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RESEARCH ARTICLE

Characterization of *Campylobacter jejuni* and *Campylobacter coli* isolates from chicken offal in Metro Manila, Philippines: Insights from virulence gene prevalence and multilocus sequence typing analysis

Subejano, M.S.E.[1,2], Penuliar, G.[1,2*]

[1]Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City 1101 Philippines
[2]Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City 1101 Philippines
*Corresponding author: gmpenuliar@up.edu.ph

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ABSTRACT

Campylobacteriosis is a human infection primarily caused by *Campylobacter jejuni* and *Campylobacter coli*. Consumption of contaminated chicken and poultry products is the main mode of transmission. These bacteria possess virulence factors, including adhesins and toxins, which contribute to their pathogenesis. Moreover, their large genomes undergo frequent genetic recombination, resulting in a high degree of genetic diversity. However, limited information is available regarding the virulence and genotypic diversity profiles of these microorganisms in the Philippines. The objective of this study was to address this knowledge gap by characterizing *Campylobacter* isolates obtained from chicken offal sold in wet markets in Metro Manila, Philippines. Multilocus Sequence Typing (MLST) analysis was performed to determine the sequence types, resulting in the identification of 13 unique sequence types, including nine previously unreported ones, and three clonal complexes. Notably, the widespread sequence type ST-305 was found in samples from different markets. Furthermore, six isolates deposited in the *Campylobacter* PubMLST database were identified as *C. coli* based on allele profiles. Profiling using 10 selected virulence genes revealed that more than half of the isolates carried these genes. The most prevalent virulence gene was *cadF* (100%), followed by *flaA* (95%), *racR*, *cdtA*, *cdtB*, and *cdtC* (85%). The genes *dnaJ* and *ceuE* were also present in 75% of the isolates. Despite the limited sample size, the findings of this study reveal a significant level of genotypic diversity among the *Campylobacter* isolates. This diversity has important implications for source attribution studies and the identification of strains involved in campylobacteriosis outbreaks. Furthermore, the investigation of virulence factors associated with colonization and invasion of the avian gut can provide insights for the development of practical applications in *Campylobacter* control strategies. Understanding and addressing these factors are crucial steps toward mitigating the risk of *Campylobacter* infections and enhancing public health efforts.

Keywords: *Campylobacter*; virulence genes; MLST.

INTRODUCTION

Campylobacter is a Gram-negative bacterium that is commonly found in the gastrointestinal tracts of various animals, including wild birds, domesticated animals, and those involved in food production (Burnham & Hendrixson, 2018). It is a zoonotic pathogen that is responsible for campylobacteriosis, a common cause of bacterial gastroenteritis, that is transmitted primarily through the consumption of contaminated poultry meat and products (García-Sanchez *et al*., 2017). *Campylobacter jejuni* and *Campylobacter coli* are major pathogens associated with campylobacteriosis, surpassing even *Salmonella* in their impact on human health (Kreling *et al*., 2020). The World Health Organization (WHO) reported that *C. jejuni* is responsible for an estimated 96 million cases of enteric infections worldwide annually (Bailey *et al*., 2018). The disease is characterized by bloody diarrhea, abdominal pain, fever, and occasionally nausea and vomiting. Given its widespread occurrence and the potential for long-term complications such as Guillain-Barré syndrome, campylobacteriosis poses a significant public health concern (WHO, 2020).

To better understand the epidemiology and transmission dynamics of *Campylobacter*, it is crucial to investigate the genotypic diversity of the isolates. By employing Multilocus Sequence Typing (MLST), a widely utilized molecular typing method, it becomes possible to analyze genetic variations in specific housekeeping genes and determine the relatedness of bacterial isolates (Wieczorek *et al*., 2020). MLST offers valuable insights into various aspects, including population dynamics, global epidemiology, and surveillance of pathogenic and antibiotic-resistant bacteria (Chan *et al*., 2001). This method enables the identification of distinct sequence types, the classification of isolates into clonal complexes, and the detection of potential transmission routes and patterns. By applying MLST

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analysis to *Campylobacter* isolates derived from chicken offal sold in public wet markets in Metro Manila, Philippines, we can gain comprehensive insights into the genotypic diversity of these specific isolates. This investigation will contribute to a better understanding of the genetic characteristics of *Campylobacter* strains circulating in the local poultry market, identify potential sources of contamination, and provide important information for the development of targeted control strategies. Furthermore, the generated MLST data can be compared with global databases, facilitating broader epidemiological studies and contributing to the international surveillance efforts on *Campylobacter*.

In addition to genotypic diversity, the virulence factors of *Campylobacter* play a crucial role in its pathogenicity. These factors, including adhesins, toxins, and other virulence-associated proteins, are key determinants in the ability of *Campylobacter* to colonize and cause disease (Ziprin *et al*., 2001). Adhesins facilitate the attachment of *Campylobacter* to host cells, promoting colonization, while toxins can damage host tissues and modulate immune responses. Other virulence factors may interfere with host cell signaling or disrupt cellular processes (Sharma *et al*., 2017). Investigating the prevalence of these virulence-associated genes provides valuable insights into the molecular mechanisms by which *Campylobacter* successfully infects and affects its hosts. It helps identify specific virulence factors that contribute to pathogenicity and understand their potential roles in disease progression (Zeng *et al*., 2016). Furthermore, studying the distribution and diversity of virulence genes in *Campylobacter* isolates from chicken offal can shed light on the potential pathogenicity and virulence mechanisms of these strains in relation to their source and origin.

Limited data exist regarding the prevalence and virulence gene profiles of *Campylobacter* in the Philippines, despite extensive global research on its genetic diversity. This knowledge gap is significant given that poultry meat, which are established primary sources of human campylobacteriosis, and other poultry-related products, such as chicken offal, are sold in wet markets. In the Philippines, the consumption of chicken offal is a prevalent and culturally significant dietary practice. Chicken intestines and other offal parts are commonly skewered, grilled, and enjoyed as a popular street food delicacy. Investigating the prevalence and genotypic diversity of *Campylobacter* isolates in chicken offal is crucial for accurately assessing transmission risks, overall food safety situation, and developing effective control strategies to reduce campylobacteriosis burden in the Philippines (Hidano *et al*., 2014). Furthermore, understanding the virulence gene profiles of *Campylobacter* isolates from chicken offal will help elucidate their pathogenic potential and ability to cause illness in humans, which can help raise awareness among consumers about the importance of safe food handling and preparation practices.

This study aimed to investigate the genotypic diversity of *C. jejuni* and *C. coli* isolates from fresh chicken offal obtained from public wet markets in Metro Manila, Philippines. Using MLST analysis, the research identified unique sequence types and clonal complexes among the isolates, providing insights into the population structure and relatedness of *Campylobacter* strains within this specific context. Additionally, the study aimed to determine the profile of virulence-associated genes in these *Campylobacter* isolates, shedding light on the potential pathogenicity and virulence mechanisms employed by these strains.

The findings of this research will enhance our understanding of the epidemiology, pathogenicity, and virulence mechanisms of *Campylobacter* in the Philippines. This knowledge will be of immense value for public health initiatives focused on controlling and preventing the transmission of *Campylobacter* infections, thereby enhancing food safety and safeguarding human health. By identifying the specific characteristics and genetic diversity of *Campylobacter* strains in fresh chicken offal from public wet markets, this study provides crucial insights that can inform the development of targeted interventions and strategies to mitigate the impact of campylobacteriosis outbreaks. Ultimately, these efforts will contribute to the overall well-being and improved health outcomes of the population, making a significant impact on public health practices and policies.

MATERIALS AND METHODS

Sample Collection

From September to December 2021, a total of 118 fresh chicken offal samples were collected from various public wet markets in Metro Manila, Philippines. The samples were stored in labeled resealable plastic bags, placed in ice boxes, and transported to the Medical Microbiology Laboratory of the Institute of Biology, University of the Philippines Diliman. The samples were processed by removing visible contaminants and homogenizing them for subsequent analysis. Standard operating procedures and aseptic technique were maintained to minimize cross-contamination.

Isolation of *Campylobacter* spp.

The isolation protocol described in ISO 10272:2006-1 (ISO, 2006) was followed with some modifications. To enrich the samples, 25 mL of homogenized sample was added to 100 mL of Bolton selective enrichment broth supplemented with cefoperazone (20 mg/L), vancomycin (20 mg/L), trimethoprim (20 mg/L), cycloheximide (50 mg/L), piperacillin-tazobactam (8 mg/1 mg/L), and 5% (v/v) mechanically-defibrinated lysed horse blood. The pre-enrichment was carried out at 37°C for 4 hours under microaerobic conditions, followed by incubation at 42°C for 48 h under the same conditions. After selective enrichment, 100 µL of the enrichment culture was spread-plated onto mCCDA plates supplemented with mCCDA Selective Supplement containing cefoperazone (32 mg/L), amphotericin B (10 mg/L), and piperacillin-tazobactam (8 mg/1 mg/L). The plates were incubated at 42°C for 48 h under microaerobic conditions. Gray, flat, and moist colonies resembling typical *Campylobacter* species were purified and maintained on Mueller-Hinton agar (MHA) plates supplemented with 5% (v/v) mechanically-defibrinated lysed horse blood. The MHA plates were incubated under the previously described culture conditions.

Molecular Identification

The boiling lysis method (Subejano & Penuliar, 2018) was utilized for the extraction of genomic DNA from the presumed *Campylobacter* isolates. Identification of the isolates was accomplished using *Campylobacter jejuni*-specific primers MDmapA1F 5'-CTATTTATTTTTGAGTGCTTGTG-3' and MDmapA2R 5'-GCTTTATTTGCCATTTGTTTTATTA-3' (Inglis & Kalischuk, 2004). PCR assays were conducted in 20-µL reaction mixtures composed of 10 µL of GoTaq® Master Mix (Promega, Wisconsin, USA), 1.2 µL of each primer (0.6 µM final concentration), 6.6 µL of nuclease-free water, and 1 µL of template DNA. The PCR amplification was performed using a MyCycler™ Thermal Cycler System (Bio-Rad, California, USA), with the following optimized cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 5 min. To confirm the accuracy of the assays, *C. jejuni* ATCC 33560 was included as a positive control.

Multilocus Sequence Typing

PCR amplification of the seven housekeeping genes was performed according to the protocol described by Dingle *et al*. (2001), which made use of the following primers: asp-A9 5'-AGTACTAATGATGCTTATCC-3', asp-A10 5'-ATTTCATCAATTTGTTCTTTGC-3', gln-A1 5'-TAGGAACTTGGCATCATATTACC-3', gln-A2 5'-TTGGACGAGCTTCTACTGGC-3', glt-A1 5'-GGGCTTGACTTCTACAGCTACTTG-3',

glt-A2 5'-CCAAATAAAGTTGTCTTGGACGG-3', gly-A1 5'-GAGTTAGAGCGTCAATGTGAAGG-3', gly-A2 5'-AAACCTCTGGCAGTAAGGGC-3', pgm-A7 5'-TACTAATAATATCTTAGTAGG-3', pgm-A8 5'-CACAACATTTTTCATTTCTTTTTC-3', tkt-A3 5'-GCAAACTCAGGACACCCAGG-3', tkt-A6 5'-AAAGCATTGTTAATGGCTGC-3', unc-A7 5'-ATGGACTTAAGAATATTATGG-3', unc-A2 5'-GCTAAGCGGAGAATAAGGTGG-3'. The amplification was carried out in 25 µL reactions composed of 12.5 µL of GoTaq® Green Master Mix, 8.5 µL of sterile water, 1.5 µL (0.6 µM) of each primer, and 1 µL of template DNA. The PCR amplification was performed using a MyCycler™ Thermal Cycler System with the following optimized cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The annealing conditions used were 56°C for 1 min for primers asp-A9/A10 and gln-A1/A2, 60°C for 1 min for primers glt-A1/A2, 65°C for 1 min for primers gly-A1-/A2, 53°C for 1 min for primers pgm-A7/A8 and tkt-A3/A6 and 57°C for 1 min for primers unc-A7/A2. *C. jejuni* ATCC 33560 was included as a positive control to confirm the accuracy of the assays.

Virulence Gene Profiling

The prevalence of 10 virulence genes (*cadF*, *cdtA*, *cdtB*, *cdtC*, *ceuE*, *ciaB*, *dnaJ*, *flaA*, *pldA*, and *racR*) were determined by PCR amplification using the following primers: cadF-F2B 5'-TTGAAGGTAATTTAGATATG-3', cadF-R1B 5'-CTAATACCTAAAGTTGAAAC-3' (Konkel *et al*., 1999), DS-18 5'-CCTTGTGATGCAAGCAATC-3', DS-15 5'-ACACTCCATTTGCTTTCTG-3' (Hickey *et al*., 2000), JEJ1 5'-CCTGCTACGGTGAAAGTTTTGC-3', JEJ2 5'-GATCTTTTTGTTTTGTGCTGC-3' (Gonzalez *et al*., 1997), cdtB-113 5'-CAGAAAGCAAATGGAGTGTT-3', cdtB-713 5'-AGCTAAAAGCGGTGGAGTAT-3', cdtC-192 5'-CGATGAGTTAAAACAAAAAGATA-3', cdtC-351 5'-TTGGCATTATAGAAAATACAGTT-3', ciaB-403 5'-TTTTTATCAGTCCTTA-3', ciaB-1373 5'-TTTCGGTATCATTAGC-3', dnaJ-299 5'-AAGGCTTTGGCTCATC-3', dnaJ-1003 5'-CTTTTTGTTCATCGTT-3', flaA-664 5'-AATAAAAATGCTGATAAAACAGGTG-3', flaA-1494 5'-TACCGAACCAATGTCTGCTCTGATT-3', pldA-84 5'-AAGCTTATGCGTTTTT-3', pldA-981 5'-TATAAGGCTTTCTCCA-3', racR-25 5'-GATGATCCTGACTTTG-3' and racR-593 5'-TCTCCTATTTTTACCC-3' (Datta *et al*., 2003). The amplification reactions were carried out in 10 µL volumes, composed of 5 µL of GoTaq® Master Mix, 0.6 µL of each primer (0.6 µM final concentration), 2.8 µL of nuclease-free water, and 1 µL of template DNA. The PCR amplification was performed using a MyCycler™ Thermal Cycler System with the following optimized cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The annealing conditions used were 53°C for 1 min for primers cadF-F2B/R1B, 60°C for 1 min for DS-18/15, 63°C for 1 min for cdtB-113/713, 55°C for 1 min for cdtC-192/351 and JEJ1/JEJ2, 44°C for 1 min for ciaB-403/1373, 47°C for 1 min for dnaJ-299/1003, 62°C for 1 min for flaA-664/1494, 49°C for 1 min for pldA-84/981, and 52°C for 1 min for racR-25/593. To confirm the accuracy of the assays, *C. jejuni* ATCC 33560 was included as a positive control and PCR amplification using primers MDmapA1F/A2R was performed as internal control.

Agarose Gel Electrophoresis

The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide in 1X Tris-Acetate-EDTA buffer. Electrophoresis was conducted at 100 V for 24 min. The gel was visualized using a UV transilluminator, and a 100-bp molecular weight marker from Vivantis Technologies (California, USA) was included as a size reference.

DNA Sequencing and Sequence Typing

PCR products were submitted to Macrogen, Inc. (Seoul, Republic of Korea) for purification and DNA sequencing. The consensus sequences were compared to the *Campylobacter* PubMLST database (https://pubmlst.org/campylobacter/) (Jolley *et al*., 2018) to assign allele numbers. The process was repeated until a seven-digit allele profile was obtained for each isolate. The allele profiles were further analyzed against the database to determine the corresponding sequence type (ST) and/or clonal complex (CC) assignment. In case of any allele numbers or profiles that did not match the existing database, they were submitted to the *Campylobacter* PubMLST database curator for the assignment of new allele numbers, sequence types, and clonal complexes. All isolates were deposited in the PubMLST *C. jejuni*/*coli* database for future reference under ID numbers 112218, 112235-112242, and 112244-112254.

MLST Phylogenetic Analysis

Full minimum spanning trees were constructed using the PubMLST plugin GrapeTree (Zhou *et al*., 2018) to visualize the genetic relationships between MLST sequence types derived from our study and those present in the PubMLST database (Jolley *et al*., 2018). The minimum spanning trees were generated to illustrate the associations of the isolates from our study with those from the database, considering factors such as geographical location and animal source.

RESULTS

Multilocus Sequence Typing

After confirming the identification of 20 isolates as *Campylobacter jejuni* using *C. jejuni*-specific primers, multilocus sequence typing (MLST) analysis was performed. The isolates were queried against the PubMLST *Campylobacter* database to assign them respective sequence types (STs) and/or clonal complexes (CCs). The MLST analysis results is summarized in Table 1. The database curator identified six isolates as *Campylobacter coli* based on the allele profiles submitted. Notably, none of these *C. coli* isolates shared the same sequence type as *C. jejuni*. In terms of novel allele numbers, a total of five were deposited, including three for *asp*, one for *gln*, and one for *gly*. Furthermore, nine novel allele profiles were designated as new sequence types, with five (ST-11910, ST-11912, ST-11913, ST-11914, ST-11915) belonging to *C. coli* and the remaining four (ST-11905, ST-11907, ST-11908, ST-11909) belonging to *C. jejuni*.

The analysis revealed a total of thirteen unique sequence types among the isolates, namely ST-305, ST-11910, ST-11915, ST-10695, ST-9968, ST-5968, ST-11907, ST-11909, ST-11905, ST-11908, ST-11912, ST-11913, and ST-11914. ST-305 was the most prevalent, accounting for 35% (7/20) of the isolates, followed by ST-9968, which represented 10% (2/20). Three distinct clonal complexes were identified, namely the ST-574 complex, ST-1150 complex, and ST-443 complex. ST-305 was classified within the ST-574 complex, while ST-10695 belonged to the ST-1150 complex. An isolate with ST-5968 clustered with the ST-443 complex.

MLST Phylogenetic Analysis

Figure 1 depicts the minimum spanning tree generated using GrapeTree (Zhou *et al*., 2018), providing a visual representation of the relationship between the investigated sequence types and those present in the database. The positions of the sequence types on the tree are determined by their reported geographical locations or continents. ST-305 is predominantly observed in Asia but also has occurrences in North America and Africa. Similarly, ST-9968 is primarily prevalent in Asia but is also found in North America and Oceania. Another sequence type, ST-10695, is distributed in both Asia and North America. In contrast, based on the tree, ST-5968 appears to be exclusive to Asia. The remaining novel STs identified in this study (ST-11905, ST-11907, ST-11908, ST-11909, ST-11910, ST-11912, ST-11913, ST-11914, ST-11915) are currently exclusive to Asia, as they have not been previously reported.

Figure 2 displays a minimum spanning tree illustrating the distribution of the investigated sequence types in this study and

Table 1. MLST of the *C. jejuni* and *C. coli* isolates

Isolate	Species	*asp*	*gln*	*glt*	*gly*	*pgm*	*tkt*	*unc*	ST	CC
CJ01	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ02	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ03	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ04	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ05	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ06	*C. coli*	32	813[a]	2	82	104	34	139	11910[b]	
CJ07	*C. coli*	33	39	2	10	113	34	17	11915[b]	
CJ08	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ09	*C. jejuni*	9	53	2	10	11	3	3	305	ST-574 complex
CJ10	*C. coli*	103	110	30	82	188	164	79	10695	ST-1150 complex
CJ11	*C. jejuni*	7	15	57	10	550	34	35	9968	
CJ12	*C. jejuni*	7	15	57	10	550	34	35	9968	
CJ13	*C. jejuni*	7	17	79	15	23	3	12	5968	ST-443 complex
CJ14	*C. jejuni*	7	15	57	919[a]	550	34	35	11907[b]	
CJ15	*C. jejuni*	599[a]	15	57	64	113	34	35	11909[b]	
CJ16	*C. jejuni*	600[a]	15	57	64	113	34	35	11905[b]	
CJ17	*C. jejuni*	601[a]	15	57	64	113	34	35	11908[b]	
CJ18	*C. coli*	33	39	1	82	113	34	6	11912[b]	
CJ19	*C. coli*	33	39	57	64	113	34	35	11913[b]	
CJ20	*C. coli*	33	53	57	82	596	164	87	11914[b]	

[a] New allele number
[b] New sequence type

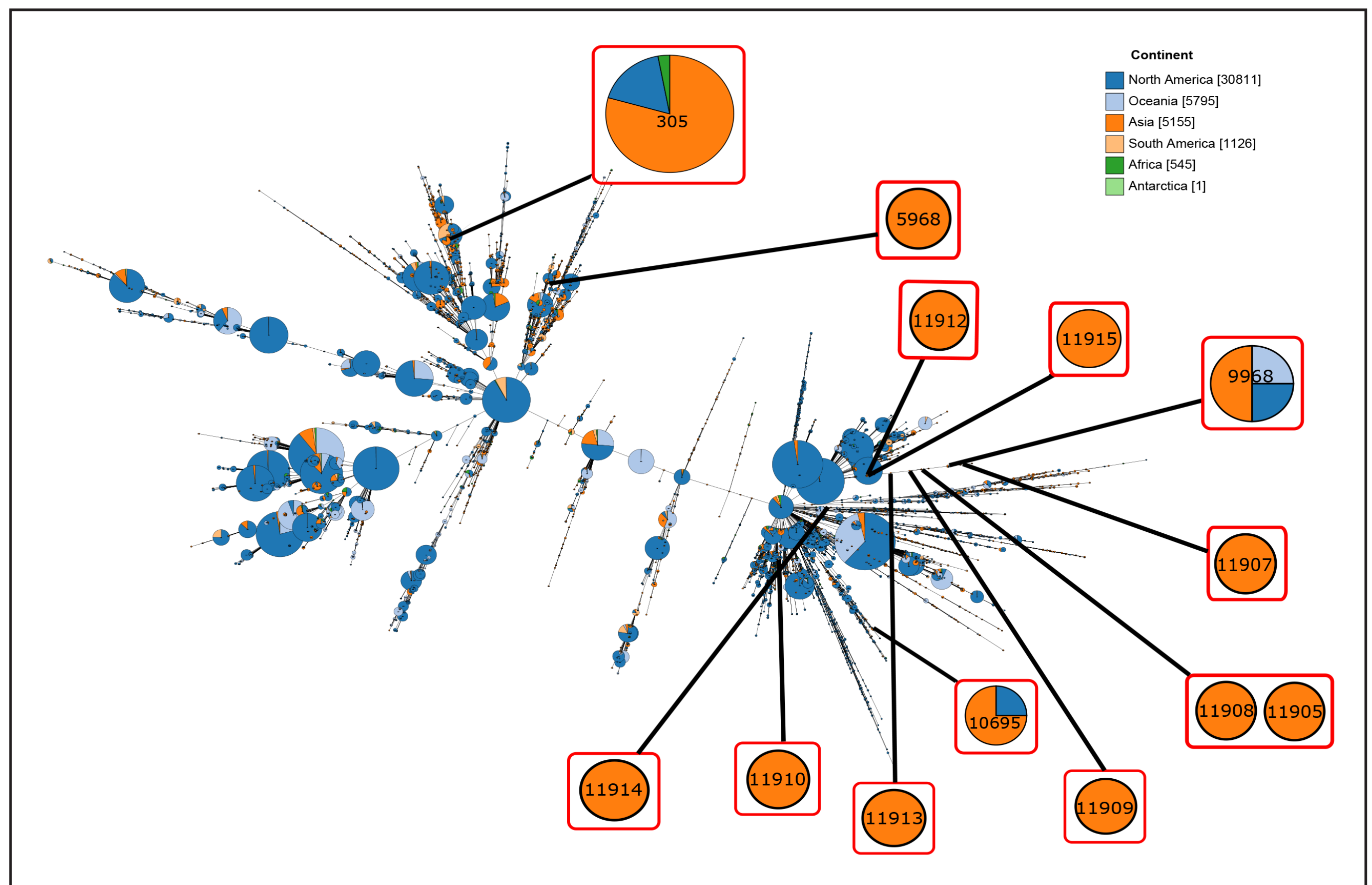

Figure 1. A minimum spanning tree visualizing the distribution of the STs in this study relative to other STs in the *C. jejuni*/*C. coli* PubMLST database based on their geographical location or continent of isolation. The tree was constructed using the MSTree V2 algorithm implemented in GrapeTree. The size of the clusters is proportional to the number of STs included in the cluster.

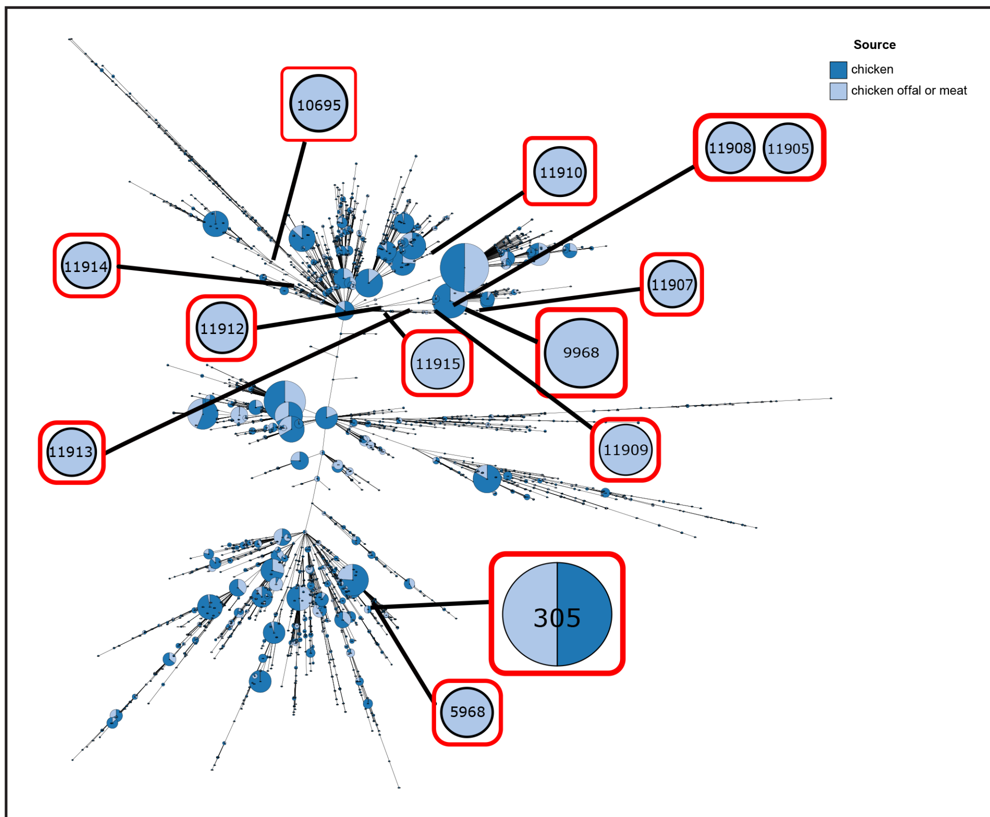

Figure 2. A minimum spanning tree visualizing the distribution of the STs in this study relative to other STs in the *C. jejuni/C. coli* PubMLST database based on whether they were isolated from a live animal source, specifically chicken, or from chicken offal or meat. The tree was constructed using the MSTree V2 algorithm in GrapeTree based on the sequences available in the MLST database. The size of the clusters is proportional to the number of STs included in the cluster.

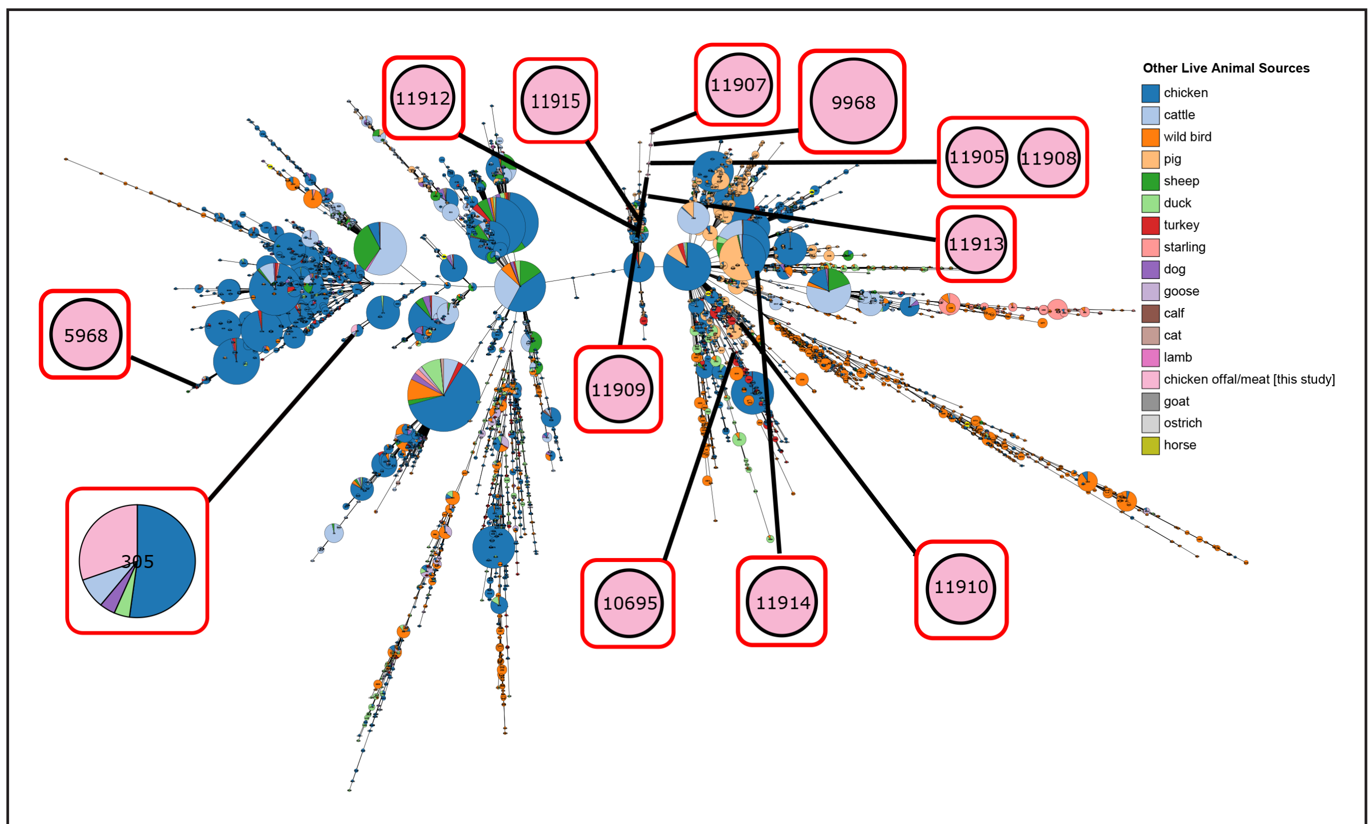

Figure 3. A minimum spanning tree visualizing the distribution of the STs in this study relative to other STs in the *C. jejuni/C. coli* PubMLST database based on the live animal from which they were isolated. The tree was constructed using the MSTree V2 algorithm in GrapeTree based on the sequences available in the MLST database. The size of the clusters is proportional to the number of STs included in the cluster.

Table 2. Virulence gene profiling of the *C. jejuni* and *C. coli* isolates

Isolate	Virulence Genes									
	ciaB	*dnaJ*	*pldA*	*racR*	*cadF*	*cdtA*	*cdtB*	*cdtC*	*ceuE*	*flaA*
CJ01	+	+	+	+	+	+	+	+	+	+
CJ02	+	+	+	+	+	+	+	+	+	+
CJ03	+	+	+	+	+	+	+	+	+	+
CJ04	+	+	+	+	+	+	+	+	+	+
CJ05	+	+	+	+	+	+	+	+	+	+
CJ06	–	–	–	–	+	–	–	–	–	–
CJ07	–	–	–	–	+	–	–	–	–	+
CJ08	+	+	+	+	+	+	+	+	+	+
CJ09	+	–	+	+	+	+	+	+	+	+
CJ10	–	–	–	–	+	–	–	–	–	+
CJ11	+	+	+	+	+	+	+	+	+	+
CJ12	+	+	+	+	+	+	+	+	+	+
CJ13	+	+	+	+	+	+	+	+	+	+
CJ14	–	+	+	+	+	+	+	+	+	+
CJ15	–	+	+	+	+	+	+	+	+	+
CJ16	–	+	–	+	+	+	+	+	+	+
CJ17	–	+	–	+	+	+	+	+	+	+
CJ18	+	–	+	+	+	+	+	+	+	+
CJ19	–	+	–	+	+	+	+	+	–	+
CJ20	–	+	–	+	+	+	+	+	–	+
Prevalence	55%	75%	65%	85%	100%	85%	85%	85%	75%	95%

those in the database, based on whether they were isolated from live chickens or chicken offal or meat. Among the analyzed sequence types, only ST-305 is observed in both live chickens and chicken offal or meat. In contrast, the non-novel sequence types (ST-10695, ST-9968, ST-5968) appear to be exclusively associated with chicken offal or meat. Figure 3, on the other hand, shows the distribution of the sequence types considering other sources of live animals. Only ST-305 is shared with other live animal sources, including cattle, dog, and duck.

Virulence Gene Profiling

The results of the virulence gene profiling are summarized in Table 2. Among the analyzed genes, the *cadF* gene displayed the highest prevalence, being detected in all the isolates (100%). Following *cadF*, the *flaA* gene was present in 95% of the isolates. The *racR*, *cdtA*, *cdtB*, and *cdtC* genes showed a relatively high prevalence, being identified in 85% of the isolates. The *dnaJ* and *ceuE* genes were the fourth most prevalent, observed in 75% of the isolates. The *pldA* gene exhibited a prevalence rate of 65%. In contrast, the *ciaB* gene had the lowest prevalence among the isolates, being detected in only 55% of the samples.

DISCUSSION

Chicken and associated products play a vital role in global food consumption, providing a significant source of animal protein for many populations. However, they are also recognized as potential sources of foodborne infections, with campylobacteriosis being one of the most common bacterial illnesses associated with chicken consumption. *Campylobacter*, particularly *Campylobacter jejuni* and *Campylobacter coli*, are commonly found in the intestinal tracts of poultry, including chickens. Contamination of chicken and its products can occur during various stages of production, processing, and preparation. This can include the presence of *Campylobacter* in live birds, cross-contamination during slaughter, improper storage and handling, and inadequate cooking practices (WHO, 2020).

This study contributes significant insights into the genetic diversity of *C. jejuni* and *C. coli* isolates obtained from chicken offal in Metro Manila, Philippines. The identification of 13 distinct sequence types (STs) and three clonal complexes within a limited sample size (n=20) highlights the substantial genetic variability present within *Campylobacter* species, in line with previous research findings (Colles *et al*., 2003; Dingle *et al*., 2001). These results underscore the importance of investigating the genetic diversity of *Campylobacter* populations, as it provides valuable information for understanding their epidemiology, transmission dynamics, and potential public health implications in the context of foodborne illness.

The predominant ST-305, identified in this study, has been previously isolated from poultry in Japan (Asakura *et al*., 2012) and broiler carcasses in Belgium (Elhadidy *et al*., 2018). The presence of ST-305 in both live chicken sources and chicken offal or meat highlights its zoonotic potential, indicating the risk of *Campylobacter* transmission through the consumption of animal food products. Furthermore, ST-305 has been detected in other live animal sources such as cattle and duck, including humans, suggesting its ability to colonize and persist in multiple hosts (Figure 3). This broad host range could be due to different animals coming into contact with the bacteria through their surroundings, food, or interactions with other animals, leading to its presence in different species. In agricultural and farming settings, animals may also share common spaces, equipment, or handlers, which can lead to cross-contamination and increase the likelihood of disease emergence in new hosts (Griekspoor *et al*., 2013).

The second most prevalent sequence type, ST-9968, was previously identified in a human isolate from a patient in Western Australia who had traveled to Southeast Asia (Varrone, 2019). While ST-5968 itself lacks published references, its clonal complex ST-443 has been reported in chicken-derived isolates (Ohishi *et al*., 2017). These findings suggest potential links between ST-9968 and human infection, highlighting the importance of monitoring and understanding the genetic characteristics of *Campylobacter* strains circulating in both human and animal populations. The presence of ST-443 in chicken-derived isolates further emphasizes the role of poultry as a potential reservoir for this clonal complex and its potential implications for food safety.

ST-10695, which is associated with *C. coli*, lacks specific references, but its clonal complex, the agriculture-associated ST-1150 complex, has been implicated in the progressive accumulation

of *C. jejuni* DNA. The ST-1150 complex has demonstrated substantial introgression, resulting in the replacement of the *C. coli* core genome and acquisition of novel DNA (Sheppard *et al*., 2013). This finding helps elucidate why isolate CJ10, belonging to the ST-1150 complex, was initially identified as *C. jejuni*. The remaining five *C. coli* isolates in this study do not belong to any existing clonal complexes, suggesting the presence of distinct genetic lineages or potential emergence of novel clonal complexes in *C. coli* populations.

Six isolates deposited in the PubMLST database were reassigned to *C. coli* by the database curator as they were found to contain *C. coli* alleles. This coincides with the work by Dingle *et al*. (2005) where a *C. jejuni* isolate was reported to have a *C. coli* sequence, a clear indication that interspecies recombination occurs within the housekeeping genes of *C. jejuni*. Expanding the analysis to include a larger number of isolates from a specific species in the primer target region would aid in the development of primer sets that are more reliable and precise. Furthermore, exploring the use of different genes that are not easily influenced by recombination and that can effectively differentiate between the closely related species *C. coli* and *C. jejuni* may potentially improve existing typing schemes (Klena *et al*., 2004). Additionally, based on the evaluation by Burnett *et al*. (2002), 16S rRNA sequence-dependent assays may not be the best approach to differentiate these closely related species. Future work may also benefit from the use of an additional assay to confirm the identity of *Campylobacter* isolates prior to conducting MLST.

The limited sample size in this study restricts conclusive findings on the host specificity and geographic distribution of ST-5968, ST-9968, and ST-10695, which are seemingly exclusive to chicken offal (Figure 2). This observation, however, can be due to several factors, including the absence of an active immune system and differences in the microenvironment, which create an environment in the chicken offal that is conducive to bacterial proliferation, allowing bacteria from the surrounding environment, including potential contamination from food handlers. Furthermore, disparities in sampling and detection techniques may also have a role, since some bacteria may persist at low levels or within specific niches in live animals, making them challenging to detect using conventional methods, in contrast to post-mortem samples like chicken offal. Additional research with a larger sample size is necessary to investigate the prevalence and distribution of these sequence types across different hosts and geographical regions. Such studies would provide more comprehensive insights into the host adaptation and epidemiology of *Campylobacter* strains associated with chicken offal.

The virulence gene profiling in this study revealed notable patterns. The *cadF* gene, which encodes the outer membrane protein CadF involved in host cell binding, was found in all isolates, indicating its widespread presence among *Campylobacter* strains. This high prevalence of the *cadF* gene aligns with previous studies that have demonstrated its conservation in both *C. jejuni* and *C. coli*. Previous research conducted by Konkel *et al*. (1999) has also reported the ubiquity of the *cadF* gene, further supporting the findings of this study.

The *flaA* gene, responsible for encoding flagellin and playing a pivotal role in motility, colonization, and pathogenesis (Merchant-Patel *et al*., 2010) was detected in 95% of the isolates, except for one *C. coli* isolate. Notably, the virulence genes *racR*, *cdtA*, *cdtB*, and *cdtC* were found in 85% of the isolates, highlighting their involvement in adherence, colonization, and toxin production (Apel *et al*., 2012). Previous research has linked these genes to the production of the cytolethal distending toxin (CDT), which has been implicated in *Campylobacter*-induced diarrhea (Talukder *et al*., 2008). The high prevalence of these genes suggests their potential significance in the pathogenicity of *Campylobacter* strains.

The *dnaJ* gene, known for its role in *C. jejuni* thermotolerance and colonization, was present in 75% of the isolates, while the *ceuE* gene, responsible for iron acquisition, was detected in the same percentage. Additionally, the *pldA* gene, associated with adhesion, was found in 65% of the isolates. Previous studies have demonstrated that mutations in these genes can impair the colonization ability of *Campylobacter* strains in chickens (Hermans *et al*., 2011). On the other hand, the *Campylobacter* invasion antigen B (*ciaB*) gene, crucial for epithelial cell invasion, was detected in 55% of the isolates. The presence of these genes suggests their potential contribution to the virulence and pathogenicity of the *Campylobacter* isolates.

The identification of these virulence genes in *Campylobacter* isolates has important practical implications, particularly in the context of food safety and human health. The presence of these genes can be utilized in the detection and surveillance of *Campylobacter* in food products, enabling timely interventions to minimize the risk of contamination and subsequent human infection. Additionally, the knowledge of colonization mechanisms in avian intestines, facilitated by the understanding of these genes, can inform the development of targeted strategies for the prevention and control of *Campylobacter* transmission, both in poultry production and in public health settings (Hansson *et al*., 2018). This can contribute to reducing the burden of campylobacteriosis, a significant public health concern worldwide (Igwaran & Okohab, 2019).

In summary, this study significantly contributes to our understanding of the genetic diversity and virulence gene profiles of *Campylobacter* isolates from chicken offal in Metro Manila, Philippines. The identification of zoonotic potential and the adaptability of *Campylobacter* strains across different hosts underscores the need for comprehensive control and prevention strategies. However, due to the limitations of the small sample size and localized scope, further research incorporating larger sample sizes and broader geographic representation is necessary to strengthen our knowledge of *Campylobacter* epidemiology and its impact on public health (Igwaran & Okohab, 2019). Such studies will help inform targeted interventions and measures to mitigate the risks associated with *Campylobacter* contamination in poultry products and prevent campylobacteriosis outbreaks.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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