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Antimicrobial Resistance Determinants Circulating among Thermophilic Campylobacter Isolates Recovered from Broilers in Ireland Over a One-Year Period

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Abstract: Campylobacteriosis is the leading cause of human bacterial gastroenteritis, very often associated with poultry consumption. Thermophilic Campylobacter (Campylobacter jejuni and Campylobacter coli) isolates (n = 158) recovered from broiler neck skin and caecal contents in Ireland over a one-year period, resistant to at least one of three clinically relevant antimicrobial classes, were screened for resistance determinants. All ciprofloxacin-resistant isolates (n = 99) harboured the C257T nucleotide mutation (confering the Thr-86-Ile substitution) in conjunction with other synonymous and nonsynonymous mutations, which may have epidemiological value. The A2075G nucleotide mutation and amino acid substitutions in L4 and L22 were detected in all erythromycin-resistant isolates (n = 5). The tetO gene was detected in 100% (n = 119) of tetracycline-resistant isolates and three of which were found to harbour the mosaic tetracycline resistance gene tetO/32/O. Two streptomycin-resistant C. jejuni isolates (isolated from the same flock) harboured ant(6)-Ib, located in a multidrug resistance genomic island, containing aminoglycoside, streptothricin (satA) and tetracycline resistance genes (truncated tetO and mosaic tetO/32/O). The ant(6)-le gene was identified in two streptomycin-resistant C. coli isolates. This study highlights the widespread acquisition of antimicrobial resistance determinants among chicken-associated Campylobacter isolates, through horizontal gene transfer or clonal expansion of resistant lineages. The stability of such resistance determinants is compounded by the fluidity of mobile genetic element.

Keywords: Campylobacter; antimicrobial resistance; ciprofloxacin; tetracycline; aminoglycosides; macrolides

1. Introduction

Campylobacter is the most commonly reported foodborne bacterial pathogen causing human gastroenteritis in the European Union (EU) and Ireland, most often associated with the broiler reservoir [1]. Ireland was found to have a 98% prevalence of campylobacter-contaminated broiler
carcasses in 2008 [2]. Frequent isolation of antimicrobial-resistant Campylobacter spp. of food animal origin continues to limit the spectrum of clinically useful antimicrobials and is internationally recognised as a major societal challenge. Veterinary antimicrobials used therapeutically and prophylactically are often the same as, or belong to the same class as those used clinically [3].

Macrolides, fluoroquinolones and aminoglycosides are classified as critically important antimicrobials, while tetracycline is considered a highly important antimicrobial [3]. Resistance to (fluoro)quinolones and tetracyclines is highly prevalent in clinical and broiler-associated Campylobacter spp. isolates, while resistance to erythromycin is typically low to moderate across Europe [4–6]. Macrolides are the first line antibiotic for the treatment of enteric gastroenteritis, while fluoroquinolones and tetracyclines remain as alternatives [7–9]. Systemic infections are routinely treated with aminoglycosides and resistance to aminoglycosides is low in clinical and broiler isolates across Europe [5]. Cross-resistance between aminoglycoside antimicrobials is incomplete and although streptomycin is not used clinically to treat campylobacteriosis, resistance can be used as an indicator for acquired aminoglycoside resistance genes.

In Gram negative bacteria, DNA gyrase is the primary target of fluoroquinolones [11]. DNA gyrase is a heterotetrameric type IIA topoisomerase, consisting of two polypeptide subunits (GyrA and GyrB, encoded by gyrA and gyrB, respectively), catalysing ATP-dependent negative supercoiling of DNA to regulate replication, repair and gene expression [12–14]. Resistance to (fluoro)quinolones among Campylobacter spp. is largely mediated by chromosomal mutations in the quinolone resistance-determining region (QRDR) of gyrA, typically conferred by the C257T nucleotide mutation (Thr-86-Ile) [15]. The QRDR is located near the Tyr-125 active site, involved in DNA-protein bridge formation during DNA strand passage [15].

Macrolides act by binding to the 50S bacterial ribosomal subunit and inhibit translational elongation, and interfere with protein synthesis and subsequent ribosomal subunit assembly [16,17]. Polymorphisms in the 23S ribosomal RNA (rRNA), mutations in 50S ribosomal proteins L4 and L22 (encoded by rplD and rplV, respectively) or the presence of the emerging ermB gene contribute to macrolide resistance [8,18]. Beta-hairpin extensions from 50S ribosomal proteins L4 and L22 are involved in the regulation of nascent peptide exit from the large ribosomal subunit [19,20]. Mutations in L4 and L22, combined with the overexpression of antimicrobial efflux genes have been reported to contribute to high-level macrolide resistance [21]. The ribosomal methylase encoded by ermB was reported recently for the first time in thermophilic Campylobacter spp., located on a chromosomal multidrug resistance genomic island (MDRGI) (likely originating from a Gram positive species) in a high-level erythromycin-resistant Campylobacter coli (C. coli) isolate (ZTC113) of swine origin in China [8,22]. ErmB dimethylates adenine at position 2074 of the 23S rRNA gene, reducing the affinity of macrolides [23].

The intrinsic, chromosomally encoded resistance-nodulation-division (RND) CmeABC (Campylobacter multidrug efflux) efflux pump in Campylobacter spp. contributes to baseline resistance against structurally diverse antimicrobials [6,24–26]. The cmeABC operon encodes a tripartite multidrug efflux pump that consists of an outer membrane channel protein (cmeC), an inner membrane efflux transporter (cmeB) and a periplasmic fusion protein (cmeA) [27]. Repressor (cmeR) binding to an inverted repeat (IR) (TGTAATAAATATTACA) in the intergenic region between cmeR and cmeA transcriptionally represses the cmeABC operon [28,29]. Consequently, polymorphisms in the repressing site induce efflux pump overexpression and enhanced resistance to antimicrobials, most notably, erythromycin [8,30].

Tetracycline resistance in Campylobacter spp. is largely conferred by a ribosomal protection protein (RPP), TetO, capable of displacing tetracycline from its primary binding site on the 30S ribosomal subunit [17,31]. Bacterial resistance to tetracycline is also associated with ATP-dependent efflux or enzymatic inactivation of tetracycline [32–34]. Campylobacter tetO can be located chromosomally but is often plasmid-mediated [31,33,35–39]. Tetracycline RPPs are widely distributed among bacterial genera and it has been reported that the tetO gene exists in at least eleven bacterial genera, including four Gram negative and seven Gram positive genera [33]. The conserved acquisition of tetO between members of
different bacterial genera indicates that conjugative plasmids, transposons, or recombination events contribute to the dissemination and maintenance of the tetO gene [37].

Although tetO acquisition is the most prevalent genetic event conferring tetracycline resistance among Campylobacter spp., mosaic tet genes (specifically tetO32/O) have also been reported within the genus [34]. Mosaic tetracycline resistance genes are derived from the interclass (double-crossover) recombination of two or more RPP-encoding gene (predominantly tetO, W, 32 [34,40–42] and tetM, S [43]) to form functional chimera [34]. Mosaic tet genes are widespread among Gram positive and Gram negative genera in human and animal isolates [41].

Aminoglycosides are broad spectrum antimicrobials and inhibit protein synthesis by binding to 16S rRNA of the 30S ribosome [44,45]. Campylobacter spp. resistance to aminoglycosides is mediated by reduced antimicrobial binding affinity for target sites due to enzymatic modification, via acetylation, phosphorylation or adenylation of amino or hydroxyl groups of the aminocyclitol nucleus or sugar moieties [44–46]. Although there are two main nomenclature systems used to identify aminoglycoside modifying enzymes [47–49], we followed the system proposed by Shaw et al. (1993), later extended to include an expanded panel of aminoglycoside 6-nucleotidyltransferases (also known as adenylyltransferases) [50,51]. The designation proposed by Shaw et al. (1993) is as follows: the type of modification (nucleotidyltransferase/adenyltransferases (ANT)); the modification site (6’); a roman numeral to denote unique resistance profiles (I), and a letter to represent unique protein sequences (b) [48].

Genes for ANT enzymes are found on transposons, plasmids or chromosome, often in associated with other resistance genes and very often as part of the transposon-associated aminoglycoside-streptothricin resistance gene cluster (ant(6)-I-sat4-aphA3), first isolated from Staphyloccoci [52,53]. ANT(6)-I encoding genes are widely distributed among clinical and animal streptomycin-resistant thermophilic Campylobacter spp. isolates [51].

Despite the high prevalence of Campylobacter in broilers on the island of Ireland, in the last twenty years, only a few reports of the molecular mechanisms contributing to resistance exist for broiler [54–59], clinical [54,56,59–61], domestic animal [62] or ruminant [56,63] isolates. We report the antimicrobial resistance determinants circulating among 158 resistant Campylobacter jejuni (C. jejuni) and C. coli isolates recovered from Irish broiler neck skin and caecal samples over a one-year period (2017–2018).

2. Results

2.1. Fluoroquinolone Resistance

Isolates resistant to ciprofloxacin/nalidixic acid were screened for mutations in the gyrA gene and 100% of isolates (n = 99) harboured a C257T point mutation, which is the dominant mutation conferring resistance among campylobacters. Resistant isolates were grouped into C. jejuni gyrA and C. coli gyrA (arbitrarily named GTJs and GTCs, respectively) sequence types based on the presence of synonymous and nonsynonymous mutations present in the portion of the gyrA gene sequenced (Table 1). Ciprofloxacin-resistant C. jejuni isolates (n = 85) were grouped into three GTJs (GTJ-I, -II, and -III). A large proportion (47.1%) carried the Thr-86-Ile substitution exclusively (GTJ1). Synonymous mutations T72C, C243T, T357C, C360T, C471T, T483C, and C622T were exclusively associated with C. jejuni and were present in both GTJ-II and GTJ-III. Nonsynonymous Ser-22-Gly (A64G) and Ala-206-Thr (G616A) mutations were present in 35.3% and 17.7% of isolates of ciprofloxacin-resistant C. jejuni isolates, respectively and were the basis of defining GTJ-II and GTJ-III, respectively. Both GTJ-II and GTJ-III were associated with the Asn-203-Ser (A608G) substitution. All CIP-resistant C. coli isolates tested (n = 14) harboured one nonsynonymous mutation only (Thr-86-Ile), but were grouped into seven GTCs based on the presence of various synonymous mutations (Table 1).
Table 1. GyrA sequence types (GTs) and associated polymorphisms distributed among 85 ciprofloxacin-resistant *C. jejuni* isolates (GTJs) and 14 ciprofloxacin-resistant *C. coli* isolates (GTCs). Polymorphisms causing an amino acid substitution (nonsynonymous mutations) are highlighted in black.

| GTJ  | n  | Nucleotide Position (Base Indicated in Brackets) of Wild-type Strain *C. jejuni* NCTC 11168 gyrA (GenBank Accession Number: L04566.1 and AL111168.1) |
|------|----|----------------------------------------------------------------------------------|
|      |    | 64 (A) 72 (T) 243 (C) 257 (C) 357 (T) 360 (C) 471 (C) 483 (T) 608 (A) 616 (G) 622 (T) |
| GTJ-I | 40 (47.1%) | . . . . . . T . . . . . . |
| GTJ-II | 30 (35.3%) | G C T T C T G . T . . . . . . |
| GTJ-III | 15 (17.7%) | C T T C T C T C G A T T . . |

| GTC  | n  | Nucleotide Position (Base Indicated in Brackets) of Wild-type Strain *C. coli* NCTC 11366 gyrA (GenBank Accession Number: AF092101.1 and NZ_UIGM01000003.1) |
|------|----|----------------------------------------------------------------------------------|
|      |    | 117 (T) 252 (C) 257 (C) 297 (C) 342 (T) 471 (T) 498 (G) |
| GTC-I | 1 (7.1) | . . . . . . T C . . . . . . |
| GTC-II | 7 (50) | C . . . . . . T C . . . . . . |
| GTC-III | 1 (7.1) | C . . . . . . T C . . . . . . |
| GTC-IV | 1 (7.1) | . . . . . . T C . . . . . . |
| GTC-V | 2 (14.3) | . T T . T C . . . . . . |
| GTC-VI | 1 (7.1) | . . . . . . T C . . . . . . |
| GTC-VII | 1 (7.1) | . . . . . . T C . . . . . . |
No high-level moxifloxacin resistance was detected among the 99 (fluoro)quinolone-resistant isolates tested and minimum inhibitory concentrations (MICs) ranged from 0.5–8 mg/L.

2.2. Erythromycin Resistance

Five erythromycin-resistant isolates were screened for mutations contributing to erythromycin resistance. All five erythromycin-resistant isolates harboured the A2075G mutation in the 23S rRNA gene. A T82C mutation (Ser-28-Pro substitution) in the rplD gene was detected in the five isolates, and the partial sequences shared 100% homology with the rplD gene of erythromycin-sensitive C. coli isolates (GenBank accession numbers: MH084640.1 and MH084639.1) [64]. Identical mutations were also observed in the rplV sequence in all erythromycin-resistant isolates: double point mutation at positions 308 and 309 (Ala-103-Val), A325G (Thr-109-Ala), a double point mutation at positions 332 and 2020.

Aminoglycoside 3'-N-acetyltransferase, SatA IPR008125 EOO12820.1

The antimicrobial resistance rates of this pool of broiler-associated thermophilic Campylobacter spp. (GenBank accession numbers: WP_052855148.1) and Serratia marcescens (GenBank accession number: Q7B9H0), S. suis, L. monocytogenes and P. interleukinosa (GenBank accession numbers: WP_052786298.1) were also observed in the tetO gene (900 bp), with almost identical sequences (99.89%) (Table 2). Equally, the TetO/32/O gene was not detected among the erythromycin-resistant isolates tested.

2.3. Tetracycline Resistance

A portion of the tetO gene was detected in 100% of tetracycline-resistant isolates (n = 119). Three isolates (CITCj625-18, CITCj727-18, and CITCc3448-18), accounting for 2.5% of the tetracycline-resistant isolates, harboured the mosaic tetO/32/O type II gene, confirmed by PCR/partial sequencing and genomic sequencing (Figure 1B). Isolates CITCj625-18 and CITCj727-18 carried identical mosaic tetracycline genes, but differed from CITCCc3448-18 by Thr-118-Ile and Glu-176-Asp substitutions and A684G and A789G point mutations. The mosaic tetracycline gene detected among the Irish broilers isolates in this study was very similar to a Streptococcus suis (S. suis) (GenBank accession number: KY994102.1) tetO/32/O gene (Table 2). Equally, the TetO/32/O sequences were >99.5% identical to Campylobacter spp. (GenBank accession numbers: WP_052855148.1) and Clostridiales (WP_117823345.1) TetO/32/O sequences.

Figure 1. **A** Schematic of *Campylobacter jejuni* isolate CITCj625-18 multidrug resistance genomic island. **B** Schematic of the mosaic tetO/32/O type II gene detected in CITCj625-18 and CITCj727-18. Figure adapted from Warburton et al. (2016). White bars are tetO and central, checked bar is tet32 (297 bp).
Table 2. Percentage identity (percentage cover in brackets) of mosaic tetracycline resistance gene (tetO/32/O) type II in three tetracycline-resistant Campylobacter spp. isolates detected in this study and Streptococcus suis tetO/32/O gene (GenBank accession number: KY994102.1).

| Species/Strain | C. jejuni CITCj625-18 tetO/32/O | C. jejuni CITCj727-18 tetO/32/O | C. coli CITC727-18 tetO/32/O | KY994102.1 S. suis tetO/32/O |
|----------------|---------------------------------|---------------------------------|----------------------------|-----------------------------|
| C. jejuni CITCj625-18 tetO/32/O | 100% (100%) | 100% (100%) | 99.78% (96%) | 99.73% (96%) |
| C. jejuni CITCj727-18 tetO/32/O | 100% (100%) | 100% (100%) | 99.78% (96%) | 99.73% (96%) |
| C. coli CITC727-18 tetO/32/O | 99.78% (100%) | 100% (100%) | 100% (100%) | 99.95% (100%) |
| KY994102.1 S. suis tetO/32/O | 99.73% (100%) | 99.73% (100%) | 99.95% (96%) | 100% (100%) |

2.4. Streptomycin Resistance

C. jejuni isolates CITCj625-18 and CITCj727-18 were found to harbour multiple resistance genes, including a truncated tetO (873 bp, truncated at the 3’ end), mosaic tetO/32/O type II (1920 bp), aminoglycoside-6-nucleotidyltransferase (ant(6)-Ib), and streptothricin acetyltransferase (satA). These antimicrobial resistance genes were located circumjacent to proteins involved in replication and recombination (Table 3, Figure 1A). The MDRGI contained 10 open reading frames in a region of 35.9% GC content, compared to the average genomic GC content of 30.5%. Genomic sequences of C. jejuni isolates CITCj625-18 (first thin) (1,630,363 bp) and CITCj727-18 (final thin, isolated a week later) (1,636,524 bp) were nearly identical with an average nucleotide identity (ANI) of 99.99% and 30.5% GC content, indicating that the isolates had been circulating within and had been maintained by the flock. The pair belonged to sequence type ST-45 clonal complex (ST-137) and harboured LOS locus class C.

Table 3. Description of CITCj625-18 (representative of CITCj727-18) multidrug resistance genomic island.

| Locus Tag       | Gene Annotation            | InterProScan Protein Family or Domain | GenBank Accession Number of Closest (%) Identity/% Coverage | Predicted Function                                                                 |
|-----------------|----------------------------|---------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------------------------------|
| HBF06_00624     | Holliday junction ATP-dependent DNA helicase, RuvB | IPR004605 WP_002856258.1 100/100 | Holliday junction helicase (strand exchange reactions in homologous recombination) |
| HBF06_00625     | Histidine phosphotransferase Autotransporter adhesion, CapC | IPR036641 MPA99107.1 100/90 | Signal transduction                                        |
| HBF06_00627     | Truncated tetracycline resistance ribosomal protection protein, TetO | IPR005546 EAK6247206.1 99.16/94 | Protein secretion                                          |
| HBF06_00628     | Aminoglycoside-6-nucleotidyltransferase, ANT(6)-Ib | IPR035650 AUA17601.1 99.59/92 | Ribsosomal protection protein conferring tetracycline resistance (TetO) |
| HBF06_00629     | Replication protein, RepB | IPR007530 WP_001255868.1 100/100 | Adenyllytransferase activity conferring resistance to aminoglycosides |
| HBF06_00630     | DNA topoisomerase            | IPR002631 WP_002777339.1 100/100 | Plasmid replication protein                                |
| HBF06_00631     | Topoisomerase                | IPR000380 WP_139985553.1 99.52/100 | Topoisomeration and single stranded breakage during transcription, DNA replication and recombination. |
| HBF06_00632     | Streptothricin acetyltransferase, SatA | IPR008125 ECO12620.1 96.86/100 | Acetylation of streptothricin conferring resistance |
| HBF06_00633     | Mosaic tetracycline resistance ribosomal protection protein, TetO/32/O | IPR005560 WP_002855148.1 100/100 | Ribosomal protection protein conferring tetracycline resistance |
| HBF06_00634     | Plasmid replication protein | None detected EDP4862066.1 99.21/97 | Plasmid replication                                        |

CITCc1631-18 and CITCc3448-18 belonged to ST-828 clonal complex (ST-6543 and ST-1096, respectively) and harboured the ant(6)-le gene (900 bp), with almost identical sequences (99.89% identity). ANT(6)-le in this study shared 99.66% amino acid identity with each other, where CITCc3448-18 harboured a nonsynonymous G820A mutation (Val-274-Ile) and was identical to a C. coli ANT(6)-le protein (GenBank: WP_052786298.1). CITCc1631-18 and CITCc3448-18 ANT(6)-le shared between 29.7-36.1% identity to ANT(6)-Ia, ANT(6)-Ib, ANT(6)-Ic, and ANT(6)-Id amino acid sequences (GenBank: AF97257.1, AF97264.1, AAR10415.1, and WP_001258597.1, respectively) [50,51,65].
Aminoglycoside 3-N-acetyltransferase (AAC(3)) (261 amino acids) was also detected in both CITCJ625-18 and CITCJ727-18, 44.3% identical to a aminoglycoside 3-N-acetyltransferase (AAC(3)) variant (239 amino acids) in both CITC1631-18 and CITC3448-18. However, these isolates were gentamicin-susceptible, although this protein may confer resistance to other aminoglycoside antibiotics [66]. The C. coli variant was identical to AAC(3) genes reported in Campylobacter spp., 37.5% identical to AAC(3)-Ia described in Serratia marcescens (GenBank accession number: Q7B9H0), and 19–32.25% similar to AAC(3) orthologous, including types AAC(3)-Ib/Id/Ie/Ila/Ilb/Ilc/Ild/Ile/Ill/Ila/Illb/Illc/Iva/Vla/VIIIa/Ixa/Xa/Xla. The AAC(3) variant in CITCJ625-18 and CITCJ72-18 was identical to Campylobacter AAC(3) variants and was 19.3–25.84% similar to orthologues reported in other bacterial genera.

2.5. Overall Distribution of Antimicrobial Resistance

The antimicrobial resistance rates of this pool of broiler-associated thermophilic Campylobacter spp. isolates (resistant to at least one antimicrobial; n = 158) have been detailed previously [67], and are summarised below (Figure 2).

![Figure 2. Distribution of antimicrobial resistance among 158 thermophilic Campylobacter spp.](image)

3. Discussion

This study reports the antimicrobial resistance determinants circulating among Irish broiler-associated Campylobacter isolates, collected throughout the Republic of Ireland, from the three largest poultry processors, over a one-year period (2017–2018).

The Thr-86-Ile mutation is the predominant genetic alteration conferring (fluoro)quinolone resistance among Campylobacter spp. [68], and was detected in all (fluoro)quinolone-resistant isolates (n = 99) tested in the current study, similar to reports published worldwide [6,69–71]. Some studies have not detected the Thr-86-Ile mutation universally in the QRDR of (fluoro)quinolone-resistant Campylobacter spp. isolates [58,62,72], indicating that other factors are responsible for, or contribute to, (fluoro)quinolone resistance.

Fluoroquinolone-resistant Campylobacter spp. are ecologically competitive and persistent even in the absence of antimicrobial selective pressure [73–75]. Despite a distinct rise in ciprofloxacin resistance among broiler Campylobacter isolates (3.1% to 28.9%) in Ireland between 1998 and 2000 [54,76], in 2017–8 resistance to ciprofloxacin remained stable (28.3%) [67]. Clonal expansion of resistant lineages has likely contributed to the persistence of ciprofloxacin resistance in Ireland, considering that fluoroquinolones typically account for less than 1% of all veterinary antimicrobials sold in Ireland [77–79].

C. jejuni isolates harbouring multiple amino acid substitutions in the gyrA QRDR (GTJ-II and GTJ-III) (Table 1) had ciprofloxacin MICs ranging from 8–16 mg/L, except one GTJ-II isolate had an MIC of 4 mg/L, while isolates harbouring the Thr-86-Ile mutation exclusively (GTJ-I) had MICs ranging...
from 4–16 mg/L. Similarly, Ekkapobyotin et al. (2008) observed varying ciprofloxacin/nalidixic acid MICs in isolates harbouring identical GyrA amino acid substitutions [80]. Moreover, the Ser-22-Gly, Asn-203-Ser and Ala-206-Thr mutations have been reported in fluoroquinolone-sensitive strains [81–83].

To confirm the apparent lack of involvement of these accessory gyrA mutations in the development of fluoroquinolone resistance, the introduction of these mutations in fluoroquinolone-susceptible strains could be investigated. Authors have previously reported that double mutations in gyrA (at amino acid positions 86 and 90) were necessary to produce high level moxifloxacin resistance [84,85]. Moxifloxacin is a potent fluoroquinolone with activity against fluoroquinolone-resistant campylobacters that harbour a single mutation in gyrA [86]. In this study, no high-level resistance to moxifloxacin was observed among the (fluoro)quinolone-resistant isolates. These data indicate that mutations outside the gyrA QRDR have a negligible effect on (fluoro)quinolone resistance.

Variation in gyrA alleles within a population of Campylobacter isolates have been identified as epidemiological markers and may serve as a supplementary approach to classical epidemiological typing methods [81,83,87,88]. In our study, the GTs detected were species specific, although Ragimbeau et al. (2014) reported the presence of a typical C. coli gyrA type in 0.23% (n = 1) of 430 C. jejuni isolates tested, and 1.4% (n = 4) C. coli isolates harboured a typical C. jejuni gyrA type. The amino acid substitutions present in each of the three GTJ (Table 1) lineages have been associated with poultry Campylobacter isolates previously [89–91]. Only one nonsynonymous mutation (Thr-86-Ile) was detected among the seven GTCs detected (Table 1), similar to previous studies reporting a single nonsynonymous mutation (Thr-86-Ile substitution) present in (fluoro)quinolone-resistant C. coli QRDR sequences [83,89,90]. It is likely that additional variants of Campylobacter spp. gyrA alleles exist, and may reflect ecological evolution [83].

Identical mutations in the 23S rRNA, rplD, and rplV genes were detected in erythromycin-resistant isolates (n = 5), while MICs ranged from 128 mg/L to ≥ 128 mg/L. Three of these isolates were collected from the same flock in north-central Ireland while one isolate was collected from a farm approximately 10 km away, the following week. The fifth erythromycin-resistant isolate was recovered from a farm in the mid-south-west of Ireland, two months previously, but all birds from these farms were processed in the same processing plant.

The A2075G point mutation in the 23S rRNA gene remains the most prevalent genetic event conferring macrolide resistance [8,92] and was detected in all erythromycin-resistant isolates in this study. The 23S rRNA A2074G and A2074C mutations were not detected. Mutations in the rplV gene, encoding the 50S ribosomal protein L22 were detected in the C-terminal region (amino acids 109–142) [18], including Thr-109-Ala, Ala-111-Glu, Ala-114-Thr, and Pro-120-Thr. Nonsynonymous mutations (T282A and C294T) were also observed in the region encoding the highly conserved β-hairpin loop at amino acids 78–98 [18]. Mutations in the RplD β-hairpin (spanning amino acid positions 55–77 [18,93]) are often associated with bacterial macrolide resistance, and such mutations were not observed among the Irish erythromycin-resistant isolates tested in this study. Moreover, polymorphisms were detected outside the cmeR regulatory IR. The effects of mutations detected in this study in rplD, rplV, and the intergenic region of cmeR-cmeA are unknown, but may contribute to erythromycin resistance.

The ermB gene was not detected among the erythromycin-resistant isolates tested. Resistance mediated by ermB in Campylobacter spp. is largely confined to China, which may reflect the extensive use of antimicrobials in food producing animals in China [8]. Three reports of genetically distinct ermB-positive C. coli isolates recovered from poultry exist in Europe [94–96] and an ermB-positive isolate was detected for the first time in the United States in a C. jejuni isolate of clinical origin [97], while the ermB gene was recently detected in 18.3% of 240 thermophilic Campylobacter spp. retail meat associated-isolates in South Africa [98]. Mutations in C. jejuni 23S rRNA has been associated with a fitness cost and reduced doubling times [99–101], although tolerance to low temperatures may facilitate persistence in the environment and transmission of resistant strains through the food supply [100].
All tetracycline-resistant isolates harboured a portion of the *tetO* gene, while three isolates harboured the mosaic *tetO32/O* type II gene were detected. Mosaic tetracycline resistance genes in *Campylobacter* spp. are typically derived from *tetO* and *tet32* sequences in the type II conformation, with a shorter central *tet32* segment [34], although there are limited reports of these resistance genes circulating among *Campylobacter* spp. The first mosaic tetracycline RPP gene was detected in *Megasphaera elsdonii*, composed of a central *tetW* region flanked by two *tetO* regions [102]. However, the progenitors of these mosaic genes are based only on the order in which they were discovered, and the current classification system does not adequately reflect the variable nature of tetracycline RPPs [34,43]. The *tetO* primers [103] used in this study amplify a region at the beginning of the *tetO* gene and enable the detection of mosaic *tet* genes with a central portion flanked by an initial *tetO* region until position 228. The *tetO32/O* type II gene here reported was associated exclusively with an MIC of 64 µg/L. However, 27 of 119 (22.7%) tetracycline-resistant *Campylobacter* spp. isolates tested had MICs of 64 µg/L or ≥ 64 µg/L, indicating that other factors contribute to enhanced tetracycline resistance. It should be noted in this study, all three isolates harbouring mosaic tetracycline genes were also co-resistant to streptomycin, enabling co-selection and persistence of these antimicrobials. The burden of mosaic tetracycline resistance genes within the genus should be considered as part of the approach to elucidate developing and newly acquired antimicrobial resistance determinants within the genus.

All streptomycin resistant (n = 4) isolates had MICs of ≥ 16 µg/L. Streptomycin-resistant *C. jejuni* isolates CITCj625-18 and CITCj727-18 (ST-137) harboured sialylated LOS locus class C, which has been identified as a risk factor for post-infectious Guillain-Barré syndrome and increased severity of enteric disease [104]. CITCj625 and CITCj727-18 belonged to ST-137 and members of the diverse *C. jejuni* ST-45 clonal complex [105,106]. ST-137 is frequently isolated from cases of enteric campylobacteriosis [105] and broilers/avian [107–109], porcine [105], and bovine [110] hosts. The ST-137 genotype is widely dispersed and represents an ecologically successful clone [106]. A study by Dearlove et al. (2016) reported that ST-45 clonal complex was a generalist lineage capable of frequent transmission between hosts.

Genomic sequencing of tetracycline-/streptomycin-resistant *C. jejuni* isolates (CITCj625-18 and CITCj727-18, isolated from the same flock, first and final thin) revealed identical genes in a multidrug resistance genomic island (Table 3, Figure 1A). Both isolates harboured a truncated *tetO* gene and a mosaic *tetO32/O* type II gene, homologous that of Gram positive (GenBank accession number: KY994102.1) and *Campylobacter* spp. (GenBank accession number: WP_002823161.1). The presence of multiple *tet* genes (coding for similar or different mechanisms) in Gram negative isolates has also been documented [33]. However, the truncated form detected in this study (CITCj625-18 and CITCj727-18) is likely a remnant of a region of insertion or recombination. Truncated *tetO* genes have also been reported in *C. coli* MDRGI containing *aadE* (*ant(6)-Ib*) and *ermB* [94,111]. Aminoglycoside (*ant(6)-Ib* (867 bp)) and streptothricin resistance (*satA*) genes were also located within the multidrug resistance island (Figure 1A). Streptothricin acetyltransferase A (*satA*) is frequently reported in Gram positive bacilli [112] and shares less than 40% identity with the streptothricin acetyltransferase A (*sat4*) reported in *Campylobacter* [52,111,113]. Plasmid replication proteins within the MDRGI suggest a plasmid as the insertion vehicle of the resistance genes.

CITCc3448-18 belonged to ST-828 clonal complex (ST-1096). ST-1096 has been isolated from a case of gastroenteritis in the United Kingdom (UK) in 2016 [114] and has also been previously reported in *C. coli* of swine origin in Spain, America, and Grenada [51,115,116]. CITCc1631 was ST-6543 (ST-828 clonal complex), which has been associated with clinical and chicken-associated isolates [117]. There are a total of fifteen depositions (all are UK associated) of *C. coli* ST-6543 on the PubMLST *Campylobacter* database at the time of writing [114], including eleven clinical isolates (faeces), two chicken-associated isolates, and two isolates with no source allocation.

Both streptomycin-resistant *C. coli* isolates (CITCc1631-18 and CITCc3448-18) harboured the emerging *ant(6)-le* gene (900 bp), found widely disseminated among clinical and animal *C. coli* isolates [51,65]. Unlike other *Campylobacter ant(6)* genes, *ant(6)-le* appears to be inherent to *C. coli* and does not have a Gram positive ancestor [65]. The *ant(6)-le* gene was originally detected in a hypervariable
genomic region, unaccompanied by other resistance genes [65]. Similarly, the CITCc1631-18 and CITCc3448-18 ant(6)-le genes were located chromosomally, and were not located near other resistance determinants. Both streptomycin-resistant C. coli isolates were co-resistant to ciprofloxacin/nalidixic acid (GTC-V) and tetracycline. C. coli isolate 1631-18 harboured tetO (1920 bp), while C. coli isolate CITCc3448-18 also harboured the mosaic tetracycline resistance gene, tetO/32O (1290 bp), highly homologous with that detected in CITCj625-18 and CITCj727-18.

4. Materials and Methods

4.1. Bacterial Isolate Culture Conditions and Susceptibility Testing

A total of 350 thermophilic Campylobacter isolates (296 C. jejuni and 54 C. coli) were recovered from free range and intensively-reared broiler carcasses (neck skin and caecal contents) using ISO 10272-2:2017 [2]. Isolates were collected between September 2017 and September 2018, from the three largest poultry processing plants in the Republic of Ireland. The collection of isolates was speciated using matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker, Billerica, MA, United States). Isolates were previously tested [67] for their MIC to six clinically relevant antimicrobials, namely ciprofloxacin, nalidixic acid, erythromycin, tetracycline, gentamicin, and streptomycin according to ISO 20776:2006 and EC Decision 2013/652/EU [118,119]. Overall, 158 (140 C. jejuni and 18 C. coli) isolates tested were resistant to at least one antimicrobial and were subsequently tested for resistance determinants.

4.2. DNA Extraction

Briefly, isolates were recovered from −80 °C on Columbia blood agar (Fannin Ltd, Dublin, Ireland) and incubated for 24 h at 42 °C, under microaerobic conditions (5% O2, 10% CO2, 85% N2) and subcultured. DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer’s instructions and DNA was standardised to 50–100 ng/µL.

4.3. Genotypic Characterisation of Antimicrobial Resistance—PCR Amplification and Sequencing

Primer sets, target genes and annealing temperatures are listed in Table 4. Primers to detect mosaic tetracycline resistance genes (tetO/32O and tetO/W/O) were designed on SnapGene2.3.2 software (from Insightful Science; available at snapgene.com) and regions of primer complementarity were assessed on Primer-BLAST [120].

PCRs were performed with 2.5 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA), 1× PCR buffer I and 2.5 mM magnesium chloride (Applied Biosystems), 0.2 mM of each deoxyribonucleotide (Sigma Aldrich, St Louis, MO, USA), 0.2 pmol/µL of each primer, and 1 µL (1–2 ng/µL) of DNA. Reaction conditions were denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing (Table 4) for 30 s and extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min. PCR products were purified using the High Pure PCR Purification Kit (Sigma Aldrich). Purified PCR products were Sanger sequenced (forward and reverse reads) (Table 4) by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany). Consensus sequences were aligned and assembled on SeqMan Pro (Lasergene) (DNAStar, Madison, WI, USA).
Table 4. Primer sequences and target genes used for the detection of antimicrobial resistance determinants in resistant thermophilic *Campylobacter* spp. isolates and sialylated lipooligosaccharide (LOS) locus classes A, B and C in streptomycin-resistant *C. jejuni* isolates.

| Primer          | Type     | Sequence (5' - 3')                                                                 | Target                                      | Amplicon Size (bp) | Annealing (°C) | Reference |
|-----------------|----------|----------------------------------------------------------------------------------|---------------------------------------------|--------------------|----------------|-----------|
| GZgyrA5         | A + S    | ATT TTT AGC AAA GAT TCT GAT                                                       | QRDR region of *gyrA*                       | 673                | 50            | [15]      |
| GZgyrA6         | A + S    | CCA TAA ATT ATT CCA CCT GT                                                        |                                             |                    |                |           |
| TetO-FW         | A        | ACG GAT AGT TTA TGG TAT ACC                                                        | tetO                                        | 171                | 52            | [103]     |
| TetO-RV         | A        | TGG GGT ATC TAT AAT GTT GAC                                                        |                                             |                    |                |           |
| TetO/W/O-F      | A        | ATC CAG ACA GCA GTG ACA TC                                                         |                                             |                    |                |           |
| TetO/W/O-R      | A        | ATG ATA GAC CGG AAA CAG GG                                                         |                                             |                    |                |           |
| TetO/32/O-32/F  | A        | GAT ACA ATG AAT TTT GAG CG                                                         |                                             |                    |                |           |
| TetO/32/O-32/R  | A        | AAT TGT CTT TGG CAC TCC C                                                         |                                             |                    |                |           |
| TetO/32/O-32/F  | A        | CGG GCA GGT TTA AGT G                                                            |                                             |                    |                |           |
| TetO/32/O-32/R  | A        | CTG TAT CAG CAA TCT CTG GG                                                        |                                             |                    |                |           |
| F2-campy-23S    | A + S    | AAT TGA TGG GGT TAG CAT TAG C                                                     | Domain V of 23S rRNA                       | 316                | 55            | [121]     |
| R2-campy-23S    | A + S    | CAA CAA TGG CTC ATA TAC AAC TTA                                                   |                                             |                    |                |           |
| L4c-F           | A + S    | TTA TCC TTC TTT TGT AAT AGA TTC TAA                                               | rplD                                        | 614                | 48            | [61]      |
| L4c-R           | A + S    | ATG AGT AAA GTA GTT TTA AAT GAT                                                    |                                             |                    |                |           |
| L22c-F          | A + S    | TTA GCT TTC CTT TTT CAC TGT TGC TTT                                              | rplV                                        | 425                | 48            | [61]      |
| L22c-R          | A + S    | ATG AGT AAA GCA TTA ATT AAA TTC ATA AG                                               |                                             |                    |                |           |
| ermB(B)-F       | A        | GGG CAT TTA ACG AGG AAA CTG G                                                      | ermB                                        | 421                | 52            | [111]     |
| ermB(B)-R       | A        | CTG TGG TAT GCC GGG TAA GT                                                         |                                             |                    |                |           |
| CmeclF3         | A + S    | AACACGCTTACTGGTCGTG                                                             | cmeABC                                      | 428                | 45            | [28]      |
| CmeclR4         | A + S    | AAATGTGTTAGCCGATCT                                                              |                                             |                    |                |           |
| cst-II-F        | A        | ATG AAA AAA GTT ATT ATT GGT GGA AAA TTA                                           | LOS locus class A/B                        | 885/876            | 50            | [122,123] |
| cst-II-R        | A        | TTA TTT TCC TTT TGG AAT ATT TTT TAT                                              |                                             |                    |                |           |
| orf14c-F        | A        | CAA GTG TGG AAA ATG TTA TCT ATT AAT                                              | LOS locus class C                          | 995                | 50            | [123]     |
| orf14c-R        | A        | ATG CAA ATA CAA AAC AAT TCC                                                       |                                             |                    |                |           |

A, amplification; A + S, amplification and sequencing.
Ciprofloxacin-resistant isolates (n = 99) were screened for mutations in the QRDR of the \textit{gyrA} gene \cite{15}. Products were purified and sequenced, as described above. Consensus sequences were aligned to the \textit{gyrA} of the reference \textit{C. jejuni} (GenBank accession number: L04566.1 and AL111168.1) and \textit{C. coli} sequences (GenBank accession number: AF092101.1 and NZ_UIGM01000003.1) on SnapGene 2.3.2.

The primers used for the amplification and sequencing of domain V of 23S rRNA, \textit{rplD}, and \textit{rplV} (encoding L4 and L22 ribosomal proteins, respectively) and the regulatory site of the \textit{cmeABC} operon in five erythromycin-resistant \textit{C. coli} isolates are listed in Table 4. Partial multiple alignment to reference \textit{C. coli} type strain NCTC 11366 (ATCC 33559) 23S rRNA (GenBank accession number: GQ167698.1), \textit{rplD} (GenBank accession number: DQ639752.1 and UIGM01000003.1), \textit{rplV} (GenBank accession number: UIGM0100003.1) and \textit{cmeABC} operon (GenBank accession number: FJ797670.1) sequences was performed on SnapGene 2.3.2. Isolates were also screened for the presence of \textit{ermB}, according to Wang et al. \cite{14}.

Tetracycline-resistant isolates (n = 119) were screened for the presence of \textit{tetO}, according to Aminov et al. \cite{2001} and products were visualised on 2% agarose gel electrophoresis. The \textit{tetO} amplicon of a tetracycline-resistant \textit{C. jejuni} isolate (CITCj382-18) from this study was purified and sequenced (as described above) as a positive control. Consensus sequences were aligned to the \textit{C. jejuni} \textit{tetO} gene (GenBank accession number: M18896.2).

Primers were designed to target \textit{tetO}/W/O (Table 4) based on alignments between \textit{tetO} (GenBank accession number: M18896.2) and mosaic \textit{tetO}/WO genes (GenBank accession numbers: EF065524.1 and AJ196921.1). Two \textit{tetO}/32/O-targeting primers (Table 4) were designed based on regions of homology between \textit{tetO} (GenBank accession number: M18896.2) and \textit{tetO}/32/O type I genes with a longer central \textit{tet}32 region (GenBank accession numbers: AJ295238.3 and JQ740052.1) and \textit{tetO}/32/O type II genes with a shorter central \textit{tet}32 segment (GenBank accession numbers: KY994102.1, FP929050.1, AIOQ01000025.1, NZ_AUJS01000017.1 and AABYPB01000003.31) \cite{34}.

Streptomycin-resistant isolates (n = 4) were screened for sialylated LOS locus class A/B and C (Table 4) by PCR, using \textit{C. jejuni} 81-176 (ATCC BAA2151) and \textit{C. jejuni} NCTC 11168 (DSM 27585), respectively, as positive controls.

4.4. Moxifloxacin Minimum Inhibitory Concentration Testing

All (fluoro)quinolone-resistant isolates (n = 99) were tested for moxifloxacin susceptibility. Briefly, isolates were recovered from \(-80^\circ \text{C} \text{ on CBA (Fannin Ltd) for 24 h at 42 }^\circ \text{C under microaerobic conditions, and subcultured to CBA for 20 \pm 2 h at 42 }^\circ \text{C under microaerobic conditions. In microtiter plates, 100 } \mu\text{L serial dilutions of moxifloxacin (Sigma Aldrich) were prepared in of Mueller Hinton broth with lysed horse blood (Thermo Fisher Scientific, Waltham, MA, USA) ranging from 0.125–16 mg/L. A 0.5 McFarland inoculum was prepared in 5 mL demineralised water (Thermo Fisher Scientific) and 100 } \mu\text{L was transferred to 11 mL of Mueller Hinton broth with lysed horse blood (Thermo Fisher Scientific). Moxifloxacin serial dilutions were inoculated with 100 } \mu\text{L of cell suspension and incubated for 20 \pm 2 hat 42 }^\circ \text{C under microaerobic conditions.}

4.5. Genome Sequencing and Genomic Analysis

The genomes of four streptomycin-resistant isolates (\textit{C. jejuni} isolates CITCj625-18 and CITCj27-18 and \textit{C. coli} isolates CITCc1631-18 and CITCc3448-18) were sequenced. DNA was quantified in triplicate with the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific). Genomic DNA libraries were prepared using the Nextera-XT protocol (Illumina, San Diego, CA, USA), with changes including 2 ng of input DNA and a minute PCR elongation time. DNA quantification and library preparation were performed on a Hamilton Microlab STAR system. Pooled libraries were quantified using the Kapa Biosystems library quantification kit on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250 bp paired-end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 \cite{124}. De novo assembly was performed using SPAdes version 3.7 \cite{125} and assembly quality was assessed using QUAST \cite{126}. 

Genomes were annotated using Prokka 1.14.3 [127]. Similarity searches were performed using the BLAST suit of programs [128] and InterProScan [129]. Multi locus sequence typing (MLST) patterns was determined using PubMLST [114]. ANI was calculated using EzBioCloud [130].

4.6. Data Availability

This whole-genome project has been deposited at DDBJ/ENA/GenBank under the accession number PRJNA612628. CITCj625-18, CITCj727-18, CITCc1631-18, and CITCc3448-18 genomes can be accessed using SAMN14379027, SAMN14379028, SAMN14379029, and SAMN14379030. The partial and complete gene sequences deposited in GenBank are listed in Table 5. Erythromycin-resistant isolates harboured identical ribosomal mutations and CITCc1303-18 partial sequences were submitted. One representative gyrA GT was submitted for each type.

Table 5. Accession numbers of partial sequences submitted to GenBank in this study.

| Isolate          | Gene                   | GenBank Accession Number | Sequence Length (bp) |
|------------------|------------------------|--------------------------|----------------------|
| C. coli (CITCc1303-18) | 23S ribosomal RNA, partial sequence | MT155934 | 262       |
| C. coli (CITCc1303-18) | 50S ribosomal protein L4 (rplL) gene, partial cds | MT155935 | 519       |
| C. coli (CITCc1303-18) | 50S ribosomal protein L22 (rplV) gene, partial cds | MT155936 | 319       |
| C. jejuni (CITCj493-17) | gyrA (GTJ-I), partial cds | MT176407 | 644       |
| C. jejuni (CITCj999-18) | gyrA (GTJ-II), partial cds | MT176408 | 644       |
| C. jejuni (CITCj193-18) | gyrA (GTJ-III), partial cds | MT176409 | 644       |
| C. coli (CITCc3796-B-18) | gyrA (GTJ-I), partial cds | MT176400 | 550       |
| C. coli (CITCc3521-18) | gyrA (GTJ-II), partial cds | MT176401 | 550       |
| C. coli (CITCc3118-17) | gyrA (GTJ-III), partial cds | MT176402 | 550       |
| C. coli (CITCc1631-18) | gyrA (GTJ-IV), partial cds | MT176403 | 550       |
| C. coli (CITCc3790-18) | gyrA (GTJ-V), partial cds | MT176404 | 550       |
| C. coli (CITCc1303-18) | gyrA (GTJ-VI), partial cds | MT176405 | 550       |
| C. coli (CITCc1303-18) | gyrA (GTJ-VII), partial cds | MT176406 | 550       |
| C. jejuni (CITCj625-18) | tetO/32/O type II, complete cds | MT176410 | 1920       |
| C. jejuni (CITCj727-18) | tetO/32/O type II, complete cds | MT176411 | 1920       |
| C. coli (CITCc3448-18) | tetO/32/O type II, complete cds | MT176412 | 1920       |
| C. jejuni (CITCj625-18) | ant(6)-Ib, complete cds | MT176413 | 867       |
| C. jejuni (CITCj727-18) | ant(6)-Ib, complete cds | MT176414 | 867       |
| C. coli (CITCc3448-18) | ant(6)-Ie, complete cds | MT176415 | 900       |
| C. coli (CITCc1631-18) | ant(6)-Ie, complete cds | MT176416 | 550       |

5. Conclusions

Although non-poultry sources contribute to campylobacteriosis incidence, poultry are natural thermophilic Campylobacter spp. hosts. The broiler industry serves as a reservoir for the dissemination of resistant campylobacters. The enrichment and stability of Campylobacter spp. resistance determinants is noteworthy but the natural competence and potential of recombination or acquisition of mobile genetic elements contributes to the Campylobacter. Taken together, the data collected in this study point to the diversity of resistance determinants circulating among Irish broilers, contributing to the development of resistance to clinically relevant antimicrobials.

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