Prevalence of Campylobacter Species in Diarrheal Samples of Children Less than 10 Years

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Abstract

Introduction: Campylobacter spp. is one of the most common causes of diarrheal diseases all over the world, with a rapid acquisition of antibiotic resistance.

Objective: Detect the frequency of Campylobacter species in diarrheal stool of children under ten years by conventional and molecular methods, and detect the bacterial ability to produce biofilm.

Methods: A total of 200 children presented with diarrhea had been enrolled in this study. Campylobacter was isolated and diagnosed primarily by characteristic features on Gram stain, culture media, biochemical tests, and tested against 13 antibiotics by disc diffusion method. The ability of biofilm production was tested by crystal violet quantitative ELISA microtiter plate assays. Then Campylobacter spp was detected by Multiplex PCR using species specific genes.

Result: The prevalence of Campylobacter spp. was 17% by conventional methods and 15% by molecular method. The results of antibiotic susceptibility test showed that, there is complete resistance (100%) to cephalothin, ampicillin, and clindamycin for both species, full resistance (100%) to trimethoprim-sulfamethoxazol and erythromycin by *C.coli*, and high resistance (92.3%) to trimethoprim-sulfamethoxazol and erythromycin by *C.jejuni*, while the lowest resistance was to nalidixic acid and amikacin (7.7%) by *C.jejuni*, and (12.5%) to tetracyclin, amoxicillin, amikacin, chloramphenicol, and ciprofloxacin by *C.coli*. The frequency of biofilm production in all positive Skirrow's culture was (35.29%) as 12 out of 34 positive isolates, ranging from mild to severe biofilm formation. By PCR assay, 64.7 % (22 of 34) positive Skirrow's culture were also positive based on *hipO* gene specific for *C.jejuni*, while the prevalence of *asp* gene was (23.5%).

Conclusion: The prevalence of Campylobacter spp. was 17% by conventional methods and 15% by molecular methods, most of *Campylobacter jejuni* and *Campylobacter coli* isolates were MDR, and sensitive only to limited number of antibiotics, many Campylobacter isolates produce biofilm, there was highly significant correlation between hippurate hydrolysis results and molecular detection of Campylobacter spp. depending on *hipO* and *asp* genes.

Keyword: *Campylobacter jejuni*, *Campylobacter coli*, *hipO* gene, *asp* gene, biofilm.

Introduction

Genus Campylobacter consists of about 26 species, the most important disease-causative agents are *Campylobacter jejuni* and *Campylobacter coli* [1], Campylobacteriosis is characterized by watery and/ or bloody diarrhea, fever, malaise, abdominal pain, cramps, and vomiting. It is more dangerous for young children due to risk of dehydration resulted from loss of nutrients and essential components of food; like proteins and salts [2].

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Laboratory diagnosis can be done on stool samples, rectal swabs, and even blood in case of *Campylobacter fetus,* the diagnosis relay on culture using selective media, molecular detection, or rapid diagnostic test like Gram stain and biochemical tests. Many studies have investigated the genes responsible for differentiation between species including *hipO* and *asp* genes. The *hipO* gene region is the hippuricase gene specific for *C. jejuni* [3], and the *asp* gene region, the aspartokinase gene specific for *C. coli* [4].

Patients and Methods

A total of 200 patients were included in this study. Stool samples were collected during a period from the first of September 2019 to the end of January 2020 from Al-Imamain Al-Kadhimain Medical City,Baghdad. Campylobacter was isolated and identified by ordinary methods according to morphological characteristics on cultures, biochemical, and molecular methods in the laboratories of microbiology department /College of Medicine/ Al-Nahrain University. This study was approved by the ethical committee of the College of Medicine-Al-Nahrain University.

Antibiotic susceptibility testing

Campylobacter isolates were tested for their susceptibility to thirteen antimicrobial agents in accordance to CLSI 2016 recommendations [5]. These antibiotics are amickacin(AK) 30 µg/disk, amoxicillin (AX) 30µg/disk, ampicillin (AM) 10 µg/disk, cephalothin(KF) 30 µg /disk, chloramphenicol(C) 10 µg/disk, ciprofloxacin(CIP) 5 µg/disk, clindamycin(DA) 5 µg/disk, erythromycin(E) 15 µg/disk, gentamicin(CN) 10, nalidixic acid(NA) 30 µg/disk, rifampin(RA) 5 µg/disk, Sulfamethoxazole/trimethoprim(SXT) 25 µg/disk, and tetracycline(TE) 30 µg/disk.

Biofilm formation

Detection of biofilm formation was performed by crystal violet quantitative ELISA microtiter plate assay as described by Fields *et al*. (2008) [6].

DNA Extraction

DNA was extracted according to the manufacture instructions using Geneaid Presto Stool DNA Extraction Kit Quick Protocol.

Molecular Detection of *hipO* and *asp* genes

Sequences of primers had been designed and synthesized in Alpha-DNA (Canada) as showed in tables (1 & 2). For preparation of the PCR mixture reaction, DNA template (5-50 ng), forward and reverse primers were diluted by adding nuclease free water to the desired concentrations and mixed by pipetting with master mix (Accupower Bioneer PCR premix kit , Korea) as illustrated in table (3). PCR mixture without DNA template was used as negative control.

Table (1): Sequences and product size of *hipO* gene

hipO gene	Nucleotide sequences (5' to 3›)		Products bp	References
hipO	F R	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	735	Linton et. al, 1997 [7]

Table (2): Sequences and product size of *asp* gene

asp gene	Nucleotide sequences (5' to 3›)		Products bp	References
asp	F R	GGTATGATTTCTACAAAGCGAG ATAAAAGACTATCGTCGCGTG	500	Harzandi et. al, 2015 [8]

Table (3): The mixture of PCR working solution for detection of *hipO* and *asp* genes in Campylobacter spp.

Component	Concentration	Volume µl
Master mix	1X	0 µl
Primer F.	10pm/ µl	3.0 µl
Primer R.	10pm/ µl	3.0 µl
DNA Template	5-50 ng	4 µl
Deionized D.W.	-	34 µl
Total volume		50 µl

The reaction was carried in Thermal cycler (Cleaver Scientific Thermal Cyclers- TC32/80, UK) according to the program showed in table (4).

Table (4): PCR program for amplification of Campylobacter genes by thermal cycler:

No.	Steps	Temperature	Time	No. of cycles
1	Initial denaturation	96˚C	6 minutes	1
2	Denaturation	95˚C	40 seconds	35
3	Annealing	60 ˚C	45 seconds	
4	Extension	72 ˚C	1 minutes	
5	Final extension	72 ˚C	10 minutes	1

Gel electrophoresis

A 6 µL of each amplified sequence and 100 bp ladder resolved by electrophoresis according to Sambrook and Russell (2001) [9]. The products were visualized in UV trans-illuminator (LKB, Sweden).

Results

In regard to culture on Skirrow's medium, Catalase, and Oxidase tests; out of 200 samples, there were 34 (17.0 %) positive and 166 (83.0%) negative, while according to hippurate hydrolysis test, 26 (13.0%) were positive and 174 (87.0%) were negative, within culture the percentage of female was 55.9% while the male was 44.1% and according to the age group, the highest percentage of Campylobacter isolates was (35.29%) in age group (7.5-9 years), while the lowest percentage was (8.8%) in age group (≤ 1 year).

Antibiotic susceptibility test

The results of antibiotic susceptibility test showed that, there is complete resistance (100%) to cephalothin, ampicillin, and clindamycin for both species, full resistance (100%) to trimethoprim-sulfamethoxazol and erythromycin by *C.coli*, and high resistance (92.3%) to trimethoprim-sulfamethoxazol and erythromycin by *C.jejuni*, while , there was low resistance to chloramphenicol and gentamicin (34.61%), ciprofloxacin (26.92%), nalidixic acid and amikacin (7.7%) by *C.jejuni*, and low resistance to rifampin (37.5%), tetracyclin, amoxicillin, amikacin, chloramphenicol, and ciprofloxacin (12.5% for each) by *C.coli*.

Biofilm detection of Campylobacter isolates

With reference to measurement of biofilm optical density (O.D.) by ELISA reader, it has been shown that cut-off value was equal to (0.165) nanometers, the isolate which had OD less than the cut-off value was considered non-biofilm producer while the isolate that had OD equal or higher than the cut-off value was considered as biofilm producer. This study showed that (35.29%) of isolates produce biofilm while (64.7%) were not biofilm producer.

The association between biofilm and antibiotic resistance

The current study showed that all of the 12 biofilm producing isolates (100%) were resistant to cephalothin, clindamycin, and ampicillin, while 11 (91.66%) of them were resistant to trimethoprim and erythromycin. Statistically there is no significant difference (*P*= 0.7) between biofilm formation and antibiotic resistance in Campylobacter species.

Multiplex Polymerase Chain Reaction screening for *hipO* and *asp* genes

Sequences amplification of Campylobacter species-specific genes (*hipO* and *asp*) was done by multiplex PCR technique with product size 735bp of *hipO* gene and 500bp of *asp* gene, as shown in figure (1).

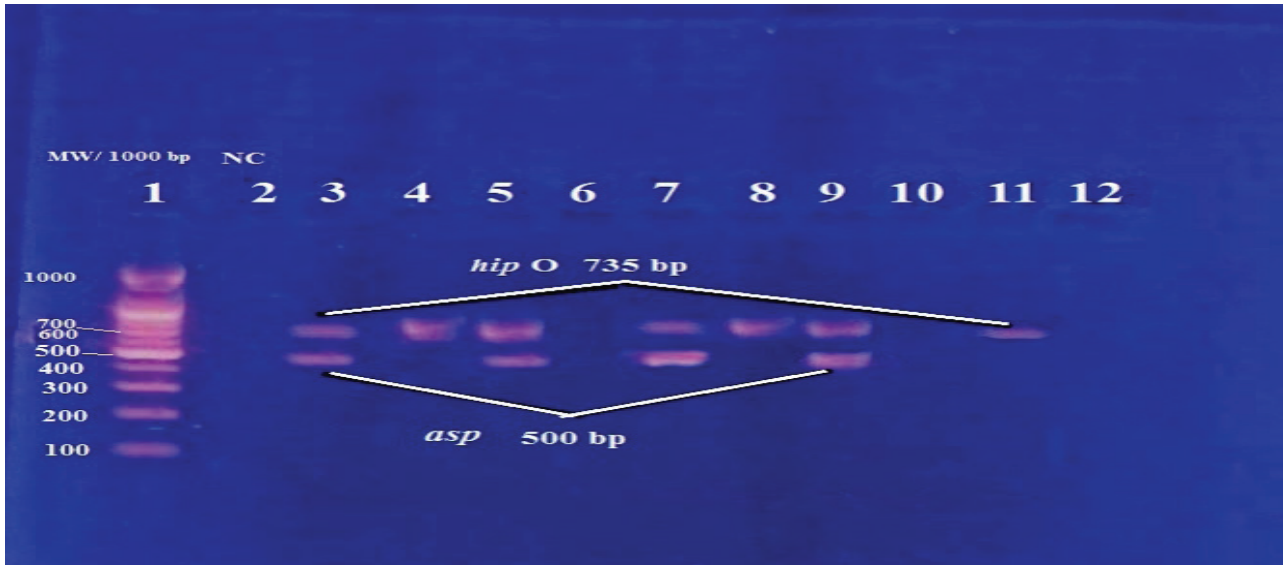

Figure (1): Gel electrophoresis of PCR products (735 bp) for *hipO* gene and (500 bp) for *asp* gene. Lane 1: 100bp ladder. Lane 2: Negative control. Lanes 3-12: PCR products of clinical stool samples. (2% agarose, 7 v/cm^2, 45 min).

The current result showed that, 16 (72.7%) of *C.jejuni* isolates detected by PCR according to presence of *hip*O gene had no biofilm , while only one isolate had low-level (4.5% within PCR *hipO*), 3(13.6%) of isolates had mid-level and 2 had high-level (9.1%), as appeared in table (5). The biofilm degree according to PCR *hipO* result for *C.jejuni* revealed that there is no significant differences between them (*P*=0.26).

Table (5): Biofilm degree in correlation with PCR *hipO* gene

P=0.26			PCR hipO for C.jejuni		Total %
			Negative %	Positive %	
Biofilm degree	No biofilm	Count	6	16	22
		% within Biofilm degree	27.3	72.7	100.0
		% within PCR hipO	50.0	72.7	64.7
		% of Total	17.6	47.1	64.7
	Low level biofilm	Count	3	1	4
		% within Biofilm degree	75.0	25.0	100.0
		% within PCR hipO	25.0	4.5	11.8
		% of Total	8.8	2.9	11.8
	Mid-level biofilm	Count	1	3	4
		% within Biofilm degree	25.0	75.0	100.0
		% within PCR hipO	8.3	13.6	11.8
		% of Total	2.9	8.8	11.8
	High level biofilm	Count	2	2	4
		% within Biofilm degree	50.0	50.0	100.0
		% within PCR hipO	16.7	9.1	11.8
		% of Total	5.9	5.9	11.8
Total		Count	12	22	34
		% within Biofilm degree	35.3	64.7	100.0
		% within PCR hipO	100.0	100.0	100.0
		% of Total	35.3	64.7	100.0

This result also touched on the frequency of biofilm degree according to PCR asp for C.coli and showed that, there were 4 (50.0%) of C.coli isolates positive for asp gene with no biofilm, 2(25.0%) had low-level and one isolate had mid and high-level of biofilm formation.

Discussion

This study revealed that Campylobacter distribution in clinical samples by Skirrow's media was with percentage of (17%) in Iraqi patients, these results agreed with the study conducted in Northern Poland by Szczepanska *et al*., 2017 [10], who reported that Campylobacter distribution was 15.5% (306/1973) of all analyzed samples. The current study disagreed with a study conducted in South Africa by Martina O. *et al.*, 2019 [11], who reported that out of 505 paediatric stool

specimens; (46.9%) 237 Campylobacter isolates were collected.

The present study also exhibited that infected females were more than males, with a percentage of isolates (44.1%) in males and (55.9%) in females, which agreed with a study conducted in Ecuador by Toledo *et al*., 2017 [12], who reported that out of the 127 children studied, 17 harbored Campylobacter sp. corresponding 12.9% (9/70) to boys and 14.0% (8/57) to girls. Whereas the current result disagreed with a study done in Nigeria by Adekunle *et al*., 2019 [13], who reported that from the 815 subjects with diarrhea, 347 (42.6%) were females and 468 (57.4%) were males. The reason of the present result is that; the number of samples were collected from females more than males.

According to the Age group, this study showed that, the highest percentage of Campylobacter isolates (35.29%) was in age group (7.5-9 years), while the lowest percentage was (8.8%) in age group (≤ 1 year). The infection with Campylobacter occurs with high percentage in young children due to outdoor activities and contact with animals. This study agreed with a study done in Sub-Saharan Africa by Noel *et al*., 2020 [14], who reported that the country-level mean prevalence in totally-all ages and under-five children was 18.6% and 9.4%, respectively.

Emergence of antibiotic resistance is a never-ending process due to its capacity to resist and acquire various resistance mechanisms against antibacterial drugs. The results of this study showed that 100% of *Campylobacter jejuni* and *Campylobacter coli* isolates were resistant to cephalothin, clindamycin, and ampicillin when tested by standard disc diffusion method. Similar findings in a study conducted in South Africa by Otigbu *et al*., 2018 [15], who reported that, the high rates of resistance to clindamycin (84.2%) make this antibiotic a poor choice for treatment of infections with Campylobacter.

Also the current study showed that the *Campylobacter jejuni* and *Campylobacter coli* developed low resistance (7.7 %) and complete sensitivity (0%) to Nalidixic acid respectively, and this result agreed with a study conducted in Lebanon by Ibrahim *et al*., 2019 [16], who proved by disc diffusion that a low level of resistance to nalidixic acid (17.2%) was found in *Campylobacter* isolates. But the present study disagreed with a study published in Korea by Park *et al*., 2019 [17], who reported that higher resistance rates of *Campylobacter jejuni* (90.0%) developed against Nalidixic acid.

In this study among 34 clinical isolates, there were 12 (35.3%) Campylobacter spp. showed biofilm formation ability. Within positive biofilm the percentage was 66.7% and 33.3% for *Campylobacter jejuni* and *Campylobacter coli* respectively. And within positive culture it was 23.5% and 11.8% respectively. This study agreed with a research was done in Malaysia by Huei *et al*., 2014 [18], who reported that out of 17 *Campylobacter jejuni* isolates, 3 (17.6%) were positive for biofilm formation within positive culture. The present findings were higher than results of a study done in Korea by Kim *et al*., 2017 [19], who reported that 18% (14 of 78) Campylobacter isolates were able to produce biofilm.

Two species-specific genes (*asp* and *hipO*) were identified in Campylobacter isolates using Muliplex PCR technique. The result of this study revealed that the frequency of Campylobacter isolates by Muliplex PCR was 15%. In contrast to cultural identification which revealed that Campylobacter spp. frequency was 17% (34 positive culture out of 200 samples), this difference could be due to the low concentration of DNA tested in molecular methods.

The current study coincides with a study published in Thi-Qar Governorate by Harb *et al*., 2019 [20], who reported that the frequency of Campylobacter spp. by molecular technique (PCR) was 10.9% (17/155). Whereas this study disagreed with a study published in China by Ying *et al*., 2018 [21], who reported that molecular isolation ratio of Campylobacter was 7.0% (26/370).

According to the polymerase chain reaction results of Campylobacter genes, the percentage was 11% for *hipO* gene (*Campylobacter jejuni*-specific gene) and 4% for *asp* gene (*Campylobacter coli*-specific gene), which resembles a study done in Italy by Bianchini *et al.,* 2014 [22], who published that *C. jejuni* was detected in 34 from total 282 samples (12%) of dairy products by PCR. But it is much lower than a study result conducted in Iran by Mryam *et al*., 2018 [23], who reported that detection rates for the *hipO* gene was 91% of samples.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

Acknowledgment : I express my deepest gratitude to all staff of Microbiology Department /Al- Nahrain University /College of Medicine and staff of laboratory department of Al- Imamain Al-Kadhumian Medical city for their endless cooperation by equipments and experiences.

Author's contribution: Ghada N. Khalaf: Preparation, performing and doing the tests of the research.

Dr. Thanaa Rasheed Abdulrahman: Advise and interpretation the results, research done under her supervision.

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