ISOLATION, IDENTIFICATION AND TOXIGENIC ASPECTS OF *Campylobacter jejuni* ISOLATED FROM SLAUGHTERED CATTLE AND SHEEP AT BASRAH CITY

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ABSTRACT

The study was conducted from (December 2010 to June 2011). During this study 330 samples were collected including 210 bile samples (130 of sheep and 80 of cattle) and 120 fecal samples (70 of cattle and 50 of sheep) from Basrah abattoir. These samples were analyzed for checking the presence of *Campylobacter jejuni*using selective media, biochemical, serological and biotyping tests. The final isolation rate of *Campylobacter jejuni*was 13.3% from total samples, isolation rate from bile was 17.6% while from fecal samples was 5.8% (X^2=7.24, P=0.007, DF=1). The isolation rate from sheep was 14.5% while from cattle was 11.5%,the differences in isolation rates was statistically significant **(**X^2=0.46, P=0.5, DF=1).

Eight isolates of *C. jejuni*were investigated for the presence of genes encoded the cytolethal distending toxin *(cdt*A*, cdt*Band *cdt*C) by using PCR assay. The results revealed that *cdt*Bwas detected in 6 (75%) of the isolates and *cdt*Cof 2 (25%).

All *C. jejuni*isolates were subjected to antimicrobial susceptibility assays using disc diffusion method. The results showed that the percentage of isolates sensitivity togentamycine, ciproflaxacine and nitrofurantuone were(95.5%, 90.9%, and 88.6%) respectively, whereas, the percentage of susceptibility to azithromycine ,erythromycine and chloramphenicol were (68.2%,52.3% and 34.1%) respectively, on the other hand, all isolates were resistant to tetracycline.

INTRODUCTION

Campylobacter infection is a zoonotic disease observed in most parts of the world. The disease is caused by *Campylobacter jejuni*, or less commonly *C. coli*, and it is estimated to cause 5-14% of diarrhoea, worldwide, (1).*C. jejuni*is a Gram-negative, microaerophilic and thermotrophic spiral rod that

is unable to multiply at temperatures below 30°C. *C. jejuni*is sensitive to various environmental stresses, including oxygen, UV, heat, drying, high salt concentrations and low pH values (2).

Cytolethal distending toxin (*cdt*) consists of three subunits encoded by the *cdt*A, *cdt*B, and *cdt*Cgenes, which are 30, 29, 21 Kbp in size, respectively, and are also arranged as an operon. It is considered that *cdt*B, which is encoded by the *cdt*Bgene, is the active subunit of the holotoxin and may block the cell cycle in G2 phase through inhibition of CDC2 kinase and causes cell distention (3; 4 and 5).

Campylobacter enteritis is self-limited, but antibiotic therapy must be considered in severe cases. However, erythromycin, tetracycline and fluoroquinolones have been used for treatment of acute *Campylobacter* gastroenteritis, but recently some resistant strains have been isolated in developed and undeveloping countries (6). These resistance in *Campylobacter* spp. are related to the antibiotic usage in veterinary medicine and prophylaxis.In such cases, we will face to bacteria with increased levels of antibiotic resistances, by which the treatment process will be more difficult (7).

This study aimed to 1) Isolation and identification of *C. jejuni*in slaughtered cattle and sheep by using enrichment and selective media; 2) Detecting the cytolethal distending toxin(*cdt*) genes in *C. jejuni*isolates; 3) Determination the susceptibility patterns of *C. jejuni*isolates to the antimicrobial agents.

MATERIALS AND METHODS

-Samples collection

The total number of collected samples was330; samples were composed of 210 gallbladder samples and 120 fecal samples which were collected from normally slaughtered animals (cattle130 samples and sheep 200 samples). The bile fluid was collected by a sterile syringe inserted aseptically into gallbladder; however, the fecal swabs were prepared by insertion and rolling a sterile swabs in fecal samples that were collected in sterile containers. Fecal swabs and bile fluid were inserted in Cary-Blair media tubes (8). Samples were immediately transported by ice bag to the laboratory**.**

-Laboratory Diagnosis

The transported specimens were directly inoculated to Preston enrichment brothsupplemented with Preston selective supplement (Himedia, India) and were incubated at 42C^{o} in a microaerophilic atmosphere by using Campy-Gen(Oxoid) (5%O2,10%CO2 ,and N2 85%) for 48hrs (9). The growth in preston broth wassubcultured on selective solid media modified Charcoal CefoperazoonDeoxycholat

Agar (mCCDA) and a selective supplement from (Oxoid), and incubated at 42 Cº in a microearophilic atmosphere for 48 hrs.

The characteristic colonies are grayish, flat and moistened, with a tendency to spread, and may have a metal sheen (10).The suspected colonies were picked up and subcultured again onto mCCDA to isolate pure colonies for further processing. The isolates were diagnosed to genus level by using Gram stained smear, characteristic corkscrewlike motility, oxidase test, catalase test. The identification of thermophilic*Campylobacter*s was done by using latex agglutination test HiCampylobacter Latex Test Kit(Himedia, India).

Biotyping of isolates was done according to the scheme proposed by (11). Rapid hippurate hydrolysis test was used according to (12), rapid H2S test was done according to (13), DNA hydrolysis test and susceptibility to nalidixic acid disk (30 µg).

-Molecular detection of *cdt*A, B and Cgenes by usingPCR

The DNA was extracted and purified according to the instructions of reagent genomic DNA kit (Geneaid / UK). A PCR assay targeting the *cdt* genes was usedaccording to (14),by using the primerssequencesillustrated in table (1), the PCR assay was done individually for each gene. The PCR amplification mixture (20µl) includes 5µl of green master, 5µl of template DNA, 1 µl of each primer given in table (1), and 8 µl of nuclease free water to complete the amplification mixture to 20 µl.

The PCR tube containing amplification mixture was transferred to preheated thermocycler and the program showed in table (2) was started.

Table (1): Oligonucleotide primers sequences used for PCR amplification of *cdt* (A, B, and C) genes.

Primer name	Description	Sequence ($5^- \rightarrow 3^-$)	Target gene	Size of PCR amplicon (bp)
*cdt*A	forward primer	CCTTGTGATGCAAGCAATC	*cdt*A	370
	reverse primer	ACACTCCATTTGCTTTCTG		
*cdt*B	forward primer	CAGAAAGCAAATGGAGTGTT	*cdt*B	620
	reverse primer	AGCTAAAAGCGGTGGAGTAT		
*cdt*C	forward primer	CGATGAGTTAAAACAAAAAGATA	*cdt*C	182
	reverse primer	TTGGCATTATAGAAAATACAGTT		

Table (2): Thermocycler program used for amplification procedure.

Step	Tempertuer	Time	Number of cycle
Initial denaturation	94 ºC	5 min	1
Denaturation	94 ºC	1 min	30
Annealing	55 ºC	1 min	
Extension	72 ºC	1 min	
Final extension	72 ºC	5 min	1

-Antibiotic susceptibility test

Antibiotic susceptibility was done according to (15). Seven antibiotic discs used in this study were from Bioanalyse-Turkey, including: gentamycin (30µg), nitrofurantoin (100 µg), azithromycin (15 µg), ciproflaxacin (5 µg), tetracyclin (10 µg), erythromycin (15 µg), chloramphnicol (30 µg).

-Statistical analysis

The results were analyzed statistically by using Minitab v.14.

RESULTS

According to the results of isolation and identification, out of 330 tested samples , 44 isolates were identified as *C. jejuni*. The characteristic colonies are grayish, flat and moistened, with a tendency to spread, and may have a metal sheen on mCCDA as showed in figure (1). Thehighest isolation rate was from bile 37/210 (17.6%) and low rate was detected from fecal samples 7/120(5.8%) (Table3). However, the isolation rates of *C. jejuni* from slaughtered sheep and cattle were 29/200 (14.5%) and 15/130 (11.5%) respectively (table 4), there is a statistically not significant.

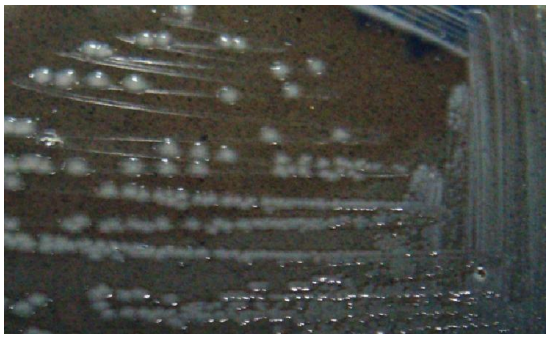

Figure (1): Colonial morphology of *C. jejuni* on mCCDA, at 42 ºC for 48h (gray, small, sheen appearance, moist, spreading on the media and have special odor)

Table (3): Occurrence of*C. jejuni* according to type of sample.

Type of sample	Total	Positive	%
Bile	210	37	17.6
Fecal	120	7	5.8

X^2=7.24, P=0.007, DF=1 (The Chi-square statistic is 7.243. The P value is 0.007118. This result is significant at $p < 0.05$.).

Table (4): Occurrence of *C. jejuni* according to animal's species.

Animal species	Total	Positive	%
Sheep	200	29	14.5
Cattle	130	15	11.5

X^2=0.46, P=0.5, DF=1 (The Chi-square statistic is 0.4599. The P value is 0.497661. This result is *not* significant at $p < 0.05$.)

Table (5) illustrates the biotyping results of 44 isolates, according to (11), all the isolates of *C. jejuni* were considered as biotype IV.

Table (5): Result of biotyping tests which used to identified of *C. jejuni* isolates.

Tests	Total number of examened isolates	Number of positive isolates	Percent
Hippurat hydrolysis	44	44	100
DNase production	44	44	100
Rapid H2S test	44	44	100
Susceptibility to Naldixic acid	44	44	100
Total number of sampels	330	44	13.3

Cytolethal distending toxin genes were determined by using PCR assay that contained a primer sets. Amplification of *cdt*B produced bands corresponding to their respective molecular size (620bp) target as showen in figure (2), whereas, amplification of ctdC gene produced bandscorresponding to

approximately (182bp) target as shows in figure (3). The *cdt*A gene was not detected in examined isolates.

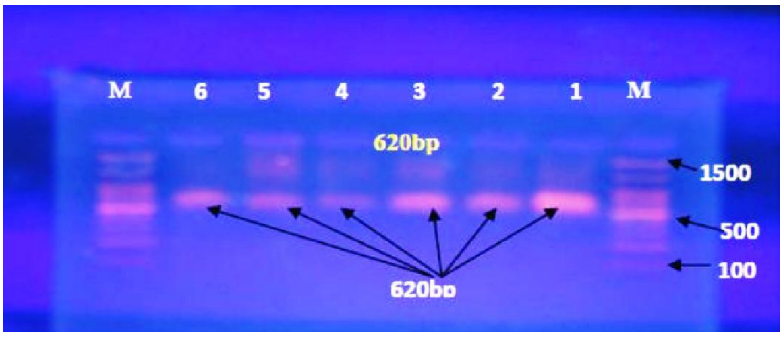

Figure (2): PCR amplification of *cdt*B gene. Lane M, Molecular size marker; Lanes 1-6, *cdt*B genes approximately (620bp).

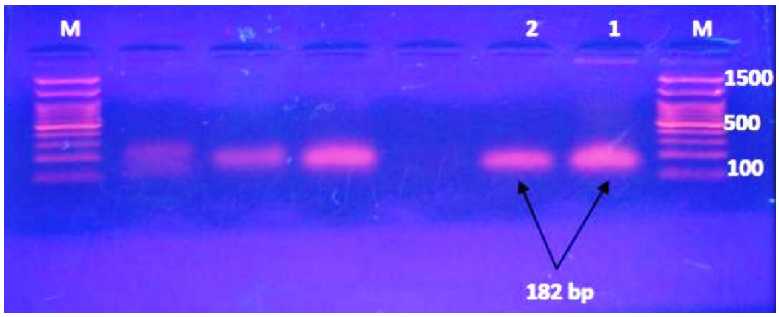

Figure (3): PCR amplification of *cdt*C gene. lane M, molecular size marker; lanes 1-6, *cdt*C genes approximately (182bp).

The results of antimicrobial susceptibility testing of *C. jejuni* isolates against 7 chosen antimicrobial agents are presented in table (6). The highest percentages of antimicrobial susceptibilities were to gentamicin, ciprofloxacin and nitrofurantoin (95.5%, 90.9% and 88.6%) respectively, however, the susceptibility to tetracycline was (0%).

Table (6): Antimicrobial susceptibility of 44*C. jejuni* isolates against seven antimicrobials.

Antimicrobials	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Azithromycin	30	68.2	14	31.8	-	-
Chloramphenicol	44	100	-	-	-	-
Ciprofloxacin	40	90.9	4	9.1	-	-
Erythromycin	23	52.3	8	18.2	13	29.5
Gentamicin	42	95.5	2	4.5	-	-
Nitrofurantoin	39	88.6	-	-	5	11.4
Tetracycline	-	-	-	-	44	100

DISCUSSION

In the present study, all the samples were taken from normal slaughtered sheep and cattle which attained Basrahabattoir. The isolation rate of *C. jejuni* was 44/330 (13.3%) from all tested samples, tables (3 and 4). The higher isolation rate was obtained from bile samples of normal sheep and cattle with (17.6%) of tested bile samples, whereas, the lower isolation rate was obtained from fecal samples (5.8%) of normal slaughtered sheep and cattle. The result of isolation rate from sheep is slightly lower than that reported by(16). However, the isolation rate of *C. jejuni* from sheep samples was similar to that reported by (17), who detected it in 17.5% of slaughtered sheep. Moreover, (18) were isolate *C. jejuni* from total samples of sheep in rate (16.9%). The isolation rate of *C. jejuni* from cattle was (11.5%), this result similar to that reported by (19), who isolated it in percent (15.5%) from total tested samples.

Cytolethal Distending Toxin, composed of three subunits encoded by the *cdt*A, *cdt*B and *cdt*C genes, causes eukaryotic cells to arrest in the G2/M phaseof the cell cycle, preventing them from entering mitosis and consequently leading to cell death (20). The *cdt*A and *cdt*C subunits have roles in binding to the host cell, and the *cdt*B subunit has a nuclease activity (21). In the present study, based on the PCR technique results of eight *C. jejuni* isolates, rate of *cdt*B was 75%. The results of this study about *cdt*B detection rate are in agreement with (22 and 23), who detected this gene in rates 70.2% and 88% respectively.Whereas, (24) found the gene in 97% of isolate, on the other hand, (25) found that the all *C. jejuni* strains isolated from bovine were positive to *cdt*B gene.

Cytolethal distending toxin *cdt*C gene was detected in 25%of tested isolates, however, the all tested isolates were negative to cdtA gene. These results are in agreement with (26) who found out of 16 detected *C. jejuni*, 11isolates (68.75%) harbored the various subunits of *cdt*A, *cdt*B and *cdt*C genes, and 5 (31.25%) were negative for all tested *cdt* genes, moreover, 4 (25%) harbored only *cdt*B and *cdt*C. Also, (27) recorded that the *cdt*A, *cdt*B and *cdt*C were detected from human *C. jejuni* in following percent 50%, 27.77% and 27.77% respectively; however, the detection rates from chicken *C. jejuni* were 13.33%, 20% and 25% respectively. Deletion or mutations in the *cdt*gene cluster have been reported by (28 and 29).

The antimicrobial resistance becomes a major public health concern in both developed and developing countries in recent years(30).Thus, direct contacts with infected animals and consumption of

animal food products can cause enteritis in human beings (31). Results of this study regarding antimicrobial susceptibility of *C. jejuni* isolates illustrated that all pathogenic *Campylobacter* isolates were sensitive tochloramphenicol, whereas, the susceptibility to gentamicin, ciprofloxacin, nitrofurantuine, azithromycine and erythromycine(95.5%, 90.9%, 88.6%, 68.2%, 52.3%, and 34.1%) respectively.

Result of chloramphenicol susceptibility similar to that discovered by (32), gentamicin susceptibility test is in agreement to that recorded by (33), who detected that 100% of *C. jejuni* isolates were sensitive, also (32 and 34) are in accordance with this result. Result of ciprofloxacinsusceptibility test is in accordance with that found by (33), and differed from that recorded by (33and 34).Susceptibility to nitrofurantuine comparative to that reported by (35). Sensitivity to azithromycine is in agreement with that reported by (33). Susceptibility of isolates to erythromycine similar to that of (33), moreover, this result may be interpreted by (36 and 37), who noted that in some developing countries the resistant to macrolides is much higher.

All isolates were resistant to tetracycline, similar result was reported by (38) frombroiler, whereas, other researchers reported range of resistance from 18.2% to 78% (32 and39).

Based on results of this study presence of thermophilic*Campylobacters* (*C.jejuni*) hold possible risks of infection to people through the consumption of contaminated animal products or through contact with infected animals.Antimicrobial resistant isolates constitute a threat to workers in slaughterhouses or consumers in the case of infection.

عزل وتشخيص و السمات الذيفانية للمنحنيات الصائمية المعزولة من الابقار والاغنام المذبوحة في مدينة البصرة

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الخلاصة

أجريت هذه الدراسة للفترة من كانون الاول ٢٠١٠ لغاية حزيران ٢٠١١ ، حيث تم جمع ٣٣٠ عينة من مجزرة البصرة تضمنت ٢١٠ عينة صفراء (١٣٠ من الاغنام و ٨٠ من الابقار) و ١٢٠ عينة براز (٧٠ من الابقار و ٥٠ من الاغنام). استخدمت هذه العينات للتحري عن وجود المنحنيات الصائمية من خلال استخدام الاوساط الانتقائية والاختبارات الكيموحيوية والمصلية والتنميط الحيوي. كانت نسبة العزل النهائية للمنحنيات الصائمية ١٣.٣ % من العدد الكلي للعينات، كانت نسبة العزل من الصفراء ١٧.٦ % بينما من عينات البراز كانت ٥.٨ % (قيمة مربع كاي = 7.24، الاحتمالية= 0.007 و درجة الحرية = ١). كانت نسبة العزل من الاغنام ١٤.٥ %

بينما من الابقار ١١.٥ % الاختلافات في نسبة العزل كانت هامة احصائيا (قيمة مربع كاي = 0.46، الاحتمالية= ٠.٠٥ و درجة الحرية = ١).

تم استخدام ٨ عزلات من المنحنيات الصائمية للتحري عن وجود الجينات التي تشفر للذيفان النافخ و القاتل للخلايا (*cdt*A, *cdt*B, *cdt*C)من خلال استخدام فحص البلمرة المتسلسل . أظهرت النتائج اكتشاف الجين *cdt*B في ٦ (٧٥%) من العزلات وجين *cdt*C في ٢ (٢٥%) .

كل عزلات المنحنيات الصائمية تم فحص حساسيتها اتجاه سبعة من المضادات الميكروبية باستخدام طريقة الانتشار القرص. أظهرت النتائج ان العزلات كانت حساسة للجنتاميسين والسبروفلاكساسين والنتروفيورانتون بنسب مئوية (٩٥.٥ % ، ٩٠.٩ % ، ٨٨.٦ %) على التعاقب. بينما النسبة المئوية للحساسية للأ زيثرومايسين و ايرثروميسين و كلورامفنيكول كانت (٦٨.٢ %، ٥٢.٣ %، ٣٤.١ %) على التعاقب، من جانب أخر كل العزلات كانت مقاومة للتتراسايكلين.

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