



OPEN ACCESS

EDITED BY

Ramona Iseppi,
University of Modena and Reggio Emilia, Italy

REVIEWED BY

Orhan Sahin,
Iowa State University, United States
Laura Espina,
University of Zaragoza, Spain
Aditya Upadrasa,
mBio, Ireland

*CORRESPONDENCE

Richard A. Stabler
✉ richard.stabler@lshtm.ac.uk

†These authors have contributed
equally to this work and share
first authorship

RECEIVED 13 December 2023

ACCEPTED 06 February 2024

PUBLISHED 21 February 2024

CITATION

Lehri B, Navoly G, Corser A, Nasher F,
Willcocks S, Pham TN, Wren BW, Luu QH and
Stabler RA (2024) Phenotypic and genotypic
characterization of *Campylobacter coli*
isolates from the Vietnamese poultry
production network; a pilot study.
Front. Ind. Microbiol. 2:1355079.
doi: 10.3389/finmi.2024.1355079

COPYRIGHT

© 2024 Lehri, Navoly, Corser, Nasher,
Willcocks, Pham, Wren, Luu and Stabler. This is
an open-access article distributed under the
terms of the [Creative Commons Attribution
License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or
reproduction in other forums is permitted,
provided the original author(s) and the
copyright owner(s) are credited and that the
original publication in this journal is cited, in
accordance with accepted academic
practice. No use, distribution or reproduction
is permitted which does not comply with
these terms.

Phenotypic and genotypic characterization of *Campylobacter coli* isolates from the Vietnamese poultry production network; a pilot study

Burhan Lehri^{1†}, Georgina Navoly^{2†}, Abigail Corser¹,
Fauzy Nasher¹, Sam Willcocks¹, Thi Ngoc Pham³,
Brendan W. Wren¹, Quynh Huong Luu³ and Richard A. Stabler^{1*}

¹Department of Infection Biology (DIB), London School of Hygiene and Tropical Medicine (LSHTM), London, United Kingdom, ²UCL Genetics Institute, University College London (UCL), London, United Kingdom, ³National Institute of Veterinary Research, Hanoi, Vietnam

Introduction: Changing farming practices and the associated increase in the use of antibiotics are amongst the main drivers shaping the global increase of *Campylobacter* infections. The effects farming practices have on *Campylobacter* species, need to be studied at the global scale, particularly in emerging middle-income countries, where the demand for low-cost poultry meat is rising. While *Campylobacter jejuni* causes the majority of poultry associated diarrhoea, *Campylobacter coli* causes a significant amount of disease but are relatively understudied. In this study we characterised seven *C. coli* strains isolated from chicken faeces and chicken meat in Thai Nguyen province, Vietnam.

Methods: Whole Genome Sequencing and phenotypic assays (growth, motility, antimicrobial resistance testing, virulence assay) were performed to reveal the genetic relatedness and pathophysiological characteristics of the isolates.

Results: All isolates were resistant to ciprofloxacin and nalidixic acid but susceptible to phenicols. Three were resistant to macrolides azithromycin and erythromycin. Six isolates were classified as multi-drug resistant. All isolates had similar growth rates in laboratory culture media, while five were hyper-motile. Lethality towards a tractable host-model system, larvae of the greater wax moth *Galleria mellonella*, often used to determine *Campylobacter* virulence, was demonstrated for the first time for *C. coli*.

Discussion: Multilocus sequence typing data identified five ST's all within the *C. coli* ST-828 clonal complex and were previously reported in North American (ST-829), European (ST-1586), and Asia (ST-5511) from patients suffering from gastroenteritis, emphasising the global spread of these strains. This work highlights the importance of further research into this understudied global threat.

KEYWORDS

Campylobacter, *C. coli*, Vietnam, antimicrobial resistance, *Galleria mellonella*

1 Introduction

The demand for low-cost poultry meat and eggs are rapidly rising globally. To satisfy the increasing demand for meat and egg production the use of antimicrobial agents in animal food production has become widespread (Mulchandani et al., 2023). The global use of antibiotics is also projected to rise (Van Boeckel et al., 2017). Farmers, particularly in rapidly developing countries often rely on antibiotics as growth promoters and to improve the health of animals, however, this largely contributes to the generation of antibiotic resistant bacteria and the subsequent global increase in antimicrobial resistance (AMR) (Agyare et al., 2018). The seriousness of antibiotic overuse has been exacerbated further by difficulties in implementing antibiotic regulation policies in several countries (WHO, 2017; Tangcharoensathien et al., 2018). The interplay between the increasing incidence of foodborne illnesses, bacterial infections and AMR is strongly suggesting that the expansion of poultry production is presenting a growing public health risk (Kaakoush et al., 2015; Skarp et al., 2016).

The rapid intensification of poultry and egg has been linked with increasing incidence of bacterial food poisoning, mostly caused by *Campylobacter* species (Shane, 2000). *Campylobacter* spp are estimated to cause globally approximately 166M cases of disease [92M-300M 95% UI (uncertainty intervals)] and 37K (27-55k 95% UI) deaths annually (Kirk et al., 2015). This equates to 1,390 (752-2,576 95% UI) Disability Adjusted Life Years (DALYs) per 100,000 persons and 31 (22-46 UI) deaths per 100,000 persons (Kirk et al., 2015). However, LMIC regions carry significantly more burden of disease with *Campylobacter* spp being the most prevalent foodborne disease in the WHO South-East Asia region (1,152 [200-3,372 95% UI] DALYS per 100,000 persons and 0.4 [0.1-0.9 95% UI] deaths per 100,000 persons (Kirk et al., 2015). While species of *Campylobacter* are often combined in epidemiological data *C. jejuni* receives the majority of study. *C. coli*, the second most common *Campylobacter* species, is also implicated in bacterial gastroenteritis, contributing to a quarter of *Campylobacter* related gastroenteritis (Kaakoush et al., 2015), and one of the leading cause of foodborne diarrhoea (Laaveri et al., 2018).

Vietnam has undergone rapid economic and population growth, along with urbanisation leading to changes in agricultural and food production practices. Poultry is most popular livestock in Vietnam and the second-largest producer of meat (Birhanu et al., 2021). Vietnam had 512.7 million birds in 2020 producing 1.505 million tonnes of meat and 15.1 billion eggs (Birhanu et al., 2021). There is a paucity of data on *Campylobacter* prevalence and epidemiological data in Vietnam, in particularly for *C. coli*. A previous Vietnamese study conducted, identified *C. coli* being more resistant to antibiotics, such as ciprofloxacin, nalidixic acid and tetracycline, than *C. jejuni*, even though both species belong to the same ecological niche (Nguyen et al., 2016).

The aim of this pilot study was to characterise understudied *C. coli* isolated from poultry production networks, sampling from farms and point-of-sale retail outlets in Hanoi, Vietnam. The isolates were investigated for their phenotypic AMR profile and their draft genomes were compared against each other.

Additionally, the *Galleria mellonella* virulence infection model was adapted for *C. coli*, which has not been previously reported. This pilot study paves the way for the large in-depth One Health Poultry Hub investigation of *Campylobacter* across Vietnam and provides much needed data on *C. coli* present in the Vietnamese poultry meat production network.

2 Materials and methods

2.1 Bacterial isolates

A prevalence survey of 30 chicken farms and 30 retail markets in Thai Nguyen province, Vietnam was performed in between December 2019 and January 2020. A total of 30 conventional farms were visited and a single faecal sample taken from six-week-old broilers. Five retail markets were visited three times and each occasion five raw chicken meat sample (approximately 300g meat) were collected. Five supermarkets were visited three times and each occasion five raw chicken meat sample (approximately 500g meat) were collected. A total of 36 putative *Campylobacter* isolates were from farms 25/30 (83.3%) and from retail markets 11/30 (36.7%) following the ISO 10272-1: 2017. Multiplex-PCR based identified revealed that 80.6% were *C. jejuni* (29/39) and 19.4% *C. coli* (7/36). The *C. coli* were selected for further study (Table 1; Supplementary Table S1).

2.2 Growth conditions

C. coli isolates were routinely cultured for 48 hours at 37°C on Columbia Blood Agar (CBA) (Oxoid, UK) supplemented with 5% defibrinated horse blood (Oxoid, UK) and Skirrow supplement (Oxoid, UK) under microaerobic conditions in a variable atmospheric incubator (Don Whitley Scientific, UK). *C. coli* isolates were further cultured on CBA/blood/skirrow plates for 24 hours prior to experimentation. Oxidase, indole test and gram staining were used to confirm the presence of *Campylobacter* sp. (Supplementary Table S1). Hippurate test was used to distinguish *C. jejuni* (positive) from *C. coli* (negative) (Supplementary Table S1). Growth curve experiments were also conducted to compare *C. coli* growth rate with well-studied *C. jejuni* 81-176, (a human isolate with a putative plasmid containing a type IV secretion and verified as highly virulent through testing in macaque monkeys (Russell et al., 1989). Growth curves were averaged from two biological repeats, in 10 ml Brucella broth (Oxoid, UK), containing $\sim 10^8$ colony-forming units (CFU), cultured for 24 hours under microaerobic conditions at 37°C with optical density readings (OD600nm), being taken at 2-hour intervals.

2.3 Motility assay

Overnight *C. coli* cultures were diluted to 8×10^8 CFU/ml bacteria in Brucella broth (Oxoid, UK). *C. coli* (3 μ l) was added into 0.4% (wt/vol) Brucella agar and incubated for 72 hours at 37°C,

TABLE 1 Multi-locus sequence type (MLST) profile for *C. coli* isolates.

Isolate	Source	Location	Date	ST (CC)	Allele						
					<i>aspA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkt</i>	<i>unca</i>
C57	Chicken faeces	Farm 03	9/12/2019	ST511 (ST-828)	33	39	30	82	113	47	139
C75	Chicken faeces	Farm 25	23/12/2019	ST1586 (ST-828)	33	176	30	82	113	43	17
M8	Chicken faeces	Farm 26	23/12/2019	ST6184 (ST-828)	32	39	30	82	113	43	17
M10	Chicken meat	Retail market 01	30/12/2019	ST966 (ST-828)	33	39	30	79	112	47	17
M14	Chicken meat	Supermarket 05	30/12/2019	ST966 (ST-828)	33	39	30	79	112	47	17
C76	Chicken meat	Retail market 02	06/1/2020	ST829 (ST-828)	33	39	30	82	113	43	17
M39	Chicken meat	Retail market 04	13/1/2020	- (ST-828)	33	291*	30	82	113	47	17

ST, sequence type; CC, clonal complex. *M39 had a novel *glnA* allele that was one SNP different to the *glnA* (291) allele. MLST data was extracted from draft genome sequences using the PubMLST *Campylobacter jejuni/coli* database.

under microaerobic conditions. Cell motility was observed at 24, 48 and 72 hours of growth. For experiment controls the representative strains *C. jejuni* 81-176 and *C. jejuni* 11168H [a hyper-motile variant of the standard original sequenced strain *C. jejuni* NCTC11168 (Parkhill et al., 2000)] were used. *C. jejuni* strains were used as they have well characterised motility and allow relative motility between *C. coli* and *C. jejuni*.

2.4 Antimicrobial susceptibility testing

Minimum inhibitory concentration (MIC) was determined based on EUCAST (version 3.0 Jan 2021), ISO 20776-1:2019 and CLSI (M45-A2) guidelines, following the broth microdilution method in untreated 96 well Nunc Sterile round bottom plates (ThermoFisher Scientific, UK). 5×10^5 CFU/ml of overnight bacterial cultures were inoculated with varying concentrations of antibiotics, described below. Antibiotics were prepared in correlation with EUCAST, ISO 20776-1:2019 guidelines. The samples were incubated for 48 hours at 37°C in filter sterilised cation adjusted MH broth, supplemented with 2.5% defibrinated horse blood (Oxoid, UK) along with 20 mg/L β -Nicotinamide adenine dinucleotide sodium salt (Sigma Aldrich, UK), at 120 revolutions per minute (RPM), under microaerobic conditions. Growth was determined visually by comparing to the negative control, as well as by using a SpectraMax iD5 microplate reader (Molecular Devices, UK).

Antibiotics tested with ranges and MIC values as described by National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) (<https://www.cdc.gov/narms/antibiotics-tested.html>) unless stated. Briefly gentamicin (0.125-32 mg/L), kanamycin (0.5-16 mg/L), streptomycin (0.0625-64 mg/L), clindamycin (0.0156-32 mg/L), azithromycin (0.015-64 mg/L), erythromycin [0.25-64 mg/L (EUCAST, 2023)], chloramphenicol (0.0156-256 mg/L), florfenicol (0.03-64 mg/L), ciprofloxacin [0.0156-64 mg/L (EUCAST, 2023)], nalidixic acid (2-64 mg/L), tetracycline (0.0625-64 mg/L), telithromycin (0.0156-8 mg/L), ampicillin (0.015-125 mg/L). Tentative epidemiological cut-off values (T

ECOFF were used for streptomycin, ampicillin, as no breakpoints were available during the study (EUCAST, 2020). Kanamycin had no breakpoint or T(E)ECOFF values available. *C. jejuni* ATCC 33560 with known MIC values was used as a control (EUCAST, ISO 20776-1:2019).

2.5 Virulence testing using *Galleria mellonella* model

Galleria larvae were sourced from LiveFood Direct and selected to be of matched size (~20 mm length), colour (cream) and maturity (~35-40 days old). *Galleria* are reared under controlled conditions and receive organic nutritional support including honey and wheatgerm. Prior to experimentation, *Galleria* were stored at 10°C within a breathable container half-filled with wood shavings. *Campylobacter* isolates were diluted to 10^8 CFU/ml in phosphate-buffered saline (PBS) and 10 μ l was injected into *G. mellonella* larvae. *G. mellonella* was incubated at 37°C with phenotypic effect being monitored over the course of nine days. Three biological replicates (using 2 x n=5 *G. mellonella*, 1 x n=10 *G. mellonella*) were conducted over 9 days. Phosphate-buffered saline (PBS) was used as a negative control and virulent *C. jejuni* 81-176 was used as a positive control. *C. jejuni* 81-176 was used as a control as the species has previously been used for the *G. mellonella* model (Champion et al., 2010; Senior et al., 2011) while, to our knowledge no *C. coli* strains have previously been studied in the *G. mellonella* model.

2.6 Genomic DNA extraction and sequencing

Genomic DNA was extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen, UK), following manufacturer guidelines. Prior to sequencing DNA quality and concentration was measured using a NanoDrop 2000/2000c Spectrophotometer (ThermoFisher Scientific, UK). Illumina Miseq library preparation and sequencing were prepared according to manufacturer's protocol. Libraries were sequenced on a MiSeq (Illumina, USA) with 2 x 150

bp reagent kit v2. Additional sequencing was conducted using a MinION sequencer (Oxford Nanopore, UK). A R10 flow cell (FLO-MIN111) was used for sequencing, alongside ligation sequencing kit (SQK-LSK110) and the rapid barcoding kit (SQK-RBK004) for multiplexing, manufacturer recommended PCR conditions were used with eight minutes amplification using NEB LongAmp Taq 2X Master Mix (NEB M0287). DNA end repair was performed using NEBNext Ultra II End repair/dA-tailing Module (E7546), while adapter ligation was done using the NEBNext Quick Ligation Module (E6056).

2.7 Genome assembly

Raw fastq files were quality controlled using TrimmomaticPE with settings: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36 (Bolger et al., 2014). Sequenced reads were *de novo* assembled using St. Petersburg genome assembler (SPAdes version 3.15.3) (Nurk et al., 2013). The draft genome assemblies were assessed using quality assessment tool for genome assemblies (Quast) (Gurevich et al., 2013). For *C. coli* isolates that were sequenced on both MinION and MiSeq, draft genomes were assembled using Unicycler v0.4.9 (Wick et al., 2017). Once assembled, contigs were annotated by Prokaryotic Genome Annotation (Prokka) (Seemann, 2014) using a bespoke *Campylobacter* database using reference genomes (accession AF226280, AL111168, CM000854, CM000855, CP000025, CP000538, CP000768, CP000814, CP001876, CP001900, CP001960, CP002029, CP003871, CP006729, HE978252, HG428754).

2.8 AMR gene identification, MLST and virulence analysis

Abricate with the ResFinder database (update date 10-03-2022) (Zankari et al., 2012; Seemann, 2018) was used to identify AMR genes. Additionally, PathogenWatch (<https://pathogen.watch/>) and ResFinder (<https://cge.food.dtu.dk/services/ResFinder/>) identified AMR related single point mutations within the *C. coli* genomes. Multi-Locus Sequence Typing (MLST) analysis was conducted with MLST 2.0 from the Centre for Genomic Epidemiology using PubMLST.org database version 2.0.0. PubMLST database was also used to identify clonal complex of the sequenced isolates. ChewBBACA (version 16.04) (Silva et al., 2018) with the *C. jejuni* schema 'INNUENDO_wgMLST' (update date 20-05-2021) having 2794 loci was used for core genome multi locus sequence typing (cgMLST), by selecting loci that are present in 95% of *C. coli* and *C. jejuni* isolates within RefSeq database. PHYLOViZ was used to generate a minimum spanning tree, the Newick tree format, which was imported to Interactive tree of life (iTOL) to generate the tree in Figure 1. For ChewBBACA and Abricate *C. coli* isolates were compared against complete RefSeq *C. jejuni* and *C. coli* isolates. Pre-annotated sequence files were imported onto the *Campylobacter* Virulence Factors of Pathogenic Bacteria database (VFDB) (<http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Campylobacter>).

2.9 Statistical analyses

Survival curves were generated using Prism version 9.1 software, and survival rates of *G. mellonella* models were analysed using the Kaplan-Meier method. Tests were performed using a two-sided probability, and survival rates were considered statistically significant when $p < 0.05$. For motility and growth rate analysis, three biological replicates were conducted, and statistical significance was conducted using one way ANOVA test, with $p < 0.05$ being considered statistically significant.

3 Results

3.1 Phenotypic analysis

3.1.1 Growth rate analysis of *C. coli*

C. coli were isolated from poultry faeces (C57, C75, C76) and meat (M8, M10, M14, M39) from farms, retail markets or supermarkets in Hanoi, Vietnam (Supplementary Table S1). All isolates were catalase and oxidase positive but Indole and Hippurate negative (Supplementary Table S1). *C. coli* growth was also compared with *C. jejuni* 81-176 in brucella broth for 24 hours, demonstrating no significant difference in growth rate between the *C. coli* isolates and *C. jejuni* 81-176 (Supplementary Figure S1).

3.1.2 Motility

To assess motility of the *C. coli* strains, the isolates were compared to two *C. jejuni* with known motility phenotypes; hyper-motile *C. jejuni* 11168H (Karlyshev et al., 2001; Macdonald et al., 2017) and motile *C. jejuni* 81-176 (Korlath et al., 1985; Hofreuter et al., 2006). After 72hrs, *C. coli* C75 ($p=0.001$), M14 ($p=0.01$) and M39 ($p = 0.04$) had disseminated through soft Brucella agar significantly further than hypermotile *C. jejuni* 11168H and were deemed more motile (Figure 2). *C. coli* C57 ($p=0.15$) and C76 ($p=0.06$) motility was not significantly different from the *C. jejuni* 11168H control (Figure 2). *C. coli* M8 ($p=0.02$) and M10 (0.03) were less motile than *C. jejuni* 11168H but similar to the motile *C. jejuni* 81-176 (Figure 2).

3.1.3 Antibacterial susceptibility testing

All *C. coli* isolates were resistant to quinolones (ciprofloxacin/nalidixic acid) and tetracycline (Table 2). For aminoglycosides, *C. coli* C57 and C76 were resistant to gentamycin. C57 and C76 also had kanamycin MIC of >16 mg/L and streptomycin MIC of 64 mg/L, which was greater than the (T)ECOFF of 4 mg/L (Table 2). All isolates were susceptible to phenicols, however, C75 and M8 were a single doubling dilution below the resistance cut-off (Table 2). *C. coli* C57, C76 and M8 were resistant to macrolides (erythromycin and azithromycin) and lincosamide (clindamycin) (Table 2). Isolates C57 and M8 were also resistant to telithromycin (Table 2). *C. coli* C57, M39 and C75 demonstrated very high level of ampicillin insensitivity (MIC 125 mg/L), higher than (T)ECOFF (16 mg/L) while *C. coli* M8 (1.9 mg/L) was acutely susceptible

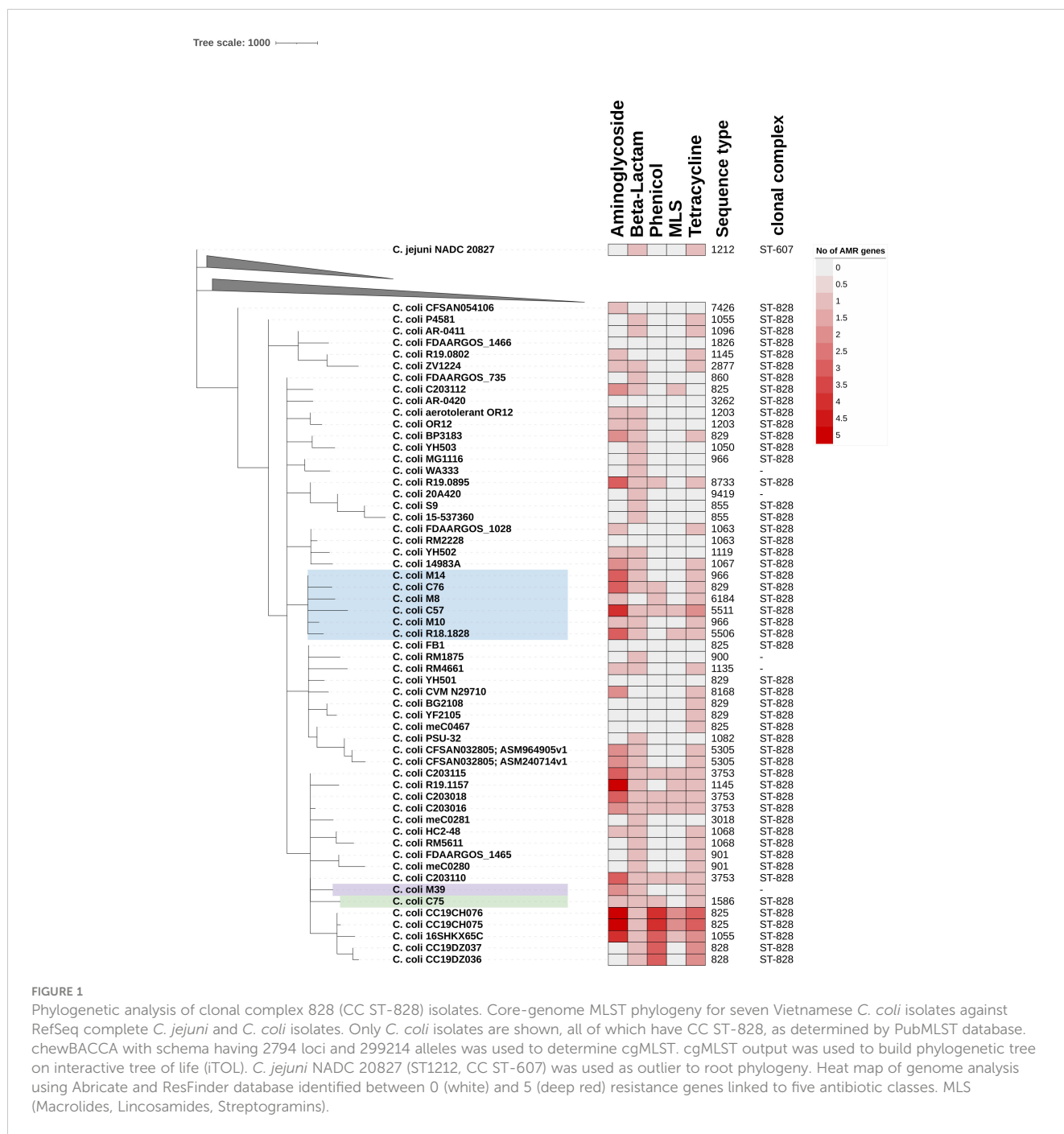


FIGURE 1 Phylogenetic analysis of clonal complex 828 (CC ST-828) isolates. Core-genome MLST phylogeny for seven Vietnamese *C. coli* isolates against RefSeq complete *C. jejuni* and *C. coli* isolates. Only *C. coli* isolates are shown, all of which have CC ST-828, as determined by PubMLST database. chewBACCA with schema having 2794 loci and 299214 alleles was used to determine cgMLST. cgMLST output was used to build phylogenetic tree on interactive tree of life (iTOL). *C. jejuni* NADC 20827 (ST1212, CC ST-607) was used as outlier to root phylogeny. Heat map of genome analysis using Abricate and ResFinder database identified between 0 (white) and 5 (deep red) resistance genes linked to five antibiotic classes. MLS (Macrolides, Lincosamides, Streptogramins).

(Table 2). Multi-drug resistance (MDR) of thermophilic *Campylobacter* strains is defined as resistance to at least three different antimicrobial classes (Magiorakos et al., 2012). All isolates, except *C. coli* M14, match the MDR definition. *C. coli* M14 was only resistant to quinolones and tetracycline, however, was also above the (T)ECOFF for streptomycin (aminoglycoside; (16 vs 4 mg/L) (Table 2). *C. coli* C57 was resistant to all classes tested, except phenicol (Table 2).

3.1.4 *Galleria mellonella* pathogenicity

Campylobacter does not naturally infect *G. mellonella*, however, due to the lack of readily available models for testing *Campylobacter*

virulence, it is an established surrogate infection model to assess virulence and it has been used for numerous bacterial infections, including *C. jejuni* (Kurstak and Vega, 1968; Dudziak and Jozwik, 1969; Champion et al., 2010; Insua et al., 2013; Viegas et al., 2013; Bojanic et al., 2020). Pathogenicity of *C. coli* isolates was tested using a *G. mellonella* wax moth infection model (Figure 3). The results showed that disease in *G. mellonella* infected with *C. coli* M10 and M14 are significantly attenuated ($p=0.0438$ and $p=0.0290$ respectively, Gehan-Breslow-Wilcoxon test) compared to the positive control *C. jejuni* 81-176, with many *G. mellonella* surviving beyond 9 days. Survival of *G. mellonella*, infected with *C. coli* C75, C76, M8 and M39, was not significantly different from

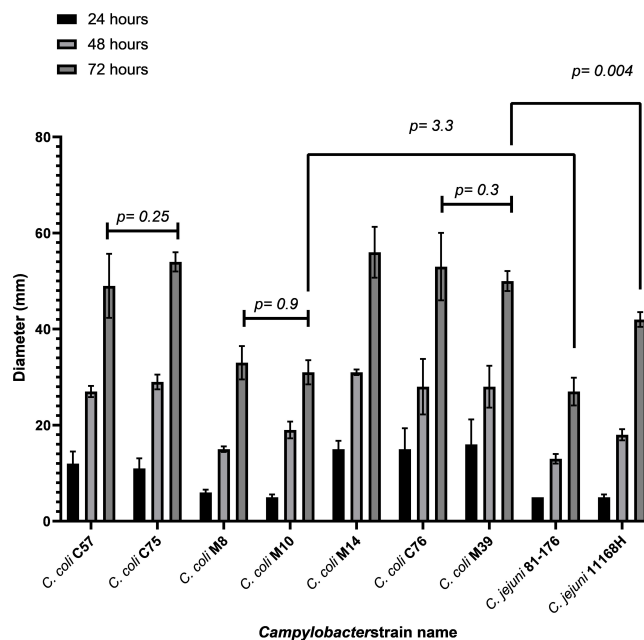


FIGURE 2

Motility analysis of *C. coli* isolates along with *C. jejuni* 81-176 and 11168H. The data shows an average of three biological replicates. Data was obtained by inoculating Brucella 0.4% (wt/vol) agar with 8×10^8 CFU/ml bacteria. Growth was observed over three days with measurement time points being 24, 48 and 72 hrs. P-values are generated using one-way ANOVA. *C. coli* isolates were compared against *C. jejuni* 81-176 and hyper-motile isolate *C. jejuni* 11168H.

C. jejuni 81-176; $p = 0.593, 0.117, 0.692$ and 0.164 respectively (Figure 3). More *C. coli* C57 infected *G. mellonella* died than *C. jejuni* 81-176 over the initial 4 days, but this was not significant over the course of 9 days, $p = 0.224$ (Figure 3).

3.2 Genotypic analysis

3.2.1 *C. coli* genomes

All isolates were whole genome sequenced using Illumina short read sequencing technology with *C. coli* isolates C57, C76, M8 and M39 further sequenced using a Minion sequencer (Supplementary Table S2). Draft genomes ranged from 1.64 – 1.77 Mbp, which is comparable to *C. coli* 76339 accession HG326877 (1.58 Mbp) and *C. coli* RM4661 accession CP007181 (1.82 Mbp). Assembly contig numbers ranged from 1 (M8) to 21 (C75) contigs of length ≥ 1 kb. The mean number of predicted coding sequences (CDSs) for sequenced *C. coli* strains was 1773 (SD ± 50.2) (Supplementary Table S2).

3.2.2 Identification of a novel *C. coli* plasmid.

A circularised plasmid of length 27,557 bp was identified for isolate *C. coli* M39, named pCCM39-Huong. Annotation of pCCM39-Huong identified 37 coding sequences; 22 had predicted function, but 15 (40%) were hypothetical of unknown function (Supplementary Table S3). Annotated genes included *ssb2* (single stranded DNA binding protein) (Jeon and Zhang, 2007), potentially

involved in DNA processing during conjugation. Genes *traG* and *traL* both involved in conjugal transfer of genetic material, a toxin component (*vbhT*) (Sprenger et al., 2017), *virB1*, *virB4*, *virC*, *trbB*, *traM*, *trbI*, *trbF*, *trbE*, *trbM* and *trbD* all related to type IV secretion system were also found (Zatyka and Thomas, 1998; Mace et al., 2022) (Supplementary Table S3).

pCCM39-Huong had sequence similarity to two unnamed plasmids found in *C. coli* isolates 20A420 (accession CP058341) and *C. coli* meC0281 (CP027635) (Figure 4). *C. coli* 20A420 plasmid was of length 26,486 bp, with 97.36% identity and 90% coverage compared to pCCM39-Huong. Plasmid meC0281 was of length 30,719 bp and had 96.33% identity and 96% coverage when compared to pCCM39-Huong (Figure 4). When the two plasmids p20A420 and meC0281 were aligned to plasmid pCCM39-Huong, the difference between the two plasmids and pCCM39-Huong related to non-contiguous hypothetical protein encoding genes (Figure 4). *C. jejuni* 81-176 contains two plasmids (pTet [AF226280] and pVir [AY394561]) both of approximately 35kb. pCCM39-Huong reads did not map to any of the pVir gene sequence, demonstrating that these plasmids are highly different to plasmid pCCM39-Huong. Comparison between pCCM39-Huong assembly and pTet only identified a single shared complete gene (a putative DNA primase) and partial matches to *cpx32* (phage/Rha family transcriptional regulator) and *cmgB5* (putative type IV secretion system component). Mapping of the M39 genome fastq data showed that pTet *tetO* (tetracycline resistance) was highly similar to the chromosomal *tetO* present within the M39 genome.

TABLE 2 Antibiotic susceptibility of *C. coli* isolates.

Abx class	Abx	MIC	C57	C75	M8	M10	M14	C76	M39	ATCC 33560
		Cut-offs (S/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)	MIC (S/I/R)
Aminoglycoside	GEN	2/4	>32 (R)	>32 (R)	0.25 (S)	4 (R)	<0.125 (S)	32 (R)	>32 (R)	0.5 (S)
Lincosamide	CLN	1/2	≥8 (R)	0.125 (S)	16 (R)	1 (S)	0.5 (S)	8 (R)	0.5 (S)	0.25 ⁻ (S)
Macrolide	AZN	0.5/1	16 (R)	≤0.015 (S)	>64 (R)	0.125 (S)	<0.015 (S)	64 (R)	0.06 (S)	0.5* (S)
	ERY	8/>8	32 (R)	1 (S)	>64 (R)	1 (S)	1 (S)	64 (R)	0.5 (S)	0.5 (S)
Phenicol	CHL	16/32	1 (S)	16 (S)	16 (S)	1 (S)	1 (S)	8 (S)	2 (S)	2 ^S (S)
	FLO	4/8	2 (S)	0.5 (S)	1 (S)	1 (S)	1 (S)	0.5 (S)	4 (S)	2 (S)
Quinolone	CIP	0.001/0.5	16 (R)	8 (R)	16 (R)	16 (R)	8 (R)	4 (R)	32 (R)	0.0625 (I)
	NAL	16/32	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	16 ^S (S)
Tetracycline	TET	2/4	32 (R)	≥64 (R)	>64 (R)	32 (R)	64 (R)	64 (R)	64 (R)	1 (S)
Ketolide	TEL	4/8	>8 (R)	2 (S)	>8 (R)	2 (S)	4 (S)	4 (S)	1 (S)	2 (S)
		(T)ECOFF	MIC (T)	MIC (T)	MIC (T)	MIC (T)	MIC (T)	MIC (T)	MIC (T)	MIC (T)
β-lactam	AMP	>16	125 (>)	125 (>)	1.9 (<)	15.625 (=)	7.8125 (<)	62.5 (>)	125 (>)	3.9 (<)
Aminoglycoside	KAN	N/A	>16	>16	>16	4	8	>16	>16	16
	STR	>4	64 (>)	1 (<)	1 (<)	16 (>)	16 (>)	64 (>)	4 (=)	4 (=)

S (susceptible), I (intermediate), R (resistant), Abx (antibiotics tested), GEN (gentamycin, S ≤ 2 mg/L, R ≥ 4 mg/L), CLN (clindamycin, S ≤ 1 mg/L, R ≥ 2 mg/L), AZN (azithromycin, S ≤ 0.5 mg/L, R ≥ 1 mg/L), ERY (erythromycin, S ≤ 8 mg/L, R > 8 mg/L), CHL (chloramphenicol, S ≤ 16 mg/L, R ≥ 32 mg/L), FLO (florfenicol, S ≤ 4 mg/L, R ≥ 8 mg/L), CIP (ciprofloxacin, S ≤ 0.001 mg/L, R > 0.5 mg/L), NAL (nalidixic acid, S ≤ 16 mg/L, R ≥ 32 mg/L), TET (tetracycline, S ≤ 2 mg/L, R ≥ 4 mg/L), TEL (telithromycin, S ≤ 4 mg/L, R ≥ 8 mg/L), AMP (ampicillin, no break points), KAN (kanamycin, no break points), STR (streptomycin, no break points). T (tentative epidemiological cut-off values [(T)ECOFF]); AMP [16 mg/L] and STR [4 mg/L], KAN has no EUCAST or CLSI breakpoint or (T)ECOFF value for *Campylobacter* strains, > (greater than (T)ECOFF), = (equivalent to (T)ECOFF), < (less than (T)ECOFF). *C. jejuni* ATCC 33560 within expected MIC range unless one 2-fold dilution above expected MIC (*) or below expected MIC (°) or no given MIC (°). MIC values are in mg/L for *C. coli* strains C57, C75, M8, M10, M14, C76, M39 and *C. jejuni* ATCC 33560.

3.2.3 *C. coli* epidemiological information

Analysis of the sequenced *C. coli* genomes identified five different sequence types (STs), with M39 having a novel ST, due to a novel *glnA* allele that was one single-nucleotide polymorphism

(T198C) different to *glnA*(291) (Table 1). All STs belonged to the ST-828 clonal complex (Table 1). In the PubMLST database (accessed 22-6-2022) of the 16,083 ST-828 clonal complex isolates, 15,974 (99.3%) were identified as *C. coli*, 97 *C. jejuni* and

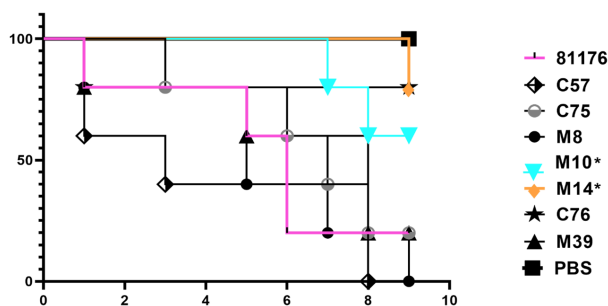
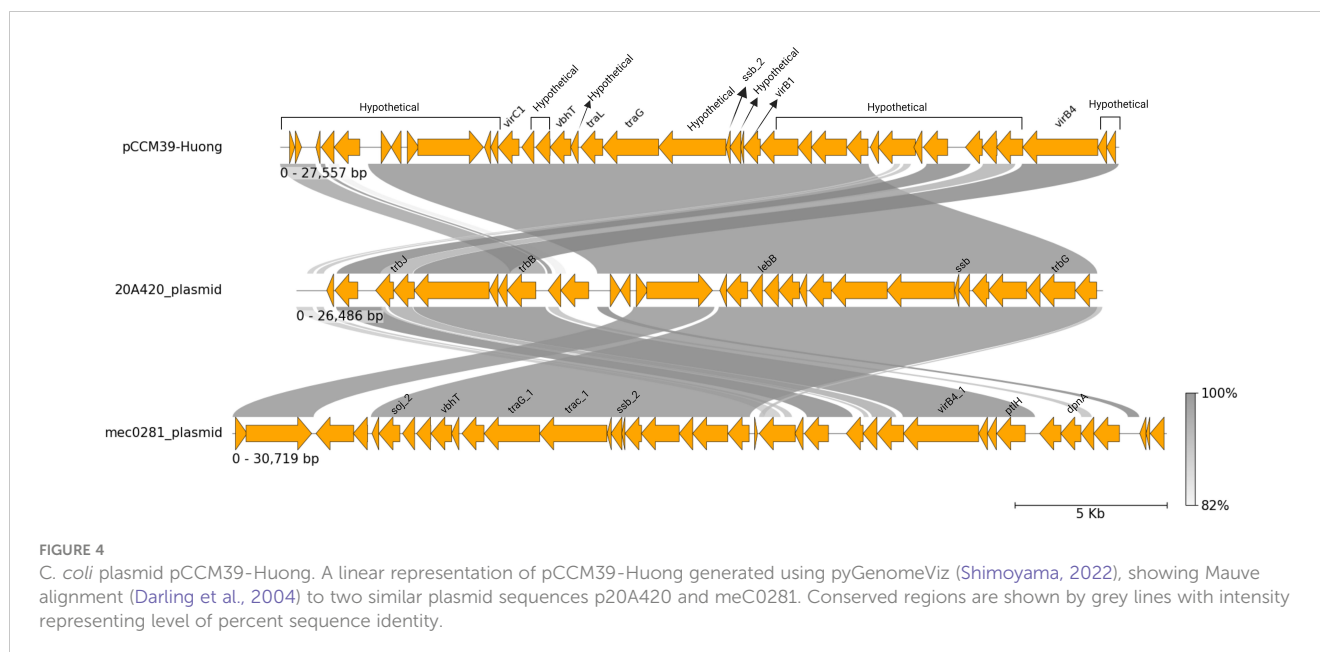


FIGURE 3 Virulence testing of Vietnamese *C. coli* isolates using *Galleria mellonella* model. Representative survival plot showing probability of survival of *G. mellonella* after infection with *C. coli* isolates (C57, C75, M8, M10, M14, C76, M39). *C. jejuni* 81-176 was used as a positive reference standard and PBS negative control. Data from an independent biological repeat, representative of the three independent experiments conducted is shown. *C. coli* isolates M10 and M14 were significantly attenuated ($p=0.0438$ and $p=0.0290$ respectively, Gehan-Breslow-Wilcoxon test) compared to the *C. jejuni* 81-176 reference control.



12 as *Campylobacter* spp. cgMLST further confirmed that the isolates were *C. coli*, as these were grouped away from *C. jejuni* (Figure 1) and clustered with reference ST-828 clonal complex isolates (Figure 1). The sequenced isolates were not clustered based on whether they were isolated from farm or supermarket/retail market (Figure 1). *C. coli* M39 and C75 belonged to the same subclade (Figure 1) and shared 5 MLST alleles (Table 1). M10 and M14 (from supermarket-bought chicken meat) were both ST-966, however cgMLST data indicated that these were very similar, but not identical isolates with a lowest harmonic mean allelic distance of 268. *C. coli* M14, C76, M8, C57, M10 belonged to the same subclade and were related to *C. coli* R18.1828, isolated from Taiwan (Figure 1).

3.2.4 AMR genotypes

Analysis of the draft genomes identified potential AMR genes to all classes of antibiotics tested. The β -lactamase gene *bla*_{OXA-193} was present in all sequenced genomes except M8 and 142 out of 284 (50%) reference genomes (Supplementary Table S4). Four aminoglycoside genotypes were identified with isolates containing between one (M8, M10, C75) and three (C57, C76, M14) CDSs (Supplementary Table S4). All isolates had a copy of *tet* (O) linked to tetracycline resistance, 4 out of 7 sequenced isolates had *cat2* phenicol resistance gene, but only C57 had the *erm*(B) MLS (macrolide, lincosamide and streptogramin) resistance gene (Figure 1; Data Sheet 2).

Sequence analysis showed that only *C. coli* C57 was predicted to have nine resistance genes, covering aminoglycosides, β -lactam, phenicol, MLS and tetracycline resistance (Figure 1; Supplementary Table S4). Analysing the *C. coli* M8 genome identified aminoglycosides, β -lactamase and phenicol AMR genes (Figure 1; Supplementary Table S4). The ResFinder database did not identify any resistance genes for the control susceptible strain *C. jejuni* ATCC 33560.

3.3 Correlation between AMR phenotype and genotypes

Prediction of phenotypic resistance to antibiotics from the presence of genes linked to resistance has proven complex (Doyle et al., 2020). All isolates, except M8 and M14 were resistant to gentamycin. Four isolates had an MIC above streptomycin (T)ECOFF and five had an MIC of >16 mg/L for kanamycin, however all isolates contained at least one aminoglycoside resistance gene. Only the isolates (*C. coli* C57, M10, M14 and C76) with *ant*(6)-Ia were resistant to streptomycin. *C. coli* M8 possessed only *aph*(3')-III and had a kanamycin MIC > 16, similar to other three other *aph*(3')-III positive isolate (*C. coli* C75, C76 and M39). The fifth *aph*(3')-III positive isolates, *C. coli* M14, had a kanamycin MIC of 8. While C57 did not possess an *aph*(3')-III and had a kanamycin MIC >16 this isolate did have four alternative aminoglycoside genes. All isolates, except *C. coli* M8 possessed *bla*_{OXA-193} as the only β -lactamase yet MIC values ranged from 1.9 (*C. coli* M8) to >125 (*C. coli* C57, C75 & M39). *C. coli* C57, M8 and C76 were resistant to erythromycin, the *erm*(B) inducible erythromycin resistance was only present in *C. coli* C57 however M8 and C76 had a 2075A>G mutation within the 23S rRNA gene associated with macrolide resistance. All isolates were non-susceptible to ciprofloxacin and SNP analysis identified an identical gyrase subunit A (*gyrA*) (T86I) mutation in all isolates known to confer resistance.

3.4 Correlation between virulence related genes and genotypes

Genotypic data for each isolate showed that motility genes (*flaA*, *flaB*, *flaC*, *flaG*, *flgB*, *flgC*, *flgD*, *flgE2*, *flgE*, *flgG2*, *flgG*, *flgH*, *flgI*, *flgK*, *flgL*, *flgR*, *flhA*, *flhB*, *flhF*, *flhG*, *fliA*, *fliD*, *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliL*, *fliM*, *fliN*, *fliP*, *fliQ*, *fliR*, *fliS*, *fliY*, *motA*, *motB*, *pflA*) were present in all *C. coli* isolates. All *C. coli* isolates also had the adhesion

(*cadF*, *jlpA*, MOMP, PEB1) (Krause-Gruszczynska et al., 2007), cytolethal toxin (*cdtABC*) and *Campylobacter* invasion related genes (*ciaB*) (Konkel et al., 1999). The well-characterised O- and N-linked general glycosylation systems were also present.

C. coli M10 (and M14) demonstrated significantly lower virulence in the *G. mellonella* infection model and was the least motile (along with M8), whereas *C. coli* C57 was the most virulent isolate. To identify any genetic cause for the virulence of *C. coli* C57, we isolated C57 fastq reads that did not match the M10 and M14 assembled draft genomes. These unmapped reads were assembled to represent CDSs in *C. coli* C57 that were absent or highly divergent from *C. coli* M10 and M14. The unmapped reads assembled in to 39 genome fragments containing 158 predicted CDSs. To remove any CDSs that were present in M10 and M14, but were not currently in these draft genomes, the raw reads from M10 and M14 were mapped against this assembly, which identified six contigs containing 16 CDSs that were present in M14 but not M10. 106 of 142 CDS were annotated as hypothetical proteins, two were IS1595 family transposases, 28 were annotated as enzymes, with many linked to sugar metabolism (e.g. dTDP-glucose 4,6-dehydratase), modification methylase (*dpnIIA* and *dpnIIB*), ribosomal silencing factor (RsfS) and sugar efflux transporter. Additionally, both flagellin FlaA (highly like the residues 102-572 of 11168 FlaA) and FlaB (67 amino acid fragment, identical to last 67 residues of 11168 FlaB) were present, demonstrating significant variation between *C. coli* C57 and M10/M14 flagellin genes.

4 Discussion

4.1 Is *C. coli* antimicrobial resistance a concern?

C. coli has emerged as a significant public health concern due to its high rates of antimicrobial resistance (van Vliet et al., 2022). There is evidence of genetic convergence between *C. coli* and *C. jejuni*, due to human activity, such as intensive farming of poultry, where both species can inhabit, allowing increased horizontal transmission of DNA between the species (Sheppard et al., 2008).

The antibiotic of choice for *Campylobacter* associated gastroenteritis are macrolides (Wieczorek and Osek, 2013), however, macrolide resistance is more common in *C. coli* isolates than *C. jejuni* (Luangtongkum et al., 2009). Macrolides resistance in North America is around 10% for both *C. jejuni* and *C. coli* from humans/broilers. Across Europe *C. jejuni* from humans had lower levels of erythromycin resistance (1.1%) compared to *C. coli* (8.5%) (European Food Safety, A and P. European Centre for Disease and Control, 2023). While in Asia, less than 5% of *C. jejuni* were macrolide resistant, as opposed to 14 – 62% of *C. coli* (Luangtongkum et al., 2009). In this study, three out of seven isolates were found to be resistant to both macrolides, presumptively linked to the presence of either an acquired gene or 23S rRNA gene mutations, reflecting previous studies in this

country. Fluoroquinolones are also commonly used to treat *Campylobacteriosis* but again reduced susceptibility (ciprofloxacin and nalidixic acid) was demonstrated in all isolates and linked to a known *gyrA* mutation (Ohishi et al., 2017; Devi et al., 2019). This was unsurprising, as previous studies have indicated *gyrA* related resistance to fluoroquinolones being common in Vietnam, with *Campylobacter* isolates rapidly acquiring resistance (Luangtongkum et al., 2009). Tetracycline resistance was also observed for all isolates due to *tet(O)* (Pratt and Korolik, 2005), although a duplicate copy of *tet(O)* in *C. coli* C57 was not linked with higher MIC. Tetracycline is seldom used for the treatment of acute gastroenteritis (Garcia-Fernandez et al., 2018), but is the most frequent antibiotic in the treatment and control of various poultry diseases, leading to high resistance rates in a number of zoonotic pathogens.

The presence of multiple AMR phenotypes meant that all but *C. coli* M14 were resistant to at least three classes of antibiotics, however no isolates demonstrated resistance to the phenicols tested. Florfenicol is used for agricultural purposes and is also thought to be more active than chloramphenicol (Sutli and Gressler, 2021). Yet, the borderline resistance for chloramphenicol in two of the *C. coli* strains C75 and M8 should be concerning, as resistance to this group of antibiotics may arise without judicious use.

4.2 Do *C. coli* from South-East Asia present a global threat to human health?

MLST is an epidemiological tool to link isolates with a similar genetic background together and can be used to understand transmission of pathogens on both a local and global scale. All the MLST profiles, apart from M39 with a novel *glnA* mutation, were in the public database (25th Jan 2022) and were therefore previously reported. The MLST profiles of *C. coli* C75 (ST-1586), M8 (ST-6184), M10 (ST-966) and M14 (ST-966) have previously been found in human stool samples, many of which were linked to sporadic cases of gastroenteritis, demonstrating that the isolates could potentially be of clinical importance. ST-1586 (*C. coli* C75) have been identified in 'North America' (6 isolates), USA (5) and the UK (10), all of which were identified from human faeces, and some documented as a sporadic case of gastroenteritis. ST-1586 (*C. coli* C75) has been reported from Vietnamese clinical samples between 2014-2019. ST-966 (M14 and M10) has mostly been documented from the USA, except a single UK clinical isolate. ST-829 (*C. coli* C76) was primarily from the USA (1021, 83%) with many of which from carriers such as poultry. ST-5511 (*C. coli* C57) was rare with only being identified in three *C. coli* isolates from China, with two identified from chicken. ST-6184 (*C. coli* M8) has been identified previously in poultry in the USA and China, with a single case from China isolated from human stool, linked to an outbreak. ST-829 (*C. coli* C76) has been submitted over 1200 times to the MLST database, mostly identified in high-income countries (HICs), mainly from the USA (1021, 83% of the documented cases).

ST-829 was identified in England (44) and Scotland (33), all of which were isolated from human faeces, relating to gastroenteritis. The MLST database is heavily skewed towards isolates from Europe, UK and USA so the true spread of these clones is unknown but it does suggest that the global transport of food is carrying these isolates far and wide to the detriment of human health.

4.3 Are *C. coli* more virulent than *C. jejuni*?

Within the limited number of isolates common *Campylobacter* sp. virulence related genes were identified and it was demonstrated *C. coli* can be equally motile as the reference hyper-motile *C. jejuni* 11168H strain and equally lethal as the reference strain *C. jejuni* 81-176 in the *G. mellonella* infection model. Although no strain was significantly more virulent than the *C. jejuni* reference, two strains were significantly less virulent. Comparison between these identified 142 CDSs present in the more virulent isolate however with the majority of these with unknown function it was not possible to link genotypes to virulence. Genome sequencing identified a potential novel virulence related plasmid in *C. coli* M39, carrying genes related to the type IV secretion system. The type IV secretion system is involved in DNA conjugation, helping bacteria in adapting to environmental stress and is a potential mechanism of spreading AMR, allowing the uptake of DNA from the environment (Walden et al., 2010) although this strain was not more resistant than other isolates. The type IV secretion system can also play a role in increased host colonisation, biofilm formation and transfer of toxins to host cells and thus can have a direct involvement in virulence (Kienesberger et al., 2011), although this strain was no more virulent than other *C. coli* isolates in the Galleria model.

Despite the limitations of the study in terms of isolate numbers, narrow graphical and temporal range it was demonstrated that *C. coli* warrants further study to understand the global relevance compared to the highly studied *C. jejuni*. Although *C. jejuni* is the most common *Campylobacter* species to infect humans, other *Campylobacter* species, such as *C. coli* need to be studied for several reasons, including interspecies horizontal gene transfer (Mourkas et al., 2022), which may contribute the emergence of multi-drug resistant pathogens. This, together with the expanding global poultry production networks and the intensification of production, may pose a global public health risk. This highlights the urgent need for a coordinated global effort to combat AMR.

Declaration of generative AI in scientific writing

During the preparation of this work the authors did not use any generative AI and AI-assisted technologies in the writing process.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, JAKGTW000000000, JAKGTV000000000, JAKGTS000000000, JAKGTU000000000, JAKGTT000000000, JAKGTR000000000, CP091310.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

BL: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Data curation. GN: Data curation, Formal analysis, Writing – original draft. AC: Formal analysis, Investigation, Writing – review & editing. FN: Investigation, Writing – review & editing, Methodology, Resources. SW: Investigation, Methodology, Writing – review & editing, Formal analysis. PN: Writing – review & editing. BW: Writing – review & editing, Funding acquisition. LH: Writing – review & editing, Conceptualization, Formal analysis, Methodology, Writing – original draft. RS: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Investigation, Project administration, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by the UKRI GCRF One Health Poultry Hub (Grant No. BB/S011269/1), one of 12 interdisciplinary research hubs funded under the UK government's Global Challenges Research Fund Interdisciplinary Research Hub initiative. BL, LH and RS and this project was supported by the BBSRC GCRF One Health Poultry Hub grant number BB/S011269/1. GN was supported by BBSRC grant number BB/M009513/1.

Acknowledgments

We would like to thank Abdi Elmi and Sherif Abouelhadid for their technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the

reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/finmi.2024.1355079/full#supplementary-material>

SUPPLEMENTARY TABLE 4

Analysis of the draft genomes identified potential AMR genes. Abricate with the ResFinder database (update date 10-03-2022) was used to identify AMR genes. Red highlighting indicates study samples demonstrating resistance to a specific class of antibiotics.

References

- Agyare, C., Etsiapa Boamah, V., Ngofi Zumbi, C., and Boateng Osei, F. (2018). "Antibiotic use in poultry production and its effects on bacterial resistance," in *Antimicrobial Resistance - A Global Threat*, ed Y. Kumar (IntechOpen, online). <http://dx.doi.org/10.5772/intechopen.79371>.
- Birhanu, M. Y., Geremew, K., Esatu, W., Worku, S., Getachew, F., Viet Don, N., et al. (2021). *Poultry production, marketing and consumption in Vietnam: A review of literature*. ILRI Research Report 80 (Nairobi, Kenya).
- Bojanic, K., Acke, E., Roe, W. D., Marshall, J. C., Cornelius, A. J., Biggs, P. J., et al. (2020). Comparison of the Pathogenic Potential of *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus* and Limitations of Using Larvae of *Galleria mellonella* as an Infection Model. *Pathogens* 9. doi: 10.3390/pathogens9090713
- Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170.
- Champion, O. L., Karlyshev, A. V., Senior, N. J., Woodward, M., La Ragione, R., Howard, S. L., et al. (2010). Insect infection model for *Campylobacter jejuni* reveals that O-methyl phosphoramidate has insecticidal activity. *J. Infect. Dis.* 201, 776–782. doi: 10.1086/650494
- Darling, A. C., Mau, B., Blattner, F. R., and Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403. doi: 10.1101/gr.2289704.
- Devi, A., Mahony, T. J., Wilkinson, J. M., and Vanniasinkam, T. (2019). Antimicrobial susceptibility of clinical isolates of *Campylobacter jejuni* from New South Wales, Australia. *J. Glob. Antimicrob. Resist.* 16, 76–80. doi: 10.1016/j.jgar.2018.09.011.
- Doyle, R. M., O'Sullivan, D. M., Aller, S. D., Bruchmann, S., Clark, T., Coello Pelegrin, A., et al. (2020). Discordant bioinformatic predictions of antimicrobial resistance from whole-genome sequencing data of bacterial isolates: an inter-laboratory study. *Microb. Genom.* 6. doi: 10.1099/mgen.0.000335.
- Dudziak, B., and Jozwik, Z. (1969). The virulence of some *Escherichia coli* strains for larvae of *Galleria mellonella* L. *Acta Microbiol. Pol. B* 1, 137–141.
- EUCAST (2020) The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 10.0, 2020. (Sweden: EUCAST). Available online at: http://www.eucast.org/clinical_breakpoints/.
- EUCAST (2023). The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 13.1. (Sweden: EUCAST). Available online at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_13.1_Breakpoint_Tables.pdf.
- European Food Safety, A and P. European Centre for Disease and Control (2023). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2020/2021. *EFSA J.* 21, e07867. doi: 10.2903/j.efsa.2023.7867
- Garcia-Fernandez, A., Dionisi, A. M., Arena, S., Iglesias-Torrens, Y., Carattoli, A., and Luzzi, I. (2018). Human *Campylobacteriosis* in Italy: emergence of multi-drug resistance to ciprofloxacin, tetracycline, and erythromycin. *Front. Microbiol.* 9, 1906. doi: 10.3389/fmicb.2018.01906.
- Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUASt: quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075. doi: 10.1093/bioinformatics/btt086.
- Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., et al. (2006). Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infect. Immun.* 74, 4694–4707. doi: 10.1128/IAI.00210-06.
- Insua, J. L., Llobet, E., Moranta, D., Perez-Gutierrez, C., Tomas, A., Garmendia, J., et al. (2013). Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. *Infect. Immun.* 81, 3552–3565. doi: 10.1128/IAI.00391-13.
- Jeon, B., and Zhang, Q. (2007). Cj0011c, a periplasmic single- and double-stranded DNA-binding protein, contributes to natural transformation in *Campylobacter jejuni*. *J. Bacteriol.* 189, 7399–7407. doi: 10.1128/JB.01012-07.
- Kaakoush, N. O., Castano-Rodriguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi: 10.1128/CMR.00006-15.
- Karlyshev, A. V., McCrossan, M. V., and Wren, B. W. (2001). Demonstration of polysaccharide capsule in *Campylobacter jejuni* using electron microscopy. *Infect. Immun.* 69, 5921–5924. doi: 10.1128/IAI.69.9.5921-5924.2001.
- Kienesberger, S., Schober Trummler, C., Fauster, A., Lang, S., Sprenger, H., Gorkiewicz, G., et al. (2011). Interbacterial macromolecular transfer by the *Campylobacter fetus* subsp. *venerealis* type IV secretion system. *J. Bacteriol.* 193, 744–758. doi: 10.1128/JB.00798-10.
- Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleeschauwer, B., et al. (2015). World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases 2010: A data synthesis. *PLoS Med.* 12, e1001921. doi: 10.1371/journal.pmed.1001921.
- Konkel, M. E., Kim, B. J., Rivera-Amill, V., and Garvis, S. G. (1999). Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol. Microbiol.* 32, 691–701. doi: 10.1046/j.1365-2958.1999.01376.x.
- Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C., and Robinson, R. A. (1985). A point-source outbreak of *Campylobacteriosis* associated with consumption of raw milk. *J. Infect. Dis.* 152, 592–596. doi: 10.1093/infdis/152.3.592.
- Krause-Gruszczynska, M., van Alphen, L. B., Oyarzabal, O. A., Alter, T., Hanel, I., Schliephake, A., et al. (2007). Expression patterns and role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol. Lett.* 274, 9–16. doi: 10.1111/fml.2007.274.issue-1.
- Kurstak, E., and Vega, C. E. (1968). Bacterial infection due to *Salmonella typhimurium* in an invertebrate, *Galleria mellonella* L. *Can. J. Microbiol.* 14, 233–237. doi: 10.1139/m68-039.
- Laaveri, T., Vilkkman, K., Pakkanen, S. H., Kirveskari, J., and Kantele, A. (2018). A prospective study of travellers' diarrhoea: analysis of pathogen findings by destination in various (sub)tropical regions. *Clin. Microbiol. Infect.* 24, 908 e909–908 e916. doi: 10.1016/j.cmi.2017.10.034.
- Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C. M., and Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol.* 4, 189–200. doi: 10.2217/17460913.4.2.189.
- Macdonald, S. E., Gundogdu, O., Dorrell, N., Wren, B. W., Blake, D., and Stabler, R. (2017). Draft genome sequence of *Campylobacter jejuni* 11168H. *Genome Announc.* 5. doi: 10.1128/genomeA.01556-16.
- Mace, K., Vadakkepat, A. K., Redzej, A., Lukoyanova, N., Oomen, C., Braun, N., et al. (2022). Cryo-EM structure of a type IV secretion system. *Nature* 607(7917):191–196. doi: 10.1038/s41586-022-04859-y.

- Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x.
- Mourkas, E., Yahara, K., Bayliss, S. C., Calland, J. K., Johansson, H., Mageiros, L., et al. (2022). Host ecology regulates interspecies recombination in bacteria of the genus *Campylobacter*. *eLife* 11, e73552. doi: 10.7554/eLife.73552.sa2.
- Mulchandani, R., Wang, Y., Gilbert, M., and Van Boeckel, T. P. (2023). Global trends in antimicrobial use in food-producing animals: 2020 to 2030. *PLoS Glob Public Health* 3, e0001305. doi: 10.1371/journal.pgph.0001305.
- Nguyen, T. N., Hotzel, H., El-Adawy, H., Tran, H. T., Le, M. T., Tomaso, H., et al. (2016). Genotyping and antibiotic resistance of thermophilic *Campylobacter* isolated from chicken and pig meat in Vietnam. *Gut Pathog.* 8, 19. doi: 10.1186/s13099-016-0100-x.
- Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., et al. (2013). Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J. Comput. Biol.* 20, 714–737. doi: 10.1089/cmb.2013.0084.
- Ohishi, T., Aoki, K., Ishii, Y., Usui, M., Tamura, Y., Kawanishi, M., et al. (2017). Molecular epidemiological analysis of human- and chicken-derived isolates of *Campylobacter jejuni* in Japan using next-generation sequencing. *J. Infect. Chemother.* 23, 165–172. doi: 10.1016/j.jiac.2016.11.011.
- Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088.
- Pratt, A., and Korolik, V. (2005). Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *J. Antimicrob. Chemother.* 55, 452–460. doi: 10.1093/jac/dki040.
- Russell, R. G., Blaser, M. J., Sarmiento, J. I., and Fox, J. (1989). Experimental *Campylobacter jejuni* infection in *Macaca nemestrina*. *Infect. Immun.* 57, 1438–1444. doi: 10.1128/iai.57.5.1438-1444.1989.
- Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153.
- Seemann, T. (2018). *Abricate*.
- Senior, N. J., Bagnall, M. C., Champion, O. L., Reynolds, S. E., La Ragione, R. M., Woodward, M. J., et al. (2011). *Galleria mellonella* as an infection model for *Campylobacter jejuni* virulence. *J. Med. Microbiol.* 60, 661–669. doi: 10.1099/jmm.0.026658-0.
- Shane, S. M. (2000). *Campylobacter* infection of commercial poultry. *Rev. Sci. Tech* 19, 376–395. doi: 10.20506/rst.issue.19.2.14.
- Sheppard, S. K., McCarthy, N. D., Falush, D., and Maiden, M. C. (2008). Convergence of *Campylobacter* species: implications for bacterial evolution. *Science* 320, 237–239. doi: 10.1126/science.1155532.
- Shimoyama, Y. (2022). *pyGenomeViz: A genome visualization python package for comparative genomics*.
- Silva, M., MaChado, M. P., Silva, D. N., Rossi, M., Moran-Gilad, J., Santos, S., et al. (2018). chewBBACA: A complete suite for gene-by-gene schema creation and strain identification. *Microb. Genom.* 4. doi: 10.1099/mgen.0.000166.
- Skarp, C. P. A., Hanninen, M. L., and Rautelin, H. I. K. (2016). *Campylobacteriosis*: the role of poultry meat. *Clin. Microbiol. Infect.* 22, 103–109. doi: 10.1016/j.cmi.2015.11.019.
- Sprengr, H., Kienesberger, S., Pertschy, B., Polt, L., Konrad, B., Bhutata, P., et al. (2017). Fic Proteins of *Campylobacter fetus* subsp. *venerealis* Form a Network of Functional Toxin-Antitoxin Systems. *Front. Microbiol.* 8, 1965. doi: 10.3389/fmicb.2017.01965.
- Suttili, F. J., and Gressler, L. T. (2021). “Chapter 3 - antimicrobial agents,” in *Aquaculture pharmacology*, eds F. Kibenge, B. Baldisserotto and R. Chong (Amsterdam, Netherlands: Elsevier), 131–168. doi: 10.1016/B978-0-12-821339-1.00004-0.
- Tangcharoensathien, V., Witthayapipopsakul, W., Panichkriangkrai, W., Patcharanarumol, W., and Mills, A. (2018). Health systems development in Thailand: a solid platform for successful implementation of universal health coverage. *Lancet* 391, 1205–1223. doi: 10.1016/S0140-6736(18)30198-3.
- Van Boeckel, T. P., Glennon, E. E., Chen, D., Gilbert, M., Robinson, T. P., Grenfell, B. T., et al. (2017). Reducing antimicrobial use in food animals. *Science* 357(6358), 1350–1352. doi: 10.1126/science.aao1495.
- van Vliet, A. H. M., Thakur, S., Prada, J. M., Mehat, J. W., and La Ragione, R. M. (2022). Genomic screening of antimicrobial resistance markers in UK and US *Campylobacter* isolates highlights stability of resistance over an 18-year period. *Antimicrob. Agents Chemother.* 66, e0168721. doi: 10.1128/aac.01687-21.
- Viegas, S. C., Mil-Homens, D., Fialho, A. M., and Arraiano, C. M. (2013). The virulence of *Salmonella enterica* Serovar Typhimurium in the insect model *Galleria mellonella* is impaired by mutations in RNase E and RNase III. *Appl. Environ. Microbiol.* 79, 6124–6133. doi: 10.1128/AEM.02044-13.
- Wallden, K., Rivera-Calzada, A., and Waksman, G. (2010). Type IV secretion systems: versatility and diversity in function. *Cell Microbiol.* 12, 1203–1212. doi: 10.1111/j.1462-5822.2010.01499.x.
- WHO (2017) WHO guidelines on use of medically important antimicrobials in food-producing animals. Available online at: <http://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf>.
- Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). “Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13, e1005595. doi: 10.1371/journal.pcbi.1005595.
- Wieczorek, K., and Osek, J. (2013). Antimicrobial resistance mechanisms among *Campylobacter*. *BioMed. Res. Int.* 2013, 340605. doi: 10.1155/2013/340605.
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261.
- Zatyka, M., and Thomas, C. M. (1998). Control of genes for conjugative transfer of plasmids and other mobile elements. *FEMS Microbiol. Rev.* 21, 291–319. doi: 10.1111/j.1574-6976.1998.tb00355.x.