ORIGINAL ARTICLE

Phenotypic and Genotypic Heterogeneity of *Campylobacter coli* Within Individual Pigs at Farm and Slaughter in the US

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Impacts

- Multiple *Campylobacter* strains (heterogeneity) were detected within individual pigs at farm and on the carcasses at slaughter which highlights the importance of testing more than one isolate per positive sample to make valid interpretations.
- Multidrug resistant *Campylobacter coli* exhibiting resistance to fluoroquinolones and macrolides were isolated from pigs at different stages of production.
- The observed phenotypic diversity within pigs and carcasses was also detected at the genotypic level.

Keywords:
Campylobacter; heterogeneity; antimicrobial resistance; swine; minimum inhibitory concentration; multi locus sequence typing

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Summary

The aim of this study was to determine the phenotypic and genotypic diversity of multiple *Campylobacter* isolates ($n = 3$ per sample) present within individual (heterogeneity) pig faecal and carcass samples at farm and slaughter, respectively. We isolated 1459 *Campylobacter coli* (1110 on farm and 349 from slaughter) from 908 pigs and 757 carcasses and characterized them for their antimicrobial susceptibility profile to a panel of six antimicrobials using the agar dilution method. Overall, we detected a significantly higher *Campylobacter* prevalence at the farm (54.7%) than at slaughter (19%) level ($P < 0.05$). *C. coli* isolates were resistant most commonly to tetracycline (66.2%) and erythromycin (53.6%) while fluoroquinolone resistance was detected in isolates ($n = 17$) only from the farm level. Phenotypic diversity of *C. coli* isolates at the 4-fold minimum inhibitory concentration levels within the same sample was detected in 38.6% ($n = 192$) pigs and 40.2% ($n = 58$) carcass swabs with no significant difference between the two sources ($P = 0.72$). Phenotypic heterogeneity based on the antimicrobial resistance patterns was observed in 32.5% ($n = 162$) of the farm samples and in 30.5% ($n = 44$) carcass swabs at slaughter ($P = 0.64$). A subset of 40 isolates representing ten pigs and eight carcass samples (originating from separate pigs) were further genotyped by multi locus sequence typing. The observation of phenotypic diversity was replicated at the genotypic level, as it was highlighted by the 22 sequence types which represented the 40 isolates. In conclusion, we detected multiple *C. coli* subtypes from individual pig or carcass samples indicating unprecedented level of heterogeneity. Our study clearly signifies the importance of testing multiple colonies to make appropriate and valid conclusions in epidemiological-based studies.

Introduction

Campylobacter is recognized as a leading cause of bacterial foodborne infections in both developing and developed countries. Recent reports from the US indicate that *Campylobacter* was responsible for 5825 cases with an annual incidence of 12.7 per 100 000 individuals (Morbidity and Mortality Weekly Report, 2009). Within the European Union, *Campylobacter* has an annual incidence of 40.7 cases per 100 000 populations which is even higher than the 26.4 cases for *Salmonella* (EFSA, 2010). Even though *Campylobacter jejuni* is recognized as the leading causes of campylobacteriosis in humans, recent studies done in Spain and UK have highlighted the importance of *Campylobacter coli* as a human pathogen due to its more potent antimicrobial resistance and virulence gene profile and in its ability to cause more indigenously acquired foodborne diseases than *C. jejuni* (Saenz et al., 2000; Tam et al., 2003; Thakur et al., 2009). The majority of the epidemiological-based studies that are aimed to determine the diversity of *Campylobacter* and other bacterial pathogens are centred on isolating and characterizing a single isolate from a positive sample. However, a few studies conducted to determine the genotypic diversity of *Campylobacter* in dogs, sheep, pigs and broilers have revealed the importance to characterizing multiple isolates from the individual host to obtain a better understanding of the diversity and epidemiology of the pathogen (Weijtens et al., 1999; Hald et al., 2004; Açik and Cetinkaya, 2006; De Cesare et al., 2008; Hunter et al., 2009). A single study has been conducted in pigs housed under experimental conditions where the authors highlighted the presence of multiple *Campylobacter* strains with individual pigs (Weijtens et al., 1999). However, no study has been conducted in pigs to determine both the phenotypic and genotypic diversity of *C. coli* in pigs at farm and slaughter in commercial herds. A single experimental study on *C. coli* diversity in specific pathogen-free pigs has been conducted (Leblanc-Maridor et al., 2008). One of the characters that can be used for phenotypic characterizing includes determination of the antimicrobial resistance profile. It is also important to substantiate this phenotypic diversity with genotypic data. There are different genotypic approaches that can be employed to determine the pathogen diversity in a host. For genotyping *Campylobacter*, the preferred methods include pulsed field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) (Dingle et al., 2001; Ribot et al., 2001; Thakur et al., 2006, 2009).

In this study, the antimicrobial susceptibility profile and genotype of *C. coli* isolated from the same cohort of pigs sampled at farm and slaughter were determined. The specific aims of this study were (i) to investigate whether pigs were co-colonized with different strains of *C. coli* and (ii) to determine whether phenotypic diversity is also detected at the genotypic level. We analysed the MIC levels and antimicrobial resistance patterns at the phenotypic level and MLST data at the genotypic level to answer the above questions.

Material and Methods

Source of *C. coli* isolates

The isolates in this study originated from pigs that were sampled as part of a longitudinal study conducted in North Carolina. These pigs originated from different unique production systems (conventional and the Antimicrobial free) and were sampled at farm and at slaughter over a 2-year period extending from October 2002 to October 2004 (Thakur and Gebreyes, 2005a). Details of the farm and representative pig population selection process have been described in detail elsewhere (Thakur and Gebreyes, 2005a,b). Briefly, a total of 21 groups of pigs were sampled at the nursery farms (6 weeks of age) and finishing farms (within 48 h of slaughter). The same cohort of pigs was also sampled at the slaughter plant at three stages including pre-evisceration (before viscera is taken out), post-evisceration (after viscera is taken out) and post-chill. Three presumptive *Campylobacter* colonies from each sample (faecal/carcass sample) were isolated in this study. Overall, a total of 1459 *C. coli* (1110 on farm and 349 from slaughter) isolated from 908 pigs and 757 carcasses were characterized at the phenotypic level and representative 40 isolates were further characterized at the genotypic levels (Thakur and Gebreyes, 2005b).

Antimicrobial susceptibility testing

For phenotypic characterization, we determined the minimum inhibitory concentration (MIC) of the isolates against a panel of six antimicrobials using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). These antimicrobials were representative of drugs used in the humans and swine industry. The list of antimicrobials with their abbreviations, range of concentrations (mg/l) and breakpoint levels (mg/l) used are: chloramphenicol (Chl; 0.25–128; 32), ciprofloxacin (Cip; 0.008–4; 4), erythromycin (Ery; 0.06–32; 8), gentamicin (Gen; 0.06–32; 8), nalidixic acid (Nal; 0.25–128; 32) and tetracycline (Tet; 0.06–32; 16). We used *C. jejuni* ATCC 33560 as the quality control organism. The MIC_{50} at which 50% of the isolates tested were susceptible were determined. Multidrug resistance (MDR) was defined as isolates exhibiting resistance to three or more antimicrobials

when tested by the agar dilution method. *C. coli* phenotypic heterogeneity within individual samples was determined based on differences in the MIC level (minimum 4-fold dilution difference) and antimicrobial resistance profiles.

Multi Locus Sequence Typing

A total of 40 *C. coli* isolates representing 10 pigs and 8 carcasses (unrelated to each other) were selected systematically representing the different antimicrobial resistance profiles, temporal and spatial attributes. We considered all the pig batches and the stage of sampling, including all the production stages on farm and slaughter, for isolate selection that were genotyped by MLST. The *C. coli* isolates were genotyped by MLST following the method described previously (Dingle et al., 2005). Briefly, all the housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) were amplified using PCR followed by sequencing of the correct size amplicon product. For sequencing, we used 2 μl of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, CA, USA) with 0.5 μl of 1 : 15 diluted primer, 5.5 μl of molecular grade deionized water and 2 μl of the purified PCR product. Sequencing 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 allelic profiles and the sequence types (STs) were then generated by blasting the correct sequence size on the MLST website from the *Campylobacter* database (www.mlst.net). We used a conservative approach to determine the *C. coli* population structure where isolates with six or more shared alleles at each locus were considered as members of the same clonal complex or lineage. The groups based on the ST data were determined using the eBURST software as recommended previously (Feil et al., 2004).

Statistical analysis

The frequency of antimicrobial susceptibility profiles and MIC of *C. coli* isolates within and between the pigs at farm and carcasses at slaughter level were compared using the χ^2 test (Minitab Inc., State College, PA, USA) and Fisher's exact two-tailed test wherever applicable. A value of $P < 0.05$ was considered statistically significant. Phylogenetic analysis and determination of variable sites in the unique alleles was done using the MEGA software version 4.2.

Results

A total of 1459 *C. coli* (1110 on farm and 349 from slaughter) that were isolated from 908 pigs at farm and 757 carcasses at slaughter were characterized at the phenotypic and genotypic levels to determine the diversity within individual samples. For this purpose, a total of three isolates per positive samples were selected and further characterized. Overall, *C. coli* isolates exhibited highest frequency of resistance against tetracycline (66.2%) followed by erythromycin (53.6%). Resistance against ciprofloxacin was detected in 17 (1.5%) isolates from on-farm samples (Conventional: 13; ABF: 4) only. These isolates were detected in five farms (two conventional and three ABF farms) and represented both the nursery and finishing stages of production. Phenotypic heterogeneity between the three *C. coli* isolates from a single positive sample at the 4-fold MIC differences was observed in 38.2% ($n = 192$) in pigs on farm (Table 1). At slaughter, similar high frequency of diversity was detected between *C. coli* isolates from individual carcass swabs in 40.2% ($n = 58$) of the carcass swabbed. There was no significant difference in the level of heterogeneity based on the 4-fold MIC level difference between samples from the farm and slaughter ($P = 0.72$).

Table 1. Phenotypic heterogeneity of 1665 *C. coli* isolates from pig faeces or pig carcasses based on the MIC (4-fold dilution) and antimicrobial resistance patterns at farm ($n = 908$) and slaughter ($n = 757$), respectively, USA, 2002–2005

Sampling stage	No. of pigs/carcasses tested	No. of pigs carcasses positive (%)	Heterogeneity, *n* (%)[a]	
			MIC (4-fold difference)[b]	Antimicrobial resistance pattern[c]
Farm	908	497 (54.7)	192 (38.6)	162 (32.5)
Slaughter	757	144 (19.0)	58 (40.2)	44 (30.5)
Total	1,665	641 (38.4)	250 (39.0)	206 (32.1)

[a] Represents the total number (%) of *C. coli* isolates exhibiting phenotypic heterogeneity which indicates the presence of *Campylobacter* isolates with different antimicrobial resistance patterns and MIC levels within the same pig/carcass.
[b] Total number (%) of isolates from the same sample exhibiting phenotypic heterogeneity at the 4-fold MIC level.
[c] Total number (%) of isolates from the same sample exhibiting phenotypic heterogeneity at the antimicrobial resistance pattern level.

We detected 19 different resistance patterns (combinations of resistance to different antimicrobials) with Ery^R-Tet^R being the most common pattern irrespective of the stage of production (farm or slaughter). We observed 11 different MDR patterns with resistance to three or more antimicrobials in 79 (5.4%) of the isolates with Ery^R-Nal^R-Tet^R (2.7%) being the predominant pattern. Overall, the heterogeneity based on the resistance patterns was 32.5% ($n = 162$) among the samples from farms and in 30.5% ($n = 44$) carcass swabs (Table 1). There was no significant difference between heterogeneity seen in resistance patterns at farm and slaughter ($P = 0.21$).

A total of 40 *C. coli* isolates, representing ten pigs and eight carcasses samples (originating from separate pigs), that exhibited heterogeneity based on the phenotypic criteria were genotyped by MLST. This subset of *C. coli* isolates was represented by a total of 22 STs including 13 STs from the farm and the remaining nine STs from slaughter (Table 2). We also found STs that were unique to the processing stage including ST-1413 was represented by isolates from the farms exhibiting the Cip^R-Ery^R-Nal^R-Tet^R MDR pattern. Overall, the phenotypic diversity observed within multiple isolates picked up from a sample was also observed at the genotypic level based on the MLST typing data. Among the isolates representing a single pig or carcass, a total of 11 isolates representing two pigs and three carcasses had a different ST but the same resistance pattern. Individual antimicrobial resistance patterns exhibited by *C. coli* isolates from different sources were represented by multiple STs. We also detected the reverse where a particular ST was represented by isolates exhibiting different resistance patterns. A total of three pigs representing six isolates had different genotypic and phenotypic profiles.

Using the eBURST software, we detected three groups within the cluster of 40 isolates that were represented by 22 STs. Group 1 was the largest including 23 isolates and represented by 10 STs. This group consisted of six STs that were unique to slaughter and the remaining four were unique to the farm level. A total of seven STs were designated as singletons and not assigned any group. MLST of *C. coli* isolates in our study highlighted the weak clonal population and the diverse genetic makeup of this species. This observation also holds true for isolates detected within individual pigs and carcasses in our study. The phylogenetic tree constructed by using the 22 unique STs after concatenating the gene sequences (3309 bp) further corroborated the above result showing lack of clustering of the STs based on the processing stage (Fig. 1). Multiple STs detected in the same sample were broadly distributed without any overlap between them. A total of two *C. coli* isolates recovered from the carcasses, including STs-1123 and 1428, and a single ST (1425) from the farm was clustered separately.

Discussion

It is important to determine and understand the diversity of *Campylobacter* in pigs at farm and at slaughter. Isolating a single isolate from a sample does not provide us with sufficient information on the true status of a pathogen in the host. The isolation of a pan-susceptible *Campylobacter* strain from a pig in no way indicates that resistant strains are not prevalent in the same animal. Relatively few studies have been conducted to determine the diversity of *Salmonella* and *Campylobacter* in dogs, pigs, broiler caeca, broiler carcasses and sheep (Weijtens et al., 1999; Hiett et al., 2003; Wonderling et al., 2003; Hald et al., 2004; Açik and Cetinkaya et al., 2006; De Cesare et al., 2008; Hunter et al., 2009). To date, no extensive study has been conducted in swine to determine the phenotypic and genotypic diversity of *Campylobacter* that exists in them.

In this study, we tested three colonies of *Campylobacter* from each of several pigs to get a better understanding of its diversity (heterogeneity) in pigs at the phenotypic and genotypic levels. Considerable phenotypic heterogeneity was observed among the *C. coli* isolated from individual samples at both the 4-fold MIC and antimicrobial resistance pattern levels. In a recent study, strain variation in *Campylobacter* within a host has also been reported from broiler carcasses (Hunter et al., 2009). A study conducted in Italy (De Cesare et al., 2008) reported the prevalence of up to three different subtypes in broiler carcasses which is similar to what has been reported in the US (Hiett et al., 2003). Phenotypic heterogeneity detected in our study indicates the presence of multiple strains of *C. coli* strains in swine necessitates the testing of multiple isolates from a single sample. This result was observed irrespective of the stage of processing (farm or slaughter). The presence of antimicrobial resistant strains in the conventional production system was not surprising. Different antimicrobials including chlortetracycline and the macrolide tylosin are used in the conventional swine production system for prophylaxis and growth promotion; therefore, the findings may imply strong association between antimicrobial use and resistance. It is also possible that other unknown environmental sources are transmitting these resistant *C. coli* strains to pigs. From a food safety perspective this has important implications, especially in scenarios where administration of a particular antimicrobial may not result in appropriate response to the therapy primarily due to infection with multiple strains exhibiting unique resistance profiles. Multiple strains of *C. coli* were detected at the farm level in individual pigs. Our results are similar to previous studies done in pigs which also reported the presence of multiple *Campylobacter* strains within individual pigs and pig herds (Weijtens et al.,

Table 2. Phenotypic and genotypic heterogeneity indicated by the MLST Sequence Type (ST) and Antimicrobial resistance profile of 40 *Campylobacter coli* isolates from ten pigs on farm and eight carcasses at slaughter as part of a study on pigs in USA, 2002–2005

Source	ID	ST[a]	Housekeeping genes							Production stage	Antimicrobial resistance pattern[b]
			aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
Pig 1	524	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	525	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	526	1441	33	38	37	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
Pig 2	548	1419	33	38	46	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	549	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
Pig 3	552	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	553	1413	33	38	30	82	104	117	17	Nursery	Chl^R-Ery^R-Tet^R
Pig 4	554	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	555	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
	556	1413	33	38	30	82	104	117	17	Nursery	Cip^R-Ery^R-Nal^R-Tet^R
Pig 5	447	1443	32	38	44	82	113	43	36	Finishing	Pansusceptible
	448	1434	32	38	44	82	104	43	17	Finishing	Tet^R
Pig 6	458	1439	33	38	30	82	104	173	17	Finishing	Ery^R-Nal^R-Tet^R
	459	1439	33	38	30	82	104	173	17	Finishing	Ery^R-Nal^R-Tet^R
Pig 7	623	1442	33	38	132	82	104	85	17	Finishing	Ery^R-Tet^R
	625	1413	33	38	30	82	104	117	17	Finishing	Tet^R
Pig 8	4516	1425	33	39	134	174	104	43	68	Finishing	Chl^R-Cip^R-Ery^R-Nal^R-Tet^R
	4517	1425	33	39	134	174	104	43	68	Finishing	Chl^R-Cip^R-Ery^R-Nal^R-Tet^R
Pig 9	3813	825	33	39	30	82	113	47	17	Finishing	Chl^R-Ery^R-Tet^R
	3814	825	33	39	30	82	113	47	17	Finishing	Chl^R-Ery^R-Tet^R
Pig 10	4075	1452	33	39	46	82	104	44	17	Finishing	Cip^R-Nal^R-Tet^R
	4076	1424	33	39	132	82	104	44	17	Finishing	Cip^R-Nal^R-Tet^R
	4077	1436	33	39	44	82	104	44	17	Finishing	Tet^R
Carcass 1	2196	1467	33	38	30	167	104	43	17	Pre-evisceration	Tet^R
	2197	1467	33	38	30	167	104	43	17	Pre-evisceration	Tet^R
Carcass 2	3490	1130	33	38	30	82	104	43	68	Post-evisceration	Tet^R
	3491	854	33	38	30	82	104	43	17	Post-evisceration	Tet^R
Carcass 3	4559	887	33	38	30	82	104	85	68	Post-evisceration	Tet^R
	4560	1455	33	38	37	82	104	85	68	Post-evisceration	Tet^R
Carcass 4	4562	887	33	38	30	82	104	85	68	Post-evisceration	Chl^R-Ery^R-Tet^R
	4563	887	33	38	30	82	104	85	68	Post-evisceration	Cip^R-Gen^R-Nal^R-Tet^R
Carcass 5	2205	1113	33	38	30	78	104	35	17	Post-evisceration	Tet^R
	2207	1449	33	38	37	78	104	35	17	Post-evisceration	Tet^R
Carcass 6	4042	1123	53	38	44	82	118	35	36	Post-evisceration	Tet^R
	4044	1123	53	38	44	82	118	35	36	Post-evisceration	Chl^R-Ery^R-Nal^R-Tet^R
Carcass 7	3635	828	33	39	30	82	104	43	17	Post-chill	Pansusceptible
	3637	828	33	39	30	82	104	43	17	Post-chill	Tet^R
Carcass 8	4443	1428	53	38	30	81	118	43	36	Post-chill	Ery^R-Tet^R
	4444	1428	53	38	30	81	118	43	36	Post-Chill	Ery^R-Tet^R
	4445	1428	53	38	30	81	118	43	36	Post-Chill	Ery^R-Tet^R

[a] Sequence types indicates the unique number assigned on the basis of the allelic profile generated based on the allele nucleotide sequence number in the MLST database (www.mlst.net).

[b] Chl, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline.

1993, 1999). The high phenotypic diversity observed in carcasses at the slaughter stage also indicates the pigs come in contact with unique *Campylobacter* strains at the processing environment. This observation was also correlated by the MLST genotypic data where unique STs were detected at the slaughter plants which were different from those detected from the same group of pigs at the farm. It clearly indicates that the slaughter plant environment gets contaminated by pigs coming from different farms which in turn contaminate new batches of pigs. This has been shown before in a study done to determine the status of multiple *Salmonella* strains in pigs at farm and slaughter (Wonderling et al., 2003). However, it is important to highlight that blast chilling had a significant detrimental effect on *Campylobacter* survivability limiting the food safety exposure.

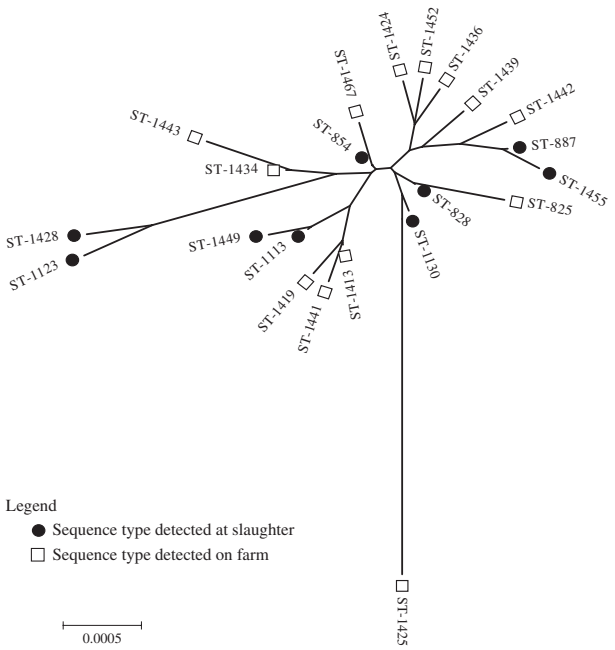

Fig. 1. Radial neighbour-joining tree highlighting the heterogeneity among 22 unique sequence types (ST) representing 40 *Campylobacter coli* isolates from 10 pigs and 8 carcasses at farm and slaughter, respectively, USA, 2002–2005.

In the present study, we report the result of genotyping 40 *C. coli* isolates that represented ten pigs and eight carcasses by MLST. The presence of unique STs within at farm or slaughter indicates that specific STs could have adapted to unique environments. None of the STs representing the 23 isolates from the farm were detected at slaughter. It is possible that multiple *C. coli* genotypes were grouped together in different clusters and that unique STs are likely associated with the processing (slaughter) environment. This could potentially indicate that the pigs get exposed to different *Campylobacter* strains in the slaughter environment by cross contamination through a number of sources including contact with pigs from different farms, during transportation or while resting in the lairage. Similar studies conducted in *C. jejuni* and *C. coli* have reported the association of ST complexes to specific niche (Miller et al., 2006; Thakur et al., 2006). The results of our study highlight the high genotypic diversity of antimicrobial resistant *C. coli* in the swine production environment.

The genotypic diversity of *C. coli* and the detection of unique STs in different production phases could provide these isolate a selective advantage over other bacterial pathogens for rapidly evolving and dominating a particular environment. The absence of shared genotype in isolates from the sow, its respective piglets and the littermates previously highlights the extent of diversity of this pathogen (McCarthy et al., 2007). Similar observations were made when we analysed the STs with respect to the antimicrobial resistance patterns. Barring a few STs that were restricted to specific resistance patterns (ST-1413 was associated with isolates with Cip^R-Ery^R-Nal^R-Tet^R pattern); most of the STs were found to be associated with various resistance patterns again highlighting the diverse genetic makeup of this species. All the STs that were members of the three different clusters in our study were unique and no overlapping STs were detected. This clearly indicates the predilection and preference of particular *C. coli* genotype dependent on either the source (pig or carcass) and origin (farm or slaughter).

In conclusion, the results of our study clearly show the high level of heterogeneity *C. coli* isolates within the individual pig faecal or carcass swab samples. This result signifies the importance of testing more than one *Campylobacter* colony from a host to perform epidemiological studies and make appropriate conclusions. The presence of multiple strains of *C. coli* in the same host may have important implications which can range from identifying the exchange of genetic material, detecting transmission sources and helping in outbreak investigations. 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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