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Highlighting on the Occurrence of *Campylobacter* in Chickens with Special Reference to its Antimicrobial Resistance

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ABSTRACT

Chickens are recognized as the primary reservoir of thermotolerant *Campylobacter* spp. and they are responsible for an expected 80% of human campylobacter infection.

A total of 113 campylobacter isolates (32.8%) were recovered from 345 different samples collected from human (children stool swabs) and chicken (cloacal swabs, cecal parts, neck skin, liver. gizzard, thigh and breast meat). It was found that 69.9% of *C. jejuni* isolates were susceptible to gentamicin, followed by kanamycin, norfloxacin and ciprofloxacin (67.3. 59.3 and 49.6%, respectively). On the other hand, it has been found that all the examined isolates were resistant to ampicillin and erythromycin (100% each). Moreover, majority of *C. jejuni* isolates were resistant to tetracycline, trimethoprim/sulfamethoxazole, nalidixic acid and cephalothin (90.3, 82.3, 80.5 and 75.2%, respectively).

Estimating the MAR indices for all *C. jejuni* isolates revealed that all the tested isolates had an index greater than 0.2 indicating a high-risk source of contamination, where the antibiotics are often used Three XDR *C. jejuni* isolates (2.7%) had an index of 0.9.

the conventional PCR assay was used to detect genes specific for genus *Campylobacter* and *C. jejuni* in 20 XDR and 5 MDR .

All confirmed *C. jejuni* isolates (25) were tested for the presence of 4 critical virulence genes those play important roles in *C. jejuni* pathogenesis (*fla*A, *vir*B11, *wla*N and *cdt*B).

The effects of SICs of Rosemary and Ginger extracts (0.0125% each) on the transcriptional modulation of *fla*A, *vir*B11 and *wla*N genes were assessed by qRT-PCR assay. The results of this study have an important clinical impact. As all tested genes were found to be markedly down regulated after exposure to SICs of tested phytochemicals.

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INTRODUCTION

Poultry, mainly chickens and its products have been recognized as the main source of campylobacteriosis in humans. Thus, there is a significant need for controlling *C. jejuni* in chickens, which leads to minimizing the prevalence of human campylobacter infections (**Upadhyay et al. 2017**).

Campylobacteriosis is a self-limited disease and antimicrobial therapy is not generally indicated in most cases. However, under specific clinical circumstances, antibiotic therapy may be necessary **(Bhunia, 2018).** In these specific cases, therapy may be complicated due to the emergence of antimicrobial resistant *campylobacter* strains as a result of the wide spread using of antibiotics in agriculture and veterinary medicine **(Silva et al. 2018).**

plant extracts are used in herbal medicine for treating various diseases due to its antimicrobial properties (**Upadhyay et al. 2017**). Giner extract and Rosemary are phytophenolic compounds that used as a food flavoring agent and exhibits significant antimicrobial properties against major foodborne pathogens

Along with the association between virulence and clinical infection, the virulence factors may also be associated with the antimicrobial resistance (**Ghunaim et al. 2015).** Molecular typing based on PCR-based techniques including enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) is developed for typing of *Campylobacter* spp. (**Malakauskas et al. 2017**).

One of the best characterized campylobacter virulence markers are the Flagellin A (*fla*A) gene which determines the flagella formation, the production of cytolethal distending toxin B (*cdt*B), *vir*B11, a plasmid related gene, is associated with host cell invasion (**Tamma, 2018**). Finally, *wla*N is involved in the β-1,3 galactosyltransferase production and biosynthesis of lipooligosaccharide (LOS) (**Malakauskas et al. 2017**).

Therefore, the aim of the present study was to assess the efficacy of subinhibitory concentrations (SICs) of Giner extractand Rosemary on the expression of critical virulence genes of multi-virulent and multi-drug resistant (MDR) *C. jejuni* isolates from poultry origin via real-time quantitative PCR (qRT-PCR) assay, these isolates were genetically correlated with the human ones.

MATERIALS AND METHODS

Samples:

A total of 345 various samples were collected from human (Elahrare hospital) and chicken sources (private farms and retail markets) from different localities in Sharkia Governorate. The samples comprise stool swabs of children with diarrhea (pediatric diarrhea) (100) and broiler chicken specimens (245) obtained from retail outlets. The data of all collected samples are presented in Table (1). The collected samples were aseptically transported in an ice box as soon as possible to the bacteriological laboratory for examination.

Table 1. Distribution of collected samples during the course the study

Source (No)	Sample (Symbol)
Human (100)	Stool swabs of children with diarrhea (H, 100)
	Clocal swabs (CcS, 35)
	Cecal parts (Ccp, 35)
Boiler chicken (245)	Neck skin (CNS, 35)
	Thigh meat (Ctm, 35)
	Breast meat (Cbm, 35)
	Liver (C1, 35)
	Gizzard (Cg, 35)
Total 345	

Isolation and Identification of *Campylobacter spp* :

Isolation was done according to (ISO 2006).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were done using the standard disc diffusion method according to **CLSI (2017)** Ten antimicrobials (Oxoid, Cambridge, UK) belonging to seven different classes were used: norfloxacin (NOR,10 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), cephalothin (KF, 30 µg), erythromycin (E, 15 µg),kanamycin (K, 30 µg), gentamicin (CN, 10 µg), tetracycline (TE, 30 µg), ampicillin (AM, 10 µg) and trimethoprim/sulfamethoxazole (SXT, 25 µg). Interpretition was determined by measuring the diameter of the inhibition zone around each disc and the results were interpreted according to the clinical and laboratory standards institute **(CLSI, 2017) .**

Determination of multiple antibiotic resistance (MAR) index

The multiple antibiotic resistance (MAR) index was determined for each isolate by using the formula MAR=a/b, where a represents the number of antimicrobials to which the test isolate depicted resistance and b represents the total number of antimicrobials to which the test isolate has been evaluated for susceptibility (**Sandhu et al. 2016**).

Molecular Grouping of MDR *C. jejuni* Isolates

All molecular investigations carried out in this study were conducted on the highly resistant C. jejuni isolates.

Extraction of DNA

Total DNA was extracted from the biochemically identified MDR *C. jejuni* isolates utilizing a Bacterial DNA Extraction Kit (QIAamp DNA Mini Kit; Qiagen, Valencia, CA,USA) according to the guidelines of the manufacture.

PCR Assays and Cycling Parameters

Uniplex PCR assays were performed for amplification of the 23S rRNA gene of genus *Campylobacter*, mapA gene of C. jejuni and three critical virulence genes (wlaN,flaA, and virB11). Moreover, ERIC-PCR was used to determine the genotypic profiles and the genetic association among the molecularly identified MDR *C. jejuni* isolates. All PCR reactions were done utilizing the Emerald Amp GT PCR master mix (Takara, Berkeley,CA, USA) according to the manufacturer's guidelines. The specific genes and the primer sequences for all PCR assays are shown in Table (2) DNA of C. jejuni ATCC 33291 was utilized as a positive control and PCR grade water (no templateDNA) was utilized as a negative control in uniplex PCR assays.

Table 2. Oligonucleotide primer sequences and amplified PCR products of seven target genes of *campylobacter* species

Primer name (target gene)	Primer sequence (5 -3)	Amplified product (bp)	Reference
23 S (23S rRNA)	TATACCGGTAAGGAG-TGCTGGAG ATCAATTAAC-CTTCGAGCAGCACCG	650	Wang et al. 2002
MapA (ma A)	CTATTTTATTTTGAG-TGCTTGTG GCTTTATTTGCCATTT-GTTTTATTA	589	Shin and lee, 2009
FlaA (flaA)	AATAAAAATGCTGA-TAAAACAGGTG TACCGAAC-CAATGTCTGCTCTGATT	855	
Vir B (virB11)	TCTTGTGAGTT-GCCTTACCCCTTTT CCTGCGTGTCCTGTGTT ATTTACCC	494	Data et al., 2003
WlaN (wlaN)	TTAAGAGCAAGA-TATGAAGGTG CCATTTGAATTGA-TATTTTTG	627	Talukder et al. 2008
Cdtb (cdtB)	GTTAAAATCCCCTGC-TATCAACCA GTTGGCACTTGGAATTT-GCAAGGC	495	Bang et al.2003
ERIC	ATGTAAGCTCCTGGG-GATTCAC AAGTAAGTGACTGGGGT GAGCG	Variable	Versaovic et al. 1991

Table 3. Cycling conditions for conventional and ERIC-PCR amplification campylobacter genes

Target gene	Specificity	Initial denaturation	No. of cycles	Amplification cycle			Final extension	Reference
				Denaturation	Annealing	Extension		
23S rRNA	Genus *Campylobacter*	95˚C 6 min	30	95˚C 30 sec	59˚C 30 sec	72˚C 30 sec	72˚C 7 min	**Wang *et al.* 2002**
***map*A**	*C. jejuni*	95˚C 5 min	35	95˚C 30 sec	58˚C 90 sec	72˚C 1 min	72˚C 10 min	**Shin and Lee, 2009**
***fla*A**	The major flagellin protein	94˚C 5min	30	94˚C 1 min	53˚C 1 min	72˚C 1 min	72˚C 10 min	**Datta *et al.***
***vir*B11**	Type IV secretion system of *C. jejuni* virulence plasmid	94˚C 5 min	30	94˚C 1 min	53˚C 1 min	72˚C 1 min	72˚C 10 min	**2003**
***wla*N**	β-1,3 galactosyltransferases involved in LOS production	94˚C 5 min	35	94˚C 1 min	46˚C 1 min	72˚C 1 min	72˚C 10 min	**Talukder *et al.* 2008**
	Cytolethal distending	94˚C		94˚C	42˚C	72˚C	72˚C	**Bang *et al.***

Assessment of the efficacy of Giner and Rosemary extract SICs on the expression of critical virulence genes of C. jejuni Phytochemicals

Two phytochemicals were used to control C. jejuni virulence. Giner (Sigma-Aldrich, St. Louis, MO, USA) and Rosemary extracts (Sigma-Aldrich, St. Louis, USA) Determination of Subinhibitory Concentrations(SICs) of used phytochemicals was carried out according to **Upadhyay et al. 2017**.

Evaluation of Phytochemicals' Effects on C. jejuni Virulence Genes' Expression by Real-Time Quantitative Reverse Transcription PCR Assay

The qRT-PCR assay was used to determine the efficacy of Rosemary and Ginger SICs on *C. jejuni* virulence genes' expression according to **Upadhyay et al. 2017**

Briefly, each selected *C. jejuni* strain was separately inoculated with the SICs of the tested phy-tochemicals in Campy-Thio broth to mid-log phase (8 h) at 42◦C under microaerophilic conditions. After incubation, aliquots of suspended cells were centrifuged and the su-pernatant was decanted and the pellet was used immediately for RNA extraction using QiampRNease Mini Kit (Qiagen, Valencia, CA, USA) following the guidelines of the manu-facturer. Transcript levels of the investigated virulence genes of *C. jejuni* were determined in the presence or absence of Rosemary and Ginger. Real-time PCR amplification was performed, in triplicates, in the Stratagene MX3005P real-time PCR machine (Thermo Fisher, CA, USA) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA,USA) according to the guidelines of the manufacturer. A melting curve analysis was done to differentiate between the specific and non-specific amplification products. The 23S rRNA gene was used as a normalizing agent (endogenous control). The relative expression levels of the investigated genes in *C. jejuni* cells exposed to the used phytochemicals compared to the non-exposed (control) cells were obtained by the delta-delta Ct (2-ΔΔCt) method (Yuan et al,2006) whereas fold changes (relative quantification, RQ) = 2−ΔΔCt, ΔΔCt = ΔCt treatment − ΔCt control, ΔCt = Ct tested gene − Ct endogenous control. If a ΔΔCt is equal to 0, the fold changes (RQ) will be 1, which indicates no change in gene expression between control and treatment. Moreover, RQ values below 1 indicate down-regulation in the gene expression.

Statistical analysis

ERIC-PCR genotypic patterns were converted to numeric bp by using the BioDocAnalyze (Biometra, Germany) program and then the ERIC-PCR data were converted to the binary code according to the absence or presence
of each band. The Jaccard coefficient was used to determine the profiles similarity **(Jaccard, 1912).** The dendrogram was achieved through the sequential hierarchical analysis and an unweighted pair group method with an arithmetic average (UPGMA). The dendrogram and the cluster analysis were achieved through the SPSS Inc. version 26 (IBM Corp., Armonk, NY, USA). Additionally, the Fisher's exact was used to study the differences in the prevalence of *C. jejuni*, antimicrobial resistance patterns and the prevalence of virulence genes from different sources. We also applied Pearson chi-square to identify the association between different typing methods. Moreover, weused independent samples t-test to compare the Rosemary and Giner extracteffects on C. jejuni virulence genes'expression. Both tests were done using the SPSS Inc. version 26 (IBM Corp., Armonk, NY, USA). The *P* values of less than 0.05 were considered statistically significant. Finally, the antibiotyping, virulotyping and ERIC-PCR discriminatory powers (*D* values) were calculated using Simpson's index of diversity **(Hunter, 1990).** The *D* value above 0.9 reflects good discrimination.

RESULTS

Table 4. prevalence of *C. jejuni* isolates from different samples in Sharkia

Samples source (No)	Sample type (symbol, No)	No. of *C. jejuni* isolates (%) *
Human (100)	Stool swabs (H, 100)	30 (30)
	Cloacal swabs (Ccs, 35)	19 (54.3)
	Cecal parts (Ccp, 35)	14 (40)
	Neck skin (Cns, 35)	9 (25.7)
Broiler chicken (245)	Thigh meat (Ctm, 35)	12 (34.3)
	Breast meat (Cbm, 35)	10 (28.6)
	Liver (Cl, 35)	11 (31.4)
	Gizzard (Cg, 35)	8 (22.9)
Total 345		**113 (32.8)**

The isolation rate was calculated in relation to the total number of the examined samples from different sources

Antimicrobial susceptibility testing of *C. jejuni* isolates

Table 5. Antimicrobial susceptibility patterns of 113 *C. jejuni* isolates from different sources

AMA	No. of *C. jejuni* isolates (%)						
	Chicken origin (83)			Human origin (30)			Total(%)
	R	I	S	R	I	S	R
AM	30 (100)	-	0	83 (100)	-	0	113 (100)
E	30 (100)	-	0	83 (100)	-	0	113 (100)
NA	22 (73.3)	-	8 (26.7)	69 (83.1)	-	14 (16.9)	91 (80.5)
CIP	14 (46.7)	-	16 (53.3)	43 (51.8)	-	40 (48.2)	57 (50.4)
NOR	7 (23.3) 23 (20.4)	5 (16.7) 67 (59.3)	18 (60)	16 (19.3)	18 (21.7)	49 (59)	23 (20.4)
KF	23 (76.7)	0	7 (23.3)	62 (74.7)	6 (7.2)	15 (18)	85 (75.2)
CN	8 (26.7) 11 (9.7)	4 (13.3) 79 (69.9)	18 (60)	15 (18.1)	7 (8.4)	61 (73.5)	23 (20.4)
K	1(3..3) 34 (30.1)	14 (46.7) 76 (67.3)	15 (50)	2 (2.4)	20 (24.1)	61 (73.5)	3 (2.7)
TE	26 (86.7)	1(3.3)	3 (10)	76 (91.6)	1 (1.2)	6 (7.2)	102 (90.3)
SXT	27 (90)	-	3 (10)	66 (79.5)	-	17 (20.5)	93 (82.3)

AMA: antimicrobial agent, AM: ampicillin, E: erythromycin, NA: nalidixic acid, CIP: ciprofloxacin, NOR: norfloxacin, KF: cephalothin, CN: gentamicin, K: kanamycin, TE: tetracycline, SXT: trimethoprim/ sulfa-methoxazole, S: sensitive,
I: intermediate, R: resistant.

Table 6. Multiple antibiotic resistance indices and resistance profiles of *C.jejuni* isolates from different source

MAR index	No. of antimicrobial agents to which *C. jejuni* were resistant	No. of resistant *C. jejuni* isolates from different origins (%)		
		Human (30)	Chicken (83)	Total (113)
0.3	3	0	1(1.2)	1 (0.9)
0.4	4	0	3 (3.6)	3 (2.7)
0.5	5	7 (23.3)	17 (20.5)	24 (21.2)
0.6	6	15 (50)	35 (42.2)	50 (44.2)
0.7	7	2 (6.7)	13 (15.7)	15 (13.3)
0.8	8	5 (16.7)	12 (14.5)	17 (15)
0.9	9	1 (3.3)	2 (2.4)	3 (2.7)

PCR results

Molecular confirmation of genus *Campylobacter* and *C. jejuni*

Out of the 113 isolates, 20 XDR and 5 MDR isolates recovered from human (8 isolate) and broiler chicken sources (17 isolate).The isolates were submitted for PCR amplifications of 23S rRNA and *map*A genes for molecular confirmation of genus *Campylobacter* and *C. jejuni*, respectively. The 17 avian *C. jejuni* isolates were recovered from (3)chicken thigh meat ,(3) chicken cecal parts ,(3) chicken cloacal swabs, (2) chicken liver, (2) chicken gizzard ,(2) chicken breast meat and(2) chicken neck skin .

All the 25 screened isolates (100%) were identified as genus *Campylobacter* and confirmed to be *C. jejuni*. They yielded the predicted product sizes for 23S rRNA gene (650 bp) (Figure 6A) and *map*A gene (589 bp) Figure (1).

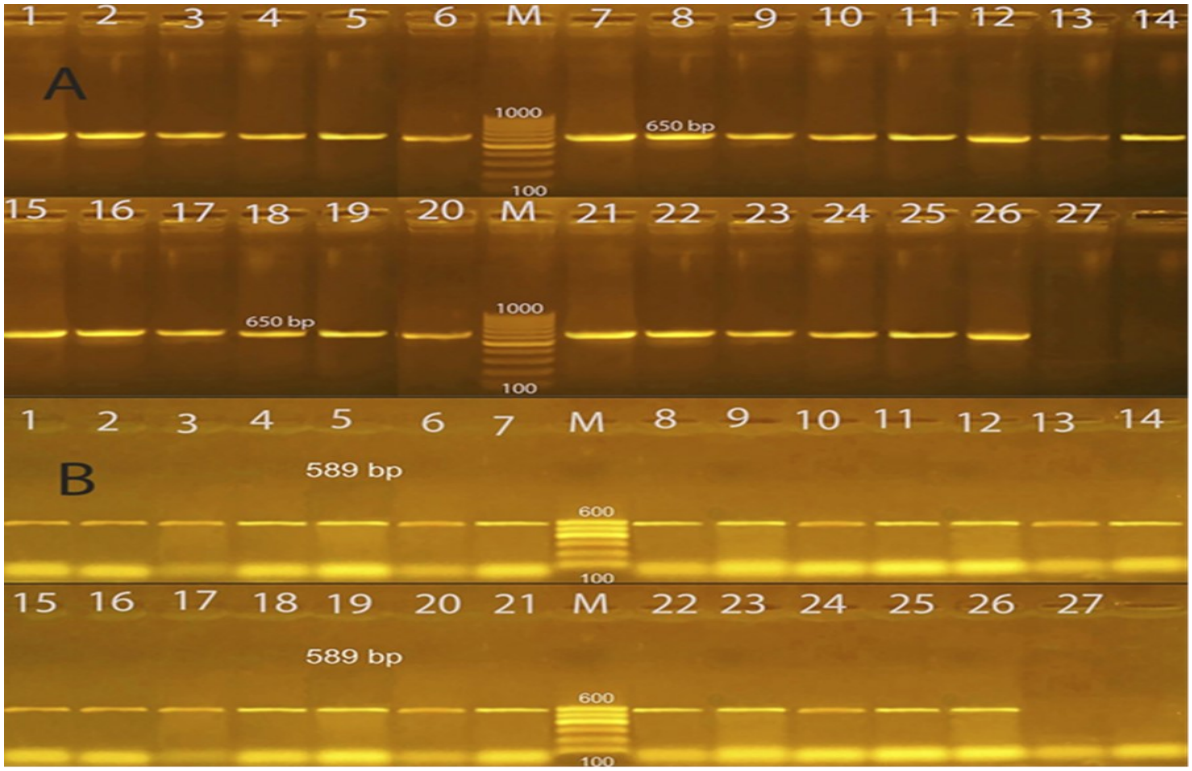

Figure (1): Agarose gel electrophoresis showing typical amplification products of 23S rRNA gene for confirmation of genus *Campylobacter* (A) and *mapA* gene of *C. jejuni* (B). M: 100-bp DNA ladder; lanes 1-8: *C. jejuni* from human stool swabs (isolate no. 1H-8H); lanes 9, 10: *C. jejuni* from chicken liver (isolate no. 95Cl, 96Cl); lanes 11, 12: *C. jejuni* from chicken gizzard (isolate no. 106Cg, 107Cg); lanes 13, 14: *C. jejuni* from chicken breast meat (isolate no. 85Cbm, 86Cbm); lanes 15-17: *C. jejuni* from chicken thigh meat (isolate no. 73Ctm-75Ctm); lanes 18-20: *C. jejuni* from chicken cecal parts (isolate no. 50Ccp-52Ccp); lanes 21-23: *C. jejuni* from chicken cloacal swabs (isolate no. 31Ccs-33Ccs); lanes 24, 25: *C. jejuni* from chicken neck skin (isolate no. 64Cns, 65Cns); lane 26: positive control (*C. jejuni* ATCC 33291) and lane 27: negative control (PCR grade water).

Molecular investigation of virulence genes among 25 MDR *C. jejuni* isolates

The confirmed *C. jejuni* isolates (25) were tested for the presence of 4 critical virulence genes those play important roles in *C. jejuni* pathogenesis (*fla*A, *vir*B11, *wla*N and *cdt*B).

Out of the 25 tested isolates, 13 (52%) were positive for *vir*B11 gene (Figure 7A), 9 (36%) were positive for *wla*N gene (Figure 2) and (table 7)(28%) were positive for *fla*A gene (Figure 2). None of the isolates were positive for *cdt*B gene. They yielded the predicted product size for *vir*B11 gene (494bp) (Figure 7A), *wla*N gene (627bp) (Figure 7B) and *fla*A gene (855 bp) (Figure 7C). No significant differences in the distributions of (*fla*A, *vir*B11 and wlaN) genes were observed among *C. jejuni* isolates from different sources (P= 0.64, 1 and 0.661, respectively).

Regarding the distribution of the investigated virulence genes among both sources, it was found that *vir*B11 and *wla*N genes were more prevalent among avian *C. jejuni* isolates (52.9 and 41.2%, respectively) than human ones (50 and 25%, respectively). Meanwhile, *fla*A gene was highly distributed among human *C. jejuni* isolates (37.5%) than avian ones (23.5%) (Figure 2).

It was found that *C. jejuni* isolates from broiler chicken origin were more virulent than *C. jejuni* isolates from human origin. Ten avian (58.8%) and 4 human (50%) *C. jejuni* isolates harbored at least 1 virulence gene. Moreover, 4 avian (23.5%) and only 1 human (12.5%) *C. jejuni* isolates contained 3 virulence genes (Figure 2).

Interestingly, 5 virulence profiles were presented among the examined *C. jejuni* isolates. Five MDR *C. jejuni* isolates (20%) exhibited the most common virulence profile (*vir*B11, *wla*N, *fla*A). It was also the most prevalent profile among avian *C. jejuni* isolates (23.5%), while *vir*B11, *fla*A was the most common virulence profile among human *C. jejuni* isolates (25%) (Table 11). Meanwhile, 11 (44%) of the examined isolates [7 avian (41.2%) and 4 humans (50%)] were non virulent. There were no statistically significant differences in the virulence profiles among the *C. jejuni* isolates from various sources (P> 0.05).

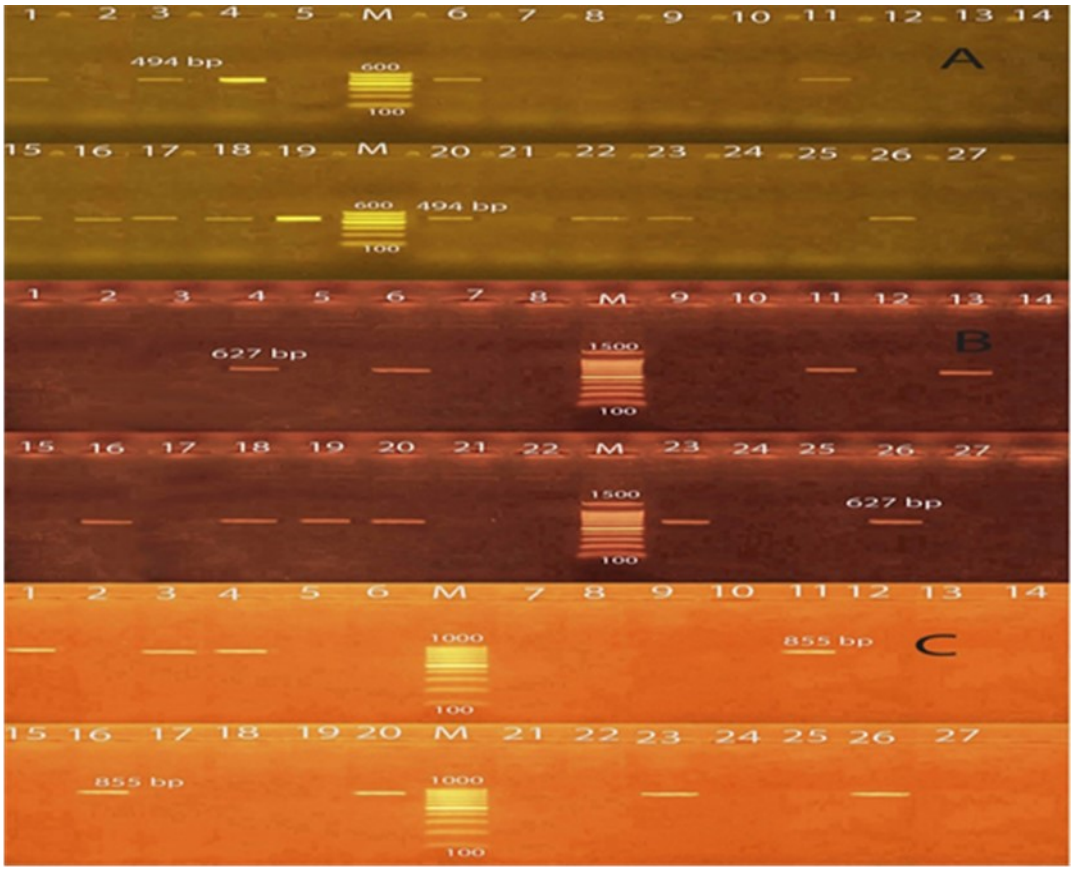

Figure (2): Agarose gel electrophoresis pattern illustrating typical amplification products of *vir*B11 (A), *wla*N (B) and *fla*A (C) genes. M: 100-bp DNA ladder; lanes 1-8: *C. jejuni* from human stool swabs (isolate no. 1H-8H); lanes 9, 10: *C. jejuni* from chicken liver (isolate no. 95Cl, 96Cl); lanes 11, 12: *C. jejuni* from chicken gizzard (isolate no. 106Cg, 107Cg); lanes 13, 14: *C. jejuni* from chicken breast meat (isolate no. 85Cbm, 86Cbm); lanes 15-17: *C. jejuni* from chicken thigh meat (isolate no. 73Ctm-75Ctm); lanes 18-20: *C. jejuni* from chicken cecal parts (isolate no. 50Ccp-52Ccp); lanes 21-23: *C. jejuni* from chicken cloacal swabs (isolate no. 31Ccs-33Ccs); lanes 24, 25: *C. jejuni* from chicken neck skin (isolate no. 64Cns, 65Cns); lane 26: positive control (*C. jejuni* ATCC 33291) and lane 27: negative control (PCR grade water).

Table 7 . Distribution of virulence profile among human and avian multi drug resistant *C.jejuni* isolates

Virulence profile	Human *C. jejuni* isolates (8)	Chicken *C. jejuni* isolates (17)	Total (25)
*vir*B11, *wla*N, *fla*A	1 (12.5%)	4 (23.5%)	5 (20%)
*vir*B11, *wla*N	1 (12.5%)	2 (11.8%)	3 (12%)
*vir*B11, *fla*A	2 (25%)	-	2 (8%)
*vir*B11	-	3 (17.6%)	3 (12%)
*wla*N	-	1 (5.9%)	1 (4%)

Genotyping and phylogenetic characterization of *C. jejuni* isolates using ERIC-PCR technique
The similarity between human and poultry isolates of the same profile calculated by Jaccard coefficient was 16.7-28.6% (18.2 and 20% in cluster II,
28.6% in cluster III, 20 and 25% in cluster IV and 16.7 and 22.2% in cluster

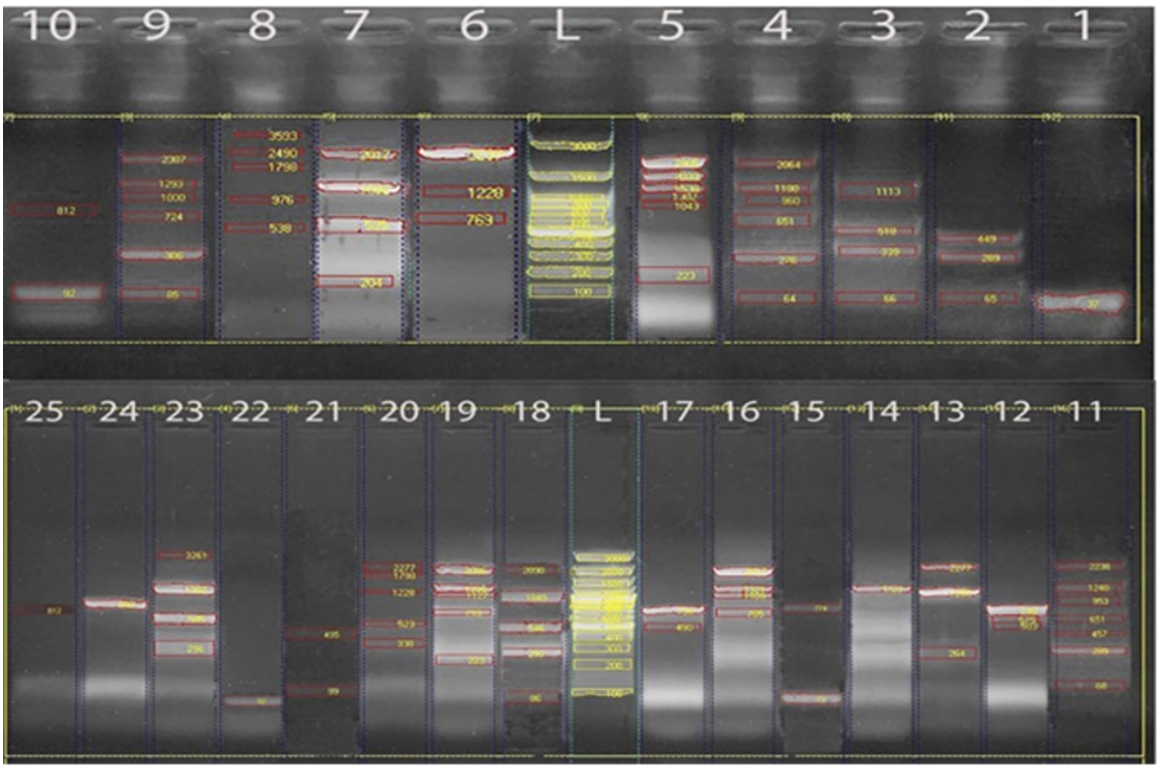

Figure (3): ERIC-PCR fingerprinting of *C. jejuni* isolates. lanes 1-8: *C. jejuni* from human stool swabs (isolate no. 1H-8H); lanes 9, 10: *C. jejuni* from chicken liver (isolate no. 95Cl, 96Cl); lanes 11, 12: *C. jejuni* from chicken gizzard (isolate no. 106Cg, 107Cg); lanes 13, 14: *C. jejuni* from chicken breast meat (isolate no. 85Cbm, 86Cbm); lanes 15-17: *C. jejuni* from chicken thigh meat (isolate no. 73Ctm-75Ctm); lanes 18-20: *C. jejuni* from chicken cecal parts (isolate no. 50Ccp-52Ccp); lanes 21-23: *C. jejuni* from chicken cloacal swabs (isolate no. 31Ccs-33Ccs); lanes 24, 25: *C. jejuni* from chicken neck skin (isolate no. 64Cns, 65Cns) and L: 100 bp DNA ladder.

Table 8. ERIC-PCR fingerprinting profiles and associated of 25 analyzed MDR *C. jejuni* isolates

ERIC-PCR profile	bp	Number of *C. jejuni* isolates	Isolates	numbers Cluster
E 1	92, 812	3	32Ccs, 65Cns and 96Cl	I
E 2	223, 296, 585, 710, 1043, 1127, 1302, 1330, 1538, 2033, 2086, 2905, 3261	3	5H, 33Ccs and 51Ccp	II
E 3	204, 535, 705, 1109, 1321, 1638, 2017	3	7H, 74Ctm and 86Cbm	III
E 4	264, 338, 523, 538, 769, 976, 1228, 1288, 1798, 2237, 2277, 2490, 3593	4	6H, 8H, 52Ccp and 85Cbm	IV
E 5	64, 65, 68, 276, 289, 449, 457, 651, 953, 960, 1180, 1248, 2064, 2238	3	2H, 4H and 106Cg	V
E 6	37	1	1H	Individual isolate
E 7	66, 339, 518, 1113	1	3H	Individual isolate
E 8	85, 306, 724, 1000, 1293, 2307	1	95Cl	Individual isolate
E 9	503, 575, 740	1	107Cg	Individual isolate
E 10	73, 774	1	73Ctm	Individual isolate
E 11	490, 736	1	75Ctm	Individual isolate
E 12	86, 292, 546, 1045, 2090	1	50Ccp	Individual isolate
E 13	99, 495	1	31Ccs	Individual isolate
E 14	892	1	64Cns	Individual isolate

bp: base pair, H: human, Ccs: chicken cloacal swab, Ccp: chicken cecal part, Cns: chicken neck skin, Ctm: chicken thigh meat, Cbm: chicken breast meat, Cl: chicken liver, Cg: chicken gizzard.

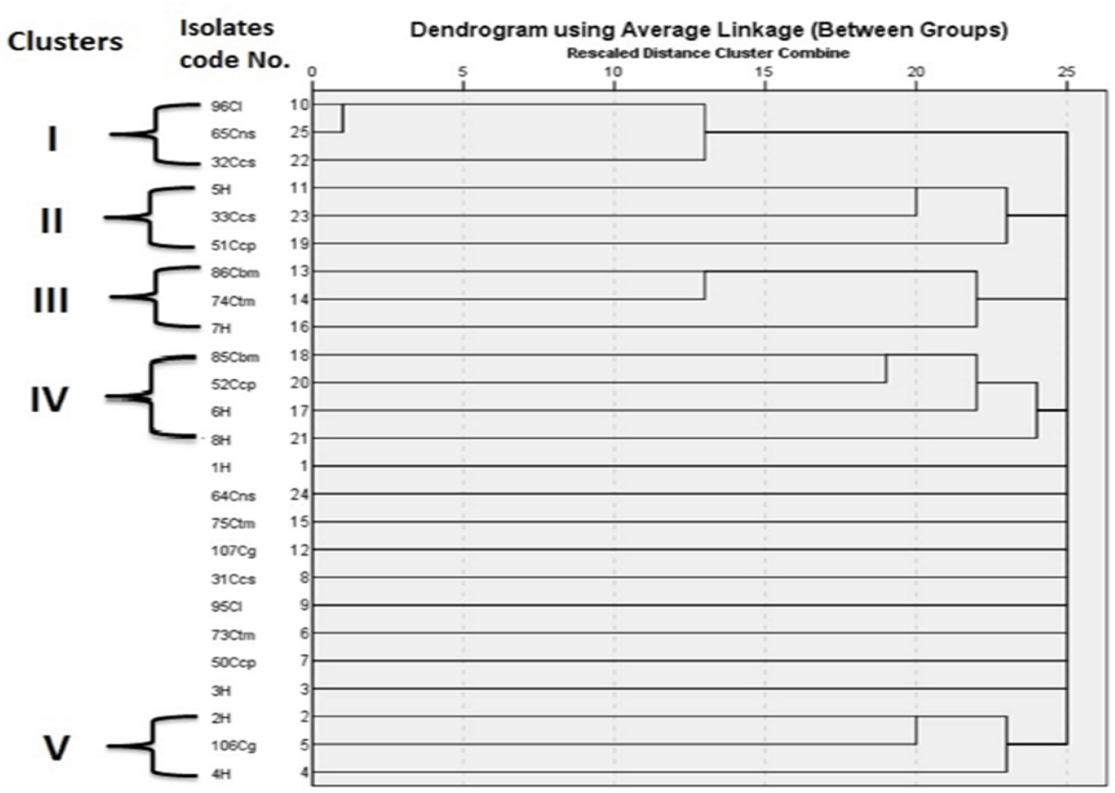

Figure (4): Dendrogram showing the relatedness of *C. jejuni* isolated from human and chicken origins as determined by ERIC-PCR fingerprinting using the SPSS computer software program.

H: human, Cl: chicken liver, Cg: chicken gizzard, Cbm: chicken breast meat, Ctm: chicken thigh meat, Ccp: chicken cecal parts, Ccs: chicken cloacal swabs, Cns: chicken neck skin.

Effects of sub-inhibitory concentrations of finger extract and Rosemary on the expression of critical virulence genes using the qRT-PCR assay

The effects of SIC of Rosemary and Ginger extract (0.0125% each) on the virulence of 4 MDR and multi-virulent *C.jejuni* isolates from chicken origins (isolate no. 106Cg, 74Ctm, 52Ccp and 33Ccs) were tested using qRT–PCR assay

Table 9. fold changes in expression of examined virulence genes after treatment of 4 C. jejuni isolates with sub inhibitory concentrations of rosemary and Ginger

	Relative gene expression *					
C. jejuni isolate number	*wlaN*		*virB11*		*flaA*	
	Rosemary	Ginger	Rosemar	Ginger	Rosemary	Ginger
106Cg	0.8526	0.5035	0.6071	0.3231	0.7071	0.5000
74Ctm	0.7071	0.5035	0.5743	0.4698	0.7220	0.5987
52Ccp	0.6071	0.3686	0.4796	0.2365	0.6690	0.3015
33Ccs	0.7270	0.4175	0.6199	0.2994	0.7631	0.4033

values represent the fold changes in comparison with the transcription levels of the control untreated isolates, which are assigned a value of 1.0

Cg: chicken gizzard, Ctm: chicken thigh meat, Ccp: chicken cecal parts, Ccs: chicken cloacal swabs

DISCUSSION

A total of 113 C. jejuni isolates were recovered from 345 different samples from various sources (32.8%). This prevalence rate is partially similar to that obtained in another study carried out in El-Fayoum, Egypt (27.3%) **(Mohammed and Aziz, 2019),** but it was higher than that reported in Qaluobya, Egypt (26.9%) (**Khalifa et al. 2013**).C. jejuni isolates were the prevalent among chicken cloacal swabs with an incidence rate of 54.3%, which is lower than that recorded in India (71%) **(Begum et al. 2015)**. In this work, C. jejuni isolates were found in 40% of chicken cecal parts. Similar results (41%) were observed in an earlier study carried out in Egypt **(Abd El-Tawab et al. 2015).** C. jejuni isolates were less prevalent in stool swabs or diarrheic children with a prevalence rate of 30%, which is higher than that obtained in China (4.8%) **(Huang et al. 2009)** .

Generally, the variation in the prevalence of C. jejuni among various sources between different studies could be due to different possible reasons like the type of examined specimens, climate factors, geographical area, health status, contamination condition, hygienic measures and the method of isolation as well as identification of the tested specimens **(Abd El-Tawab et al. 2015).** The main reasons that affect the campylobacter resistance are the indiscriminate utilization of antimicrobial agents in treatment of human infections as well as its excessive utilization in veterinary Medicine and as growth promoters in animal production (**Silva et al. 2018).**

Recently, the emergence of antimicrobial resistance in thermotolerant C. coli and C. jejuni originating from food of animal origin has become an alarming situation in both developing and developed countries with an important public health concern **(EFSA, 2013).**

The high incidence of erythromycin resistant *C. jejuni* clinical strains detected in this work is alarming, because erythromycin is often the antibiotic of choice for treating human campylobacter infection, which is unresponsive to fluoroquinolones. This leads to a significant problem, where the antimicrobial treatment become limited in numbers in case of human infection.The tetracycline resistance level observed for the examined *C. jejuni* isolates (90.3%).

majority of *C. jejuni* isolates were resistant to trimethoprim/ sulfamethoxazole and cephalothin with percentages of 82.3% and 75.2%, respectively. While *C. jejuni* isolates were susceptible to ciprofloxacin by a percentage of 49.6%.

Fluoroquinolones like enrofloxacin, norfloxacin, ciprofloxacin and nalidixic acid are significant antimicrobials as they are the primary drugs of choice for treatment of campylobacter infections in humans. For many years, these antimicrobials have been used in veterinary medicine and poultry production. Therefore, they must be used with caution to avoid the resistance of these pathogenic microorganisms to antimicrobials, which minimizes their available therapeutic options **(Chon et al. 2018).**

Moreover, 96.5% of the analyzed isolates were resistant to five or more antimicrobial agents, which collaborates with the result of a previous study in China (95.1%) **(Zhang et al. 2017).**

Antimicrobial agents have been widely utilized as supplements for growth promotion and disease prophylaxis in poultry husbandry. The overuse and misuse of the antimicrobial agents are accelerating the emergence and the incidence rate of MDR *Campylobacter* spp. at the farm level during the poultry husbandry, which can spread to humans through the food-chain leading to an increase in the human campylobacteriosis burden **(Ma et al. 2017).**

Therefore, there is an urgent need for developing preventive measures and strategies to control these bacteria and ensure the public health and safety.

Estimating the MAR indices for all examined *C. jejuni* isolates revealed that all the tested isolates (100%) had an index greater than

0.2, which is higher than the result observed in a previous study in South Korea (93.3%) **(Wei et al. 2016).** While three XDR *C. jejuni* isolates (2.7%) had an index of 0.9.

Regarding the distribution of the investigated virulence genes *vir*B11 gene was more prevalent among avian *C. jejuni* isolates (52.9%) than human ones (50%).

The difference may be attributed to the geographical variations, the virB11 plasmid nature and its invasiveness function.

These variations in the incidence of *fla*A, *vir*B11, *wla*N and *cdt*B genes may be due to the sample types and the isolates sources.

There were no statistically significant differences in the prevalence of *C. jejuni*, the resistance profiles and the prevalence of virulence genes among the *C. jejuni* isolates from chicken and human origin samples. These results are in complete harmony with the results from a previous study carried out in Poland (**Wieczorek et al. 2018).**

ERIC-PCR revealed 14 profiles with 5 main clusters corresponded to 16 isolates and 9 separate isolates. This is in agreement with **(Ahmed et al. 2015)** who reported that 18 profiles and four main clusters of the examined 31 *C. jejuni* isolates were detected.

ERIC-PCR had a higher discriminatory power than antibiotyping and virulotyping methods as it has a high discriminatory index (*D*= 0.94).

Campylobacter jejuni is an ubiquities microorganism that is found in poultry and its products. Therefore, in recent years, there is an urgent need for intervention strategies using natural compounds that have natural antimicrobial properties and are accepted by consumers such as phytochemicals including Rosemary and Giner extract (**Wagle et al. 2019)**

Recent researches have demonstrated that Giner and Rosemary extracts can change *C. jejuni* virulence and pathogenicity. This could be achieved by minimizing the *C. jejuni* attachment and invasion of the intestinal epithelial cells and also by changing the expression of its virulence factors such as motility, adhesion, invasion and cytolethal distending toxins (**Upadhyay et al. 2017)** The elects of SICs of Rosemary and Giner extracts the transcriptional modulation of virulence genes that facilitate poultry colonization such as flagellin gene (*fla*A), which is related to cell motility, adhesion and invasion, virulence plasmid gene (*vir*B11) that is related to cell invasion and β-1,3 galactosyltransferase gene (*wla*N), which is responsible for expression of Guillain-Barré syndrome were assessed by qRT-PCR assay.

All tested genes were found to be markedly down regulated after exposure to SICs of Rosemary. This is similar to a previous study in USA, which revealed that Rosemary caused down regulation in the transcription levels of *C. jejuni* virulence genes coding for motility (*fla*A) (**Wagle et al. 2019)** .Utilization of phytochemicals such as Rosemary and Ginger, which are safe to be used in food can be a safe and viable substitution to antimicrobials and chemical substances those are used to control *C. jejuni* in broiler chicken products.

CONCLUSION

The current study recommends further studied to find the proper control approaches for MDR and virulent *C. jejuni*. Moreover, Giner extract and Rosemary could potentially be used as feed supplements to control *C. jejuni* colonization in broiler chickens. Although, the results from this study are encouraging, follow-up studies investigating the efficacy of Giner extract and Rosemary in market age birds, cost benefits analysis of feed application and their effects on the organoleptic properties of products are warranted.

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