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Genetic diversity, virulence profile of *Campylobacter coli* and *Campylobacter jejuni* isolated from poultry and human in Assiut governorate, Egypt

Hager A.S. Abdelhady[1*], Asmaa A.A. Hussein[2], Omar Amen[3], Shereen M. Galal[4]

[1]*Department of Molecular Biology, Molecular Biology Research and Studies Institute, Assiut University, Egypt.*
[2]*Department of Zoonoses, Faculty of Veterinary Medicine, Assiut University, Egypt.*
[3]*Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.*
[4]*Department of Pediatrics, Faculty of Medicine, Assiut University, Assiut, Egypt.*

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***Correspondence:**

Corresponding author: Hager A.S. Abdelhady
E-mail address: hagerabdelhleem@gmail.com

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ABSTRACT

Thermotolerant *Campylobacter* genus is one of the most prevalent causes of gastroenteritis in humans, especially *C. coli* and *C. jejuni*. Despite the importance of *Campylobacter* diagnosis to public health, many laboratories continue to adopt the slow, inaccurate conventional culturing approach, which leads to false-negative/positive results. The origin, transmission, pathogenicity, and pathophysiology of *Campylobacter* spp. diseases are poorly understood. Therefore, in this study, the samples were collected over a period from August 2021 to September 2022; about 100 poultry samples and 43 stool specimens from children were collected. According to conventional culturing techniques, the overall prevalence of the *Campylobacter* genus in both poultry and humans was determined to be 31.5%, whereas PCR analysis of poultry (30) and human specimens (43) for *Campylobacter* genus revealed a 35.6% isolation rate. While *C. coli* was the only species detected in poultry-positive *Campylobacter* genus samples demonstrated by 27.3%, the human-positive *Campylobacter*'s isolates were *C. coli* with 33.3%, *C. jejuni* and mixed infection with 6.7%. Shannon and Simpson biodiversity indexes quantify genetic diversity; assuming that *Campylobacter* species express virulence genes differently, we found that *C. coli* had a higher Shannon diversity index (0.8487) and Simpson index (0.4938), while *C. jejuni* had (0.6931) for Shannon and (0.5) for Simpson index. Regarding host-virulence genes diversity, human-derived strains had a higher Shannon diversity index (1.474) and Simpson index (0.75) than poultry. This study provided evidence that the genetic profiles of circulating species of *Campylobacter* differ depending on the origin, highlighting the need for genetic diversity knowledge for effective management and prevention strategies.

Introduction

Campylobacter bacteria are gram-negative, non-spore-forming, and curved or spiral-shaped. They possess a single polar flagellum (or multiple flagella) that allows them to move in a corkscrew-like motion (Cronquist *et al*., 2012). Their optimal growth conditions include microaerophilic conditions and relatively high temperatures. Avian species serve as major reservoirs for the spread of *Campylobacter* species because their high body temperatures (41°C) offer the ideal conditions for the organism's growth (Reddy and Zishiri, 2018). The route of *Campylobacter*iosis transmission to humans by consumption of contaminated animal products and water, animal interaction, and international travel is another risk factor (Man, 2011). Patients who are infected with *C. jejuni*, *C. coli*, or both get severe diarrhea, which is watery or bloody, have high fevers, lose a lot of weight, and feel severe abdominal cramping that lasts, on average, six days. Depending on the bacterial dosages present in the consumed contaminated foods and drinks, symptoms often appear between 24 and 72 hours after consumption (Kaakoush *et al*., 2015). *Campylobacter* genus is nutritionally fastidious (needs complex nutritional conditions) and grows under strictly microaerobic and anaerobic conditions (Toplak *et al*., 2012). So, diagnostic methodologies undergo several development steps to overcome this fastidious nature and to obtain a maximum level of accuracy in the isolation of microorganisms (Ricke *et al*., 2019).

In molecular basis such as PCR, sensitivity and specificity are important factors in choosing a gene to detect *C. coli* and *C. jejuni*. The *lpxA* gene and hippuricase (*hipO*) genes are the most common *Campylobacter coli* and *Campylobacter jejuni* detection genes. These two genes have distinct features that may make one better than the other.

Like other Gram-negative bacteria, *Campylobacter* makes its outer membrane from lipopolysaccharides (LPS) by using the *lpxA* gene. The *lpxA* gene is a highly conserved housekeeping gene among *Campylobacter* species, making it a good target for detection methods that use PCR amplification (Klena *et al*., 2004). Studies have shown that the *lpxA* gene is highly specific for *Campylobacter* and lowly cross-reactive with other bacteria (Girgis *et al*., 2014).

Genetic diversity plays a role in the variation of virulence factors among *Campylobacter* strains. Once ingested, *Campylobacter* bacteria can colonize the gastrointestinal tract, particularly the small intestine. They possess various virulence factors that aid in their attachment to intestinal cells and evasion of the host immune response. These factors include motility, as *flaA* gene is represented by *Campylobacter* flagella, which allows the bacterial cells to penetrate the host's viscous intestinal mucus layer in a highly effective manner (Guerry, 2007). Adhesins such as *CadF* (*Campylobacter* adhesion protein to fibronectin), a 37 kDa outer membrane protein that attaches to its ligand fibronectin of host epithelial cells, is the most investigated adhesin factor (Kreling *et al*., 2020). Cytotoxins, such as *cdtB*, the genotoxic effects of *cdtB* on host DNA are caused by its translocation to the nucleus of host cells, which inhibits the cell cycle in the G2 or M phase and triggers DNA repair mechanisms that may result in cell cycle arrest and lead to cell death (Feola De Carvalho *et al*., 2013). Virulence factors help the bacteria penetrate and damage the intestinal epithelium, leading to inflammation and the characteristic symptoms of *Campylobacter*iosis. Different strains may possess unique combinations or variations of virulence factors, impacting their pathogenic potential. Therefore, regular investigations of *Campylobacter* prevalence in animals and humans are of significance, particularly in relation to its primary reservoir in poultry. Also, it is crucial to understand the

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virulence characteristics of the strains present in both broiler and human populations. Hence, the main aim of this study was to assess the pathogenicity capacity of *C. coli* and *C. jejuni* strains obtained from avian and human sources.

Materials and methods

Ethical approval

The research was carried out in accordance with the established guidelines of the Molecular Biology Research and Studies Institute, Assiut University, Egypt (lORG0010947-MB-21-16-A).

Collection of samples

Poultry samples

One hundred samples were randomly collected and divided between 50 cecum samples and 50 liver samples of diseased broiler chickens at 2-4 weeks old, presented to the poultry Department at Assiut University Veterinary Hospital.

Human samples

Forty-three stool swabs were collected from children who showed gastroenteritis symptoms (fever, abdominal pain, vomiting and diarrhea) admitted to the gastroenterology and hepatology unit in pediatrics department at Assuit university children hospital. All samples were collected in sterile containers, labeled, and placed in an ice box, then directly transferred into the laboratory of the poultry Department, Faculty of Veterinary Medicine, Assiut University, for bacteriological examination.

Isolation and phenotypic identification

Campylobacter spp. isolation and identification were performed according to the ISO 10272 standards. One gram of the homogenized samples was aseptically inoculated into a sterile screw-capped tube contained 9 ml of Bolton broth (HIMEDIA, M1592, USA) selective supplement and 5% lysed horse blood, which was incubated under suitable microaerophilic environments in an anaerobic jar by using the Anaero Pack System (5% O2 10% CO2 and 85% N2) at 41.5°C. After plating a loopful of the incubated broth was streaked onto Modified Charcoal cefoperazone deoxycholate agar base (mCCD) (HIMEDIA, M887I, USA), the plates were incubated for 48 hours at 41.5°C under proper microaerophilic environments, suspicious colonies were chosen and isolated. Biochemical response tests (catalase test, oxidase test, hippurate hydrolysis test) were recorded.

Polymerase chain reaction (PCR)

This part was carried out in the Molecular Biology Research Unit (MBRU), Molecular Biology Research & Studies Institute, Assiut University, Egypt.

DNA extraction

Suspected *Campylobacter* spp. colonies were subcultured onto tryptone soya broth (Oxoid, CM0129, England) and incubated overnight at 41.5°C. According to the manufacturer's instructions for QIAamp DNA Mini kit (Cat. No.51304), DNA extraction was performed. Bacterial DNA was extracted and stored at -20°C.

PCR amplification and primer design

The PCR amplification was performed in a thermal cycler (Veriti™ Thermal Cycler, Applied Biosystems, USA); according to manufacturer's instructions of master mix Cosmo PCR Red Master Mix (WF- 10203001-M), 50 µl was the total volume of the mixture prepared in a PCR tube as following: 25 µl of Master mix,15 µl DNA templet (sample), 2.5 µl of Forward primer, 2.5 µl of Reverse primer,5 µl of distilled water.

To perform a successful amplification reaction, initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 50 sec; annealing in accordance with a thermal condition of the reference of each primer's gene set cited in Table 1; extension at 72°C for 1 min; and final extension at 72°C for 10 min.

Amplified products were analyzed by using 1% agarose molecular grade, from NIPPON Genetics Europe (Cat.:AG02). Prepared according to the manufacturer's instructions, then mixed with ethidium bromide solution 1% (Carl Roth Co, Sweden) for electrophoresis and visualized on a UV transilluminator.

Statistical and genetic analysis

The frequency of *Campylobacter* genus, species *C. coli* and *C. jejuni*, and virulence genes were analyzed using SPSS software, using Chi square (χ2) test with a P-value < 0.05. Simpson's (D1) and Shannon's (H) tests were used to calculate and quantify *Campylobacter* spp. isolate's genetic diversity using Past 4 software (version 4.14). Combining bacterial species, hosts, and virulence gene distributions as input data.

Table 1. shows oligonucleotides primer's sequence used for *Campylobacter* genus, *Campylobacter* species, virulence genes identification.

Target gene	Primer's sequence (5`–3`)	Amplicon Size (bp)	Annealing Temp	References
16SrRNA for *Campylobacter* genus	Forward: GGATGACACTTTTCGGAGC Reverse: CATTGTAGCACGTGTGTC	816	56 °C	Linton *et al*. (1996)
lpxA.coli	Forward: AGA CAA ATA AGA GAG AAT CAG Reverse: CAATCATGDGCDATATGA SAA TAH GCC AT	391	55 °C	Klena *et al*. (2004)
lpxA.jejuni	Forward: ACAACT TGG TGA CGATGTTGT A Reverse: ACA GGR ATT CCR CGY TTT GTY TC	726	57 °C	Klena *et al*. (2004)
flaA	Forward: AATAAAAATGCTGATAAAACAGGTG Reverse: TACCGAACCAATGTCTGCTCTGATT	855	53 °C	Datta et al. (2003)
cadF	Forward: TTGAAGGTAATTTAGATATG Reverse: CTAATACCTAAAGTTGAAAC	400	44 °C	Konkel *et al*. (1999)
cdtB.coli	Forward: TTTAATGTATTATTTGCCGC Reverse: TCATTGCCTATGCGTATG	413	49 °C	Asakura *et al*. (2008)
cdtB.jejuni	Forward: GTTAAAATCCCCTGCTATCAACCA Reverse: GTTGGCACTTGGAATTTGCAAGGC	495	59 °C	Bang *et al*. (2001)

Results

The conventional culture method showed that prevalence rates were 21% (30/143) and 10.5% (15/143) of *Campylobacter* genus in poultry and humans, respectively, while a total prevalence was 31.5% (45/143). On the other hand, all poultry samples that were previously diagnosed positive by culture (30) and all human specimens (43) were PCR-tested, resulting in a higher isolation rate of 35.6% (26/73), divided as 15.1% (11/73) for poultry and 20.5% (15/73) for human, respectively, (Fig. 1).

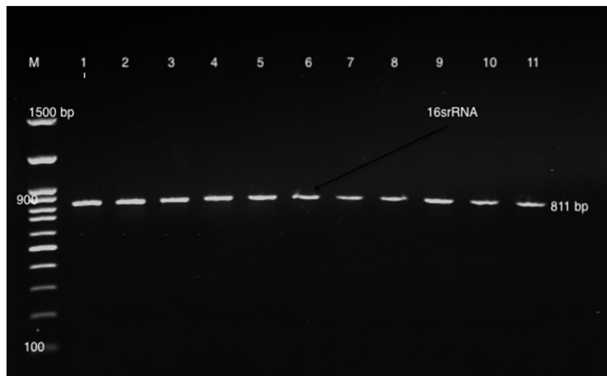


Fig 1. *Campylobacter* genus identification by polymerase chain reaction (PCR) for poultry and humans; (M) DNA ladder 100 bp; isolates produced an amplified fragment of 16srRNA gene at lanes (1-11) at the expected positions (812 bp); lane (12) was the negative control.

Regarding species level, among *Campylobacter* spp. positive isolates, *C. coli* was 30.8% (8 out of 26), and the prevalence of *C. jejuni* and mixed infection was equal, accounting for 3.8% (1/26) for each. About 27.3% (3/11) of poultry-positive *Campylobacter* genus samples were *C. coli* positive. In poultry, only *C. coli* was detected, while human-positive *Campylobacter* genus isolates were *C. coli*, *C. jejuni* and mixed infections with 33.3% (5/15), 6.7% (1/15), and 6.7% (1/15) respectively (Fig. 2).

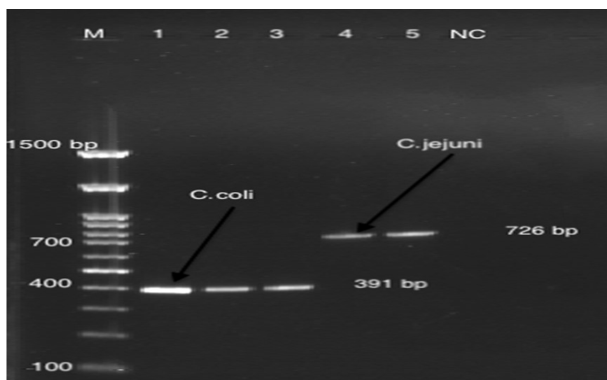


Fig. 2. *Campylobacter* species identification: lane (M) DNA ladder 100 bp, *C. coli lpxA* primer produced fragments at lanes (1,2,3) at the expected positions (391bp), while *C. jejuni lpxA* primer produced fragments at lanes (4,5) at the expected positions (726bp); lane (6) was negative control.

In order to assess host-*Campylobacter* species relationships, Simpson and Shannon's genetic diversity indices submit that humans have a higher diversity (0.4793-0.8789) than poultry (0.3369 - 0.5196), respectively (Fig. 3).

Regarding Table 2 mixed infection, *C. coli* and *C. jejuni* had 100% distribution and incidence of *flaA*, *cadF*, and *cdtB*. *C. coli* isolates had 25%, 12.5%, and 75% *flaA*, *cadF*, and *cdtB* genes, respectively. The *C. jejuni* isolate exhibited 100% expression of only two virulence genes, *cadF* and *cdtB*; *flaA* was not detectable.

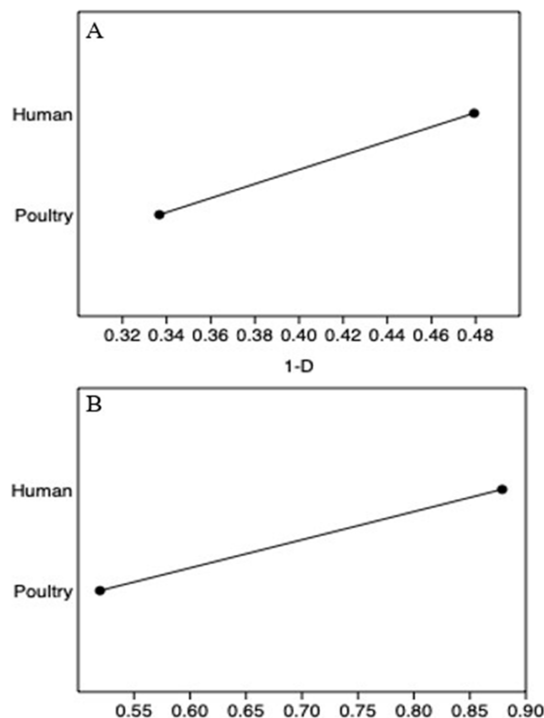


Fig. 3. Showing Genetic diversity between *Campylobacter* spp., *C. coli* & *C. jejuni* based on poultry and human sources using. (A) Simpson 1-D index; (B) Shannon index.

According to species and virulence genes genetic diversity, Mixed infection had the highest Simpson index (0.75), indicating high dominance of all virulence genes, followed by *C. jejuni* (0.5) and *C. coli* (0.4938). Shannon index, like Simpson index, offers Mixed infection with the highest result with 1.386; however, *C. coli* and *C. jejuni* values vary because Shannon index considers a number of (genes) rather than dominance; so, *C. jejuni* had 0.6931, because it lacked the *flaA* gene, but *C. coli* had 0.8487, because it had all virulence genes (Fig. 4).

Based on isolation source, 55.5%, 44.4%, and 33.3% of human isolates had all virulence genes *cdtB*, *cadF*, and *flaA*; on the other hand, poultry isolates lack all virulence genes except *cdtB.coli* demonstrated 27.3% (Fig. 5).

Regarding host-based virulence gene, *Campylobacter* spp. population in poultry source had 0% genetic diversity, while the human source was (0.75) for Simpson index and (1.474) for Shannon index.

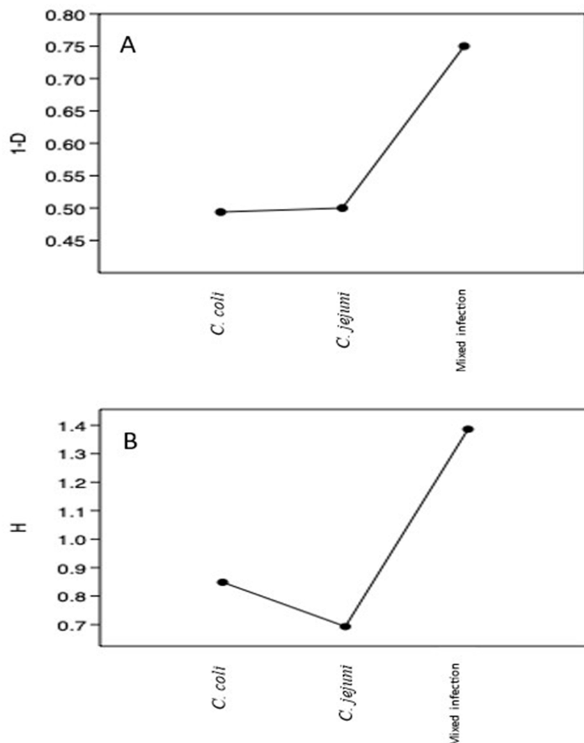
Discussion

Campylobacter spp. are important etiological agents of gastroenteritis, especially in children living in developing countries (do Nascimento Veras *et al*., 2016). Since 2008, there has been a steady increase in human *Campylobacter*iosis cases, which may be attributed in part to increased surveillance and awareness. However, due to the nature of this multi-host infection and its widespread presence in the environment, it is challenging to comprehend all facets of its epidemiology and the potential causes

Table 2. Prevalence of virulence genes according to species (*C. coli* and *C. jejuni*).

Virulence genes	*C. coli*		*C. jejuni*		Mixed infection		Total	
	No	%	No	%	No	%	No	%
flaA	2	25%	0	0%	1	100%	3	30%
cdtB.coli	6	75%	0	0%	1	100%	7	70%
cdtB.jejuni	0	0%	1	100%	1	100%	2	20%
cadF	1	12.50%	1	100%	1	100%	3	30%

for the increase in human cases (EFSA, 2012).



ig. 4. Showing genetic diversity between *Campylobacter* species (*C. coli*, *C. jejuni* and mixed infection) based on virulence genes prevalence. (A) Simpson 1-D index; (B) Shannon index.

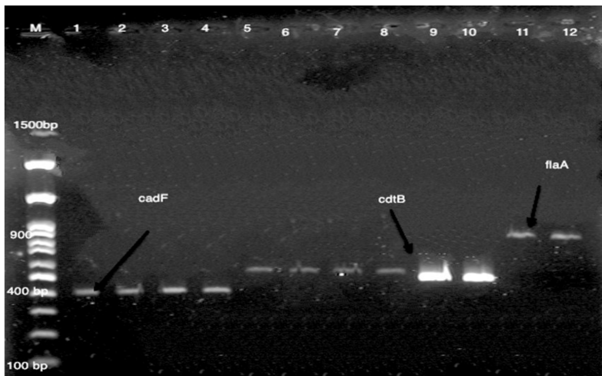


Fig. 5. Showing total prevalence profile of virulence gene in poultry and humans; *cadF* gene lanes (1,2,3,4) amplified at (400) bp. *cdtB* gene lanes (5,6,7,8,9,10) amplified at (413) bp, (495) bp. *flaA* gene lanes (11,12) amplified at (855) bp.

The current investigation applied a combination of classic culturing techniques and PCR assays to detect *Campylobacter* in broilers and humans in the Assiut Governorate; Overall 143 samples were collected (100 from poultry and 43 from humans) and bacteriologically tested, a total prevalence of *Campylobacter* genus was 31.5% (45/143). On the other hand, all human specimens (43) and (30) previously culture-positive poultry samples were subjected to PCR testing, resulting in a higher isolation rate of 35.6% (26/73). A comparable incidence of isolation was observed in poultry (Gahamanyi *et al*., 2021), amounting to 35.9%. Furthermore, an extensive epidemiological investigation was conducted to find out the prevalence of *Campylobacter* in individual animals was 38.1% (Torralbo *et al*., 2014). Regarding humans, a study confirmed that nearly one-third of the patients (31.5%) were found to be positive for *Campylobacter* spp (Rahman *et al*., 2021). On contrary, higher isolation rates in poultry were recorded by Anderson *et al.* (2012) with 86.0% prevalence for the presence of thermotolerant *Campylobacter* spp., another higher percentage was recorded by Walker *et al*. (2019) investigations of the prevalence and distribution of *Campylobacter* which were performed over a 2-year sampling period (October 2016 to October 2018) to detect *Campylobacter* spp. positive results with 90% of chicken meat and 73% of chicken offal products (giblet and liver) were recorded. In contrast, in Assiut governorate, Egypt, a similar study was performed by Abushahba (2018), but lower prevalence rates were recorded. Another lower rate of isolation is found by Mohakud *et al.* (2019) and Girgis *et al.* (2014).

In our investigation, we found that among the (26) isolates of *Campylobacter* spp. that tested positive in both poultry and humans, *C. coli* was the predominant species, followed by *C. jejuni*. We also observed mixed infections in human source. The overall prevalence of *C. coli* was 30.8% (8 out of 26). The prevalence of *C. jejuni* and mixed infection was equal, accounting for 3.8% (1/26) for each. *C. coli* was the only species found in poultry sources at 27.3% (3/11). *C. coli*, *C. jejuni*, and mixed infection were found in humans at 33.3% (5/15), 6.7% and 6.7% (1/15), respectively. A similar study found that *C. coli* dominated *C. jejuni* (Henry *et al*., 2011). A further investigation was conducted in Mansoura, Egypt (Awad *et al*., 2019). Several other studies have provided partial confirmation of this observation, including the one conducted by Vinueza-Burgos *et al*. (2017) shows that prevalence at batch level of poultry *Campylobacter* spp. positive samples were positive for *C. coli* with 68.7%, for *C. jejuni* with 18.9%, and for *C. coli* and *C. jejuni* with 12.4%, to complete agreement with the extreme result represented by Dipineto *et al*. (2017), which shows *Campylobacter coli* was the only *Campylobacter* species detected in avian samples. On contrary, other studies showed a dominance of *C. jejuni* prevalence over *C. coli* obtained by Rozynek *et al*. (2005).

One reason for an increase in *Campylobacter* spp. and *C. coli*, could be the use of molecular technology to identify pathogens. According to Ricke *et al*., (2019), these methodologies facilitate fast and accurate identification of *Campylobacter* species, including *C. coli* and *C. jejuni*. The frequency of *Campylobacter coli* may have been boosted by the rise in travel and worldwide food trade. *Campylobacter* infections are prevalent in different countries, and individuals who travel may come with strains that are uncommon in their country of origin (Mughini-Gras *et al*., 2014). Agricultural and land use changes may increase *Campylobacter coli*; according to Kougblénou *et al*. (2019), intensive livestock farming using poultry manure fertilization has led to an increase in *Campylobacter* spp. contamination of food and water.

The Shannon Diversity Index provides a measure of both species' richness (the number of different species in a population) and species evenness (the distribution of genes among species) (Kim *et al*., 2017) .A higher Shannon index value indicates higher diversity; on the other hand, the Simpson Diversity Index focuses more on dominance or concentration of species. A higher Simpson index value indicates lower diversity. Applying genetic diversity indices (Simpson & Shannon) can analyze the richness and evenness of species, genes, and communities and reveal the complexity and stability of distinct populations. So, in a related context, genetic diversity indices Simpson and Shannon reveal the connection between host and type of *Campylobacter* species; the human result shows a higher diversity (0.4793- 0.8789), more than poultry (0.3369 - 0.5196), respectively.

In the context of *Campylobacter* species pathogenicity, which depends on the presence of virulence factors that differ according to hosts and different *Campylobacter* species; in our study *flaA* gene found in mixed infection, *C. coli* and *C. jejuni* with 100%,25% and 0%, respectively. In related studies lower and higher isolation rate of each virulence gene varies from one study to another; for example, *flaA* gene was demonstrated 5% (Abdel Hafez, 2018). in contrast, a higher isolation rate was obtained from Sharika governorate, Egypt by Ammar *et al*. (2020). In our study *cadF* gene demonstrated 100% in mixed infection and *C. jejuni* while represented with12.5% in *C. coli* isolates; a lower isolation rate of *cadF* gene was recorded by Ghoneim *et al*. (2021), which showed lower percentages of *cadF* found in *C. jejuni* which were 20.58%, 10.52% and 7.69% of isolates from broiler chickens, layer chickens and human stool samples, respectively, with a total percentage of 15.15%. In contrast higher isolation rate of *cadF* gene and *cdtB* gene conducted by Hadiyan *et al*. (2022) which showed the frequent virulence factors among the *C. jejuni* isolates were *cdtB* (81.48%) and *cadF* (74.07%), while among the *C. coli* isolates had *cadF* with (61.53%) and *cdtB* (19.23%); whereas our study prevalence rate of *cdtB* genes were 100% in mixed infection and *C. jejuni* and 75% in *C. coli* isolates.

It was crucial to use genetic diversity indices to figure out the complex relationships between species and virulence genes; the variation in virulence genes harborage among *Campylobacter* species is reflected in genetic diversity values. Therefore; Simpson index highest result was found in Mixed infection with (0.75) which means high dominance of all virulence genes followed by *C. jejuni* with (0.5) and the lowest dominance demonstrated by *C. coli* with (0.4938), While Mixed infection still shows the highest result with (1.386) by Shannon index as same as Simpson index, the result differs in case of *C. coli* and *C. jejuni* because Shannon index taking into account the number of individuals (genes) more than dominance of each gene; accordingly *C. coli* isolates have a higher result with (0.8487) than *C. jejuni* which demonstrated result with (0.6931) as a result of complete absent of *flaA* gene in *C. jejuni* isolate. While all virulence genes are present in *C. coli* isolates.

Our investigation found 55.5%, 44.4%, and 33.3% of human isolates with all virulence genes *cdtB*, *cadF*, and *flaA*. Poultry isolates lack all vir-

ulence genes except 27.3% *cdtB.coli*. The human source has (0.75) for Simpson index and (1.474) for Shannon index; In contrast, poultry source has zero. In agreement with (Lima *et al*., 2022); human-derived strains showed a higher Shannon diversity index and Simpson index than poultry. Generally, many investigations indicated that virulence genes are prevalent based on *Campylobacter* spp. type (Karmi, 2019), host/source type (Barakat *et al*., 2020), or both (Reddy and Zishiri, 2018). Various *Campylobacter* genus and host cell factors influence the expression of virulence-associated genes. Virulence factor expression reduces bacterial fitness due to the excessive use of energy of bacterial cells. Therefore, due to their fitness cost, pathogens may not always activate virulence factors to overcome commensal-mediated colonization resistance of the host cells and establish infection. Dynamic virulence factor expression is a significant invasion strategy for enteric infections (Kitamoto *et al*., 2016)

Conclusion

The outcomes of this investigation demonstrate that the genetic profiles of circulating *Campylobacter* species differ depending on whether they were isolated from poultry or humans; so, understanding the genetic diversity of *Campylobacter* species is crucial for developing effective control and prevention strategies against *Campylobacter* genus related infections. It allows researchers to identify high-risk strains, track the sources of contamination and monitor the emergence of antibiotic resistance. Additionally, studying *Campylobacter* genetic diversity contributes to our broader understanding of bacterial evolution, host-pathogen interactions, and the dynamics of infection.

Conflict of interest

The authors declare that they have no conflict of interest.

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