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ORIGINAL ARTICLE

Genomic diversity, virulence and source of *Campylobacter jejuni* contamination in Irish poultry slaughterhouses by whole genome sequencing

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Abstract

Aims: The aim was to exploit whole genome sequencing (WGS) to assess genomic diversity, identify virulence genes and deduce the proportion of *Campylobacter* colonized broilers that directly contaminate their carcasses.

Methods and Results: *Campylobacter jejuni* isolates (107) from caeca and carcass neck skin samples (50 pairs from the same batch plus 7 individual caeca) sampled at three poultry slaughterhouses over a one-year period were selected for sequencing (MiSeq; Illumina). FastQ files were submitted to BioNumerics for analysis using the wgMLST scheme for allele calling. *Campylobacter* cgMLST and hierarchical clustering was performed by applying the single linkage algorithm. Sequence types (STs) were determined in silico from the WGS data and isolates were assigned into clonal complexes (CCs) using the *Campylobacter* PubMLST.org database. Virulence genes were determined by downloading core sequences from the virulence factor database (VFDB) and the National Center for Biotechnology Information (NCBI). A high degree of diversity was observed with 23 different STs identified. ST257 and CC-21 were the most common STs and CCs, respectively. cgMLST analysis suggested that 56% of carcass contamination was a direct result of contamination from caeca from the same batch. Virulence genes known to play a role in human *C. jejuni* infection were identified such as the *wlaN* gene and the genes associated with lipooligosaccharide synthesis, which were identified in 30% of isolates.

Conclusions: Caecal colonization was the more plausible occurring source of *C. jejuni* contamination of broiler carcasses, compared with cross-contamination from another batch or the environment. The high rate of genetic diversity observed amongst caecal isolates is consistent with a wide variety of *Campylobacter* strains circulating in poultry flocks in Ireland.

Significance and Impact of Study: The results will further inform broiler processors and regulators about the influence and importance of on-farm colonization

versus slaughterhouse cross-contamination and the relationship between *C. jejuni* in caeca and carcasses during processing.

KEYWORDS

Campylobacter jejuni, contamination, diversity, poultry, slaughterhouse, whole genome sequencing

INTRODUCTION

Campylobacter is the main bacterial cause of gastroenteritis in the European Union, with *Campylobacter jejuni* responsible for most human cases and frequently associated with poultry (EFSA and ECDC, 2019; Hakeem & Lu, 2021). *C. jejuni* colonizes the caeca of broilers and can cross-contaminate broiler carcasses after slaughter during the processing steps, notably at evisceration. Contaminated carcasses may pose a risk of exposure to consumers and *C. jejuni* may be acquired by humans through the handling and consuming of raw or undercooked contaminated poultry (Cawthraw et al., 1996; Kaakoush et al., 2015).

During 2008, an EU-wide baseline survey which included 26 Member States (MSs), was conducted at slaughterhouse level to determine the prevalence of *Campylobacter* in broiler batches presented for slaughter (caeca) and in the broiler carcasses (EFSA, 2010). That study reported a community-level prevalence of *Campylobacter* colonized broiler batches at 71.2% and *Campylobacter* contaminated broiler carcasses at 75.8%, and it was noted that the prevalence in individual MSs varied from 2.0% to 100.0% and from 4.9% to 100.0%, for caecal contents and carcasses, respectively (EFSA, 2010). Amongst the 26 MS, Ireland reported the fourth highest prevalence of *Campylobacter* in colonized broiler batches (83.1%) and the second highest prevalence on broiler carcasses (98.3%). A more recent national study conducted by Lynch et al. (2022) provided a useful update on *Campylobacter* in broilers in Ireland, for which monthly samples were collected from the three largest broiler processing plants in the Republic of Ireland over a 12-month period (September 2017 to August 2018). This study reported a positive rate of 66% from caecal contents and 53% from carcass samples and demonstrated that carcasses were more likely to be *Campylobacter* positive if the caecal contents of the same batch were positive (Lynch et al., 2022).

Much research has been conducted to date on the prevalence and persistence of *C. jejuni* in poultry (Desmonts et al., 2004; Frazão et al., 2017; Talukder et al., 2008). In addition, various genotyping methods, for example, multilocus sequence typing (MLST) have been used successfully for molecular differentiation of *Campylobacter* isolates (Colles & Maiden, 2012; Wieczorek et al., 2020). Few studies have been conducted to determine the most likely source of contamination of those carcasses by comparing the genetic sequences of isolates. Whole genome sequencing (WGS) is increasingly used in surveillance, epidemiological studies and source attribution investigations of foodborne pathogens (Brown et al., 2019; Llarena et al., 2017). The higher resolution of WGS in comparison with traditional molecular sub-typing methods provides the greatest discriminating ability to differentiate microbial pathogen strains and clonal lineages for use in foodborne outbreak investigations, hazard identification and source attribution (EFSA, 2019). Therefore, through the use of WGS, the aims of this study were to further evaluate a selection of *C. jejuni* isolates from the study of Lynch et al. (2022) to; (a) assess the genomic diversity of *C. jejuni* isolated from caeca, (b) deduce the proportion of carcass isolates which could be attributed to *Campylobacter* arising from the intestinal carriage or colonization of broilers from the same batch of broilers and (c) identify virulence genes that may play a role in *Campylobacter* survival and environmental adaptation during key processing stages, in addition to genes associated with human infection and colonization of broilers. The second aim would assess the risks of contamination coming from broiler batches and from the slaughterhouse environment (persistent contamination) that could guide the establishment or revision of risk management options.

MATERIALS AND METHODS

Selection of isolates for whole genome sequencing

From the previous national study of Lynch et al. (2022), we selected all available pairs of *C. jejuni* caeca isolates from two stages of processing, namely caeca contents and carcass neck skin from the same respective broiler batches, and this yielded a total of 50 pairs. To examine genetic diversity, in addition to the 50 pairs, an extra seven single *C. jejuni* caecal isolates were selected from other flocks (these did not have a matching neck skin isolate). For this study, the term flock refers to broilers reared at the same time in the same poultry house. The term batch refers to broilers reared together in the same poultry house, on the same farm holding, and which were also processed on the same date, time and location. To retain anonymity of the flocks, actual flock identities were pseudo anonymised and allocated a number instead.

Culture preparation and DNA extraction

C. jejuni isolates were recovered from frozen stocks on Columbia Agar and Horse blood (E & O Laboratories LTD) and incubated for 48 h at 37°C, under microaerobic conditions. Using a 1 µl inoculation loop, a loopful of pure culture was taken from the plate and re-suspended in 100 µl nuclease free water. A volume of 100 µl MagNA Pure 96 bacterial lysis buffer (Roche diagnostics) and 20 µl proteinase k (Roche Diagnostics) was added and vortexed for 20 s. Samples were incubated at 65°C for 10 min followed by 95°C for 10 min. DNA was extracted using the MagNA Pure 96 system (Roche Diagnostics) using MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Diagnostics). The protocol was followed exactly, and the final elution volume was 100 µl.

DNA quantification and qualification

DNA purity was determined by reading the whole absorption spectrum (220–750 nm) using the NanoDrop ND-1000 spectrometer (Thermo Scientific). A 260/280 ratio between 1.75 and 2.05 (including a 0.05 error rate**)** was considered acceptable quality. The bacterial DNA concentrations were measured using Qubit fluorometric quantitation with Qubit dsDNA HS Assay Kit (Qubit 3.0; Thermo Fisher Scientific).

Library preparation and amplification of tagmented DNA

Sample libraries for all isolates were prepared using the Illumina Nextera™ DNA Flex Library Prep kit (Illumina, Inc.), and the protocol was followed exactly. MagNA Pure DNA extraction typically yielded between 8 and 41 ng μl^{-1} therefore 12 µl of extracted DNA was used to give an input range of between 100 and 500 ng. The tagmented DNA was amplified in a working volume of 50 µl with the following settings: heated lid, initial cycle at 68°C for 3 min followed by 98°C for 3 min and 5 cycles of (98°C for 45 s, 62°C for 30 s and 68°C for 2 min) with a final run at 68°C for 1 min followed by a hold temperature of 10°C.

Normalization, denaturing and sequencing of libraries

To achieve optimal cluster density, equal library volumes (5 µl) of individual libraries were pooled into a sterile 1.5 ml tube and the pool was quantified in triplicate by Qubit fluorometric quantitation with the Qubit dsDNA HS Assay Kit before sequencing. It was assumed that the library size was 600 bp. The pooled libraries were diluted in resuspension buffer (RSB; Illumina) to give a final starting concentration of 4 nM. Libraries were denatured in NaOH and were further diluted in Hybridization Buffer (HT1; Illumina) from 4 nM to 20 pM and diluted again in HT1 to give a final loading concentration of approximately 10–14 pM. The sequencing was performed on a MiSeq platform (Illumina) using v3 chemistry, as 300-cycle paired-end runs. The pool was spiked with 1% PhiX, loaded at 20 pM and an average of 32 samples were loaded per MiSeq flow cell. The isolates included in this study were distributed over four sequencing runs.

Analysis of whole genome sequencing data

The following run metrics were used to check that the run passed basic quality metrics for raw sequence data, that is, >70% bases higher than Q30 at 2×300 bp and cluster density of 1100–1400 k/mm^2. In addition, the % of reads that aligned to the phiX was also checked to ensure that the starting concentration of the libraries were not over or underestimated. The generated FASTQ files were imported directly from Illumina BaseSpace to BioNumerics (Version 8.0; Applied Maths, Belgium). FASTQ files were assembled using the BioNumerics software and its integrated calculation engine. The FASTQ files were submitted to the BioNumerics wgMLST scheme where allele calling was performed using two processes: (i) assembly free allele calling, which was done directly on the reads by comparing kmer frequency tables of the reads to kmer frequency tables of all known alleles and (ii) assembly-based allele calling. For this latter process, first a de novo assembly was performed using SPAdes followed by a mismatch correction with the Burrow-Wheeler Aligner (BWA-AM), removal of contigs below 300 bp, and a consensus calling on the output. A consensus allele call was performed with the assembly free and the de novo assembly-based calling. Results found with both algorithms or with only one algorithm were maintained and discrepant results were removed.

The sequence quality of each individual genome was evaluated using BioNumerics to include the following information: Number of contigs, N50, coverage, genome length and core genome (%). Within BioNumerics, conventional MLST types (STs) of the *Campylobacter* isolates were determined in silico from the WGS data. To do this, the seven MLST loci were obtained using the sequence extraction tool and the MLST plugin that is linked to the PubMLST.org public scheme for *C. jejuni/coli* i.e., the 7 gene allele profile was determined from the sequence. For those isolates where STs were not defined within BioNumerics,

genome sequences (FASTA format) were submitted to the *Campylobacter* public database for molecular typing and microbial genome diversity (https://pubmlst.org/organisms/campylobacter-jejunicoli/) using the automated system for submission of new alleles (Jolley et al., 2018). On the basis of their STs, isolates were assigned into clonal complexes (CCs) using the *Campylobacter* PubMLST.org database (Jolley et al., 2018) and described as STX or CC-X where X is the ST and CC number, respectively, that the isolate was assigned.

In addition to assignment of isolates into CCs defined by their respective complement of the seven housekeeping genes, BioNumerics permitted the much more detailed discrimination of the relatedness of isolates to each other by analysis of the entire set of genes in the core genomes (cgMLST). Core genome MLST was performed using the *Campylobacter* cgMLST scheme, which is based on 1343 core loci. Phylogeny was inferred by creating a dendrogram based on cgMLST allelic differences using the single linkage algorithm with the allele calls considered categorical data. A scaling factor of 1 was used for creating dendrograms and a scaling factor of 10 was used to create the similarity matrix. The similarity matrix was exported from BioNumerics to Excel® (Microsoft® Corp) and the heat map was created in Microsoft Excel using conditional formatting. In the context of the diversity amongst the isolates examined, clusters or matches by cgMLST were defined as a distance measure of ≤14 alleles. It is likely that these isolates came from the same source (Schurch et al., 2018). Those isolates described as indistinguishable were 100% identical (zero allele difference) and closely related were 1–6 allele different.

In addition to genetic diversity analysis, the sequences of 57 caecal isolates selected from the 57 batches were analysed for virulence genes. To do this, a virulence genes database was created by downloading core sequences from the virulence factor database (VFDB) (Liu et al., 2019) and the National Center for Biotechnology Information (NCBI) as previously described (Emanowicz et al., 2022). Fasta files for all virulence genes were uploaded to a virulence genes database in BioNumerics. The aim was to remove hits with less than 80% identity and/or coverage (Truccollo et al., 2021). Character data was exported from BioNumerics to Excel® where a heat map was generated.

RESULTS

All genomes passed the basic quality metrics for raw sequence data from the MiSeq. On average, a cluster density of 1100 (K/mm^2) was achieved with 90.88% of clusters passing filter (PF) specifications. The average number of reads, yield and error rate over the eight runs was 24.035 (M) reads PF, 14.79 Gbp and 2.4% respectively. In each run, the index reads were evenly distributed across all samples.

Sequencing data produced in this study were deposited to the NCBI Sequence Read Archive (SRA) repository and are available through the BioProject accession number PRJNA854907. Accession numbers along with quality metrics of assemblies are detailed in Table S1. The de novo assemblies consisted of an average of 25 contigs with an average N50 of 197,020 bp. Core % ranged from 96%–99% with a mean core percent of 98.1%. The average coverage was 117X. The minimum, average and maximum number of alleles called over the entire data set was 1334, 1337 and 1339 alleles, respectively.

A high degree of diversity was observed amongst the 57 *C. jejuni* caecal isolates. The diversity through analysis of cgMLST data is shown in Figure 1. In total, nine clusters accounting for 26 isolates were identified and these nine clusters are highlighted in different colours. In terms of diversity, 31 unrelated (>14 allele different by cgMLST) *C. jejuni* strains were identified amongst the 57 *C. jejuni* isolates and in silico MLST typing using the traditional MLST scheme identified 23 different STs (Figure 1). ST257 was the most common ST identified in 10 *C. jejuni* isolates followed by ST50, ST21, ST45, ST6209, ST42, ST19, ST230 and ST814 identified in 8, 6, 5, 5, 3, 2, 2 and 2 *C. jejuni* isolates respectively. Fourteen *C. jejuni* STs were each represented by a single isolate. Isolates that were grouped into clusters were of the same ST however, in some instances isolates that were highly diverse by cgMLST were of the same ST using the traditional MLST naming scheme, for example, ST42, ST45, ST50, ST257 and ST814 (Figure 1). The 23 different STs were grouped into 12 known CCs. ST3149, ST4430 and ST9393 were not defined as a CC. Amongst the 12 CCs, CC-21 was the most prevalent followed by CC-45 and CC-354 encompassing 5, 3 and 2 STs each, respectively. CC-21 accounted for 18 isolates of ST50 (8 isolates), ST21 (6 isolates), ST19 (2 isolates), ST53 (1 isolate) and ST806 (1 isolate).

A visual representation of the wide range of diversity amongst the *C. jejuni* isolates shown as a heat map, that is, gradient with different shades of three colours (red, yellow, and green) is provided in Figure 2. This was generated as a result of cgMLST clustering within BioNumerics. Green is denotated by highly diverse isolates. The maximum number of allele differences between the two most different isolates was 1290 alleles (Figure 2) with 30, 19 and 15 isolates showing allele differences of >200, >500 and 1000, respectively (data not shown).

When the paired caecal and neck skin isolates cgMLST comparison was made, 29 distinctly different clusters as defined by a relationship threshold of ≤14 alleles were observed (Figure 3). Twenty-eight (56.0%) of a total of 50 neck skin isolates were closely related (up to 6 alleles) to the caecal isolates from the same batch. Occasionally, but

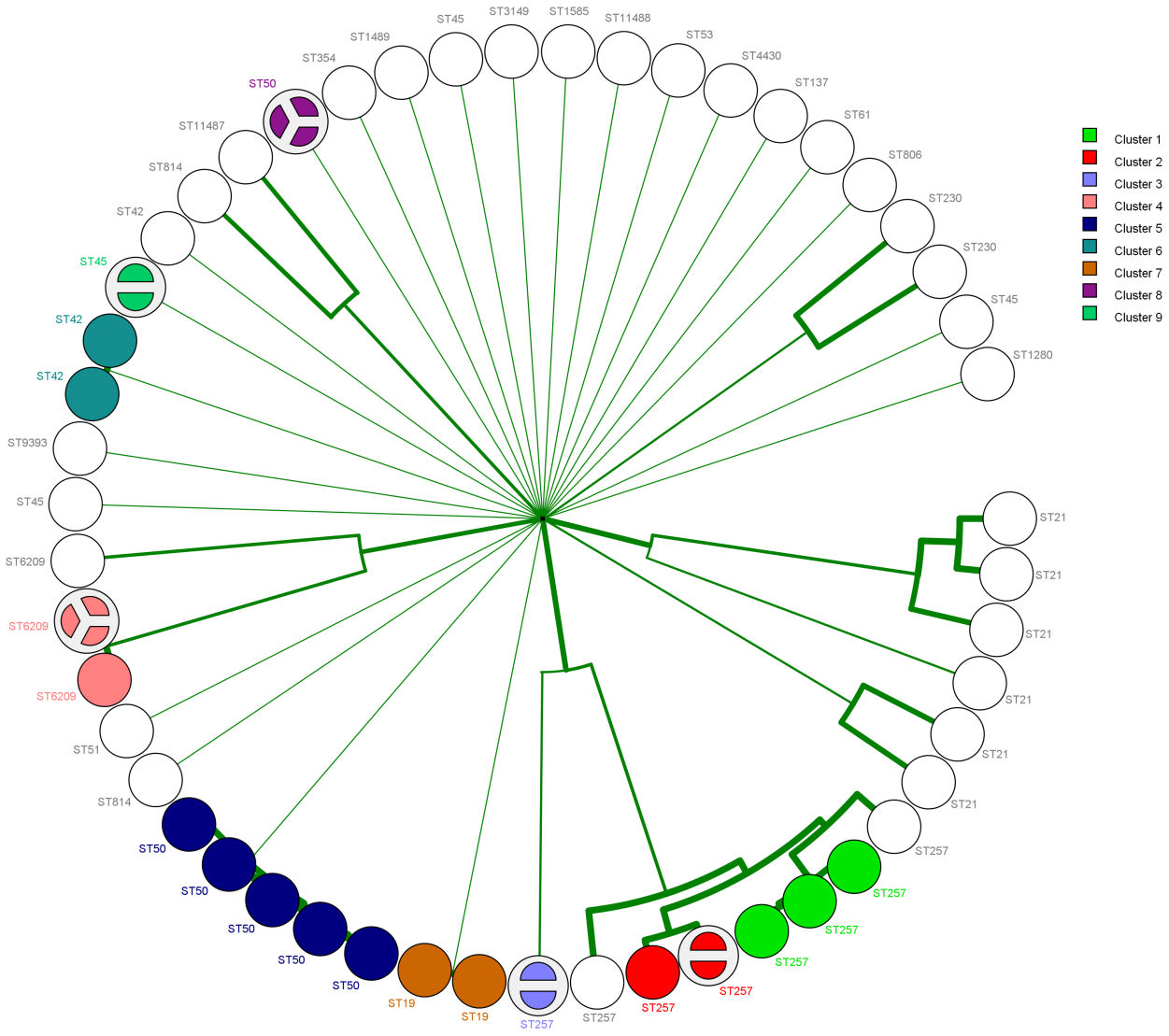


FIGURE 1 Diversity of 57 *Campylobacter jejuni* isolates from broiler caecal contents estimated by the distribution of allelic differences in the cgMLST scheme. Nine clusters are highlighted in different colours and multiple indistinguishable isolates are represented as segmented discs. A cluster in this instance, is defined as a group of isolates of the same ST and ≤14 allele different by cgMLST. The STs using the PubMLST scheme are also shown. Scaling factor = 10; Red = 100% identity (zero allele difference).

less frequently, other isolates from different batches also formed part of a cluster where neck skin and caeca from the same batch clustered (Figure 3). For one cluster, isolates from caeca and neck skin isolated 11 December 2017 (flock 25) were indistinguishable, that is, 100% identical to isolates from caeca and neck skin isolated at the same processing plant a month later, on 10 January 2018 from another flock (flock 36). Situations were also observed where closely related isolates were recovered from different processing plants from different flocks, for example a neck skin isolate from plant C (flock 16) was only one allele different to a caecal isolate from plant A (flock 28). Neck skin and caecal isolates from plant B (flocks 10, 41 and 45) were only one allele different to each other and to another caecal isolate from plant A (flock 34) as shown in Figure 3, and all of these were processed on different dates.

The distribution of virulence and survival genes amongst 57 different batches of *C. jejuni* isolates along with their associated function is shown in Figure 4. There were no hits less than 80%. Amongst the 55 genes identified, these are associated with functions including adherence, capsule synthesis, cytotoxin production, glycosylation system, hemolytic activity, invasion, lipooligosaccharide synthesis (LOS); major outer membrane protein associated with adherence, motility, outer membrane heme/haemoglobin receptor, oxidative stress resistance, regulatory system, stress response, type IV secretion system (T4SS) and toxin production. Isolates of ST19, ST21, ST50 and ST51 (and which all belonged to CC-21) harboured the highest number of virulence genes (81.8% to 89.1%).

In general, the distribution of virulence genes followed the same pattern amongst the different CC's i.e., CC-21

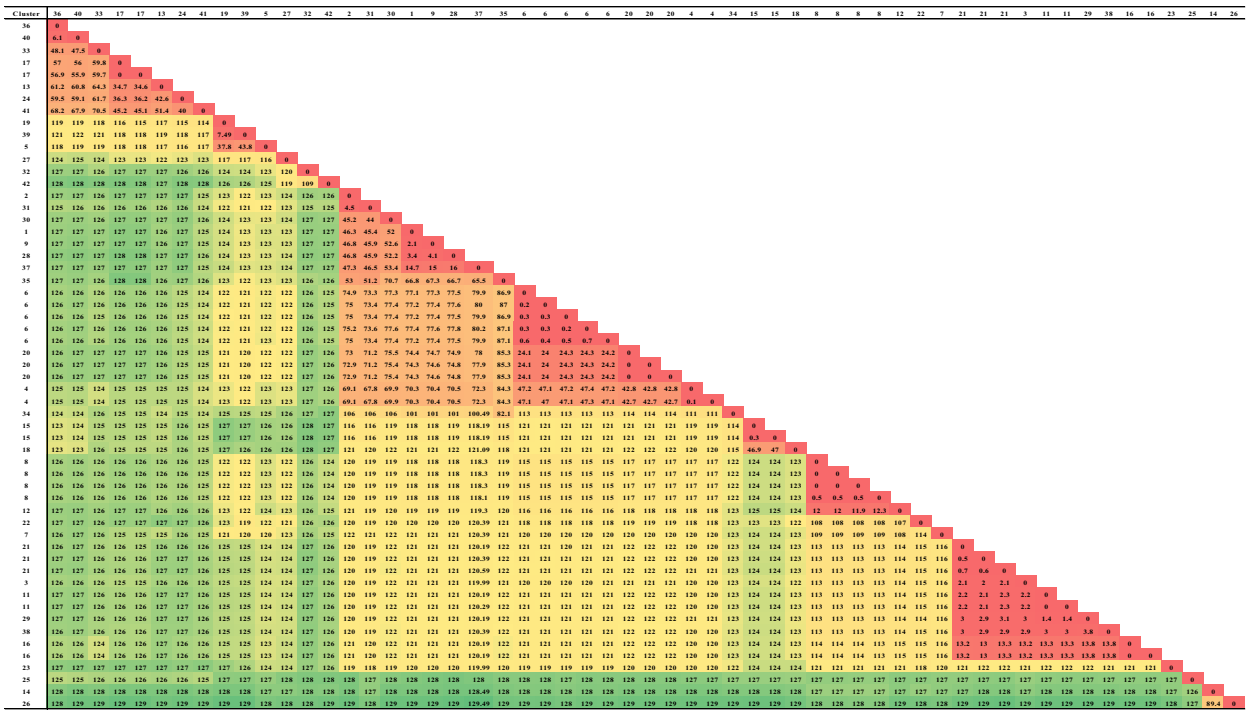


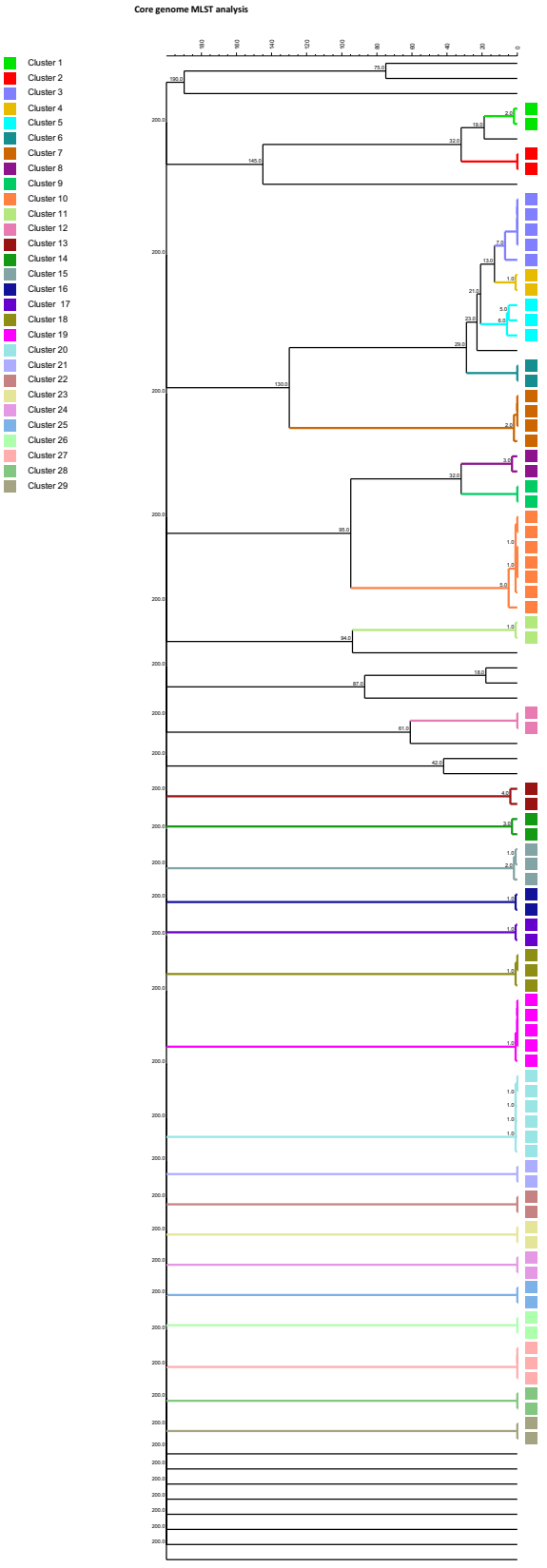
FIGURE 2 Similarity matrix of 57 broiler caecal *Campylobacter jejuni* isolates analysed by cgMLST. The numbers represent allele differences between the clusters which were defined as isolates of the same ST and ≤14 allele different by cgMLST. Scaling factor = 10; Red = 100% identity (zero allele difference).

harboured the *wlaN* gene only and all isolates within each ST generally displayed the same virulence profile. Amongst all 57 caecal isolates, only two isolates (one of ST814 and one ST11487) harboured T4SS genes and both isolates belonged to CC-661. Twenty-two virulence genes were identified in all *C. jejuni* isolates and these were associated with adherence *(cadF*, *pebA)*, glycosylation (*pseG*), invasion (*ciaB*, *ciaC iamA*), LOS (*waac*, *waaF*, *waav*), motility (*Cj0371*, *flgE*, *flgH*, *flgL*, *fliA*, *fliF*, *fliM*, *fliY*, *motA*), oxidative stress resistance (*perR*), the regulatory system (racR) and stress response (*cj0358*, *cj1371*). All isolates of the most prevalent sequence type, ST257, carried the same virulence profile harbouring 67.3% (37/55) of virulence genes. The eight isolates from the second most prevalent ST, ST50, carried a higher number of virulence genes with seven carrying 89.1% (49/55) genes and one also carrying the *neuBA1* gene. A high prevalence of virulence genes was also identified amongst the two new STs where the genomes were submitted to PubMLST (isolates of unknown ST within BioNumerics) with ST11487 and ST11488 harbouring 44/55 (80%) and 39/55 (71%) virulence genes, respectively.

DISCUSSION

This study revealed a high degree of genetic diversity within the population of *C. jejuni* originating in Irish commercial broilers, which agrees with other European studies (Griekspoor et al., 2015; Joensen et al., 2020; Messens et al., 2009; Pergola et al., 2017; Vidal et al., 2016). Amongst the 57 *C. jejuni* isolates from caecal contents there were 31 cgMLST genotypes, 23 different STs with 12 grouped into known CCs and three that did not fall into a defined CC. ST257 (CC-257), which represented 17.5% of isolates, and ST50 (CC-21), representing 14.0% of isolates, were the most dominant STs, while CC-21 was the most prevalent CC, accounting for 31.6% of isolates that were spread over five different STs. Similar to our results, widespread diversity of *C. jejuni* isolates has been described amongst clinical isolates in Ireland, with CC-21 and CC-48 the most prevalent CCs, present in 12.1% and 10.1% of cases, respectively (Brehony et al., 2021; Redondo et al., 2019). This high degree of diversity could be due to genomic variation (Bae et al., 2014) combined with the wide range of reservoir host species in which various strains of *C. jejuni* can evolve, that could potentially be a source of *Campylobacter* for each new broiler flock, or it could be due to the fact that *Campylobacter* are naturally competent meaning they can easily import DNA from their surroundings into their genomes, which influence genetic diversity (Wieczorek et al., 2015; Wiesner et al., 2003).

It has been previously reported that some CCs such as CC-21, CC-45, CC-48 and CC-257 are mostly associated with human campylobacteriosis in Scotland, Canada and Poland (Levesque et al., 2013; Sheppard et al., 2009a,



Flock	Matrix	Date	Processor
33	Caeca	2018-01-16	Plant B
29	Caeca	2018-07-04	Plant A
43	Neck Skin	2017-09-04	Plant B
37	Caeca	2017-09-04	Plant A
37	Neck Skin	2017-09-04	Plant A
31	Neck Skin	2018-06-26	Plant B
27	Caeca	2018-07-03	Plant A
27	Neck Skin	2018-07-03	Plant A
2	Caeca	2018-03-14	Plant C
36	Caeca	2018-01-10	Plant A
36	Neck Skin	2018-01-10	Plant A
25	Caeca	2017-12-11	Plant A
25	Neck Skin	2017-12-11	Plant A
29	Neck Skin	2018-07-04	Plant A
16	Neck Skin	2017-10-24	Plant C
28	Caeca	2018-05-23	Plant A
26	Caeca	2018-06-12	Plant A
41	Caeca	2018-05-08	Plant B
8	Caeca	2017-11-01	Plant B
22	Neck Skin	2018-04-11	Plant A
6	Caeca	2018-07-18	Plant B
6	Neck Skin	2018-07-18	Plant B
7	Caeca	2018-02-19	Plant B
40	Caeca	2018-01-23	Plant B
40	Neck Skin	2018-01-23	Plant B
33	Neck Skin	2018-01-16	Plant B
44	Neck Skin	2018-01-17	Plant A
34	Neck Skin	2018-04-11	Plant A
1	Caeca	2017-09-27	Plant B
1	Neck Skin	2017-09-27	Plant B
10	Neck Skin	2018-02-12	Plant B
45	Neck Skin	2018-05-22	Plant B
34	Caeca	2018-04-11	Plant A
41	Neck Skin	2018-05-08	Plant B
45	Caeca	2018-05-22	Plant B
42	Neck Skin	2018-06-12	Plant B
24	Caeca	2018-07-25	Plant A
50	Caeca	2018-09-04	Plant B
50	Neck Skin	2018-04-08	Plant B
17	Neck Skin	2018-02-28	Plant C
10	Caeca	2018-02-12	Plant B
15	Neck Skin	2017-10-24	Plant C
24	Neck Skin	2018-07-25	Plant A
30	Neck Skin	2018-08-03	Plant C
30	Caeca	2018-08-03	Plant C
22	Caeca	2018-04-11	Plant A
7	Neck Skin	2018-02-19	Plant B
28	Neck Skin	2018-05-23	Plant A
20	Neck Skin	2018-04-17	Plant C
20	Caeca	2018-04-17	Plant C
35	Caeca	2018-05-09	Plant A
35	Neck Skin	2018-05-09	Plant A
23	Neck Skin	2018-02-28	Plant C
23	Caeca	2018-02-28	Plant C
17	Caeca	2018-02-28	Plant C
3	Neck Skin	2018-07-03	Plant B
3	Caeca	2018-07-03	Plant B
39	Caeca	2017-10-31	Plant B
39	Neck Skin	2017-10-31	Plant B
14	Neck Skin	2017-10-23	Plant C
15	Caeca	2017-10-24	Plant C
14	Caeca	2017-10-23	Plant C
13	Neck Skin	2017-11-27	Plant C
19	Caeca	2017-12-05	Plant C
13	Caeca	2017-11-27	Plant C
46	Caeca	2018-01-23	Plant C
46	Neck Skin	2018-01-23	Plant C
8	Neck Skin	2017-11-01	Plant B
32	Caeca	2017-10-16	Plant A
32	Neck Skin	2017-10-16	Plant A
19	Neck Skin	2017-12-05	Plant C
18	Neck Skin	2017-11-28	Plant C
18	Caeca	2017-11-28	Plant C
47	Neck Skin	2018-05-30	Plant C
47	Caeca	2018-05-30	Plant C
21	Caeca	2018-04-17	Plant A
21	Neck Skin	2018-04-17	Plant A
48	Caeca	2018-05-09	Plant A
48	Neck Skin	2018-05-09	Plant A
5	Neck Skin	2018-05-09	Plant B
5	Caeca	2018-05-09	Plant B
4	Neck Skin	2018-05-14	Plant B
4	Caeca	2018-05-14	Plant B
11	Caeca	2017-10-31	Plant B
11	Neck Skin	2017-10-31	Plant B
49	Caeca	2018-02-06	Plant B
49	Neck Skin	2018-02-06	Plant B
31	Caeca	2018-06-26	Plant B
9	Caeca	2017-09-25	Plant B
9	Neck Skin	2017-09-25	Plant B
12	Neck Skin	2017-10-16	Plant A
12	Caeca	2017-10-16	Plant A
42	Caeca	2018-06-12	Plant B
43	Caeca	2017-09-04	Plant B
44	Caeca	2018-01-17	Plant A
16	Caeca	2017-10-24	Plant C
38	Caeca	2018-03-13	Plant A
2	Neck Skin	2018-03-14	Plant C
38	Neck Skin	2018-03-13	Plant A
26	Neck Skin	2018-06-12	Plant A

FIGURE 3 Dendrogram of cgMLST profiles of 50 pairs of *Campylobacter jejuni* isolates recovered from broiler neck skin and caecal samples. A cluster is defined as a group of isolates with ≤14 allele differenct by cgMLST.

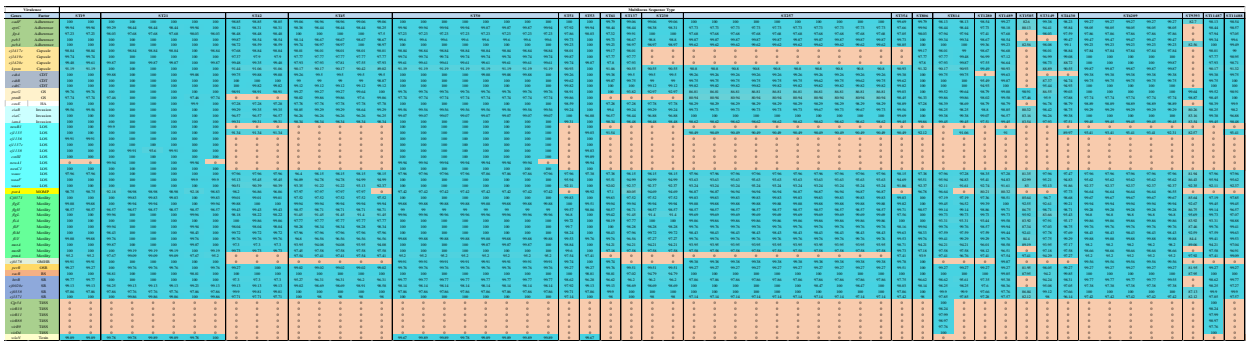


FIGURE 4 Heat map showing the distribution of virulence genes amongst different sequence types of *Campylobacter jejuni*. The numbers in each cell refer to % identity and therefore 0 = absent. CDT, cytolethal distending toxin; GS, glycosylation system; HA, hemolytic activity; LOS, lipooligosaccharide synthesis; MOMP, major outer membrane protein; OMHR, outer membrane heme/haemoglobin receptor; OSR, oxidative stress resistance; RS, regulatory system; SR, stress response; T4SS, type IV secretion system.

2009b; Wieczorek et al., 2016). In our study CC-21 was the most prevalent CC but CC-48 was not detected. Thépault et al. (2018) isolated *C. jejuni* of CC-48 from cattle in France and also reported that defined isolates of CC-21 and CC-48 are frequently isolated from various reservoirs including poultry. While a direct comparison between human and poultry isolates was not within the original scope of this study, sequences from the work of Brehony et al. (2021) were downloaded and compared with the isolates in this present study and although isolates could not be epidemiologically linked due to the differences in time (approximately 1 year), on some occasions there was only one allele difference through cgMLST between the broiler and clinical isolates (data not shown). This finding suggests that certain STs causing illness in humans in Ireland appear to be frequently occurring in broilers. Linking with other partners in a 'One Health' framework would provide a better understanding on sources of *Campylobacter* infection in Ireland as concluded by Brehony et al. (2021).

Previous work by this group detected a higher percentage of *Campylobacter* in neck skin samples obtained from processed carcasses originating from confirmed caecal positive broiler batches (68.2%), relative to batches of broilers where *Campylobacter* was not detected in their caecal contents (23.3%) (Lynch et al., 2022). Other studies have also demonstrated a relationship between the colonization of broilers and the contamination of carcasses, with smaller proportions of *Campylobacter* contaminated carcasses and lower concentrations of these bacteria on processed carcasses produced from broiler batches that were not colonized or had low *Campylobacter* colonization in their caeca before slaughter and conversely greater concentrations of contamination reported on carcasses originating from heavily colonized broiler batches (Emanowicz et al., 2021; Rasschaert et al., 2020; Reich et al., 2008; Rosenquist et al., 2006). Using WGS, our study provides further evidence that the intestinal tract of broilers of the same batch was the most frequent source of *Campylobacter* contamination to the carcasses of colonized broiler batches, as more than 50% of the neck flap isolates (28 out of 50) were indistinguishable or were closely related to the isolate obtained from the caeca sample from the same broiler batch. The association between caeca as a source for contamination on neck skin may be an underestimation since just one caecal and one carcass isolate from within each batch was tested and it is well known that there may be multiple strains (co-colonization) in caeca and even multiple strains on carcasses due to contamination during processing. To make a more accurate estimate, more work would be required on a larger sample set which could include the testing of more than one isolate from neck and caeca per batch. The majority of carcass neck skin samples from colonized batches are contaminated despite strict processing controls (Emanowicz et al., 2021; Lynch et al., 2022), which implies that preventing the colonization of flocks during rearing is a key critical control measure for production of *Campylobacter*-free chicken carcasses.

The use of other molecular biology techniques including pulsed-field gel electrophoresis (PFGE) and *fla*A sequencing has been previously documented (Iannetti et al., 2020; Natsos et al., 2021), with some observations on cross-contamination during processing and persistence on farms reported. An Italian study (Iannetti et al., 2020) used PFGE to investigate cross-contamination in a poultry slaughterhouse and it observed similar profiles in isolates recovered at both the early and later stages of processing, which was attributed to survival and contamination by flock-specific strains along the processing line. This study however did not estimate the proportion of carcass isolates directly attributed to contamination from *Campylobacter* derived from caecal carriage in broilers from within the same batch. A more recent study by Natsos et al. (2021) used *fla*A sequencing to look at the molecular diversity of *Campylobacter* spp. isolated from broiler flocks in Greece, and showed the presence of the

same *fla* types and antimicrobial resistance profiles in isolates collected from the same farm at different times, adjacent houses of the same farm and different farms and concluded persistence of strains with further contamination of subsequent batches and/or contamination via equipment, working clothes and vehicles. To the best of the authors' knowledge, our study is the first one to use WGS to provide an estimate of the significance of caecal carriage as a source of contamination of broilers carcasses from the same batch.

Regarding virulence, isolates belonging to CC-21 (ST19, ST21, ST50 and ST53) harboured the *wlaN* gene, which is associated with toxin production, and the genes associated with LOS. This is concerning as *C. jejuni* isolates carrying the *wlaN* gene and genes associated with LOS are presumably involved in the expression of ganglioside mimicry which is thought to be a critical factor in the triggering of Guillain–Barre´and Miller Fisher syndrome after *C. jejuni* infection in clinical cases (Epping et al., 2019; Gargiulo et al., 2011; Kolehmainen et al., 2019; Linton et al., 2000). A previous study did not detect any of these virulence genes in a subset of 15 Irish poultry *C. jejuni* genomes mostly isolated in 2008 but did detect them amongst 15 Irish human clinical isolates from 2016 (Truccollo et al., 2021). A more recent study by Emanowicz et al. (2022) reported these genes to be present in *Campylobacter* from an Irish poultry processing plant during 2018 and 2019 which agrees with the results of our study. Emanowicz et al. (2022) reported the prevalence of these and other genes in 3%–20% of isolates which is less than our results with a prevalence 29.8% for *cstIII*, *NeuB1*, *NeuC1* and *wlaN* and 22.8% for *NeuA1*. We observed the highest number of virulence genes in isolates of CC-21 as has been previously observed in Ireland and Poland (Emanowicz et al., 2022; Fiedoruk et al., 2019).

Another gene of particular significance is the Type IV secretion system (T4SS), encoded by a plasmid (*pVir*), which is important for both adherence and invasion of *Campylobacter* in intestinal epithelial cells (Tracz et al., 2005) and previously found in human isolates from routine stool samples (both diarrheic and non-diarrheic) in Poland (Wysok et al., 2020). T4SS is used by bacteria to transport proteins or protein-DNA complexes across the cell and help bacteria to adapt to changes in their environment (Wallden et al., 2010). Plasmid p*Vir* and its virulence *vir* genes have a very low prevalence globally (below 5%) (Panzenhagen et al., 2021) which is in keeping with this study that found it in only 2 isolates (3.5% prevalence), and this seems to suggest they are not relevant for the virulence of *C. jejuni*.

The *eptC* gene was detected in all isolates with the exception of one (ST9393). It has been reported that this gene may have a role in adherence and biofilm formation on food contact surfaces (Emanowicz et al., 2022; Lim & Kim, 2017) and therefore is potentially of significance for its contribution to cross-contamination (Bridier et al., 2015). Genes associated with capsule synthesis, that is, *cj1417c*, *cj1419c*, *cj1420c* and *kpsm* were also identified in the present study, and these have previously been reported to be associated with colonization (Truccollo et al., 2021).

In conclusion, our results using WGS revealed a high genetic diversity amongst *C. jejuni* broiler isolates, and it was observed that certain types that cause human illness appear to frequently occur in broilers. Virulence genes previously reported to be associated with survival and environmental adaptation during the processing stages in addition to some implicated with the development of more severe human illness were also identified. WGS allowed us to confirm that the major factor contributing towards contamination of broiler carcasses at abattoirs in Ireland is the contaminated caeca from birds from the same batch. While the prevention and control of *Campylobacter* contamination during processing is important for the reduction of *Campylobacter* contamination in chicken carcasses, implementation of critical control measures at farm level is required to reduce the number of colonized flocks entering the slaughter plant. The results from this present study will provide further insight on the influence and relative importance of on-farm colonization versus slaughterhouse cross-contamination on the presence of *C. jejuni* in carcasses.

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CONFLICT OF INTEREST

No conflict of interest declared.

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SUPPORTING INFORMATION

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