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Zoonotic and Molecular Characterizations of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Beef Cattle and Children

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Abstract: Campylobacteriosis -caused principally by *Campylobacter jejuni (C. jejuni*) and *Campylobacter coli* (*C. coli*) - is among the main causes of bacterial gastroenteritis worldwide. This work was done to investigate the molecular characterization of zoonotic *C. jejuni* and *C. coli* isolated from fecal samples of beef cattle, retail beef meat and beef liver and stool of children with diarrhea. Fecal samples were collected from 50 apparently healthy cattle, 60 of retail beef meat and beef liver (30 of each) as well as 50 stool samples from pediatric diarrhea were subjected to standard isolation and phenotypic identification of *Campylobacter* isolates. The prevalence of *Campylobacter* isolate was 17(34%) in fecal sample of cattle, 5(16.66%) beef meat, 8(26.66 %) beef liver and 13 (26%) in pediatric diarrhea. Out of 43 identified isolates, 26(60.46%) *C. jejuni* isolates were higher than 14(32.55%) *C. coli*, two samples were mixed infection and one *Campylobacter upsaliensis*. A multiplex-PCR method was developed for the detection of *C. jejuni* and *C. coli*. Primers were the hippuricase gene *(hipO*) characteristic of *C. jejuni*, a sequence partly covering an aspartokinase gene (*asp*) characteristic of *C. coli* and a universal 16S rDNA gene sequence serving as an internal positive control. All *Campylobacter* isolates expressed identity with 16S rDNA (genus specific gene) at 1062 pb. Multiplex PCR demonstrated one false- positive and one false-negative hippurate activity test. PCR method was incapable to identify biochemically identified *C. upsaliensis*. Amplification of *hipO* gene of *C. jejuni* and *asp*- gene of *C. coli* isolated from cattle, beef and liver have shown identical fingerprints with human *C. jejuni* and *C. coli* at 344bp and 500bp respectively, indicating the public health importance of the isolates.

Key words: Campylobacteriosis · Molecular Characterizations · Multiplex PCR *Campylobacter jejuni* · *Campylobacter coli*

INTRODUCTION

Campylobacter jejuni and Campylobacter coli are the most frequent causes of acute bacterial gastroenteritis in humans, representing an unrelenting worldwide public health problem. *C. jejuni* accounts for over 90% of cases, with the majority of the remainder caused by *C. coli* [1]. Campylobacteriosis manifested by diarrhea that is often bloody, abdominal cramping, fever and vomiting [2].

In Egypt, *Campylobacter* is the second leading cause of pediatric diarrhea with infants and one year olds experiencing 1.2 and 0.4 episodes per year, respectively [3]. Although most *Campylobacter* associated diarrhea is self-limited, complications can occur. One complication is Guillain-Barre´ Syndrome (GBS), an acute, symmetric, ascending paralysis that is estimated to occur 30 times for every 100, 000 *Campylobacter* cases [4] and the case fatality ratio approaches 10% [5].

Human campylobacteriosis occurs sporadic, making it hard to trace the sources and routes of transmission [6]. Humans can be infected either via direct contact with animals, from contaminated faeces [7] or during slaughtering and dressing, or indirectly by consumption of contaminated water [8] unpasteurized milk [9] contaminated food [10], as poultry meat [11] and cattle and sheep meat contaminated at the abattoir [12].

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Studies on the occurrence of *Campylobacter*in retail foods Zhao *et al*. [13] and Whyte *et al*. [14] reported much higher prevalences in raw poultry than in retail meat samples from other animal species.

Many wild and farmed avian and mammalian species carry *Campylobacters* as commensal members of the gastrointestinal microbiota, Sodium hippurate hydrolysis reaction is the only biochemical test used to differentiate *C. jejuni* and *C. coli*. Hippurate hydrolysis is time consuming and sometimes difficult to interpret when the enzymatic activity is impaired under the methodological condition [15]. PCR techniques differentiated *Campylobacter* isolates from field studies in Upper Egypt of pediatric diarrhea on the basis of sensitivity to boiling water [16], in addition, multiplex PCR was conducted for *Campylobacter* detection and speciation [17-22].

Although poultry meat is considered to be the major source of human infection [23], it is important to establish the significance of other reservoirs to assess their relative contribution to human disease. Most of the available studies are concerned mainly with the prevalence of *Campylobacter* in retail beef while a limited number of them discussed the prevalence of the isolates in live animals, meat and patients. The objectives of this study were to determine the prevalence of *C. jejuni* and *C. coli* in cattle, retail beef meat, beef livers and children suffer from diarrhea in Toukh, Kaliobia governorate. With the aim to assess strain diversity, a selection of isolates was characterized by multiplex PCR the most widely used typing method to detect three genes. The 16S NA gene conserved among isolates of diverse sources [23]. The *hipO* gene is specific for *C. jejuni* strains [24, 25]. The *asp* gene encodes aspartokinase specific for *C. coli* [23] to further characterize the zoonotic importance of isolates obtained through molecular testing.

MATERIALS AND METHODS

Sampling: Fecal samples were collected from (50) apparently healthy cattle in different farms in Toukh, Kaliobia governorate. Beef meat and beef liver (30 of each) were purchased from different butcher stores in the same locality. As well as stool samples were collected from (50) children (1-14 years old) with diarrhea inhabitant from rural area of Toukh and admitted in Pediatric Department, Toukh hospital. All samples were aseptically placed in separate sterile plastic bags and were immediately transported to the laboratory in a cooler with ice packs and processed immediately upon arrival for isolation of *Campylobacter.*

Isolation and Identification of *C. jejuni*: About 10 g of each fecal sample were homogenized in sterile thioglycolate broth. Meat samples were rinsed with buffered peptone water and massaged briefly by hand for five minutes, next 10 ml of the rinsate was added to 10 ml of thioglycolate broth. Broth samples were incubated at 42°C for 48 hrs under microaerobic condition (5% O_2, 10% CO_2 and 85% N_2) [26]. A loopful of enrichment broth were plated on modified charcool cefoperazone deoxycholate agar (MCCDA) (Oxoid) and incubated in microaerophilic atmosphere at 42°C for 48 hrs [22]. Suspected colonies of *Campylobacter* were identified under phase contrast microscope for detection of characteristic motility and morphological character according to Smibert, [27]. *Campylobacter* isolates were subcultured and identified by biochemical tests described by Gossens *et al*. [28] and Frost *et al*. [29] Identified colonies were stored at -70°C in nutrient broths with 15% glycerol until subjected to molecular identification [20].

Isolation of DNA: DNA was prepared for PCR by 8 min. boiling colonies in 10% Chelex 100 (Bio-Rad) in 10 mM Tris/HCl, 1 mM EDTA, pH 8. The crude DNA preparation was stored at 4°C until used add reference.

Multiplex PCR: PCR reaction contained 5 µl template DNA was performed in a total reaction volume of 25 µL containing PCR buffer [50 mM Tris / HCL, 10 mM KCL, 5 mM $(NH_4)_2SO_4$, pH 8.3], 2.6 mM $MgCL_2$, 260 µM dATP, dGTP and dCTP, 520 µM dUTP, 0.15 U UNG, 1.25 U Taq Polymerase, 0.2 µM *hipO* primers (hippuricase gene for *C. jejuni*) [22], hipO - F (5`-GACTTCGTGCAGATAT GGATGCTT) and hipO-R( 5`-GCTATAACTATCCGAAGAAGCCATCA) giving a 344 bp product, 0.4 uM *asp*- primers (aspartokinase gene for *C. coli*) *asp* -F (5`-GGTATGATTTCTACAAAGCGAG-3`) and *asp*-R(5`-ATA AAAGAC TAT CGT CGC GTG-3`) giving a 500 bp product [23] and 0.05 µM universal primers (16S- rDNA gene) [22] 16S-F(5`GGAGGCAGCAGTAGGGAATA) and 16S-R (5`-TGACGGGCGGTGAGTACAAG) giving a1062 bp product. Thermocycler conditions were 94°C for 6 min, followed by 35 cycles of 94°C for 50 s, 57°C for 40 s and 72°C for 50 s and finally 72°C for 3 min. PCR product were analyzed in 1.5 % agarose gel electrophoresis under standard conditions and stained by ethidium bromide.

RESULTS

In this study samples were obtained from feces of 50 apparently healthy cattle, 60 from retail beef meat and beef liver (30 of each) in Toukh, Kaliobia governorate in addition to 50 stool samples from pediatric diarrhea inhabitant from rural area of Toukh and admitted to hospital. Samples were tested for *Campylobacter* using traditional phenotypic characterization and discriminate between isolates by catalase negative *C. upsaliensis*, hippurase positive *C. jejuni* and positive H_2S production *C. coli*, Table (1). The prevalence of positive *Campylobacter* species was 34%in fecal sample of cattle, 16.66% beef meat, 26.66 %beef liver and 26% in children with diarrhea. Among the isolates 60.46% *C. jejuni*, 32.55% *C. coli* and two samples were mixed infection and one *C. upsaliensis* isolated from pediatric diarrhea, Table (2) as identified by conventional cultivation technique and verified by multiplex PCR assay.

A multiplex PCR was developed for the identification of *C. jejuni* and *C. coli*. Primers included in the method are the *C. jejuni* specific *hipO*-primers developed that resulted in a 344 bp amplicon, primers designed to amplify a 500 bp fragment of the *asp*- gene characteristic of *C. coli* and universal primers used to amplify a 1062 bp fragment of the 16S rDNA gene, serving as an internal positive control for the PCR. In our study all *Campylobacter* isolates expressed identity with 16S rDNA (genus specific gene) at 1062. Multiplex PCR demonstrated one false- positive and one false-negative hippurate activity test. Figure (1) shows the PCR amplification results of four *C. jejuni*, four *C. coli* and one *C. upsaliensis* strains. The biochemically identified *C. upsaliensis* could not be identified by the PCR method and showed a negative result with *hipO* and *asp*-genes. Amplification of *hipO* gene of *C. jejuni* and *asp*- gene of *C. coli* isolated from cattle, beef meat and liver showed identical fingerprint results to those of human origin at 344 bp and 500 bp respectively.

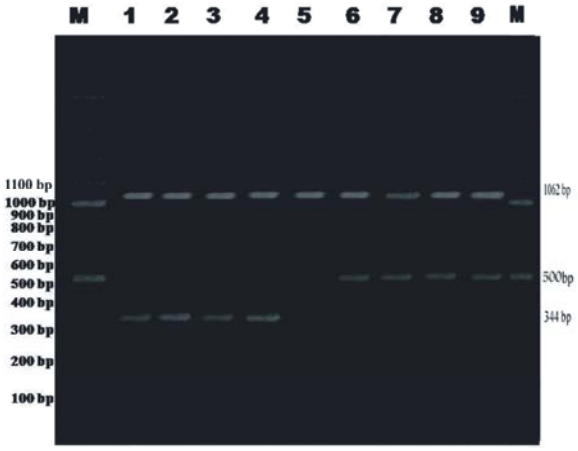

Fig. 1: PCR amplification products of *Campylobacter* isolates. Lane M: a 100bp molecular size marker. Lane 1, 2, 3 and 4, 344 bp of *C. jejuni* isolated from beef cattle, beef meat, liver and children with diarrhea respectively. Lane 6, 7, 8 and 9, 500 bp of *C. coli* isolated from beef cattle, beef meat, liver and children with diarrhea respectively. Lane 5, *C. upsaliensis* isolated from pediatric diarrhea.

Table 1: Biochemical characters of suspected *Campylobacter* isolates

Biochemical character	*C. jejuni*	*C. coli*	*C. upsaliensis*
Growth			
- at 25°C	-	-	-
- at 37°C	+	+	+
- at 43°C	+	+	+
Growth in:			
- 3.5% NaCl	-	-	-
- 1% Glycine	+	+	+
Motility	+	+	+
Catalase +	+	-	
Oxidase	+	+	+
H_2S production in TSI agar	-	+	-
Sodium hippurate hydrolysis	+	-	-
Susceptibility to			
Nalidixic acid	S	S	S
Cephalothin	R	R	S

Positive (+) Sensitive (S)
Negative (-) Resistance (R)

DISCUSSION

Campylobacter species are a major cause of bacterial gastroenteritis worldwide [10, 51] In addition to *C. jejuni* and *C. coli*, responsible for 90% and 10% of all cases of human enteric infection, respectively, other *Campylobacters* (*C. upsaliensis*) have also been implicated as gastrointestinal pathogens [1, 30, 31].

In this work the prevalence of *Campylobacter* in dependence to bacteriological and biochemical characters, was found to be 34, 16.66, 26.66 and 26% in cattle fecal samples, beef, meat, beef livers and children with diarrhea respectively. In most diagnostic laboratories at least 95% of human campylobacter isolates belong to either *C. coli* or *C. jejuni* on selective media [32]. Our finding was higher than *Campylobacter* isolated from fecal samples collected from cattle that reviously were 5% [33], 27.9%

Table 2: The prevalence of *Campylobacter* infection isolated from cattle, beef, liver and children

		Possitive Sample									
		Campulovacter isolate		*C. jejuni*		*C. coli*		Mixed infestion		*C. upsaliensis*	
Samples	No.	No.	%	No.	%	No.	%	No.	%	No.	%
1-Apparently healthy cattle	50	17	34	11	64.70	5	29.41	1	5.88	0.0	0.0
2-Beef meat	30	5	16.66	3	60	2	40	0.0	0.0	0.0	0.0
3-Beef liver	30	8	26.66	5	62.5	3	37.5	0.0	0.0	0.0	0.0
4-Children with diarrhea	50	13	26	7	53.84	4	30.76	1	7.69	1	7.69
Total	160	43	26.87	26	60.46	14	32.55	2	4.65	1.	2.32

[34] and 23.4% [35]. These differences in the prevalence of cattle associated *Campylobacter* can be attributed to several factors, including isolation methods, sample size and type (e.g. dairy versus feedlot), seasonal variations and geographical location [2]. Our results were lower than Campylobacter species isolated from 123 out of 270 (45.6%) in Turkey meat samples [36] and disagree with Hassanain [37] and Noormohamed and Fakhr [18] who failed to isolate *Campylobacter* from beef meat in Giza, Egypt and Tulsa, USA.

The overall prevalence of *Campylobacter* in beef livers in our study was 26.66% that was lower than78% [18] and 69% [38],while the isolation rate was higher than the previous study conducted in Japan and reported only 5% Campylobacter spp. in beef livers [39]. Ghafir *et al.* [40] suggested that the high level of recovery of *Campylobacter* from livers is probably due to the fact that the liver surface stays moist, which might protect this food borne pathogen. Fecal carriage of *Campylobacter* by the slaughtered cows is another possible source of contaminating beef livers in slaughter houses as well as liver location makes it easily prone to bile contamination. The risk for high prevalence of *Campylobacter* in beef liver could be magnified by under cooking livers to avoid overcooking undesired taste [18].

We discriminated between *C. jejuni* and *C. coli* isolates by hippurase test. Out of 43 tested isolates the prevalence of *C. jejuni* was 60.46% that was higher than 32.55% of *C. coli*, while mixed infection was reported in 4.65% and 2.32% was catalase negative and identified as *C. upsaliensis* and it was isolated from pediatric diarrhea. Our results were in harmony with Nielsen *et al.* [41] who found 90.9% of the isolates from fecal samples of cattle were *Campylobacter jejuni* and 6.8% were *C. coli*, Cakmak and Erol [36] identified 40.4% *C. jejuni* and 4.1%*C. coli* in Turkey meat samples, Kramer *et al.* [42] estimated in their study that 49% of their *Campylobacter* isolates from beef livers were *C. jejuni* and 2.1% were *C. coli* and speciation performed on 310 of the 366 *Campylobacter* isolates, with 81 %being *C. jejuni*, 18 % being *C. coli* in children with diarrhea in the Abu Homos district of the Beheira Governorate in Egypt [3]. *C. upsaliensis* responsible for enteritis in both adult and children [28]. Our findings were disagree with previous studies recorded the prevalence values of *C. jejuni* lower than *C. coli*, which were 23 and 45% respectively in cattle fecal samples [43], 0.5 and 4.9% respectively in surveys of retail beef [44] and 33% *C. jejuni* and 62% *C. coli* in beef liver [18].

In our study out of 43 *Campylobacter* isolates two samples were mixed infected with *C. jejuni* and *C. coli*, (5.88%) cattle fecal samples and (7.69%) children with diarrhea. *C. coli*, *C. jejuni* and *C. upsaliensis* were biochemically identified [45]. The available literature revealed that two samples of beef liver are contaminated with both species [18] and three (1 %) being mixed infections in children with diarrhea in the Abu Homos district of the Beheira Governorate in Egypt [3]. On other hand Ghafir, *et al.* [31] found that in the examined beef samples, all of the isolates were *C. jejuni*.

Prevalence values are 0.5 to 4.9% in surveys of retail beef [44].

In our study we describe a multiplex PCR to identify and discriminate between isolates of *C. coli* and *C. jejuni*. In our protocol, three genes, namely 16S rDNA (genus specific gene) and *hipO* and *asp* (species specific) for *C. jejuni* and *C. coli* respectively, were targeted. These genes and the primers used for their identification have been studied independently and reported by other workers Nayak *et al*. [46], Linton *et al*. [23] and Persson and Olsen, [22]. The biochemically identified *C. coli*, *C. jejuni* and *C. upsaliensis* strains were subjected to the multiplex-PCR method. In our work all *Campylobacter* isolates expressed identity with 16S rDNA (genus specific gene) at 1062. The presence of an internal positive PCR is required as the analysed samples may be *Campylobacter* negative and so it will eliminate false negatives, at least when the difference in copy number between the internal positive control locus and the diagnostic loci is not critical [22]. It is worth mention that all isolates showed

that the *C. coli* and *C. jejuni* strains resulted in the expected amplicons, except for two strains were identified as *C. jejuni* and *C. coli* by biochemically and then they were found to be *C. coli* and *C. jejuni* respectively by repeated PCR testing. Multiplex PCR demonstrated one false- positive and one false-negative hippurate activity test. Our findings are in agreement with other reports describing a comparison between hippurate biochemical and PCR-based speciation, with a false-positive hippurate [46] and with a false-negative hippurate activity [17]. Some *C. jejuni* strains harbour the hippuricase (hipO) gene but fail to express the enzymic activity [15] and as shown in this study, such isolates can only be correctly identified using molecular methods. We highlighted that multiplex PCR amplification of *hipO* gene of *C. jejuni* and *asp*- gene of *C. coli* isolated from cattle, beef and liver have shown identical fingerprints with human *C. jejuni* and *C. coli* at 344bp and 500bp respectively. The biochemically identified *C. upsaliensis* could not be identified by the PCR method and showed a negative result with *hipO* and *asp* genes. All strains tested were easily prepared for PCR by a simple boiling procedure of the bacterial colonies and required no special treatment to extract useful DNA for the PCR analysis. Others studies have found heat-resistant *Campylobacter* strains that could not produce template DNA by simple boiling unless treated with phenol/chloroform, proteinase K or SDS [16, 47]. The reason why no such observations were found in the present study, could be due to differences in growth conditions, DNA preparation or PCR method [22]. The present colony multiplex PCR assay proved to be accurate and simple to perform and could be completed within 3 h. It had the added advantage of detecting the *hipO* gene in *C. jejuni* strains that were hippuricase negative by phenotypic methods and therefore difficult to differentiate from *C. coli* [48]. In addition to clinical use, the method has potential as a diagnostic kit for detecting thermophilic *Campylobacters* in complex samples, such as foods in which low pathogen numbers (>10^3 CFU/ml) are frequently present. The present PCR assay offers an effective alternative to traditional biochemical typing methods for the identification and differentiation of *C. jejuni* and *C. coli* [49, 50].

We can conclud from our study that healthy cattle consider as reservoir for a number of thermophilic *Campylobacter* species, highlighting the importance of non-poultry farms as possible sources of Campylobacter infection. The high prevalence of *C. jejuni* in cattle should be of special concern and care should be taken to limit its spread during slaughtering and dressing. Efficient control measures at the farm end of the food safety continuum directed at the prevention of colonization in food animals, could contribute to reducing the risk of human infection.

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