Campylobacter* is further compounded because of the emergence of antimicrobial resistant strains that can be transmitted to humans through the food chain. High frequency of resistance to important classes of antimicrobials like the fluoroquinolones and macrolides used in the treatment of severe cases of campylobacteriosis has been reported from humans and food animals (Asai *et al.* 2007; Rozynek *et al.* 2008). Although *C. jejuni* in humans is considered to be the most important *Campylobacter* species causing infection, recent studies have highlighted the importance of *C. coli* as a human pathogen causing foodborne diseases with an inherent ability to be more frequently resistant to antimicrobials than *C. jejuni* (Gillespie *et al.* 2002; Sails *et al.* 2003; Tam *et al.* 2003; Bywater *et al.* 2004).

For epidemiological studies, genotyping of *Campylobacter* is important for outbreak investigations and to understand the epidemiology of sporadic cases acquired from various sources. The large number of *Campylobacter* strains isolated in both the developed and developing countries makes it very important to subtype them quickly and reliably with methods that have high discriminatory power. Various genotyping methods have been used to investigate the molecular epidemiology of this important foodborne pathogen and elucidate its sources and reservoirs. These include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *fla*-restriction fragment length polymorphism and amplified fragment length polymorphism (Duim *et al.* 1999; Dingle *et al.* 2005; Johnson *et al.* 2006; Thakur *et al.* 2006; Pope *et al.* 2007). Although PFGE has been considered as 'gold standard' method to subtype all the major foodborne pathogens and is highly discriminatory, inter-laboratory comparisons can be difficult because of complex protocols and accessibility to equipment. More recently, MLST methods have been developed and are now regularly used for genotyping multiple bacterial species other than *Campylobacter* including *Salmonella*, *Vibrio* and *Staphylococcus* (Kotetishvili *et al.* 2003; Alcaine *et al.* 2006; Saunders and Holmes 2007). MLST is based on indexing sequence variation in a defined set of housekeeping genes, which have limited capacities for nonlethal mutations. This overcomes limitations in other methods applied to phylogenetic analyses of bacterial populations with hypervariable genomes such as *Campylobacter* (Thakur *et al.* 2006), including strains that are temporally and geographically separated. In addition, MLST has the advantage of ease of inter-laboratory data comparisons through web-based applications.

This study was conducted to evaluate PFGE and MLST typing methods for their capacity to determine if antimicrobial-resistant *C. coli* genotypes were shared between humans with campylobacteriosis and retail meat samples that were related spatially and temporally. An important aim of this study was to evaluate the discriminatory power, throughput and group associations of MLST with PFGE using single and double restriction enzymes. Finally, we elucidated the phylogenetic makeup and evidence of sequence type (ST) association with the host or particular antimicrobial resistance profiles.

## Materials and methods

### Origin of *C. coli* strains

A total of 78 *C. coli* isolates recovered from 814 retail meat samples, including 209 samples each of chicken breast, pork chop and ground beef and another 187 ground turkey samples, were included in this study. These samples were collected as part of a retail meat surveillance study conducted in Iowa (Hayes *et al.* 2003) and *Campylobacter* was isolated and speciated using standard bacteriological and polymerase chain reaction (PCR) methods (Thakur and Gebreyes 2005). The representative isolates in this study from retail meat products included 65 isolated from chicken breast, 11 from ground turkey and two from pork chops. None of the ground beef samples were culture positive for *C. coli*. In addition, 20 *C. coli* isolates from clinically ill human patients collected in Iowa during the same time period were included for comparative analysis. Information on the intake of antimicrobials by humans prior to sample collection was not known.

### Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using the agar dilution method as recommended by Clinical and Laboratory Standards Institute (CLSI), formerly National Clinical Committee for Laboratory Standards (NCCLS) (CLSI, 2006). The list of antimicrobials with their abbreviations and range of concentrations used were: ciprofloxacin (Cip; 0·008–4 mg $l^{-1}$), erythromycin (Ery; 0·06–32 mg $l^{-1}$), gentamicin (Gen; 0·06–32 mg $l^{-1}$) and doxycycline (Dox; 0·06–32 mg $l^{-1}$). The CLSI breakpoint interpretative criteria for *Campylobacteriaceae* family were used for the antimicrobials including ciprofloxacin (Cip: 4 mg $l^{-1}$), Erythromycin (Ery:

32 mg $l^{-1}$) and Doxycycline (Dox: 8 mg $l^{-1}$) as recommended by CLSI (2006). For gentamicin (Gen), the breakpoint level of 8 mg $l^{-1}$ was used following the NARMS (2008) criteria. We used *C. jejuni* ATCC 33560 as the quality control organism. Multidrug resistance (MDR) was defined as isolates exhibiting resistance to two or more antimicrobials at the same time when tested by the agar dilution method.

### PFGE Typing

A total of 70 *C. coli* isolates were typed by PFGE following the rapid protocol for *Campylobacter* (Ribot *et al.* 2001). All human *C. coli* isolates and 50 isolates from retail meat were selected proportionally representing the source, resistance profile, time and geographic location of sampling. Briefly, 400 ml overnight culture cells were lysed and intact genomic DNA was digested in separate reactions in agarose-embedded plugs with either *Sma*I or *Kpn*I. Digested DNA was separated using a contour-clamped homogeneous electric field-DRIII (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 0·5× Tris–Borate 15 EDTA, 1% Seakem Gold agarose (FMC BioProducts, Rockland, ME, USA), 14°C, 6 V $cm^{-1}$ for 18 h with switch times ranging from 6·75 to 38·4 s with an included angle of 120°. *Salmonella* enterica serovar Braenderup 17 'Universal Marker' was used as the reference marker. Gels were stained with ethidium bromide and photographed under UV light. Interpretation of DNA fingerprint patterns was accomplished using BIONUMERICS® 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium). The banding patterns were compared using Dice coefficients with a 1·5% band position tolerance. Isolate relatedness was determined using the unweighted pair group method using arithmetic averages.

### Multilocus sequence typing

Genomic DNA purification for sequencing was done using the Qiagen DNA purification kit (Qiagen, Valencia, CA, USA) for the same 70 *C. coli* isolates typed by PFGE. MLST of seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) for *C. coli* was done following the strategy described previously for *Campylobacter* (Dingle *et al.* 2005). Sequencing reactions using the forward and reverse primers in separate wells was done using 2 $\mu$l of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, CA, USA), 1 $\mu$mol $l^{-1}$ each primer, 5·5 $\mu$l of molecular grade deionized water and 2 $\mu$l of the purified PCR product. Sequencing reaction was performed on the automated 3700 ABI capillary sequencer (Applied Biosystems) with running conditions of 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The CLUSTALW program was used for aligning the forward and reverse sequences was used (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The allelic profiles and the STs were then generated by blasting the correct sequence size on the MLST website from the *Campylobacter* database (accessible at http://www.pubmlst.org/campylobacter or http://www.mlst.net). We generated a dendrogram after concatenating the seven house keeping gene sequences using the BIONUMERICS program.

### Data analysis

The frequency of antimicrobial resistance patterns and MIC levels between isolates from different sources were compared using the chi-squared test (Minitab Inc, State College, PA, USA) and Fisher's exact two-tailed test wherever applicable. A value of $P < 0{\cdot}05$ was considered statistically significant.

Simpson's index of diversity was calculated to compare the discriminatory power of the two genotyping methods used in this study (Hunter and Gaston 1988). The formula used to calculate the index of discrimination (DI) is:

$$\mathrm{DI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

where, DI is the index of discrimination, $N$ is the total number of strains in the sample population, $S$ is the total number of types described and $n_j$ is the number of strains belonging to the $j$th type. The DI value ranges between 0·0 and 1·0. The higher the index, the more discriminatory the method is.

The ST most likely to be the founder of the clonal complex was determined using the EBURST software as recommended previously (Feil *et al.* 2004). In addition, we used the stringent group definition where isolates with six of the seven housekeeping gene alleles matching were placed in the same complex. Bootstrapping method (re-sampling 1000 times) was done to provide statistical confidence to assignment of the ST as the putative founder of the complex. The index of association ($I_A$) was determined using the START program to assess the clonality of the population (Jolley *et al.* 2001). An absolute value of zero ($I_A = 0$) indicates that the population is freely recombining and is not clonal, whereas a value of 1·0 indicates clonality of isolates. A dendrogram was built using BIONUMERICS (Applied Maths) to determine the relationship between the different STs generated. Phylogenetic analysis and determination of variable sites in the unique alleles was done using the MEGA software version 4.2 (Tamura *et al.* 2007).

**Table 1** Antimicrobial resistance phenotypes of *Campylobacter coli* isolates from humans and retail meats

| Source | No. of isolates | Resistance profile, *n* (%)* | | | | Resistance patterns, *n* (%)† |
|---|---|---|---|---|---|---|
| | | Ciprofloxacin | Erythromycin | Doxycycline | Gentamicin | |
| Pork chops | 2 | 0 | 2 (100) | 2 (100) | 0 | Ery Dox 2 (100) |
| Chicken breast | 65 | 14 (22) | 22 (34) | 31 (48) | 0 | Cip 4 (6), Ery 11 (17), Dox 12 (18), Ery Dox 8 (12), Cip Ery 1 (2), Cip Dox 7 (11), Cip Ery Dox 2 (3) |
| Ground turkey | 11 | 9 (82) | 5 (46) | 10 (91) | 0 | Ery 1 (9), Ery Dox 1 (9), Cip Ery 6 (55), Cip Ery Dox 3 (27) |
| Total meat type | 78 | 23 (29) | 29 (37) | 43 (55) | 0 | Cip 4 (5), Dox 12 (15), Ery 12 (15), Cip Dox 13 (17), Cip Ery 1 (1), Ery Dox 11 (14), Cip Ery Dox 5 (6) |
| Human | 20 | 3 (15) | 13 (65) | 12 (60) | 0 | Cip 1 (5), Ery 4 (20), Dox 3 (15), Ery Dox 6 (30), Cip Ery Dox 2 (10) |

Cip, ciprofloxacin; Ery, erythromycin; Dox, doxycycline; Gen, gentamicin.

*Antimicrobial resistance exhibited by the *C. coli* isolate to a single antimicrobial.

†Antimicrobial resistance exhibited by the *C. coli* isolate either singly or in combination with another antimicrobial.

## Results

### Antimicrobial resistance phenotypes

Ciprofloxacin resistance was observed in 15% of *C. coli* isolates recovered from ill humans and 29% of isolates obtained from retail meats (Table 1). Isolates from ground turkey were significantly more resistant to ciprofloxacin and doxycycline than isolates obtained from humans and chickens ($P < 0{\cdot}001$). Ciprofloxacin MICs among the resistant strains from humans and retail meats ranged from 8 to 64 $\mu$g ml$^{-1}$. A higher frequency of *C. coli* isolates displayed resistance to erythromycin compared with ciprofloxacin, including 65% of isolates recovered from humans and 37% of isolates obtained from retail meat samples. Erythromycin resistance was observed for both isolates originating from pork chops, and was commonly observed in isolates recovered from ill humans (65%), ground turkey (46%) and chicken breast (34%). A total of 23 *C. coli* isolate including 20 from chicken breast and three from humans were pansusceptible and all isolates were susceptible to gentamicin.

With regards to MDR phenotypes, a total of seven different antimicrobial-resistant patterns were found among the 98 *C. coli* isolates tested (Table 1). MDR *C. coli* isolates were observed from both humans and retail meats with co-resistance to erythromycin and doxycycline being the overall predominant pattern (17%). *Campylobacter coli* isolates exhibiting the MDR phenotype ciprofloxacin/erythromycin/doxycycline were recovered from humans, chicken breast and ground turkey. Also, a significantly higher frequency of *C. coli* isolates recovered from ground turkey as compared with other sources exhibited the ciprofloxacin/erythromycin and ciprofloxacin/erythromycin/doxycycline resistance patterns ($P < 0{\cdot}01$).

### *C. coli* genotypic diversity based on MLST and PFGE analysis

The overall *C. coli* population was diverse as revealed by the MLST data, with 37 STs identified among the 70 *C. coli* isolates tested. ST-829 was the most common ST, represented by 11 *C. coli* isolates from chicken breast and ground turkey followed by ST-1068 represented by seven isolates from humans (Table 2). Eight new alleles were identified resulting in the assignment of eight new STs. The new alleles for the different housekeeping genes reported include *pgm* ($n = 5$), *glt* ($n = 2$) and *gln* ($n = 1$). The majority of these new alleles were observed in isolates originating from chicken breasts (75%) with the remainder coming from ground turkey. The number of unique alleles for the different housekeeping genes varied from three for *asp* to 12 for the *pgm* gene (Table 3). Alleles varied from 0·83% for *gln* to 11·74% for *asp*. All the STs except two were found to be host specific in this study. The two nonhost-specific STs include ST-829 ($n = 9$) composed of chicken ($n = 8$) and turkey ($n = 1$) isolates, and ST-1107 ($n = 2$) represented by a single isolate each from humans and pork chops. The MLST dendrogram generated by concatenating the seven housekeeping genes sequences (3309 base pair) revealed a total of 37 clusters including 13 clonal and 24 unique profiles. Based on the Simpson's index of diversity, the MLST method had a discriminatory power of 0·95. As expected, the major groups were ST-829 (cluster 9) which included 11 isolates from chicken and turkey and

**Table 2** *Campylobacter coli* clonal complex representing sequence types (STs), source and antimicrobial resistance patterns

| Clonal complex | ST* (data) | Profile | | | | | | | Source | No. of isolates | Resistance patterns |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *asp* | *gln* | *glt* | *gly* | *pgm* | *tkt* | *unc* | | | |
| ST-828 | 825 | 33 | 39 | 30 | 82 | 113 | 47 | 17 | Chicken | 3 | Cip, Ery |
| | 829 | 33 | 39 | 30 | 82 | 113 | 43 | 17 | Chicken, turkey | 11 | Cip, Ery, Dox, Cip Dox, Ery Dox |
| | 854 | 33 | 38 | 30 | 82 | 104 | 43 | 17 | Human | 2 | Ery Dox |
| | 860 | 33 | 39 | 30 | 79 | 113 | 47 | 17 | Chicken | 3 | Ery |
| | 894 | 33 | 39 | 65 | 82 | 113 | 47 | 17 | Human | 1 | Pan-susceptible |
| | 899 | 33 | 39 | 30 | 82 | 113 | 35 | 17 | Human | 1 | Pan-susceptible |
| | 1017 | 33 | 39 | 30 | 82 | 104 | 43 | 41 | Chicken | 2 | Cip Dox |
| | 1058 | 33 | 39 | 30 | 82 | 104 | 35 | 17 | Human | 1 | Cip Ery Dox |
| | 1063 | 33 | 39 | 30 | 140 | 113 | 43 | 41 | Chicken | 1 | Ery |
| | 1068 | 33 | 39 | 30 | 78 | 104 | 43 | 17 | Human | 7 | Ery, Ery Dox, Cip Ery Dox |
| | 1082 | 33 | 39 | 30 | 82 | 211 | 85 | 17 | Human | 1 | Cip |
| | 1099 | 33 | 38 | 30 | 167 | 104 | 35 | 17 | Human | 1 | Ery Dox |
| | 1107 | 33 | 38 | 30 | 82 | 104 | 44 | 36 | Pork, human | 2 | Ery, Ery Dox |
| | 1112 | 33 | 39 | 30 | 82 | 104 | 43 | 68 | Human | 1 | Pan-susceptible |
| | 1119 | 33 | 39 | 30 | 82 | 113 | 43 | 41 | Chicken | 2 | Cip Ery, Cip Ery Dox |
| | 1148 | 33 | 39 | 122 | 82 | 113 | 43 | 17 | Turkey | 1 | Ery Dox |
| | 1175 | 33 | 39 | 65 | 82 | 216 | 47 | 17 | Chicken | 3 | Dox, Ery Dox |
| | 2625 | 33 | 39 | 30 | 82 | 209 | 47 | 17 | Chicken | 2 | Dox |
| | 2626 | 33 | 39 | 122 | 82 | 113 | 47 | 17 | Chicken | 5 | Ery Dox |
| | 2630 | 33 | 39 | 222 | 82 | 104 | 47 | 17 | Chicken | 1 | Cip Dox |
| | 2631 | 33 | 39 | 30 | 140 | 113 | 43 | 17 | Turkey | 1 | Cip Dox |
| | 2632 | 33 | 260 | 122 | 82 | 189 | 43 | 17 | Chicken | 1 | Dox |
| | 2634 | 33 | 39 | 223 | 82 | 113 | 47 | 17 | Chicken | 1 | Dox |
| | 2636 | 33 | 39 | 30 | 82 | 374 | 43 | 17 | Chicken | 2 | Cip, Cip Dox |
| | 2638 | 103 | 39 | 30 | 82 | 189 | 47 | 17 | Turkey | 1 | Cip Dox |
| | 2639 | 33 | 39 | 65 | 82 | 376 | 47 | 17 | Turkey | 1 | Cip Ery Dox |
| ST-1150 | 2633 | 103 | 110 | 103 | 140 | 104 | 164 | 120 | Chicken | 1 | Pan-susceptible |
| | 2635 | 103 | 110 | 30 | 140 | 188 | 47 | 79 | Turkey | 1 | Cip Dox |
| | 2637 | 103 | 110 | 30 | 140 | 188 | 164 | 120 | Chicken | 1 | Cip Dox |
| | 2643 | 103 | 110 | 30 | 140 | 375 | 164 | 79 | Turkey | 1 | Cip Dox |
| None | 1123 | 53 | 38 | 44 | 82 | 118 | 35 | 36 | Human | 2 | Dox |
| | 1470 | 53 | 38 | 30 | 82 | 104 | 35 | 36 | Human | 1 | Dox |
| | 2627 | 103 | 110 | 30 | 140 | 373 | 47 | 120 | Chicken | 1 | Cip Ery Dox |
| | 2628 | 33 | 39 | 30 | 140 | 113 | 47 | 41 | Chicken | 1 | Ery |
| | 2629 | 33 | 110 | 30 | 140 | 113 | 47 | 17 | Turkey | 1 | Cip Ery Dox |
| | 2640 | 103 | 110 | 122 | 140 | 104 | 164 | 41 | Chicken | 1 | Ery Dox |
| | 2644 | 33 | 153 | 44 | 161 | 104 | 44 | 17 | Human | 1 | Ery |

Cip, ciprofloxacin; Ery, erythromycin; Dox, doxycycline; Gen, gentamicin.
*Sequence type generated by the unique combination of alleles for the seven housekeeping genes.

ST-1068 (cluster 23) composed of the seven isolates from humans.

Extensive genotypic diversity of the *C. coli* isolates was also observed using PFGE. The discriminatory power of PFGE using either single or double enzymes was greater than the MLST method. Visual interpretation of the two dendrograms also reflected the ability of PFGE to discriminate between isolates that were clustered in the same group by MLST. We observed a discriminatory power (DI) of 0·98 for *Sma*I alone and 0·99 each for *Kpn*I alone and for *Sma*I plus *Kpn*I. Four clonal complexes and 57 unique clusters were identified when a dendrogram was generated using results from both *Kpn*I and *Sma*I digestions. The higher discriminatory power of PFGE was shown by the ability of this method to further partition the clonal clusters created by MLST. For example, MLST cluster 9, comprising the 11 ST-829 isolates, was further split into seven PFGE clusters (Fig. 1). Similarly, MLST cluster number 23 composed of seven human *C. coli* isolates was partitioned into three clusters by PFGE (Fig. 1). Other than two isolates (ST-1175) from chicken breast, five from humans (ST-1068) and another two (ST-2636)

**Table 3** Genetic diversity of *Campylobacter coli* based on multilocus sequence typing (MLST) data

| Locus | Fragment csize | Alleles | New alleles | No. variable sites | Per cent variable sites | $d_N/d_S$* |
|---|---|---|---|---|---|---|
| *Asp* | 477 | 3 | 0 | 56 | 11·74 | 0·0365 |
| *Gln* | 477 | 5 | 1 | 4 | 0·83 | 0·0913 |
| *Glt* | 402 | 7 | 2 | 6 | 1·5 | 0·2946 |
| *Gly* | 507 | 6 | 0 | 7 | 1·38 | 0·1527 |
| *Pgm* | 498 | 12 | 5 | 19 | 3·8 | 0·0575 |
| *Tkt* | 459 | 6 | 0 | 24 | 5·22 | 0·1185 |
| *Unc* | 489 | 6 | 0 | 8 | 1·63 | 0·00 |

*Degree of nonsynonymous base substitution to synonymous base substitution.

from chicken breast, PFGE using two enzymes was able to discriminate individuals in the rest of the ST clusters. In addition, both typing methods were able to further differentiate isolates clustered together in a single group using the alternate method. However, MLST was able to do this only in clusters created by the PFGE method using *Sma*I enzyme alone. While the use of *Kpn*I restriction enzyme alone or in combination with *Sma*I resulted in excellent discrimination (DI 0·99), *Kpn*I was unable to digest DNA from two *C. coli* isolates, one originating from chicken breast and the other from an ill person. A similar observation was seen with *Sma*I enzyme for a single isolate recovered from chicken breast.

Individual antimicrobial resistance patterns exhibited by *C. coli* isolates from different sources were represented by multiple STs and PFGE patterns. For instance, the ciprofloxacin/erythromycin/doxycycline resistance pattern ($n = 6$) was represented by multiple STs including ST-1068 (human), ST-1119, 2627 (chicken breast) and ST-2629, 2639 (ground turkey). Similarly, the erythromycin/doxycycline antimicrobial resistance pattern ($n = 12$) was represented by STs 854, 1068 and 1089 from humans, STs 829, 1175, 2626 and 2640 from chicken breast, ST-1148 from ground turkey and ST-1107 from pork chops implying strains with this resistance pattern are not necessarily clonal. PFGE analysis further confirmed these observations (Fig. 1).

### Phylogenetic analysis of the *C. coli* population

Sequence information from the MLST data was used to determine the evolutionary relationships of the 70 *C. coli* isolates tested. We used the stringent group definition of six or more shared alleles at each locus to define members of the same clonal complex. Two clonal complexes were defined in the study population with ST-828 clonal complex being the major complex representing 26 (70%) of the 37 STs (Table 2). ST-829 was predicted as the putative founder of the clonal complex. The majority ($n = 58$) of the isolates recovered from different sources clustered together in one group under this clonal complex. Four STs represented by isolates from ST-2633 (chicken), ST-2637 (chicken) and ST-2635, 2643 (turkey) were grouped under the ST-1150 clonal complex. The remaining seven STs (ST-1123, 1470, 2627, 2628, 2629, 2640 and 2644) from humans ($n = 3$), chicken breast ($n = 3$) and ground turkey ($n = 1$) did not cluster in any clonal complex.

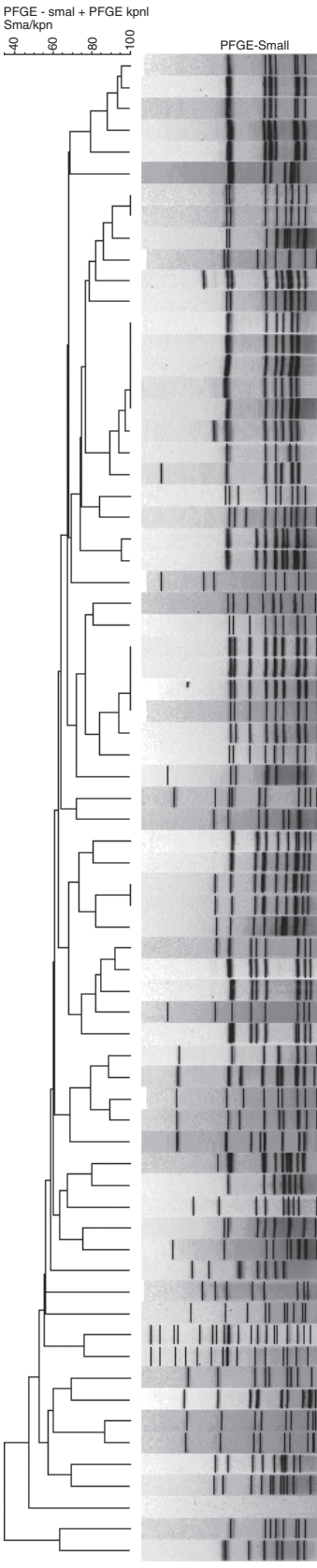
A radial neighbour-joining tree was generated by concatenating sequences (3309 base pair) from the unique STs ($n = 37$) (Fig. 2). A total of seven *C. coli* isolates including four from chicken breast and three from ground turkey clustered away from the main group of isolates. These seven STs were also reported for the first time in this study. The $d_N/d_S$ ratio (nonsynonymous to synonymous base substitution) was less than one for all the housekeeping genes and varied from 0·00 for *unc* to 0·29 for *glt* gene (Table 3). The standardized $I_A$ for the whole population was 0·14, which provides evidence of linkage equilibrium in this population.

## Discussion

Retail meat and poultry have been implicated frequently in campylobacteriosis cases in humans (Hänninen *et al.* 2000; Ge *et al.* 2002; Gillespie *et al.* 2002; Moore *et al.* 2003; Fitch *et al.* 2005; Wingstrand *et al.* 2006). Besides the role played by *C. jejuni* in causing illness in humans, *C. coli* has been recovered from humans, animals and retail meat products (Zhao *et al.* 2001; Ge *et al.* 2002; Thakur and Gebreyes 2005; Kang *et al.* 2006; Asai *et al.* 2007; Luber and Bartelt 2007; Stoyanchev *et al.* 2007). In addition, the ability of *C. coli* to exhibit resistance to multiple antimicrobials including fluoroquinolones and macrolides more frequently than *C. jejuni* has raised interest from both a scientific and a public health perspective (Ge *et al.* 2002; Luber *et al.* 2003; Fitch *et al.* 2005; Thakur and Gebreyes 2005; Kang *et al.* 2006; Thakur *et al.* 2006; Asai *et al.* 2007; D'lima *et al.* 2007; Kinana *et al.* 2007; Little *et al.* 2008).

In this study, high frequency of antimicrobial resistance was observed to ciprofloxacin and erythromycin in isolates irrespective of the host. For example, 65% of the human *C. coli* isolates were resistant to erythromycin. Among the isolates from retail meat, *C. coli* from ground turkey samples had the highest frequency of resistance to ciprofloxacin (82%) and erythromycin (46%). These results are similar to other studies which have reported high rates of resistance to fluoroquinolones and macrolides among *C. coli* recovered from turkey products (Ge *et al.* 2002; Luangtongkum *et al.* 2006; D'lima *et al.*




| CVM # | Source | ST | Clonal Complex | PFGE Cluster | MLST Cluster |
|---|---|---|---|---|---|
| 13364 | Chicken | 825 | ST-828 | 1 | 15 |
| 13656 | Chicken | 2626 | ST-828 | 2 | 15 |
| 11199 | Chicken | 825 | ST-828 | 3 | 11 |
| 16761 | Chicken | 829 | ST-828 | 4 | 9 |
| 13747 | Chicken | 1175 | ST-828 | 5 | 36 |
| 16948 | Chicken | 2634 | ST-828 | 6 | 17 |
| 11129 | Chicken | 1175 | ST-828 | 7 | 36 |
| 11130 | Chicken | 1175 | ST-828 | 7 | 36 |
| 23901 | Human | 894 | ST-828 | 8 | 18 |
| 15847 | Turkey | 2638 | ST-828 | 9 | 6 |
| 11457 | Chicken | 2626 | ST-828 | 10 | 15 |
| 13657 | Chicken | 2626 | ST-828 | 11 | 15 |
| 17767 | Human | 1068 | ST-828 | 12 | 23 |
| 17812 | Human | 1068 | ST-828 | 12 | 23 |
| 17832 | Human | 1068 | ST-828 | 12 | 23 |
| 23896 | Human | 1068 | ST-828 | 12 | 23 |
| 23913 | Human | 1068 | ST-828 | 12 | 23 |
| 23889 | Human | 1068 | ST-828 | 13 | 23 |
| 17808 | Human | 1068 | ST-828 | 14 | 23 |
| 17880 | Human | 854 | ST-828 | 15 | 22 |
| 17799 | Human | 1058 | ST-828 | 16 | 24 |
| 23904 | Human | 899 | ST-828 | 17 | 14 |
| 13757 | Chicken | 860 | ST-828 | 18 | 16 |
| 13758 | Chicken | 860 | ST-828 | 19 | 16 |
| 13827 | Chicken | 2628 | | 20 | 20 |
| 15782 | Chicken | 2626 | ST-828 | 21 | 15 |
| 16601 | Chicken | 2632 | ST-828 | 22 | 31 |
| 12865 | Chicken | 829 | ST-828 | 23 | 9 |
| 12868 | Chicken | 829 | ST-828 | 23 | 9 |
| 15661 | Chicken | 829 | ST-828 | 23 | 9 |
| 15663 | Chicken | 829 | ST-828 | 23 | 9 |
| 12912 | Chicken | 829 | ST-828 | 24 | 9 |
| 16535 | Turkey | 1148 | ST-828 | 25 | 13 |
| 23929 | Human | 1082 | ST-828 | 26 | 32 |
| 11263 | Chicken | 1017 | ST-828 | 27 | 21 |
| 23938 | Human | 1470 | | 28 | 34 |
| 13403 | Chicken | 829 | ST-828 | 29 | 9 |
| 15415 | Chicken | 829 | ST-828 | 30 | 9 |
| 17284 | Chicken | 2636 | ST-828 | 31 | 8 |
| 17285 | Chicken | 2636 | ST-828 | 31 | 8 |
| 16767 | Chicken | 1119 | ST-828 | 32 | 10 |
| 11262 | Chicken | 2625 | ST-828 | 33 | 10 |
| 11366 | Chicken | 2625 | ST-828 | 34 | 28 |
| 12908 | Chicken | 829 | ST-828 | 35 | 9 |
| 16530 | Turkey | 2631 | ST-828 | 36 | 12 |
| 11449 | Chicken | 825 | ST-828 | 37 | 11 |
| 17770 | Human | 1123 | | 38 | 33 |
| 23880 | Human | 1112 | ST-828 | 39 | 25 |
| 15419 | Pork | 1107 | ST-828 | 40 | 30 |
| 23914 | Human | 1107 | ST-828 | 41 | 30 |
| 17877 | Human | 854 | ST-828 | 42 | 22 |
| 11200 | Turkey | 2629 | | 43 | 26 |
| 16603 | Chicken | 829 | ST-828 | 44 | 11 |
| 17091 | Turkey | 2643 | ST-1150 | 45 | 3 |
| 16765 | Chicken | 2640 | | 46 | 1 |
| 17852 | Human | 1123 | | 47 | 33 |
| 16705 | Chicken | 860 | ST-828 | 48 | 16 |
| 13821 | Chicken | 1063 | ST-828 | 49 | 19 |
| 17819 | Human | 1099 | ST-828 | 50 | 25 |
| 12864 | Chicken | 829 | ST-828 | 51 | 9 |
| 17136 | Turkey | 832 | ST-828 | 52 | 9 |
| 11136 | Chicken | 2627 | | 53 | 5 |
| 16871 | Chicken | 2633 | ST-1150 | 54 | 2 |
| 15852 | Turkey | 2639 | ST-828 | 55 | 37 |
| 16449 | Chicken | 1017 | ST-828 | 56 | 21 |
| 17083 | Turkey | 2635 | ST-1150 | 57 | 7 |
| 17288 | Chicken | 2637 | ST-1150 | 58 | 4 |
| 13361 | Chicken | 1055 | ST-828 | 59 | 27 |
| 11264 | Chicken | 1119 | ST-828 | 60 | 10 |
| 17829 | Human | 2644 | | 61 | 35 |

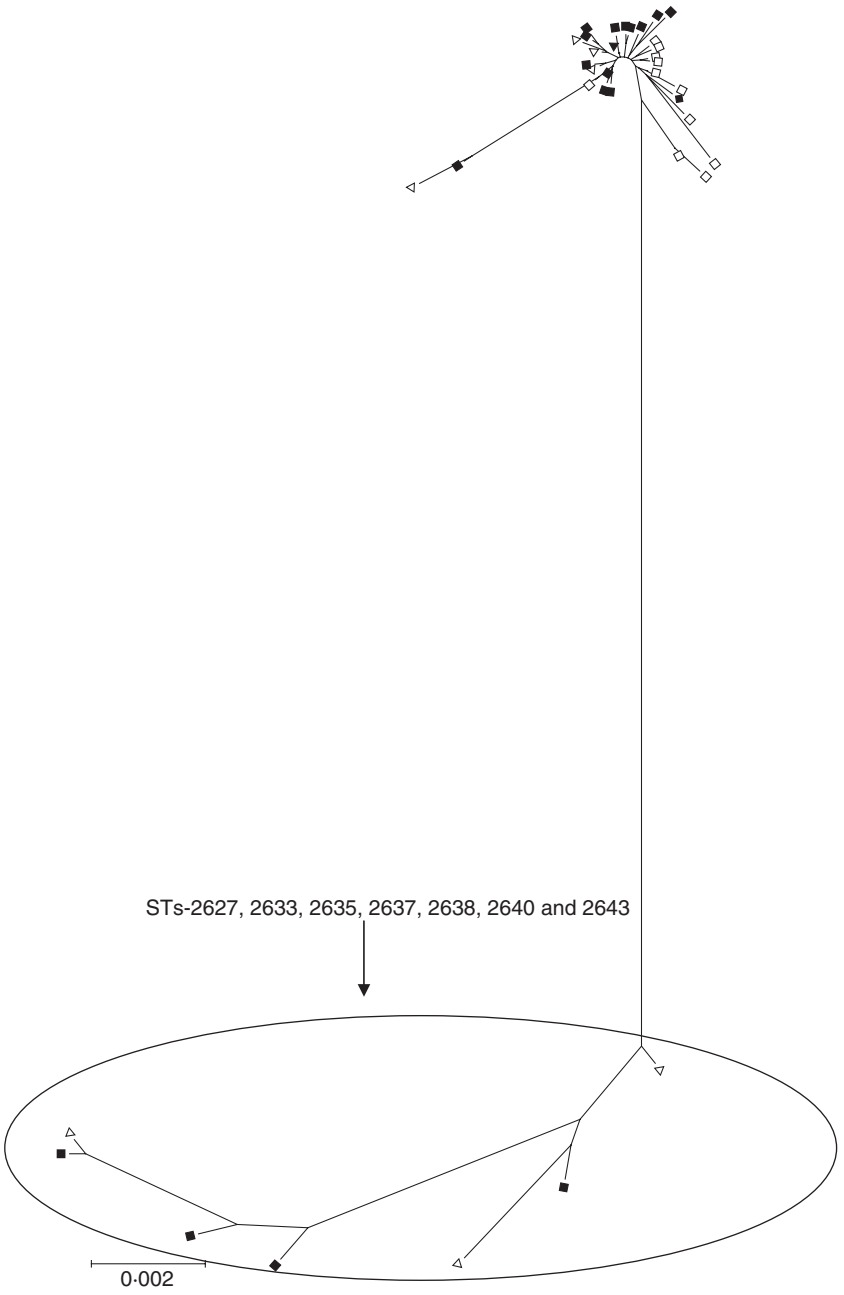



**Figure 2** Radial neighbour-joining tree representing the 37 unique sequence types identified among the 70 *Campylobacter coli* isolates from humans and retail meats. Pork chops and human (♦), human (□), chicken breast (■), ground turkey (△) and chicken breast and ground turkey (▽).

2007). This is concerning from a public health perspective because of fluoroquinolones and macrolides are the antimicrobial agents of choice for treating severe campylobacteriosis cases in humans (Butzler 2004; Yates 2005). It is however important to mention that the retail meat samples were collected in the year 2002–2003 when fluoroquinolones were licensed for use in the poultry industry. The impact of removal of these antimicrobials from the poultry industry in the US in 2005 has yet to be quantified. None of *C. coli* isolates tested showed resistance to gentamicin, which is similar to results from other studies (NARMS, 2008) including isolates originating from chicken, cattle and pigs (Bywater *et al.* 2004) and from retail pork (Ge *et al.* 2002). MDR isolates were observed

**Figure 1** Comparison of PFGE and MLST profiles among *C. coli* isolates. Black boxes indicate resistance to a particular antimicrobial agent. Empty space in the clonal complex column indicates that the particular ST could not be assigned to any complex. CIP, ciprofloxacin; ERY, erythromycin; DOX, doxycycline; GEN, gentamicin; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; ST, sequence type.

from humans and all the retail meat types including the ciprofloxacin/erythromycin/doxycycline and ciprofloxacin/erythromycin patterns. Previous studies have reported the occurrence of MDR strains of *C. coli* from animals, humans and retail meat (Cloak and Fratamico 2002; Payot *et al.* 2004; Gebreyes *et al.* 2005; Thakur and Gebreyes 2005; Little *et al.* 2008). In particular, turkey isolates have been shown to have a higher frequency of MDR compared with other sources including chicken (Ge *et al.* 2002).

In epidemiological investigations relying on molecular strain typing methods, it is very important that the right genotyping method be used. Selection of the appropriate method is challenging when pathogens with hypervariable genomes such as *Campylobacter* spp. are characterized (Parkhill *et al.* 2000; Dingle *et al.* 2001). We therefore compared the discriminatory power of MLST with PFGE and found the latter method, when using either single or double enzymes, to be more discriminatory than MLST. *Campylobacter coli* isolates with the same STs were further differentiated by PFGE typing. This is in contrast to earlier studies where MLST was reported to have a higher discriminatory power than PFGE (single enzyme only) (Thakur *et al.* 2006) or similar power (Duim *et al.* 2003). The restriction enzyme *Kpn*I was found to be a more discriminatory restriction enzyme than *Sma*I in our study. Similar observations have been made previously for typing *Campylobacter* species (Petersen and On 2000; Sahin *et al.* 2008). However, nontypeability of some strains with *Kpn*I and *Sma*I is an issue and was observed both in our study and in a recent study conducted in Puerto Rico (Oyarzabal *et al.* 2008). In addition, genetic variation seen in *Campylobacter* strains is another concern when using PFGE for genotyping (On 1998). A total of three *C. coli* isolates were untypable by either *Sma*I (ID 13361) or *Kpn*I (ID 11264 and 17829) or had too many bands (ID 12864 and 17136), so we were not able to score the bands. Based on these results, we retested them by biochemical methods and PCR and made absolutely sure they were *C. coli*. Also, all the three isolates were typable with the other restriction enzyme as shown in Fig. 1. We specifically included them in the dendrogram because we were able to reconfirm them as *C. coli* and then genotype them using MLST.

Unique STs associated with specific hosts identified in this study could indicate that different food animal rearing conditions and processing might be selecting for particular ecologically fit strains. However, this is in contrast with the results obtained by Miller *et al.* (2006) who reported similar STs shared between humans and animal sources including cattle, pigs and turkey. The wide variety of *C. coli* STs associated with chicken breast suggests that the processed samples may come in contact with different environmental sources during processing, such as rinse tanks or through shared surfaces. For example, Gormley *et al.* (2008) observed similar genotype in *Campylobacter* isolates from retail chicken to the genotype of strains prevalent in their environment. We can also speculate that the different STs found only in specific hosts may reflect host adaptation. This may be especially true for strains that circulate in a given environment, because *in vivo* passage increases the colonization potential of individual strains making them more fit to stably infect a given host.

As expected, high genotypic diversity was revealed among the *C. coli* isolates using both MLST and PFGE typing. This was clearly shown by the association of different antimicrobial resistance profiles within the same ST and host. Unlike our previous study where we found ST-1413 associated only with isolates that were ciprofloxacin resistant (Thakur *et al.* 2006), we found no such association of a specific ST with an antimicrobial resistance pattern. However, we also found ST-1068 among *C. coli* isolates only from humans. This may indicate the potential of this particular ST to cause clinical illness in humans, or reflect a predominant ST circulating in Iowa during the sampling interval. ST-1068 has previously been reported to be clonal and most often associated with cattle (Miller *et al.* 2006). As all the ground beef samples tested were negative for *C. coli*, we could not confirm this observation, however the identification of unique STs associated with human illnesses has been reported with *C. jejuni* (Dingle *et al.* 2005; Kärenlampi *et al.* 2007; McTavish *et al.* 2007; Ogden *et al.* 2007). More studies need to be conducted on *C. coli* ST-1068 to better understand its epidemiology in the environment and its association with human illness.

A group of seven STs (2627, 2633, 2635, 2637, 2638, 2640 and 2643) isolated from chicken breast and ground turkey clustered separately from the main group of isolates in the phylogenetic tree (Fig. 2). All of the STs reported in this study are being reported for the first time and could indicate that this group is evolving away from the main cluster because of recombination or mutational events which have been attributed for population divergence (Colles *et al.* 2003; McCarthy *et al.* 2007; Quiñones *et al.* 2008). Further evidence of genotypic variability was seen in some of the housekeeping genes including *asp*, *pgm* and *tkt* genes (Table 3). The variability in the housekeeping genes of our data set was higher and in a different set of housekeeping genes including the *asp* gene than what has been previously reported (Dingle *et al.* 2005; Fitch *et al.* 2005; Thakur *et al.* 2006; Kinana *et al.* 2007; Litrup *et al.* 2007). We did not find any other report highlighting such high variability in the *asp* gene in the literature. In addition, we found high variability in the *pgm* and *tkt* genes with 19 and 24 variable sites

respectively. It could be possible that this group of *C. coli* strains have recently acquired foreign genomic material due to a recombinational event that has led to such high variability in this region (Miller *et al.* 2006; Kinana *et al.* 2007; Litrup *et al.* 2007; Oyarzabal *et al.* 2008).

The major clonal complex observed in our study was ST-828 complex, which has been previously reported to be the predominant *C. coli* complex with isolates originating from a multitude of sources (Dingle *et al.* 2005; Miller *et al.* 2006; Thakur *et al.* 2006). ST-829 was predicted the clonal complex founder in our study. However, it was ultimately placed under the ST-828 clonal complex. It is quite possible that if we had a bigger pool of *C. coli* isolates and if one of these isolates was ST-828, it would have been predicted as the clonal complex founder. So, we can attribute this observation (ST-829 being the clonal complex founder) to the sample size of our study. The index of association value of 0·143 highlights the recombining nature of this population. The $I_A$ value was lower than what has been previously reported from pigs, broilers, cattle and humans (Thakur *et al.* 2006; Litrup *et al.* 2007). The neighbour-joining tree created by concatenating the sequences showed close clustering of STs from different sources. The majority of the STs that were members of this complex in our study were unique to the host and overlapping STs were observed only in two STs (ST-829 and -1107). These data perhaps indicate a host preference dependent on the *C. coli* genotype; however, further studies with additional isolates obtained from various sources need to be conducted to determine whether this is indeed the case.

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