Journal of Medicinal and Chemical Sciences 5 (2022) 1191-1199

Journal of Medicinal and Chemical Sciences

Journal homepage: http://www.jmchemsci.com/

Original Article (Special Issue)

Identification of Genetic Markers of Drug Resistance and Virulence Factor Gene in *Campylobacter Jejuni* Isolated From Children in North Iraq

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ARTICLE INFO

Article history

Receive: 2022-05-19
Received in revised: 2022-06-31
Accepted: 2022-06-05
Manuscript ID: JMCS-2205-1510
Checked for Plagiarism: **Yes**
Language Editor:
Dr. Behrouz Jamalvandi
Editor who approved publication:
Dr. Yasser Fakri Mustafa

DOI:10.26655/JMCHEMSCI.2022.7.7

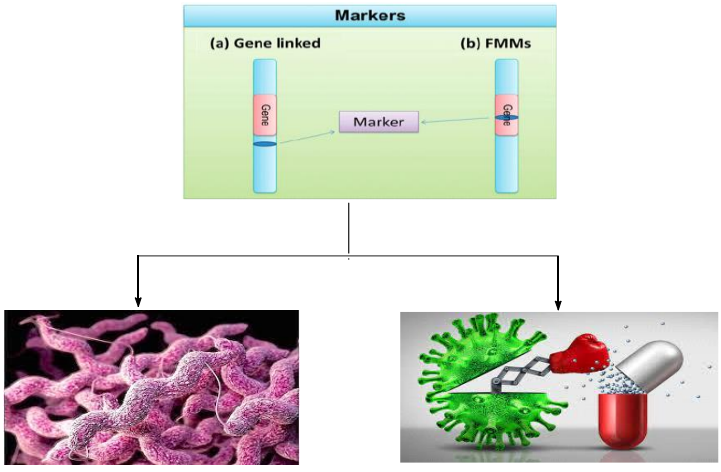
KEYWORDS

Campylobacteriosis
C. jejuni
Multidrug resist
Genetic determinants

ABSTRACT

Campylobacter(C)spp. are curved, Gram-negative. They are common prevalent causes of zoonotic disease. *C.jejuni and C.coli* are responsible of about 90% of *Campylobacteriosis*, associated with diarrheal symptoms. The collected samples included stool of children suffering from diarrhea (80,100%). The classical diagnostic methods like direct examination using gram stain or indirect examination via cultivation and purification with special medium assisted us to select 20 isolates (C1-C20, 25%), while biochemical reaction reduced the suspected samples to 9 isolates (C1, C2, C6, C7, C11, C13, C14 and C20). Antibiotic susceptibility test was carried out to show the bacterial susceptibility to the utilized antibacterial, revealing different percent as resist (tetracycline; 4samples, 45% C1, C2, C6 and C13) and sensitive 6(C6, C7, C13, C14, C17 and C20). Molecular screening was more sensitive by detecting some genetic elements responsible for drug resistance like *tetO* gene(3saples,559bp.tetracycline)and *apha-3*gene(2 samples,600bp.aminoglycosides),and *virB-11*(2 samples,708)as virulence factor gene. Finally, the sequence of *tetO* (3samples) was confirmed by diagnosing Campylobacter spp. as *C.jejuni*.

GRAPHICAL ABSTRACT



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Introduction

Campylobacter are small, spirally curved, Gram-negative. They are common prevalent causes of food-related infections over all the world. Their ability to multiply in a microaerophilic atmosphere (7-10%CO2), with a temperature range of 30-42◦C, distinguishes them from other foodborne pathogens [1].

There are currently 32 species and 13 subspecies belonging to this genus; among these taxa, the enteric pathogens *C. jejuni and C. coli* account for about 90% of *Campylobacteriosi*s [2,3]. Most clinical cases of *Campylobacteriosis* are associated with gastroenteritis, which include acute watery or bloody diarrhea, abdominal pain, vomiting, fever and dehydration, in addition to other complications, such as urinary tract infections, septicemia, arthritis, Guillain-Barre syndrome (GBS), irritable bowel and Miller Fisher syndrome, MFS [4,5,6,7].

Generally, multidrug resistance and ability to produce some virulence factors have been reported as an essential virulence determinants [8-10]. Aminoglycosides and tetracycline are antibacterial agents, commonly used against *Campylobacter* spp., which in turn, revealed variant resistance level against them [11]. Tetracyclines are used in treatment of both of animal and human infections, as a broad spectrum low economic therapy, making it a common prophylactic source incorporated with animal feeds at subtherapeutic doses to act as a growth promoter, which in turn leads to development of bacterial drug resistance [12,13]. Campylobacter tetracycline resistance is mostly conferred by a ribosomal protection protein (RPP). *Campylobacter tetO* is a gene responsible for tetracycline resistance, being capable of displacing tetracycline from its primary binding site on the 30Sribosomal subunit [14,15]. They can be located both on chromosome and plasmid [16]. RPPs are widely disseminated among bacterial genera and it has been reported that *tetO* gene was confirmed in eleven bacterial genera. Some were Gram negative and other Gram positive [33]; *tetO* acquisition among several members of bacterial isolates indicates that genetic elements like plasmids, transposons, or recombinant process events contribute to distribution and conserve it [17]. Aminoglycosides with broad antimicrobial spectrum inhibit protein synthesis via*16SrRNA* binding of ribosomal small subunit [18]. *Campylobacter* spp. resists aminoglycosides by inhibiting antimicrobial binding affinity for target sites due to enzymatic modification, like acetylation, phosphorylation or amino group adenylation [19,20]. Several virulence factor genes were identified in *Campylobacter* subspecies, including those encoding proteins involved in bacterial invasion. Genetic analysis revealed that *Campylobacter* have pathogenicity islands (PIs) harbors genes encoding bacterial type IV secretion system, *T4SS, virB-virD4* genes. In vitro studies demonstrated that the T4SS contributes to invasiveness of Campylobacter virulence factor [21].

This project focused on isolation and purification of *Campylobacter* spp., investigation of its resistance to some antibacterial and their responsible genetic determinants. In addition, we investigated virulence factor gene responsible for invasion of several species belonged to genus *Campylobacter.*

Materials and methods

Samples collection

Campylobacter isolates of this study came from kids' faeces samples (80), suffering from diarrhea in Medya Diagnostic center in Irbil /Iraq during May-October 2018.

Isolation and phenotypic identification

Purification and identification of *Campylobacter* spp. were carried out according to ISO10272-1:2006 protocol. All samples were cultivated onto modified Charcoal Cefoperazone Deoxycholate agar, mCCDA (Oxoid, UK) under micro-aerophilic condition with 5-10%CO_2 at 41 °C for 48-72 h to isolate bacterial strains in single pure form. Suspected colonies were cultivated onto Columbia Blood agar (Oxoid, UK), under the same incubation conditions. The classical macroscopic and microscopic investigation were conducted as prediagnosis. Biochemical reactions (Urease, Indolacetatehydrolysis, H_2S production and

Hippurate hydrolysis) were considered as a second diagnostic step to confirm *Campylobacter* spp**.** [1,22,23].

Antibiotic susceptibility profile

This test was conducted to distinguish antibiotic susceptibility of suspected campylobacter isolates (9) to some antibiotics utilized as therapeutic options for Campylobacter recovered from diarrheal case. Utilizing Mueller-Hinton agar, the disk diffusion method was done for ampicillin (10 µg), erythromycin (15 µg), tetra-cycline (30 µg), co-trimoxzole (25 µg), cephalothin (30 µg), gentamycin (10 µg), cefotaxime (30 µg) and oxacillin, 1 µg (Oxoid, UK). The interpretations were reported according to guidelines from Clinical and Laboratory Standards Institute [24].

Molecular screening profile

Genomic DNA (gDNA) extraction

DNA was extracted using Wizard genomic DNA (g DNA) Purification kit (Promega, USA), according to manufacturer's instructions. DNA integrity was evaluated via OWL electrophoresis system (Thermo, USA) using 5 µl of gDNA on 1% agarose gel concentration (Promega, USA). DNA concentration was estimated by Quantus Fluorometer (Promega, USA), which was standardized to 10ng/µl. and preserved at-20 until use [25].

Genetic determinants and amplification and sequencing screening

PCRs premix was 12.5 µl (Promega, USA); additional components were 1 µl (10 µM) of each of forward and reverse primer sets (Promega, USA) and 2 µl of gDNA (10 ng/µl). The final volume was completed by 25µl. with nuclease free water (8.5 µl). The primer sets used in this research are listed in Table 1. Reaction steps were denaturation at 94 °C for 4 min, and 35cycles of denaturation at 94 °C for 30 sec. Primers anneal temperatures involved 50, 52 and 55 °C for 30 sec. to hybridize *tetO, virB11*, and *aphA-3,* respectively [26,27], extension at 72 ◦C for 30sec and final extension at 72 °C for 5mins. PCR products of *tetO* gene were purified using PCR Purification Kit (Promega, USA). Then, it was sequenced and analyzed for forward by macrogen (S. Korea) using genetic analyzer (Applied Biosystems 3500, USA). The sequence was aligned (MegaX software) and assembled on the National Center for Biotechnology Information (NCBI) and the Basic Local Alignment Search Tool (BLAST).

Table 1: Sequences of primer sets used in this study

Gene	Sequence (5'- 3')		Amplicon size	reference
TetO	F	GGCGTTTTGTTTATGTGCG	559 bp.	BACON *et al. (*2000).
	R	ATGGACAACCCGACAGAAGC		
VirB11	F	GAACAGGAAGTGG AAAAACTAGC	708 bp.	BACON *et al. (*2000).
	R	TTCCGCATTGGGCTATATG		
aphA-3	F	GGGACCACCTATGATGTGGAACG	600 bp.	GIBREEL *et al*. (2004).
	R	CAGGCTTGATCCCCAGTAAGTC		

Results and Discussion

Microscopic, macroscopically and biochemical tests of Campaylobacter spp.

Twenty samples were suspected as *C. jejuni*, using routine staining depending gram staining method, under oil immersion as comain shape. On the other hand, biochemical reaction was conducted to confirm the diagnosis of *C. jejuni*. Nine selected isolates showed positive results for hyppurate hydrolysis and indole acetate hydrolysis. In addition, they revealed a negative result for urease, oxidase and H2S production, as detailed in Tables 2 and 3.

Table 2: Gram stain and two biochemical reactions for Campylobacter prediagnosis

Test \ Isolate	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
Gram stain	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Hyppurate hydrolysis	+	+	_	_	_	+	+	_	_	_	+	_	+	+	_	_	+	_	_	+
Indole acetate hydrolysis	+	+	_	_	_	+	+	_	_	_	+	_	+	+	_	_	+	_	_	+

(C)Compaylobacter spp.,(+) positive result,(-) negative result

Our results were agreed with those documented by [28] who detected *C.jujeni* utilizing traditional diagnostic methods. Another study was carried out to isolate and detect *C.jujeni*from food source contaminated with it [29], depending on biochemical reaction for determining this pathogen

Table 3: Microscopic and biochemical diagnosis of *C. jejuni*

Test	Suspected samples	Positive result	Negative result
Microscopic examination	80	20(C1-C20)	60
Biochemical tests	20	9(C1,C2,C6,C7,C11,C13,C14 and C20)	11

Antibacterial susceptibility surveillance

Nine samples were diagnosed as *C.jujeni,* revealing variant levels between resistance and sensitivity to several utilized antibacterial agents, but C1,C2 and C6 resisted most antibiotics. In contrast, C7 and C20 were susceptible to more than two antibiotics. More details are presented in Table (4) below.

Table 4: Antibacterial susceptibility test for *C.jujeni.* isolated trains

Antibacterial	Sensitive isolates	(%)	Resistance isolates	(%)	Moderate	(%)
Tetracycline	4(C7,C11,C17 and C20)	44%	4(C1,C2,C6 and C13)	45%*	1(C14)	11%
Ampicillin	0	0	9(C1-C20)	100%	0	0
Gentamycin	6(C6,C7,C13,C14,C17and C20)	67%	1(C1)	11%*	2(C2 and C11)	11%
Oxacillin	3(C6,C7 and C20)	33%	4(C1,C2,C14 and C17)	45%*	2(C11 and C13)	22%
Co-trimoxazole	2(C11 and C20)	22%	5(C1,C2,C6,C13 and C17)	56%	2(C7 and C14)	22%
Erythromycin	0	0	9(C1-C20)	100%	0	0
Cephalothin	2(C11 and C20)	22%	6(C1,C2,C13,C14 and C17)	67%	2(C6 and C7)	22%
Cefotaxime	4(C6,C13,C17 and C20)	44%	3(C1,C7 and C14)	33%	2(C2 and C11)	22%

Denotes approximately

Many studies have been carried out to check *C.jejuni* reaction towards most common antibacterial agents, like Issa.'s (2018) inquiry, reporting that this gram negative bacterium revealed high resistance percent to tetracycline (70.3%) and low percent of resistance to gentamicin (4.2%), the results of which are in agreement with those of the present study, while a study detected high percent of resistance to gentamicin (100%), and tetracycline (100%) [30], being dissimilar to our screening in this research article. Over all, resistance level was increased by *C.jejuni* to some antibacterial in the last decade. Due to use of many common antibacterial as a prophylactic option with animals feeding, acquired mutation have an important role in amino acids substitution, while the genetic elements transferring have a critical effect on acquisition of new mutations, which in

turn lead to emerge multi-drug resistance bacteria [31,32].

Molecular screening profile

Molecular assay assist in detection and classification of *C.jejuni*. In this field, the genetic determinants are responsible for antibacterial resistance, like *tetO* (559bp), *apha-3*(708bp). In addition, *virB11*(600bp) as a virulence factor gene was demonstrated via electrophoresis. Figure 1 represents the gel pages with amplicons belonging to previous genes.

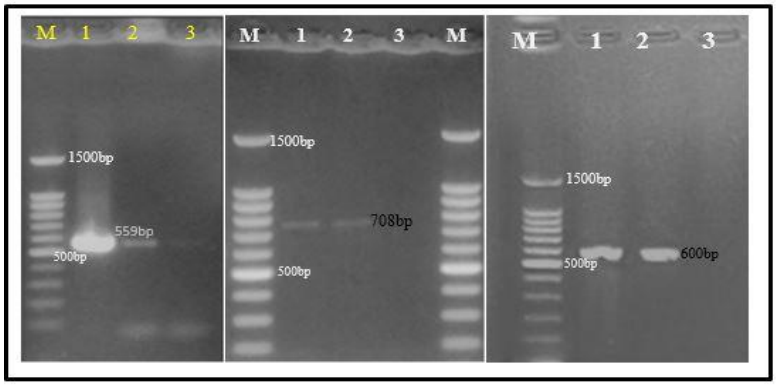

Figure 1: Genetic determinants `s amplification products. At right *TetO*(559bp.), lines 1-3(C1, C2 and C13) are positive result, in the middle*a phA-3*(708bp), lines 1&2(C1and C13) are positive results, and at left *virB11* (600bp.) lines 1& 2 (C1andC2) are positive results

The present outcomes for amplification of *tetO* (559bp.)*, aphA-3*(708bp) and *virB11* (600bp.) are in line with those detailed by [26] and [27], respectively, for detection of previous genes.

The phenotypic investigation revealed that four isolates (45%) resisted tetracycline, while molecular screening showed that three isolates (33%) represented a positive result for presence of *tetO* gene. *TetO* had a critical role in tetracycline resistance, via displacing tetracycline from its primary binding site on the small ribosomal subunit [33]. Additionally, in many tetracycline resistance cases there are other genes like *tetw* or *tet (O,32, O)* that may be play the same role in the incidence of tetracycline resistance phenomenon [34]. At the same time, two isolates (50%) were positive for the presence of *aphA-3* (708bp), as aminoglycoside resistance gene (Gentamicin) whereas one isolate resisted gentamicin phenotypically. These outcomes mean that molecular survey was more sensitive for detection of the genetic determinants of antibiotic resistance, as shown in Table (4). On the other hand, two isolates (C1&C2) were positive for presence of *virB11*gene (bp.), that were responsible for invasion of protein.

Table 4: Phenotypic and genotypic comparison of tetracycline and aminoglycosides resistance

Tests		PCR				Total
		Positive		Negative		
		No.	%	No.	%	
Antibacterial susceptibility assay (Tetracycline)	+ve 4	3	33.3	1	11	9(100%)
	-ve 5	0	0	5	56	
Antibacterial susceptibility assay (Gentamicin)	+ve 1	2	22	0	0	9(100%)
	-ve 8	0	0	8	89	

Sequence alignment and phylogenetic tree of Campylobacter*tetO* gene are presented. The sequences of the three *tetO* gene amplicons was depended on investigating the relationship among *C.jejuni* isolated strains (C1,C2andC13) in this research. Sequences outcomes revealed high

similarity among previous isolates, as that appeared in the dendogram.

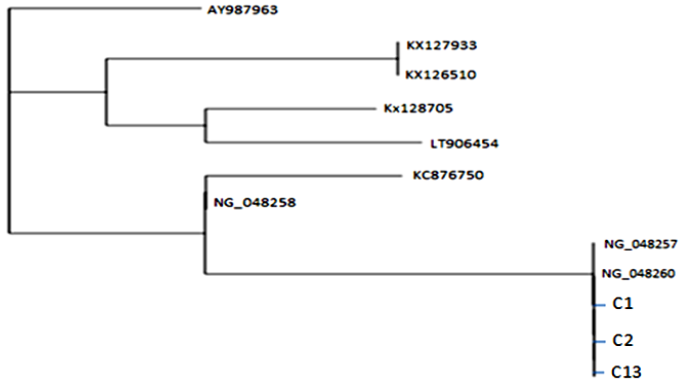

Figure 2: Phylogenetic tree show, high similarity between C-1,C2 andC-13, on the basis of the sequences alignment

The sequence of *tetO* gene was aligned using BLAST (Basic Local Alignment Search Tool). The identification percents (100%) for all sequences (C1, C1 andC13) revealed that three bacterial isolates belong to *C.jujeni* , as those documented in Figures 3, 4 and 5.

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Figure 3: Alignment of C1-*tetO* sequence with known sequence in NCBI (Accession no.: CP044169.1)

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Sbjct  1633966  AGAGCCTACTGTTATATATATGGAAAGACCGCTTAGAAAAGCAGAATATACCATCCACAT  1634025
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Figure 4: Alignment of C2-*tetO* sequence with known sequence in NCBI (Accession no.: CP044175.1)

```
>Campylobacter jejuni strain AR-0414 chromosome, complete genome
Sequence ID: CP044169.1 Length: 1714574
Range 1: 1641635 to 1642158

Score:933 bits(505), Expect:0.0,
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Figure 5: Alignment of C3-*tetO* sequence with known sequence in NCBI (Accession no.: CP044169.1)

Conclusion

According to the present results, we concluded that *C.jujeni* is an important pathogen, due to having a genetic elements like plasmid and transposon and integron. In addition to many specific sequences known as PAIs, it assists transport by livestock animals and food born, and also resists many antibiotics due to acquiring resistance genes via conjugation or transformation or by genetic manipulation; this may reduce the therapeutic options in this field, while the presence of virulence factors genes assists this bacterium to invade and colonize many organs outside the intestinal gut. The molecular screening showed high sensitivity and specificity for detection of the genetic determinants in this research in comparison to phenotypic diagnosis, whereas the sequences alignment is still as a gold standard tool to confirm the primary diagnosis foe *C.jujeni.*

Acknowledgment

I would like to thanks to the department of biology, Collage of Education for Girls, University of Mosul for laboratory facilities.

Funding

This research did not receive any specific grant from fundig agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

There are no conflicts of interest in this study.

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https://doi.org/0000-0003-4912-6045

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HOW TO CITE THIS ARTICLE

Baydaa Ghanim Mohammed. Identification of Genetic Markers of Drug Resistance and Virulence Factor Gene in Campylobacter Jejuni Isolated From Children in North Iraq. *J. Med. Chem. Sci.*, 2022, 5(7) 1191-1199

https://doi.org/10.26655/JMCHEMSCI.2022.7.7

URL: http://www.jmchemsci.com/article_153449.html