

Article

Identification of Multiple Subtypes of *Campylobacter jejuni* in Chicken Meat and the Impact on Source Attribution

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Received: 17 June 2013; in revised form: 15 August 2013 / Accepted: 29 August 2013 /

Published: 18 September 2013

Abstract: Most source attribution studies for *Campylobacter* use subtyping data based on single isolates from foods and environmental sources in an attempt to draw epidemiological inferences. It has been suggested that subtyping only one *Campylobacter* isolate per chicken carcass incurs a risk of failing to recognise the presence of clinically relevant, but numerically infrequent, subtypes. To investigate this, between 21 and 25 *Campylobacter jejuni* isolates from each of ten retail chicken carcasses were subtyped by pulsed-field gel electrophoresis (PFGE) using the two restriction enzymes *Sma*I and *Kpn*I. Among the 227 isolates, thirteen subtypes were identified, the most frequently occurring subtype being isolated from three carcasses. Six carcasses carried a single subtype, three carcasses carried two subtypes each and one carcass carried three subtypes. Some subtypes carried by an individual carcass were shown to be potentially clonally related. Comparison of *C. jejuni* subtypes from chickens with isolate subtypes from human clinical cases ($n = 1248$) revealed seven of the thirteen chicken subtypes were

indistinguishable from human cases. None of the numerically minor chicken subtypes were identified in the human data. Therefore, typing only one *Campylobacter* isolate from individual chicken carcasses may be adequate to inform *Campylobacter* source attribution.

Keywords: multiple subtypes; chicken carcass; diversity; enrichment; *C. jejuni*

1. Introduction

In 2006, New Zealand (NZ) had the highest reported rates of Campylobacteriosis in the developed world at approximately 385 per 100,000 of population [1]. Subsequent interventions in the poultry industry occurred in the years following [2] and the current rate has declined to 159 per 100,000 of population [3]. Two species, *Campylobacter jejuni* and *Campylobacter coli*, are identified in the majority of human cases of campylobacteriosis and have been isolated from environmental waters, faeces of dairy cows, cattle, sheep, chicken and their derived food products [4,5]. Well known routes of infection include the consumption of undercooked poultry, dairy products and polluted drinking water [6–8]. It has been difficult, however, to establish direct links between human cases and an animal reservoir, or transmission routes such as chicken meat or water [6,9,10]. A review of campylobacteriosis cases in England and Wales has noted an increase in cases particularly between 2004 and 2011 with marked seasonal trends associated with multiple sources of *Campylobacter* highlighting the difficulty of source attribution [11].

Difficulties in source attribution have supported the assertion that most cases of campylobacteriosis are sporadic [8,12,13]. There is concern, however, that at least some of these sporadic infections are not recognised as linked cases because not all of the available subtypes have been identified from either the clinical specimens or the suspected source [14]. For example, in an outbreak of campylobacteriosis linked to undercooked chicken liver pâté, there were multiple strains of *C. jejuni* belonging to four distinct clades isolated from the stool samples of cases [15]. Co-infection with multiple *Campylobacter* strains was documented in several of the cases and highlights the requirement for recognition of multiple infecting strains in outbreak cases. This study provides a counterexample to the “dominant outbreak strain” recognised for many foodborne outbreaks [15].

Co-infection of human cases with more than one *C. jejuni* subtype is not common, but might occur in up to 10% of campylobacteriosis cases [5,16–18]. In a review addressing co-infections by multiple strains of a pathogenic species, Balmer and Tanner suggested that the infrequency of notifications of multiple infections in a single host may change as studies increasingly apply molecular tools to identify clonal diversity [19]. Identification of minor subtypes from chicken carcasses may contribute to knowledge about the association of particular *C. jejuni* subtypes with that food and whether all subtypes identified in chickens contribute to human disease. Several studies have identified multiple clones of *Campylobacter* in chickens [20,21] and a high degree of genotypic relatedness has been noted between different subtypes isolated from individual flocks [22].

Due to the diversity of *Campylobacter* subtypes, the isolation and typing of one colony from individual environmental samples may only be valid when testing large numbers of samples over an extended time period. When investigating epidemiologically-linked cases, however, multiple colonies

from a suspected source may need to be typed to provide an accurate assessment of the presence of pathogenic subtypes [14]. The distribution of *C. jejuni* subtypes in the environment may be more accurately represented if numerically dominant and minor subtypes from the same sample are identified.

Dopfer *et al.* addressed the issue of how many isolates of a bacterial species need to be analysed when assessing the genetic heterogeneity within an individual sample [14]. Using Bayesian statistical methods, they determined that from 2–20 isolates per sample need to be characterised to ensure detection of all strains in a sample with 95% certainty. Studies of *E. coli* subtypes in human faeces discussed the importance of multiple isolations from a single stool sample to identify both dominant and minor subtypes [23]. They caution that basing a study on the isolation of numerically dominant subtypes of a bacterial species may exclude significant information, especially where the study is examining the frequency of virulence factors in a bacterial population. Schlager *et al.* [23] defined a dominant subtype as a clone which is represented by >50% of typed isolates in a sample. A minor subtype was defined as a clone that represented <10% of typed isolates in a sample. Studies of *E. coli* clonal diversity in individual samples have used a binomial formula to determine the number of randomly selected colonies required to achieve a 90% probability of identifying a minor clone: $1 - (1 - p)^n$, where p is the frequency of the minor clone and n is the number of colonies tested [23].

This current study presents subtyping data for multiple *C. jejuni* colonies isolated from individual chicken carcasses using an enrichment method. Isolates were subjected to pulsed-field gel electrophoresis (PFGE) typing with the restriction enzymes (RE) *SmaI* and *KpnI*. The binomial formula of Schlager *et al.* [23] was applied to establish a statistical basis for identifying minor clones from a single sample. Up to 25 colonies per sample were isolated to give a 93% probability of identifying numerically minor subtypes. The aim was to determine whether all subtypes isolated from chicken carcasses are pathogenic to humans by comparing them to NZ human clinical subtypes of *C. jejuni* collected nationwide. The data set from human cases included a subset of human isolates collected from the same geographical area and over the same timeframe as the chicken samples [4]. In addition, the recognition of clonally related subtypes from the same sample and/or human case was recognised as potentially significant for the epidemiological tracking of outbreak sources.

2. Results and Discussion

2.1. Identification of Multiple Subtypes of *C. jejuni* in Chicken Carcasses

To address whether identification of minor subtypes in poultry increases the likelihood of identifying *C. jejuni* subtypes relevant to the human etiology of campylobacteriosis, we determined the diversity of subtypes on fresh chicken carcasses. Twenty-five isolates per carcass were collected; during storage however, several isolates were not recovered, reducing the number of isolates for some samples to less than 25 (range 21–25 isolates per sample). Purified isolates were Gram negative, oxidase, catalase and hippurate positive, grew microaerophilically at 42 °C but not aerobically at 25 °C, and were nalidixic acid susceptible and resistant to cephalothin, confirming their identification as *C. jejuni*. In total, 227 isolates were characterized by *SmaI* PFGE to identify subtypes of *C. jejuni*. To differentiate isolates further, 15–16 isolates per chicken carcass sample (total isolates $n = 157$) were subjected to *KpnI* digestion and electrophoresis.

Nine of the chicken carcasses carried one dominant subtype, represented by >50% of the typed isolates as defined by Schlager *et al.* [23]. One of the chickens (E) carried two subtypes with similar prevalences of approximately 50%, which were referred to as co-dominant. Thirteen distinguishable subtypes were identified from the ten carcasses based on the two RE profiles; four (40%) carried more than one subtype of *C. jejuni* (Table 1, Figure 1). In these four chicken carcasses (B, C, D and E) carrying multiple subtypes, two subtypes were present in three carcasses and three subtypes present in one carcass. The percentages of minor and dominant subtypes identified in three (B, D and E) were based on the total number of isolates per sample because the *KpnI* digestion did not differentiate the *SmaI* subtype (Table 1). Chicken C carried three subtypes that could not be digested with *SmaI* RE but were resolved by *KpnI* digestion, and therefore the percentage calculations for this sample were based on the number of isolates subjected to *KpnI* digestion only ($n = 16$).

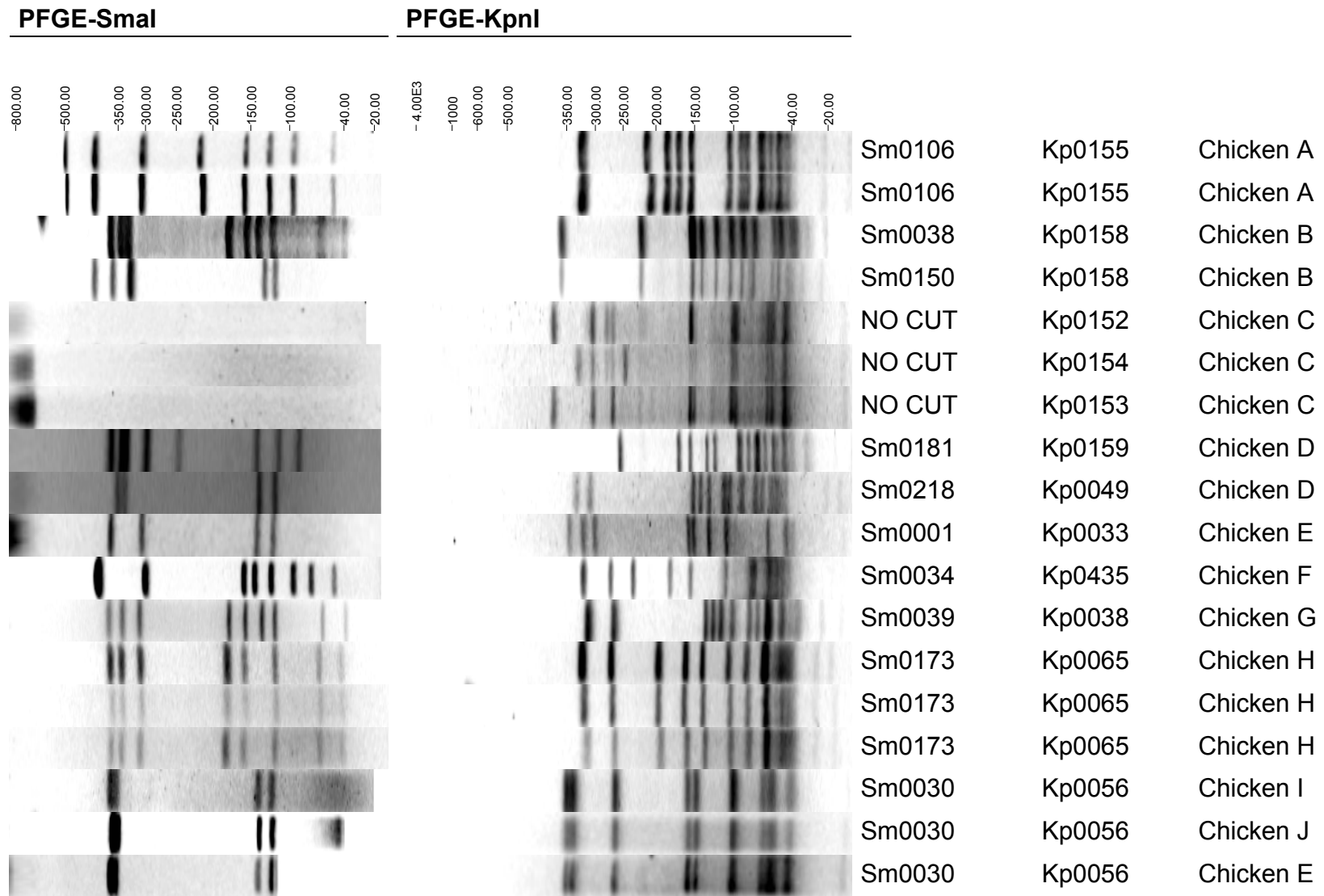
Table 1. *Campylobacter jejuni* subtypes isolated from chicken carcasses.

Chicken Sample	PFGE Subtype	Subtype Prevalence	Human Case Identity ^a	Identity to Other Sources ^a
A	Sm0106/Kp0155	Single subtype	1	None
B	Sm0150/Kp0158	Dominant (96%)	None	River water
	Sm0038/Kp0158	Minor (4%)	None	None
C	NC ^b /Kp0152	Dominant (81%)	None	None
	NC/Kp0153	Intermediate (12%)	None	None
	NC/Kp0154	Minor (6%)	None	None
D	Sm0218/Kp0049	Dominant (77%)	9	Chicken meat, sheep faecal
	Sm0181/Kp0159	Intermediate (23%)	None	Chicken meat
E	Sm0030/Kp0056	Co-dominant (52%)	3 ^c	Chicken meat
	Sm0001/Kp0033	Co-dominant (48%)	3	Chick faeces
F	Sm0034/Kp0435	Single subtype	1	None
G	Sm0039/Kp0038	Single subtype	32	Dairy cow and chicken faeces, chicken meat, pig meat and sheep offal
H	Sm0173/Kp0065	Single subtype	1	None
I	Sm0030/Kp0056	Single subtype	3 ^c	Chicken meat
J	Sm0030/Kp0056	Single subtype	3 ^c	Chicken meat

^a Isolates from chicken samples A–J compared to human and other isolates, which have *SmaI/KpnI* designations in the PulseNet Aotearoa NZ *Campylobacter* database; ^b Non-cutting by *SmaI* restriction enzyme; ^c The same PFGE profile carried by three chicken samples.

Subtype Sm0030/Kp0056 was identified in three chicken samples (E, I, J), collected at least six weeks apart. A band of approximately 25 kb was intermittently observed in the DNA fragments from subtype Sm0030/Kp0056 isolated from chicken sample J, suggesting it may represent a plasmid [24] (Figures 1 and 2). In designation of a *SmaI* banding profile, only bands between 700 and 50 kb were included, therefore the intermittent nature of this band did not affect the interpretation of members of this subtype.

Figure 1. Pulsed-field gel electrophoresis (PFGE) profiles of *Campylobacter jejuni* representing all chicken carcass isolates.



The literature reports differences in the numbers of subtypes identified in individual samples when tested by various methods. A study using enrichment methods for isolation of campylobacters, identified only one subtype per chicken carcass when up to five isolates per sample were typed [25]. In comparison, studies using direct plating or a combination of enrichment or direct plating noted higher numbers of subtypes (two–five) in *Campylobacter* positive carcasses when, on average, less than seven colonies per carcass were tested [26–28].

It is probable, given the selective nature of enrichment broths such as modified Exeter (m-Exeter), that growth characteristics of each environmental *Campylobacter* subtype in the broth will vary. The degree of this variation is unknown and consequently, if the initial sample contained equal numbers of two subtypes, it is possible that one may grow faster and be identified by subtyping as being dominant due to its growth characteristics. The same premise will hold for direct plating where injured/stressed cells have a lower probability of recovery in comparison to enrichment [26]. It is recognized that the problem of selection of dominant strains may be overcome by employing more than one procedure for each sample to enable identification of individual strains that vary under different growth conditions [5].

None of these factors undermine the use of the enrichment-PCR method as a tool for the long term isolation of campylobacters from a wide range of environments. It does suggest, however, that if studies are time-limited and sample numbers are small then enrichment should be combined with direct plating or enrichment should employ two incubation temperatures, for example, 37 °C and 42 °C [29]. This may achieve a higher diversity of subtypes from individual samples with less input of labour, as fewer colonies per method may need to be typed. A comparative study of the diversity of subtypes isolated by enrichment *versus* direct plating may be worthwhile to establish the benefits of either method and to determine if it is better to use both methods and subtype only one or two colonies isolated from each.

2.2. Comparison of *C. jejuni* Isolates from Chicken Carcasses and Human Clinical Samples

To increase understanding of the impact of chicken consumption on the number of campylobacteriosis cases, it is important to determine what proportion of *C. jejuni* subtypes identified on chicken carcasses are indistinguishable from those in human faecal specimens. Assumptions drawn from case studies and experimental data suggest up to 80% of human cases can be attributed directly to chicken preparation and consumption [11,30]. Multiple subtypes of *C. jejuni* are present in chickens [31], and it has been suggested that some of the subtypes causing disease in humans may be minor strains in the chicken microflora and therefore, not identified by conventional bacteriological techniques where only a single colony is typed [32].

This study addressed whether the numerically minor subtypes identified on chicken carcasses were associated with human cases. Confirmation of this finding would imply that numerically non-dominant subtypes in chicken meat are important in the aetiology of campylobacteriosis, and suggest that isolation techniques need to be directed toward identifying both numerically dominant and non-dominant subtypes in a sample.

Results of the comparison between *C. jejuni* isolates from humans and chicken carcasses are presented in Table 1. Additional information on the identification of the *SmaI/KpnI* profiles in other matrices and other chicken meat samples from previous studies is also shown after comparison with *Campylobacter* isolates in the PulseNet Aotearoa NZ database, 6 of the 13 *SmaI/KpnI* subtypes

identified in this study (Table 1), had not previously been observed from cases of campylobacteriosis ($n = 1248$) in NZ. Furthermore, five of these six subtypes had not been observed from any other matrix besides chicken meat. Three chicken samples (A, F and H) carried single subtypes that were each observed in one clinical case, but not from any other matrix, including chicken samples collected outside of this study. The seven subtypes which were identified in clinical samples were isolated from eight of the ten chicken carcasses analysed in this study. In addition, 2 of these subtypes (dominant and a sole subtype) were identified in 9 and 32 human clinical samples each, suggesting that these subtypes are important in the epidemiology of campylobacteriosis in NZ. In total, 50 clinical isolates in the database were indistinguishable from chicken isolates from this current study; however, none of the human isolates were collected in the same geographical location or timeframe as the chicken isolates.

In the three chickens that carried minor and/or intermediate subtypes, the numerically non-dominant subtypes did not correlate with any isolates from human cases. The fourth chicken (E), as previously mentioned, carried two co-dominant *C. jejuni* subtypes; and each of these subtypes was identified in three human cases. The subtype (Sm0030/Kp0056) was also identified in two other chicken carcasses (I and J), which were independent samples, having been collected at different times. The results from this study imply that the non-identification of minor/intermediate subtypes using the enrichment-PCR method from chicken carcasses may not be significantly limiting information relevant to tracking the source of an infection to chicken carcasses. Confirmation of this suggestion regarding numerically non-dominant subtypes would require a larger dataset of multiple subtypes collected from a large number of chickens and compared with human isolates from the same geographical area and timeframe.

2.3. Relevance of Clonally Related Campylobacter Subtypes

During this investigation, the question of the relevance of clonally related subtypes occurring in the same sample was identified as warranting further research in regards to outbreak investigations. An additional point, relevant to outbreak studies, is the identification of multiple subtypes (albeit infrequently) in individual human cases of campylobacteriosis [16,17].

The benefits of utilizing a double RE digestion were shown with Chickens B and E. For Chicken B, one isolate (labeled minor) had the same *KpnI* profile as the other isolates in the same sample, but a different *SmaI* pattern (Sm0038) (Figure 1). In Chicken E, the *SmaI* fingerprint for the two subtypes suggested that they were visually similar and could be clonally related (Figure 1). Further discrimination revealed by the *KpnI* digest, however, suggested no clonal relationship.

Chicken C carried potentially clonally related subtypes (Figure 1). In Chicken C, the three *C. jejuni* strains which produced *KpnI* profiles could not be digested by *SmaI* (subtypes: Non-cutting/Kp0152–Kp0154). The *KpnI* profiles of two of these *C. jejuni* strains were clonally related by the criteria of Tenover *et al.* in that Kp0152 had a band at approximately 270 kb which was absent in Kp0153 [33]. Profile Kp0154, although looking superficially similar to Kp0152 and Kp0153, had too many different bands from either subtype (five and four band differences, respectively) to be considered clonally related. One of the clonally related subtypes was intermediate in prevalence (12%) suggesting it would have a low probability of being identified. If the numerically dominant subtype was the only subtype isolated from the chicken, and the minor or intermediate chicken subtypes were present in the human case, then the link between the source and the case would have been missed. This

highlights the need to examine both indistinguishable and closely related banding patterns when correlating human and chicken isolates. It should be noted, however, that in the current study, none of the potential clonal subtypes from the same carcass were identified in human cases. In addition, the definition of dominant and minor subtypes in a sample may be dependent on the isolation method employed. Future comparisons of multiple subtypes in a sample could, therefore, include quantitative studies such as quantitative PCR to clarify which *C. jejuni* subtypes are numerically dominant in a sample [34].

There were two examples of subtypes identified in human clinical samples that were potentially related to chicken subtypes raising the question whether the human and chicken subtypes would be recognised as potentially clonally related in an outbreak investigation (Figures 2 and 3). Both of the co-dominant subtypes from Chicken E were clonally related to human subtypes. The *KpnI* profile of chicken isolate Sm0030/Kp0056 is presented in Figure 2 along with the three human faecal isolates that had an indistinguishable profile by *SmaI* and *KpnI* digestion. In addition, the human faecal isolate 539 (Sm0030/Kp0012) is shown in Figure 2. Clinical isolate 539 has the same *SmaI* profile as the chicken subtype Sm0030/Kp0056 but a different *KpnI* profile. This *KpnI* profile (Kp0012) is, by the criteria of Tenover *et al.* “possibly related” to the chicken isolate, because it has four band differences to Kp0056 [33]. These band differences are consistent with the occurrence of two genetic events, probable deletions in the bands occurring at approximately 350 and 175 kb in Kp0056 and appearing as bands at approximately 250 and 130 kb (respectively) in Kp0012.

Subtype Sm0001/Kp0033, which was identified in 48% of the isolates from chicken carcass E, was identified in three human cases of campylobacteriosis. In addition, this subtype is potentially clonally related to Sm0001/Kp0032 which was isolated from a human case of campylobacteriosis and from a sample of roof water (Figure 3). The band at approximately 160 kb in Kp0033 does not appear in Kp0032, but two bands appear at approximately 70 and 90 kb in Kp0032 (Figure 3). This is suggestive of a single genetic event possibly resulting in a new RE site in the 160 kb fragment (Kp0033) which is cleaved by *KpnI* into two fragments of 70 and 90 kb (Kp0032). Further genotypic and phenotypic characterisation of the potentially clonally related subtypes would be required to determine their degree of relatedness. The genotypic plasticity inferred by these isolates and their putative relatedness must be kept in context as Sm0030/Kp0056 was isolated from two other chicken samples in this study that were collected at different times, the closest interval between the three chicken samples being six weeks. Other studies have recognized that some strains of *Campylobacter* appear to remain genetically stable under both environmental and laboratory conditions [35,36].

Figure 2. Clonal relationships between *Campylobacter jejuni* human isolates and subtype Sm0030/Kp0056 from chicken meat.

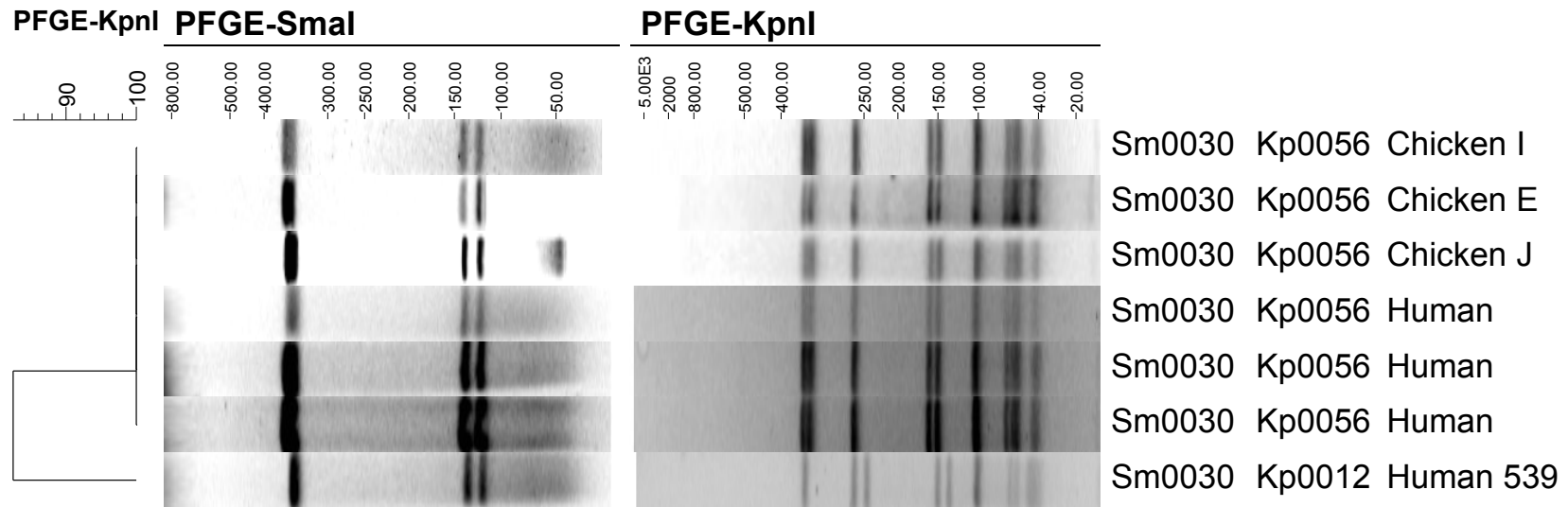
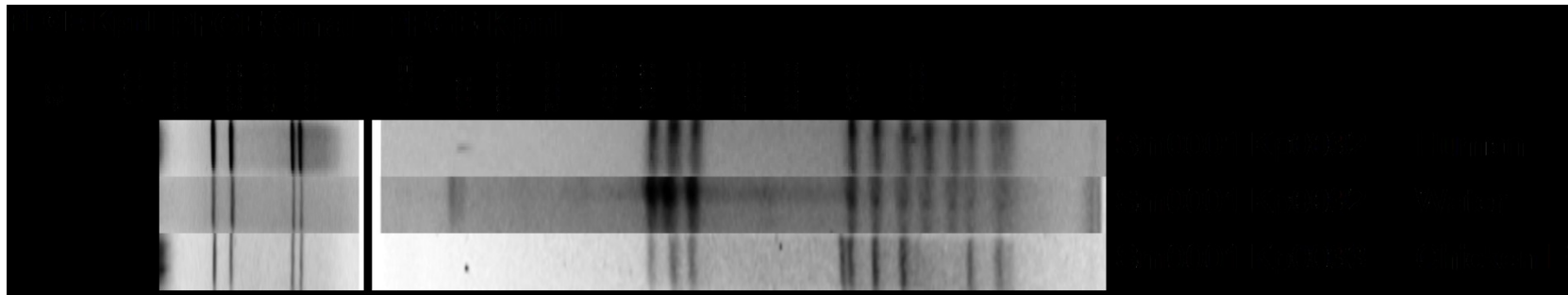


Figure 3. Clonal relationships between *Campylobacter jejuni* human and environmental isolates and subtype Sm0001/Kp0032 from chicken meat.



2.4. Chicken as a Vehicle for Transmission of *Campylobacter* to Humans

Eight of the ten chicken carcasses carried seven pathogenic subtypes, but none of the minor/intermediate subtypes were implicated in campylobacteriosis cases. Identification of non-dominant chicken subtypes such as those identified in this study; therefore, do not support the hypothesis that minor subtypes carried by retail chicken contribute to the transmission of *Campylobacter* to humans. The proportion of subtypes from chickens (6 of 13) that were not identified in human cases raises the question as to whether those subtypes are adapted to colonise chickens or the processing environment, as 5 of these were not identified in any matrix other than chicken meat. Further investigation of multiple isolates from a larger sample size of chicken carcasses would be required to confirm this proposal, and could lead to an investigation of virulence factors in pathogenic subtypes. A time-course study of the co-colonisation of chickens with multiple strains of *C. jejuni* previously isolated from chickens, reported that two of the seven *C. jejuni* strains were dominant in the *Campylobacter* flora [31]. In addition, there was a shift in the dominant strain during the investigation, which the researchers ascribed to influences from the chicken host immune response rather than differences in background flora. Studies of the co-infection dynamics of campylobacters in poultry are important for understanding *Campylobacter* epidemiology, in particular, whether pathogenic strains dominate the infection cycle in chickens or can be outcompeted by campylobacters of less significance to the human population.

The lack of correlation between subtypes in chicken carcasses and the 61 human cases from the same region and timeframe was unexpected. A factor that may have affected this result was the rural nature of the region where the study was conducted. The fact that eight of the ten chickens were contaminated with subtypes implicated in human campylobacteriosis, but not from the human cases within the study region, may suggest other routes of transmission of campylobacters in this rural area. We have previously shown that cases mainly occurred in abattoir workers, farmers, their families and farm workers, and from the largest town in the area, which had a population of 14,000 [4,9]. Subsequent analysis of human cases and subtypes isolated from the environment revealed the highest similarity to human cases was with subtypes isolated from ruminant animals (sheep, dairy cows and cattle). The greater similarity between human and ruminant isolates may suggest that close contact between farm/abattoir workers and the animals they work with is a more likely route of *Campylobacter* infection in comparison with chicken consumption for this rural community. A study in Finland typed human isolates ($n = 176$) from two geographically distinct rural and urban areas and their findings also supported a potential distinction between routes of infection in rural and urban environments [37].

3. Experimental Setup

Fresh chicken carcasses were collected from retail stores in a rural township and transported to the laboratory under refrigeration to be processed by a m-Exeter enrichment-Polymerase Chain Reaction (PCR) method within 24 h [4,38]. In brief, whole chicken carcasses were aseptically transferred into a sterile plastic bag with 250 mL of sterile buffered peptone water (pH 7.2, BPW) (1.07228, Merck, Darmstadt, Germany). After massaging the carcass with the BPW for 2 min, 10 mL of the chicken rinse was transferred to a sterile Whirl Pak bag. 90 mL of enrichment broth was added and the sample

mixed in a Colworth Stomacher 400 (A.J. Seward, London, UK) for 15 s. The primary enrichment was incubated for 4 h at 37 °C and then incubated for a further 44 h at 42 °C. All incubations were carried out in a 10% CO₂ incubator [39]. After 48 h, 100 µL of primary enrichment was transferred to 10 mL of m-Exeter broth, and incubated at 42 °C for 24 h under the same conditions. Preparation of *Campylobacter* cells for DNA extraction from enrichment broth and analysis by multiplex PCR is described by Wong *et al.* [38]. In brief, 1 mL of enrichment broth was removed, centrifuged (4000g, 20 min) and the pellet washed three times in 1 mL of sterile phosphate buffered saline (PBS; BR14a Oxoid, Hampshire, UK) by vortexing and centrifugation (4000g, 10 min). Final re-suspension of washed pellet was in 400 µL of PBS prior to heat lysis preparation for DNA amplification [38].

3.1. Characterisation of *C. jejuni* from Chicken Carcasses

Ten chicken carcass enrichments positive for *C. jejuni* by PCR were spread onto m-Exeter plates and 25 individual colonies per sample purified by streaking onto Columbia blood agar (CBA, Merck, Darmstadt, Germany) and incubated at 42 °C under 10% CO₂ [39]. Isolated colonies were stored in cryovials containing glass beads (3 mm, Ajax Finechem, Taren Point, Australia) and 20% glycerol (BDH Laboratory supplies, Poole, England) in Brain Heart Infusion broth (Difco, Detroit, MI, USA) in a –80 °C freezer. The ten chicken carcasses were collected over seven months.

3.2. Colony Identification by PCR

Identification of bacterial colonies by PCR directly from plate cultures, was achieved by removal of a portion of a single isolated colony and resuspension in 27.0 µL of sterile distilled deionized water in a 0.5 mL thin-walled PCR tube. Within 15 min from the time of colony resuspension, the tube was heated for 3 min at 100 °C and then cooled to 4 °C. Prepared premix was added to the PCR tube to obtain a final volume of 50 µL. Details of PCR premix preparation and PCR analysis is outlined in Wong *et al.* [38]. Initially, isolates from the enrichment were confirmed as *C. jejuni* by PCR analysis of a single colony per enrichment sample. Subsequently, PCR confirmation was performed on up to five isolates per PFGE subtype identified in each chicken carcass [26].

3.3. Colony Identification by Biochemical Analysis

Phenotypic characterisations of up to four isolates per PFGE subtype identified in each chicken carcass were subjected to confirmatory identification by the following biochemical tests; Gram stain, hippurate hydrolysis, catalase and oxidase reactions, nitrate reduction, susceptibility to nalixidic acid and resistance to cephalothin [40,41]. Lack of growth at 25 °C and under aerobic conditions was also verified for the isolates. Positive controls of *C. jejuni* (Enteric Reference Laboratory (ERL) 96 3376) and *C. coli* (ERL 97/454) were included for all tests.

3.4. Subtyping of *C. jejuni* by Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed using the 24 h PulseNet protocol for the analysis of Gram negative microbes [42]. *Salmonella* serotype Braenderup H9812 [43] was used as the molecular size standard and Megabase agarose (Bio-Rad Laboratories, Hercules, CA, USA) was used to make

the plugs. All *Campylobacter* isolates were subjected to restriction enzyme digestion using *SmaI* enzyme (Roche, Applied Science, Penzberg, Germany). A subset of the 25 isolates from each chicken sample was restricted with a second enzyme *KpnI* (New England Biolabs (NEB), Hitchin, UK). Due to resource constraints not all of the 25 isolates from each chicken sample were analysed by *KpnI* digestion and electrophoresis. A statistical method termed the Sampling Attribute Plan was employed to calculate the sample size required to determine 95% confidence levels for the results [44]. If the sample number was 15 from the batch of 25 indistinguishable *SmaI* subtypes, and none of the 15 isolates produced a different *KpnI* profile then there would be 95% confidence that the batch of 25 will have no more than five isolates (0%–21.8%) with a different *KpnI* profile.

Gel electrophoresis was carried out on a CHEF DRIII system (Bio-Rad) with the cooling module set at 14 °C. The image was captured with a Gel Doc 2000 gel documentation system (Bio-Rad), and band patterns analyzed and compared using BioNumerics software (Applied Maths, Kortrijk, Belgium). Only fragments in the range 700 to 50 kb were analyzed for *SmaI*, and 700 to 80 kb fragments were analyzed for *KpnI* [10]. Smaller fragments were not consistently resolved. The analytical parameters were the band-based Dice similarity coefficient and the unweighted pairs geometric matched analysis (UPGMA) dendrogram type with a position tolerance setting of 1.5% for optimization and position tolerance of 1.5% band comparison. All test isolates were normalized to the known molecular size bands of the *Salmonella* serotype Braenderup H9812 standard subtype [43]. In addition, all gels were run with a well-characterised subtype of *C. jejuni*, CPH011453, which acted as a control for the digestion reaction for both restriction enzymes.

3.5. Correlation of Chicken Carcass Isolates and Subtypes Identified in Human Clinical Samples

The PFGE profiles of isolates identified from chicken carcasses were compared with profiles in the PulseNet Aotearoa NZ *Campylobacter* database. At the time of comparison, the database contained data for 3301 isolates collected nationwide, including 1248 *C. jejuni* human clinical isolates, 581 chicken meat, 199 bovine meat, 171 river water, and 221 bovine, 93 ovine, 295 chicken and 66 duck composite/individual faecal sample isolates. The 1248 *C. jejuni* isolates from human sources included 61 human clinical isolates obtained from the same geographical area and within a one year period of the chicken carcass isolates. Human clinical isolates of *C. jejuni* in the database which had been analysed for *SmaI* but not *KpnI* and were of interest to this study were subjected to PFGE *KpnI* digestion.

3.6. Determination of Related PFGE Subtypes

Interpretative criteria for determining relatedness between isolates have been proposed by Tenover *et al.* for outbreaks of pathogens [33]. It is more difficult, however, to apply these criteria over the time period of longer-term studies. Ribot suggests that studies which collect samples over a period of more than one year require more careful interpretation of results [45].

The isolates for this study were collected over a seven month sampling period and therefore analysis of related PFGE subtypes was based on a conservative interpretation of the criteria developed by Tenover *et al.* [33]. All subtypes defined as clonally related had to have information for both restriction enzymes (RE), which suggested that both profiles were either indistinguishable or clonally

related within their respective RE digestion profile. For the purposes of this study, PFGE subtypes were considered to be “clonally related” when:

(i) The subtypes digested with the same enzyme differed by one band shift which indicated a single genetic event had occurred resulting in a DNA fragment running as a larger or smaller band due to either an insertion or deletion of DNA (respectively).

(ii) A large molecular weight band was replaced by two smaller molecular weight bands, the sum of whose DNA approximated the original larger molecular weight band. This change in PFGE pattern using the same RE, represents a single genetic event, indicative of the gain of a new restriction site resulting in the formation of two new bands, and the loss of the larger molecular weight band.

(iii) A third category corresponding to “possibly related” was applied where the changes between patterns was consistent with two separate genetic events each corresponding to an insertion/deletion of DNA (as outlined in section i) and/or the gain of a restriction site (as outlined in section ii). These two independent events together result in 4–6 band differences between patterns and have been observed in isolates collected over periods longer than six months.

4. Conclusions

The results of this study do not support the suggestion that failure to identify numerically minor subtypes in chicken carcasses is preventing the attribution of campylobacteriosis outbreaks to chicken consumption. The small sample number of chicken carcasses, however, is a limiting factor in the information generated by this study and larger surveys are required to clarify these findings. The analysis of multiple isolates from a single sample and recognition of potentially, clonally related subtypes linking human cases to an environmental matrix may help to clarify the source(s) of an outbreak.

Acknowledgements

The authors would like to thank Susan Lin for advice and assistance with paper preparation.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. *Notifiable and Other Diseases in New Zealand*; Annual Report 2006; ESR: Porirua, New Zealand, 2006; p. 13.
2. Mullner, P.; Spencer, S.E.; Wilson, D.J.; Jones, G.; Noble, A.D.; Midwinter, A.C.; Collins-Emerson, J.M.; Carter, P.; Hathaway, S.; French, N.P. Assigning the source of human campylobacteriosis in New Zealand: A comparative genetic and epidemiological approach. *Infect. Genet. Evol.* **2009**, *9*, 1311–1319.
3. *Notifiable and Other Diseases in New Zealand*; Annual Report 2012; ESR: Porirua, New Zealand, 2012; p. 25.

4. Devane, M.L.; Nicol, C.; Ball, A.; Klena, J.D.; Scholes, P.; Hudson, J.A.; Baker, M.G.; Gilpin, B.J.; Garrett, N.; Savill, M.G. The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes. *J. Appl. Microbiol.* **2005**, *98*, 980–990.
5. Kramer, J.M.; Frost, J.A.; Bolton, F.J.; Wareing, D.R. *Campylobacter* contamination of raw meat and poultry at retail sale: Identification of multiple types and comparison with isolates from human infection. *J. Food Prot.* **2000**, *63*, 1654–1659.
6. Gillespie, I.A.; O'Brien, S.J.; Adak, G.K.; Tam, C.C.; Frost, J.A.; Bolton, F.J.; Tompkins, D.S. Point source outbreaks of *Campylobacter jejuni* infection—Are they more common than we think and what might cause them? *Epidemiol. Infect.* **2003**, *130*, 367–375.
7. Kuusi, M.; Nuorti, J.P.; Hanninen, M.L.; Koskela, M.; Jussila, V.; Kela, E.; Miettinen, I.; Ruutu, P. A large outbreak of campylobacteriosis associated with a municipal water supply in Finland. *Epidemiol. Infect.* **2005**, *133*, 593–601.
8. Taylor, E.V.; Herman, K.M.; Ailes, E.C.; Fitzgerald, C.; Yoder, J.S.; Mahon, B.E.; Tauxe, R.V. Common source outbreaks of *Campylobacter* infection in the USA, 1997–2008. *Epidemiol. Infect.* **2013**, *141*, 987–996.
9. Garrett, N.; Devane, M.L.; Hudson, J.A.; Nicol, C.; Ball, A.; Klena, J.D.; Scholes, P.; Baker, M.G.; Gilpin, B.J.; Savill, M.G. Statistical comparison of *Campylobacter jejuni* subtypes from human cases and environmental sources. *J. Appl. Microbiol.* **2007**, *103*, 2113–2121.
10. Michaud, S.; Menard, S.; Arbeit, R.D. Role of real-time molecular typing in the surveillance of *Campylobacter* enteritis and comparison of pulsed-field gel electrophoresis profiles from chicken and human isolates. *J. Clin. Microbiol.* **2005**, *43*, 1105–1111.
11. Nichols, G.L.; Richardson, J.F.; Sheppard, S.K.; Lane, C.; Sarran, C. *Campylobacter* epidemiology: A descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011. *BMJ Open* **2012**, *2*, doi:10.1136/bmjopen-2012-001179.
12. Pebody, R.G.; Ryan, M.J.; Wall, P.G. Outbreaks of *Campylobacter* infection: Rare events for a common pathogen. *Commun. Dis. Rep. CDR Rev.* **1997**, *7*, R33–R37.
13. Nielsen, E.M.; Fussing, V.; Engberg, J.; Nielsen, N.L.; Neimann, J. Most *Campylobacter* subtypes from sporadic infections can be found in retail poultry products and food animals. *Epidemiol. Infect.* **2006**, *134*, 758–767.
14. Dopfer, D.; Buist, W.; Soyer, Y.; Munoz, M.A.; Zadoks, R.N.; Geue, L.; Engel, B. Assessing genetic heterogeneity within bacterial species isolated from gastrointestinal and environmental samples: How many isolates does it take? *Appl. Environ. Microbiol.* **2008**, *74*, 3490–3496.
15. Forbes, K.J.; Gormley, F.J.; Dallas, J.F.; Labovitiadi, O.; MacRae, M.; Owen, R.J.; Richardson, J.; Strachan, N.J.; Cowden, J.M.; Ogden, I.D.; *et al.* *Campylobacter* immunity and coinfection following a large outbreak in a farming community. *J. Clin. Microbiol.* **2009**, *47*, 111–116.
16. Gilpin, B.; Robson, B.; Lin, S.; Scholes, P.; On, S. Pulsed-field gel electrophoresis analysis of more than one clinical isolate of *Campylobacter* spp. from each of 49 patients in New Zealand. *J. Clin. Microbiol.* **2012**, *50*, 457–459.

17. Godschalk, P.C.R.; Gilbert, M.; Jacobs, B.C.; Kramers, T.; Tio-Gillen, A.P.; Ang, C.W.; van den Braak, N.; Li, J.; Verbrugh, H.A.; van Belkum, A.; *et al.* Co-infection with two different *Campylobacter jejuni* strains in a patient with the Guillain-Barré Syndrome. *Microbes Infect.* **2006**, *8*, 248–253.
18. Richardson, J.F.; Frost, J.A.; Kramer, J.M.; Thwaites, R.T.; Bolton, F.J.; Wareing, D.R.; Gordon, J.A. Coinfection with *Campylobacter* species: An epidemiological problem? *J. Appl. Microbiol.* **2001**, *91*, 206–211.
19. Balmer, O.; Tanner, M. Prevalence and implications of multiple-strain infections. *Lancet Infect. Dis.* **2011**, *11*, 868–878.
20. Hiett, K.L.; Stern, N.J.; Fedorka-Cray, P.; Cox, N.A.; Musgrove, M.T.; Ladely, S. Molecular subtype analyses of *Campylobacter* spp. from Arkansas and California poultry operations. *Appl. Environ. Microbiol.* **2002**, *68*, 6220–6236.
21. Simmons, M.; Hiett, K.L.; Stern, N.J.; Frank, J.F. Comparison of poultry exudate and carcass rinse sampling methods for the recovery of *Campylobacter* spp. subtypes demonstrates unique subtypes recovered from exudate. *J. Microbiol. Methods* **2008**, *74*, 89–93.
22. Denis, M.; Rose, V.; Huneau-Salaun, A.; Balaine, L.; Salvat, G. Diversity of pulsed-field gel electrophoresis profiles of *Campylobacter jejuni* and *Campylobacter coli* from broiler chickens in France. *Poult. Sci.* **2008**, *87*, 1662–1671.
23. Schlager, T.A.; Hendley, J.O.; Bell, A.L.; Whittam, T.S. Clonal diversity of *Escherichia coli* colonizing stools and urinary tracts of young girls. *Infect. Immun.* **2002**, *70*, 1225–1229.
24. Barton, C.; Ng, L.K.; Tyler, S.D.; Clark, C.G. Temperate bacteriophages affect pulsed-field gel electrophoresis patterns of *Campylobacter jejuni*. *J. Clin. Microbiol.* **2007**, *45*, 386–391.
25. Lindmark, H.; Harbom, B.; Thebo, L.; Andersson, L.; Hedin, G.; Osterman, B.; Lindberg, T.; Andersson, Y.; Westoo, A.; Olsson Engvall, E. Genetic characterization and antibiotic resistance of *Campylobacter jejuni* isolated from meats, water, and humans in Sweden. *J. Clin. Microbiol.* **2004**, *42*, 700–706.
26. Dickins, M.; Franklin, S.; Stefanova, R.; Schutze, G.; Eisenach, K.; Wesley, I.; Cave, M. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. *J. Food Prot.* **2002**, *65*, 957–962.
27. Lindmark, H.; Diedrich, I.C.; Andersson, L.; Lindqvist, R.; Engvall, E.O. Distribution of *Campylobacter* genotypes on broilers during slaughter. *J. Food Prot.* **2006**, *69*, 2902–2907.
28. Hunter, S.M.; Berrang, M.E.; Meinersmann, R.J.; Harrison, M.A. Genetic diversity of *Campylobacter* on broiler carcasses collected pre evisceration and postchill in 17 U.S. poultry processing plants. *J. Food Prot.* **2009**, *72*, 49–54.
29. Scates, P.; Moran, L.; Madden, R.H. Effect of incubation temperature on isolation of *Campylobacter jejuni* genotypes from foodstuffs enriched in Preston broth. *Appl. Environ. Microbiol.* **2003**, *69*, 4658–4661.
30. Sheppard, S.K.; Dallas, J.F.; Strachan, N.J.C.; MacRae, M.; McCarthy, N.D.; Wilson, D.J.; Gormley, F.J.; Falush, D.; Ogden, I.D.; Maiden, M.C.J.; *et al.* *Campylobacter* genotyping to determine the source of human infection. *Clin. Infect. Dis.* **2009**, *48*, 1072–1078.

31. Skanseng, B.; Trosvik, P.; Zimonja, M.; Johnsen, G.; Bjerrum, L.; Pedersen, K.; Wallin, N.; Rudi, K. Co-infection dynamics of a major food-borne zoonotic pathogen in chicken. *PLoS Pathog.* **2007**, *3*, e175.
32. Schouls, L.; Reulen, S.; Duim, B.; Wagenaar, J.; Willems, R.; Dingle, K.; Colles, F.; van Embden, J. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: Strain diversity, host range, and recombination. *J. Clin. Microbiol.* **2003**, *41*, 15–26.
33. Tenover, F.C.; Arbeit, R.D.; Goering, R.V.; Mickelsen, P.A.; Murray, B.E.; Persing, D.H.; Swaminathan, B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *J. Clin. Microbiol.* **1995**, *33*, 2233–2239.
34. Melero, B.; Cocolin, L.; Rantsiou, K.; Jaime, I.; Rovira, J. Comparison between conventional and qPCR methods for enumerating *Campylobacter jejuni* in a poultry processing plant. *Food Microbiol.* **2011**, *28*, 1353–1358.
35. Höök, H.; Fattah, M.A.; Ericsson, H.; Vagsholm, I.; Danielsson-Tham, M. Genotype dynamics of *Campylobacter jejuni* in a broiler flock. *Vet. Microbiol.* **2005**, *106*, 109–117.
36. Manning, G.; Duim, B.; Wassenaar, T.; Wagenaar, J.A.; Ridley, A.; Newell, D.G. Evidence for a genetically stable strain of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* **2001**, *67*, 1185–1189.
37. Hänninen, M.L.; Pajarre, S.; Klossner, M.L.; Rautelin, H. Typing of human *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **1998**, *36*, 1787–1789.
38. Wong, T.; Devane, M.; Hudson, J.A.; Scholes, P.; Savill, M.; Klena, J. Validation of a PCR method for *Campylobacter* detection on poultry packs. *Br. Food J.* **2004**, *106*, 642–650.
39. Fraser, A.D.; Chandan, V.; Yamazaki, H.; Brooks, B.W.; Garcia, M.M. Simple and economical culture of *Campylobacter jejuni* and *Campylobacter coli* in CO₂ in moist air. *Int. J. Food Microbiol.* **1992**, *15*, 377–382.
40. Griffiths, P.L.; Park, R.W.A. Campylobacters associated with human diarrhoeal disease. *J. Appl. Bacteriol.* **1990**, *69*, 281–301.
41. Barros-Velázquez, J.; Jimenez, A.; Villa, T.G. Isolation and typing methods for the epidemiologic investigation of thermotolerant campylobacters. *Int. Microbiol.* **1999**, *2*, 217–226.
42. Ribot, E.M.; Fitzgerald, C.; Kubota, K.; Swaminathan, B.; Barrett, T.J. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J. Clin. Microbiol.* **2001**, *39*, 1889–1894.
43. Hunter, S.B.; Vauterin, P.; Lambert-Fair, M.A.; van Duyne, M.S.; Kubota, K.; Graves, L.; Wrigley, D.; Barrett, T.; Ribot, E. Establishment of a universal size standard strain for use with the pulsenet standardized pulsed-field gel electrophoresis protocols: Converting the national databases to the new size standard. *J. Clin. Microbiol.* **2005**, *43*, 1045–1050.
44. Speck, M.L. *Sampling Plans*, 2nd ed.; American Public Health Association: Washington, DC, USA, 1984.

45. Ribot, E.; National Center for Infectious Diseases; CDC Division of Bacterial and Mycotic Diseases; Foodborne and Diarrheal Diseases Branch; Public Health Practice Program Office; CDC, Division of Laboratory Services; CDC, and Association of Public Health Laboratories. *Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis*; Centers for Disease Control and Prevention: Atlanta, GA, USA, 2002.

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