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ISOLATION AND MOLECULAR CHARACTERIZATION OF *Campylobacter jejuni* FROM LOCAL BROILER CHICKEN (LBC) AND FROZEN IMPORTED CHICKENS (IFC) IN DUHOK PROVINCE, KURDISTAN REGION- IRAQ

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ABSTRACT:

Campylobacter jejuni is one of the major foods borne pathogen that cause diarrhea in human. Consumption of undercooked poultry meats and its by-products is believed to be the main source of human campylobacteriosis. This study aims to determine the prevalence of this bacteria in LBC and IFC using conventional culture methods and conventional PCR assay, with molecular identification by sequencing of 16S rRNA. Two hundred and twenty-five samples from LBC (n=150) and IFC (n=75) were collected. Conventionally, overall, 21.3% (n = 48) were identified as *C. jejuni*. In LBC 30% (45/150), from this 9% and 72% identifies as *C. jejuni* when used enrichment and direct isolation method, respectively. Generally, the PCR confirmed 53.3% from LBC and 66.6% from IFC to be *C. jejuni*. All isolates were strongly resistant to Tetracycline, Ciprofloxacin, Nalidixic acid, and Oxytetracycline, while susceptible to Erythromycin and Gentamycin. Therefore, they were considered as Multi drug resistant strains. Based on the 16S rRNA sequencing, LBC and IFC were similar and genetically related to each other. Comparing with respective databases in NCBI, these isolates had the variation in their closeness with different strains from other countries isolated from different sources. Further study recommended, related to the virulence genes, physio-morphological characteristic, and whole genome sequencing of some isolated strains.

KEYWORDS: *C. jejuni*, *hipo*, 16S rRNA, antibiotics resistant, phylogenetic tree.

1. INTRODUCTION:

Members of the *Campylobacter* genus are gram-negative, mainly spiral-shaped, motile bacteria that grow in microaerophilic conditions (Quinn et al., 2011). Human Campylobacteriosis is the most common bacterial food-borne disease, characterized by diarrhea, fever, and abdominal cramps (Facciolà et al., 2017). *Campylobacters* can colonize the digestive tract of poultry and other farm animals as commensal and symptomless bacteria. During meat and food chain processes, they contaminate the meat and food materials (Kaakoush et al., 2015). Poultry, especially broiler chicken, has the primary role in Campylobacteriosis, and significant cases of the disease occur when dealing with raw poultry meat (Mughini Gras et al., 2012).

Campylobacteriosis is mainly caused by *C. jejuni* approximately (80- 90%) and *Campylobacter coli* (*C. coli*) (8-10%) and, at fewer rates, by *Campylobacter lari* and another *Campylobacter* species (Michael P. Doyle, 2013). *C. jejuni* is a fastidious growth bacterium that can quickly lose viability and culturability (Ghaffar et al., 2015). It needs microaerobic conditions for growth (the concentration of O_2 must be reduced to around 5%, and the concentration of CO_2 must increase to around 10%. It can grow at a temperature range of 30 to 45°C, though its optimum growth is 42°C, so it is arranged as a thermophilic *Campylobacter*. It has many selective growth media that mainly depend on composition that reduces the harmful effects of oxygen and antibiotic supplements, enhancing their chance of competition (Quinn et al., 2011). The selective medium, modified charcoal cefoperazone deoxycholate agar (mCCDA), which contains cefoperazone and amphotericin B, is perhaps the most frequently employed to isolate *C. jejuni* and *C. coli* (Griffiths, 1993). The increase of antibiotic-resistant Campylobacters worrying worldwide. The studies in the Middle East show that many Campylobacter isolates are highly resistant to quinolones and tetracycline (Mouftah et al., 2021). Increased antibiotic-resistance Campylobacter has usually been related to the excessive use of antibiotics in farm fields (Abukhattab et al., 2022; McCrackin et al., 2016).

Broiler meat is one of the familiar sources of nutrition protein in Duhok province; while people previously mainly depended on imported frozen chicken but there is a great change in inclination noticeable toward local raw broiler meat. This study aimed to open an eyelet for a better understanding of the epidemiology and antimicrobial resistance of Campylobacter in Duhok region through isolation and identification of *C. jejuni* from chickens in local traditional small poultry abattoirs and imported frozen chickens, molecular identification of isolated bacteria comparing with the standard references' strains and antibacterial sensitivity test examination.

2. MATERIALS AND METHODS:

2.1 Samples Collection:

The current study was performed from December 2022 to July 2023. Cloacal swabs were used to collect samples from one hundred and fifty broiler chickens in ten different traditional poultry abattoirs. Sterile transport swabs (Nacoxm, China) were used; the sheath of the swabs had previously been fielded aseptically with 5 ml of Buffered Peptone Water (BPW) (Himedia, India). Also, seventy-five whole frozen chickens were collected from diverse markets and supermarkets in the Duhok Region. All samples were brought to the Sumeil Veterinary Center's laboratory within two hours for primary isolation of Campylobacter.

2.2 Isolation of C. jejuni and bacteriological identification

Campylobacter was isolated using both direct and indirect methods. Hundred swabs were processed indirectly from the swabs that were taken. Briefly, one ml of BPW that is used as transport media and added to a capping vial contains 9 ml of Modified Bolton Broth (MBB), composed of Bolton broth base (Himedia, India) with CCDA selective supplements

(amphotericin B 5.0 mg and cefoperazone 16.0 mg) (Oxoid, UK) without blood. The caps of each vial were loosened and incubated in the Gas Jar (GJ) (Oxoid, UK) under microaerophilic conditions generated by a Campygen microaerophilic Gas Pack (GP) (2.5 liters) (Oxoid, UK) at 37 °C for 4 hours, followed by an extended 20 hours at 42 °C. A loopful of each enriched sample was streaked on mCCDA plates composed of *Campylobacter* blood-free selective agar base CM0739 and CCDA selective supplement(contains cefoperazone and amphotericin B) SR0155 (Oxoid, UK) agar plates, which were then put in an incubator at 42°C under microaerophilic conditions generated in the same way for 48 hours. Fifty swabs were directly streaked on mCCDA plates and incubated in microaerophilic conditions generated using gas jar (GJ) and gas pack (GP) at 42 °C for 48 hours.

The indirect method was used for imported frozen chicken (IFC). Briefly, in the laboratory with an aseptic scalpel and forceps, each chicken was removed from its original case and placed in clean, zipped food bags. 200 ml of BPW was added to each case and shaken by hands for 3 minutes; the chicken was removed, and the remaining fluid in the case was brought for examination. Three milliliter of each sample was put into a sterile glass vial that held 17 ml of MBB enrichment medium were incubated for 48 hours in microaerobic conditions generated as previously described at 42°C. A loopful of enriched media was streaked on mCCDA and incubated under the same conditions for 48 hours.

For both types of samples, mCCDA Plates that appear to have had weak growth and have undefinable colonies were sub-cultured on another mCCD agar plate by taking a loop in a cross line from the center of the growth area. One suspected separate colony is taken from each plate with growing bacteria, spreads on a Blood Agar (BA) composed of blood agar base (Liofilchem, Italy) with 10% defibrinated sheep blood plate and left to grow for 48 hours in the same conditions previously described.

Campylobacter is identified primary based on colony morphology and bacterial morphology under microscopy. Gram stain (Atom Scientific, UK) used and prepared slides were air dried and examined under light microscope at 100× oil immersion magnification. Small, Gram negative, pink colored, curved cells indicated that the isolate was likely to be *Campylobacter*. Slide based Motility test was conducted to identify the typical movement of *Campylobacter* under the light microscope. Isolated samples were stored at -20°C in brain-heart infusion broth with 15% glycerol for further testing (Gorman et al., 2004).

2.3 DNA Extraction

DNA was extracted from the bacteriologically confirmed isolates taken from fresh culture on BA agar plates. The isolation of DNA was done by using the boiling method with some modifications(Ahmed et al., 2017), briefly collected colonies suspended in 1% TAE buffer, and heated at 95°C in a heat block (company, country) for 20 min. The samples were then centrifuged at 10,000 rpm for 10 min. The supernatants are used as DNA templates for PCR. Nanodrop (Thermofisher, UK) was used to assess the quantity and quality of DNA.

2.4 Molecular Detection

DNA was extracted from all the bacterial isolates for molecular detection and identification of *C. jejuni* using polymerase chain reaction (PCR) with the indicated primers from *hipo* Species-specific gene (Table1).

The PCR reaction was made by 10 µl master mix (JenaBiosciences, Germany), 1µl of each primer (10 pmol/µl), 2µl of extracted DNA (50 ng/µl), and 6 µl of PCR-grade water. The following conditions were used to amplify PCR using (Applied Biosystem, UK) thermocycler: An initial denaturation step at 95°C for 5 minutes was followed by 35 cycles of amplification (denaturation at 95°C for 0.5 minute, annealing at 53°C for 0.5 minute, and extension at 72°C for 0.5 minute), followed by final extension at 72°C for 7 minutes.

Table 1: List of primers used in this study.

Primer Name	Gene	Sequence (5'-3')	Amplicon Size (bp)	References
515f	16S rRNA	GTGCCAGCMGCCGCGGTAA	*~ 250-300	(Boelsen et al., 2019)
806r		GGACTACHVGGGTWTCTAAT		
Hipo	*hipO* gene	ACTTCTTTATTGCTTGCTGC	323	(Kim et al., 2008)
		GCCACAACAAGTAAAGAAGC		

*Exact product size different between bacterial species

2.5 Amplification of 16S rRNA

Conventional PCR was also carried out for identification of *C. jejuni* using 16S rRNA, DNA extracts from 12 confirmed *C. jejuni* isolates from both sources and 8 non-*Campylobacter* competitive bacteria from IFC were checked; primers for the 16S rRNA ribosomal RNA were used as shown in (Table1).

The PCR reaction solution was prepared from 20µl master mix (JenaBiosciences, Germany), 2.5 µl from each primer (10pmol/µl), about 4µl of DNA extraction (50ng/µl), and PCR grade water, was added to make it 40µl. The amplification condition was as follows: Initial denaturation at 94°C for 5 minutes, then 35 cycles of amplification (denaturation at 94°C for 45 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 90 seconds), followed by a final extension at 72°C for 10 minutes. (Applied Biosystem, UK) thermocycler.

2.6 Agarose gel electrophoresis

DNA was routinely analyzed by subjecting PCR products for electrophoresis in 1.5% (w/v) agarose gel using 1X Tris-acetate, EDTA buffer (1× TAE) with nucleic acid dye, Safe dye (JenaBiosciences, Germany). Five microliters of each PCR product were loaded into each well with loading of 7 µl of 100bp DNA ladder (Jena Bioscience, Germany). The nucleic acid fragments sizes were viewed by using UV transilluminators (Fisher scientific, USA).

2.7 Sequencing of 16S rRNA and Phylogenic tree construction

To determine the similarity between the isolates from different niches, 17 isolates from LBC and three from IFC were amplified using primers targeting the conserved region of the 16S rRNA gene for identification of the *C. jejuni*. The PCR products were purified and sequenced. These samples were sequenced using sanger sequencing (Macrogen, South Korea). Chromatogram

files were received, and the quality of sequences were evaluated, bad quality sequences were trimmed out using Bioedit software. A BLAST search was performed, and the sequences were compared to known Campylobacter sequences in the GenBank. Final sequences were deposited to NCBI/GenBank, and accession numbers were received (Table 2). Multiple alignments were done with the Clustal-W method (Thompson et al., 1994), and MEGA XI was used to make a phylogenetic analysis with Neighbor-Joining. The bootstrap measures were determined from 1000 repeats of the original data (Kumar et al., 2018).

Table 2: Accession Number and isolation sources of submitted *C. Jejuni* isolates of this study.

No.	Isolation Source	Sample ID	Accession Numbers
1.	Whole frozen chicken	NC1	OP412479
2.	Whole frozen chicken	NC2	OP412445
3.	Whole frozen chicken	NC11	OP412446
4.	Chicken faecal sample	NF11	OP415426
5.	Chicken faecal sample	NF10	OP415425
6.	Chicken faecal sample	NF9	OP415387
7.	Chicken faecal sample	NF8	OP415415
8.	Chicken faecal sample	NF7	OP415417
9.	Chicken faecal sample	NF6	OP415406
10.	Chicken faecal sample	NF5	OP415398
11.	Chicken faecal sample	NF4	OP415388
12.	Chicken faecal sample	NF3	OP415394
13.	Chicken faecal sample	NF2	OP415396
14.	Chicken faecal sample	NF1	OP415411

2.8 Antibiotic Sensitivity testing (AST)

Antibiotic sensitivity test (AST) was performed using the desk diffusion method in accordance with the CLSI-M45 and BSAC Methods for Antimicrobial Susceptibility Testing Protocols (CLSI, 2015; Howe et al., 2012). The bacteria recovered from glycerol broth storage on BA were suspended in Bolton base broth to obtain the inoculated broth having suitable turbidity approximately (0.5 McFarland standard), which was streaked on Mueller Hinton Agar (MHA) plates composed from Mueller Hinton Agar I (Neogen, USA) with 5% defibrinated sheep blood agar plates and was incubated under microaerophilic condition for 48 hours. The following antibiotics desks were used : Nalidixic acid (NA) 30 µg, Oxytetracycline (OT) 30 µg, Erythromycin (E) 15 µg, Gentamycin (CN) 30 µg, Ciprofloxacin (CIP) 5 µg and Tetracycline (TE) 30 µg.

3. RESULTS:

Campylobacters were identified in 30% (45/150) of the samples collected from local broiler chicken from which 9% (9/100) were isolated using enrichment method, while 72% (36/50) were isolated using direct method without enrichment. In line with this, 75 frozen imported chickens from Brazil (30) and Turkey (45) were sampled and tested for *C. jejuni* isolation. *C. jejuni* isolated only in three imported chickens from Turkey (4/75) (Table2). The isolation of *C. jejuni* was first based on colonies morphology, Gram stain and motility test, then these samples were subjected to PCR for further confirmation using species specific gene for *C. jejuni* detection. Molecular detection by PCR showed that 24 of the isolates from LBC (4 from enrichment and 20 from direct method) and 2 of the isolates from IFC-Turkey were confirmed to be *C. jejuni* (Table 3 and Figure 2).

Table 3: Frquency and percentage of isolated and confirmed isolates of *C. jejuni* from different isolation sources.

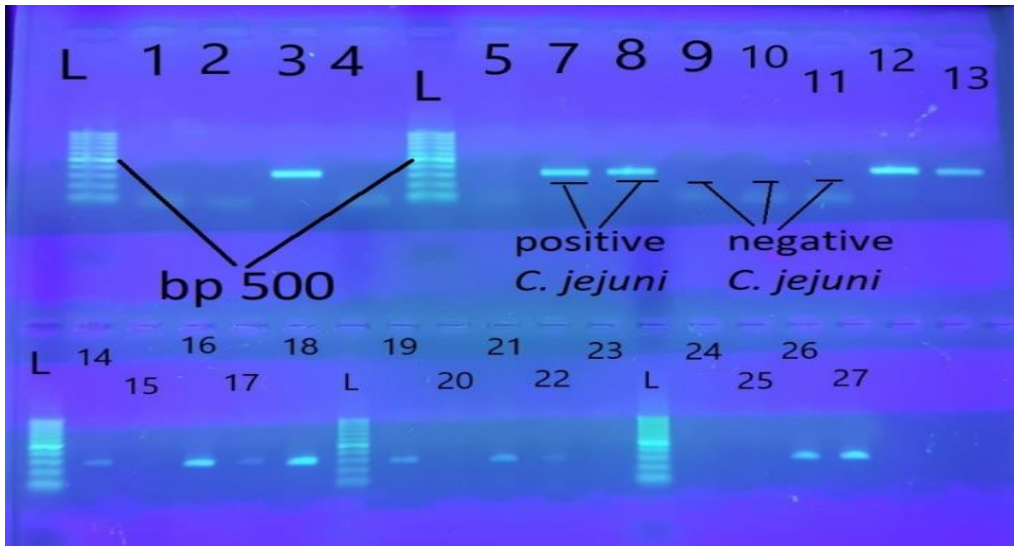| Sample type		No. of samples		% of *C. jejuni* confirmed phenotypically		% of Confirmed *C. jejuni* by PCR		
				No.	%	No.	% Per phenotypically confirmed isolates	Overall %
LBC	Enrichment method	150	100	9	9%	4	44.44%	4.00%
	Direct method		50	36	72%	20	55.56%	40.00%
IFC	From Turkey	75	45	3	7%	2	66.67%	4.44%
	From Brazil		30	0	0%	0	0.00%	0.00%

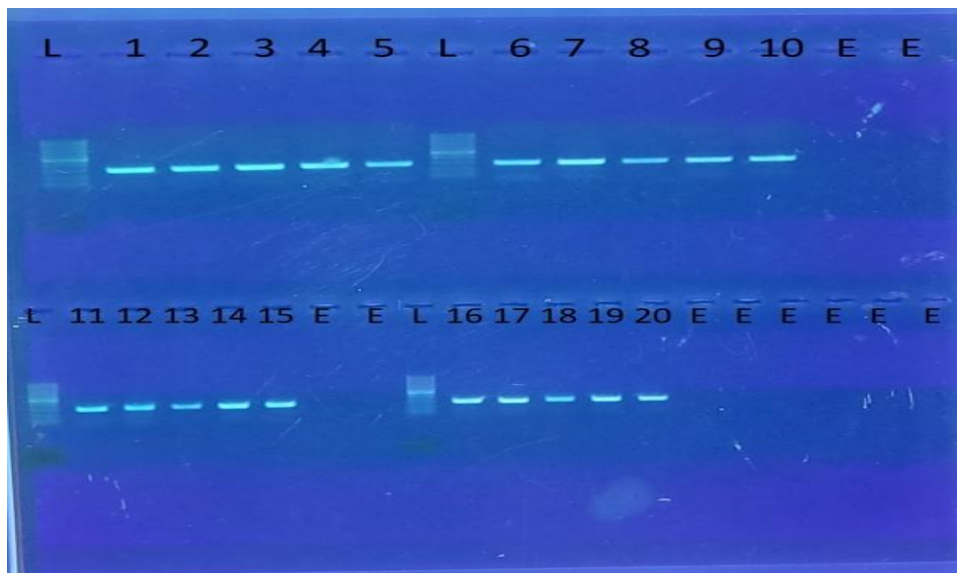Figure 1: PCR amplification of *hipo* gene for detection of *C. jejuni*, PCR product 323pb

Figure 2: PCR amplification of 16S rRNA (250-300pb), L= DNA ladder (100bp) E = Negative controls. Product size is between 250-300bp.
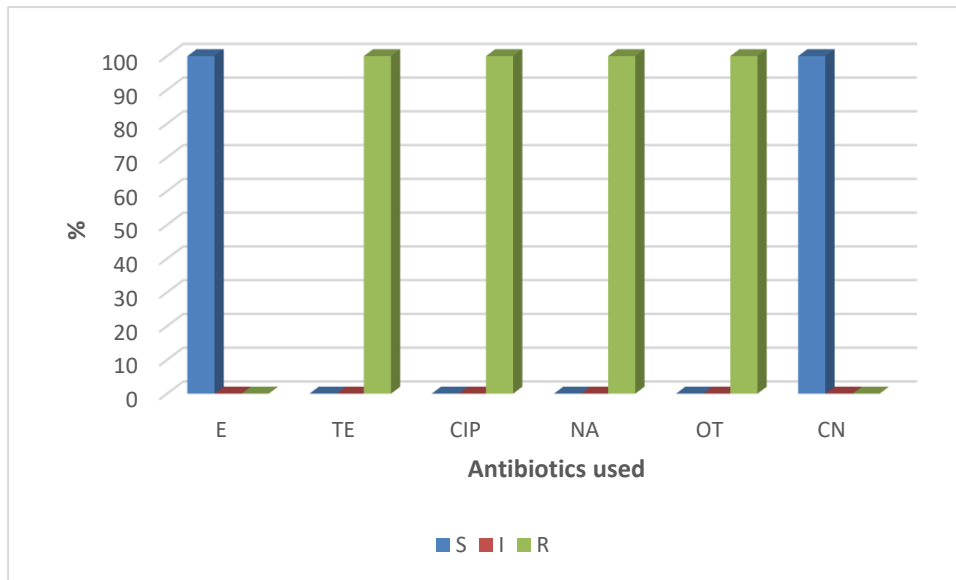
Figure 3: Antibiotics resistance percentages of the tested isolates against 6 different antibiotics.
(E, Erythromycin; TE, Tetracycline CIP, Ciprofloxacin; NA, Nalidixic acid; OT, Oxytetracycline; and CN, Gentamycin).
(S=Sensitive; I=Intermediate and R= Resistant)

Figure 4: Antibiotic sensitvity test on MHA suplenmted with blood.

AST was performed on 11 recovered isolates: 9 LBC and 2 FIC. All 11 samples (100%) from both origins were strongly resistant to TE, CIP, NA, and OT and in many cases did not have an inhibition zone. All (100%) of the 11 samples were susceptible to E and G (Figure 3, 4). These isolates were considered as multi-drug resistant strains based on the definition provided by Magiorakos et al.(2011).

The phylogenetic tree were constructed, based on the partial sequences of 16S rRNA, two trees were constructed as shown in Figure (5, A and B). The first phylogenetic tree (A) was made by using the sequences of the LBC and IFC isolates from this study. This tree was divided into two main clusters. The majority of LBC isolated were grouped under one common ancestor and are gentically related to each other. However, IFC isolates were grouped in the second ancestor with two isolates from LBC (OP415417 and OP415396).This is confirmed that some LBC and IFC-Turky isolates have a genetic relationship with each other.

The partial 16S rRNA gene sequences from different isolation sources have been compared in phylogenetic tree (B) with 12 sequences of corresponding *C. jenuni* isolates that were published in GenBank. These sequences were selected based on the isolation sources and geographical areas. This tree shows that these sequences were distinctly divided into five clusters. In the first four clusters, all the study isolates including those from IFC were grouped together with other isolates from different countries, for example, the study isolate OP415411 NF1-LBC, is more genetically related to the isolates from CP012242-Finland, OP090654- Croatia. CP030866-Chicken- from South Koreahas more relation with the present study isolates OP415417-NF7-LBC. Whereas in the second cluster, two of the isolates OP415406 NF6-LBC and OP415426 NF11-LBC are genetically related to MN736598-Chicken feces from Italy and grouped in the same cluster with the isolates from other countries.
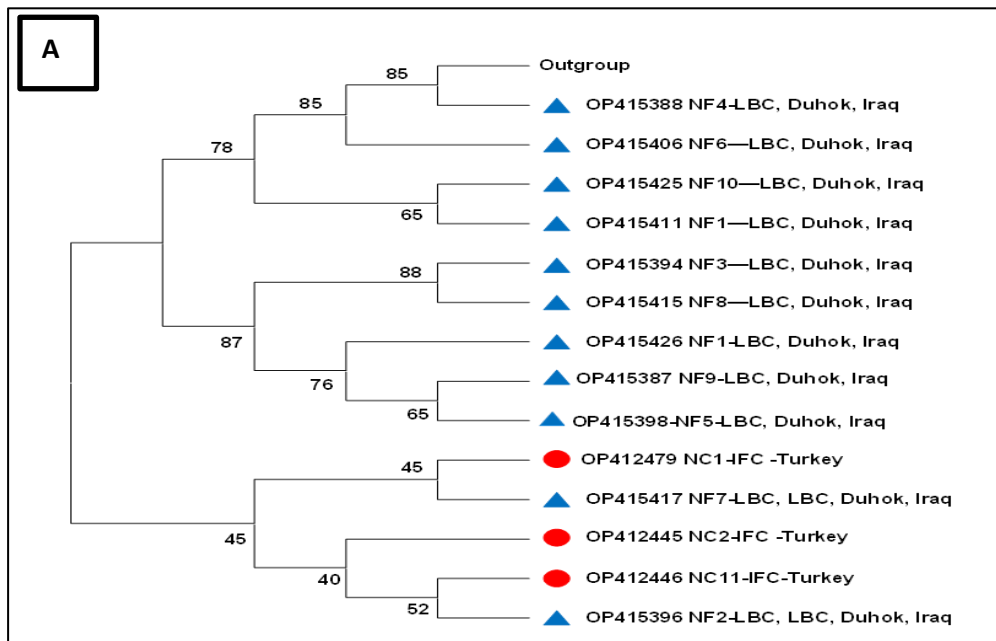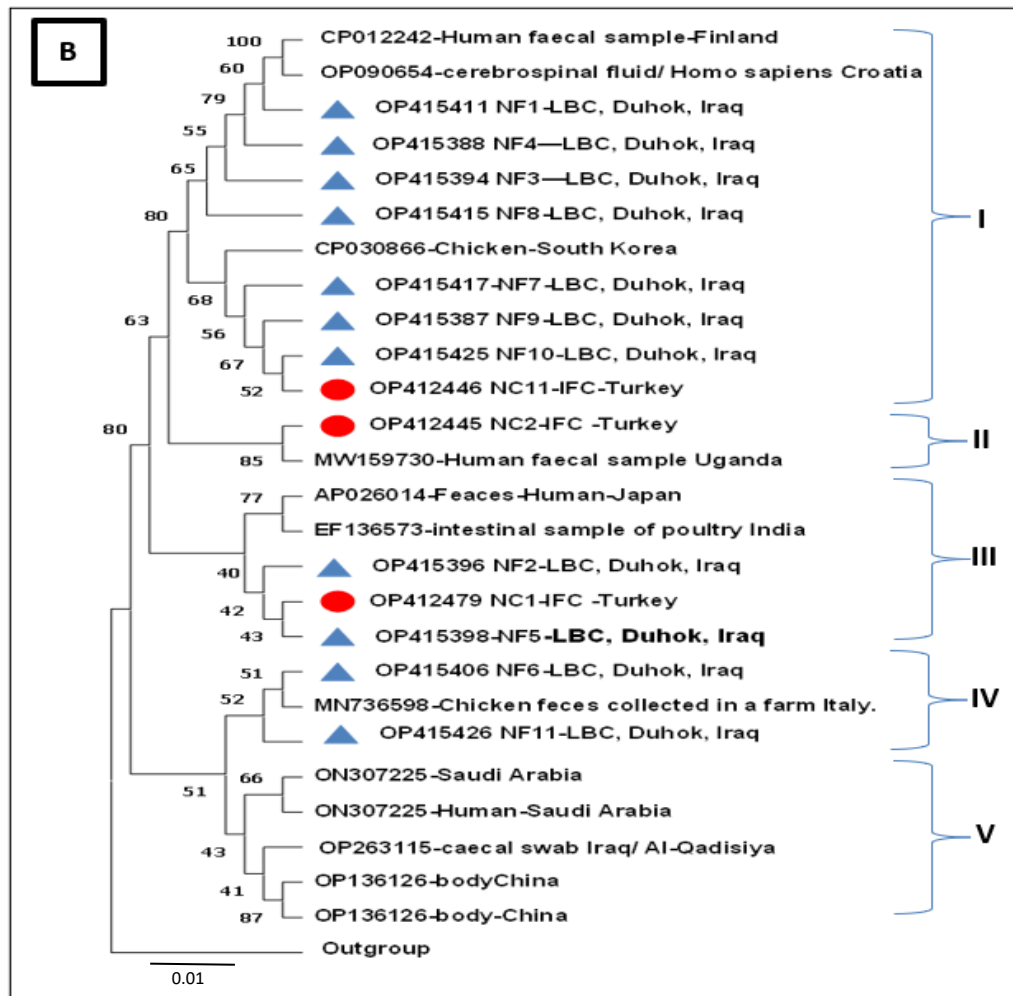
Figure 5. 16S rRNA-based phylogenetic tree of *C. jejuni* isolates from IFC and LBC (A) using 14 partial sequences from this study while tree (B) constructed to compare these isolates with other 12 respective sequences available in Database.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA11.4.

4. DISCUSSION:

Broiler chicken is considered the major source of Campylobacteriosis (Newell et al., 2017). However, molecular methods, particularly multiplex PCR, have become more simple and time-efficient, but the culture method is still the primary step for bacterial isolation and demonstration of the further evolution of epidemiology and virulence disease factors (Ricke et al., 2019). *Campylobacter* isolation rates can vary greatly depending on geographical distribution, season, and the methods of sampling and isolation (Kaakoush et al., 2015). In this study, both direct and indirect (enrichment) methods were applied for the

isolation of Campylobacter from swabs taken from LBC. Only 9% (9/100) of the sample taken from LBC through enrichment methods was identified as Campylobacter. Divsalar *et al.*, (2019) obtained a higher level of isolation by the enrichment method using Preston broth and Colombia agar in Iran. The isolation rate in 35-day-old broiler chickens was 30% (15/50) in Jordan using Bolton broth and Preston agar (Neves et al., 2018). The low rate of indirect isolation in the present study could be attributed to improper selective supplements in enrichment broth, which provide the chance for competitive bacteria to grow and adapt to the growing conditions. The rate of *Campylobacter jejuni* isolation is significantly higher in direct methods compared to enrichment methods (72% versus 9%). Previous research demonstrated that a cloacal swab and direct streaking on mCCDA could be used to obtain a higher rate of *Campylobacter* isolates (Urdaneta et al., 2015; Vaz et al., 2014). In this study, 72% (36/50) of the samples taken from LBC by direct methods had distinctive *Campylobacter* growth and appearance. The result is very similar to the result of Vaz et al. (2014) who demonstrated 72.2% (26/36) of *Campylobacter* by the use of the same direct method in Brazil, but quite different from the results of a study done in Iraq by Shakir et al. (2021) who found that the isolation rate from 150 cloacal swabs and ceca from broiler farms and chicken in live chicken markets was 24% by using a direct method on *Campylobacter* agar base with selective supplements.

In this study, from isolated *Campylobacter*, 55.56% (20/36) were confirmed as *C. jejuni* by PCR, whereas Neogi et al. (2020) found that the *C. jejuni* ratio from *Campylobacter ssp.* isolated from broiler chicken was 65% (24/37) in Bangladesh. Staji et al. (2018) detected 46.8% *C. jejuni* by using PCR from 64 isolates that were biochemically identified as *Campylobacter* species from broiler fecal samples. In the current study, from IFC, only 3 out of 75 (4%) were characterized as *Campylobacter*, and only 2 confirmed cases of *C. jejuni* were obtained. It has been proven that freezing clearly decreases the viability and capturability of *Campylobacter* (Georgsson et al., 2006; Oh et al., 2019). Industrial rapid freezing and long-term storage of broiler meat can significantly reduce the detectable *Campylobacter* percentage, even to zero (Yu et al., 2021). This information indicates the low level of detection of *Campylobacter* from IFC that was from Turkey and zero isolates from IFC that were from Brazil since products from Turkey usually reach Iraq during the first month of production while products from Brazil usually need more time to reach Iraq, usually, more than 2 months. A similar result was obtained in a study conducted by Alaboudi et al. (2020) in Jordan which examined 59 whole imported frozen chickens collected from local markets for the prevalence of *Campylobacter* species but found none.

In this study, nine recovered *C. jejuni* isolates from local broiler chickens as well as two recovered *C. jejuni* isolates from IFC showed similar antibiotic resistance patterns. All 11 samples (100%) from both origins were strongly resistant to TE, CIP, NA and OT but susceptible to E and G (Table3). In spite of the small sample size, these results are not surprising, since the high resistance of *Campylobacter* toward quinolones and tetracyclines and a low level of resistance toward macrolides and aminoglycoside have been demonstrated in other studies in the Middle East. Divsalar et al., (2019) discovered resistance to TE and CIP in all 40/40 (100%) isolated *C. jejuni* from broiler chicken and broiler meat in Iran. In Jordan, (Alaboudi et al., 2020) found 13/13 (100%) resistance toward CIP. Similarly detected in Iraq, among 36 biochemically isolated *Campylobacter*, 100% were resistant to CIP and NA and 88.8% to Tetracycline (Shakir et al., 2021). Additionally, in these three previously mentioned studies, the resistances to GE were 7.5%, 12%, and 30.5%, respectively, and the resistances to E were shown as 7.5% by (Divsalar et al., 2019) and 50% by (Shakir et al., 2021). The higher level of E resistance in the study conducted by (Shakir et al., 2021) may be due to the biochemical confirmation of *Campylobacter* to the genus level, and this high level of resistance may be related to *C. coli*, which, unlike *C. jejuni*, has a significant high resistance toward E (Authority et al., 2022).

Despite of the fact that 16S rDNA analysis enables specific identification of most Campylobacter species. However, due to the lack of discrimination among the taxa the *Campylobacter jejuni*, *C. coli* and atypical *C. lari* strains were excluded because they shared identical or nearly identical 16S rDNA sequences (Parkhill et al., 2000). Hansson et al., 2008 stated that phylogenetic analysis based on 16S rRNA sequences is not always sufficient for differentiation between *C. jejuni* and *C. coli*. Therefore, the construction of phylogenetic tree in this study was based only on confirmed *C. jejuni* strains and compared with other *C. jejuni* strains subjected to GenBank. This study has found that all LBC and IFC isolates are closely related as they shared 97-99% similarity. The study isolates also have shown a variation in their relatedness with the other isolates in other countries and with other isolation sources. The strains were grouped in five major clusters, but the majority were grouped in cluster I, including one IFC and other strains from other countries as in cluster one ,CP012242-Finland, OP090654- Croatia and CP030866-Chicken- from South Korea. While the other strains

grouped in cluster III and IV with the *C. jejuni* strains of other countries, APO26014-Japan, EF136573-India, and MN736598-Chicken feces from Italy. This could be due to the lack of risk analysis and control measures applied for the importation of eggs for hatching and importation of frozen chicken.

5. CONCLUSION

This study focused on the occurrence of *C. jejuni* in Cloacal swabs of broiler chicken and imported frozen chicken samples collected from different sources. These can serve as a possible source for transmitting of *C. jejuni* and increase the risk of campylobacteriosis in humans through the consumption of undercooked meat. The current study has identified that *C. jejuni* isolated from LBC and IFC were developed an antimicrobial resistance for certain antibiotics and considered as multidrug resistant strains, which increases the concerns for the authorities. Therefore, necessary measures at different stages in the food chain are required to minimize or remove the risks caused by this pathogen. In addition, using of antibiotics must be regulated and careful in both veterinary and human treatment. The isolated strains are genetically closely related from different sources. This study had some limitations due to the fund and time restraint . Thus additional molecular epidemiology, phenotypical and molecular identification for this microorganism are needed.

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