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

Nashwa O. Khalifa, Jehan S.A. Afify and Nagwa S. Rabie

Department Zoonoses, Faculty Vet. Med., Benha University, Egypt
Department of Food Hygy, Faculty Vet. Med., Benha University, Egypt
Department Poultry Diseases, National Research Centre, Dokki, Egypt

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].

Corresponding Author: Nagwa Rabie, Department Poultry Diseases, National Research Centre, Dokki, Egypt.
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 suffering 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 was characterized by multiplex PCR 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% O2, 10% CO2 and 85% N2) [26]. A loopful of enrichment broth were plated on modified charcoal 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 broth 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 (NH4)SO4, pH 8.3], 2.6 mM MgCl2, 260 µM dATP, 2.6 mM dTTP 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'-GACTTCTGAGATATGGAATCGTATGC-3') and hipO - R (5'-GCTATAACTATCCGAGGAAGCCATCA) giving a 344 bp product, 0.4 uM asp- primers (aspartokinase gene for *C. jejuni*) [22], asp-F (5'-GGTATGATTCTACAAAGCGAG-3') and asp-R (5'-ATAAAAGAC TAT CGT CGT CGC GTG-3') giving a 500 bp product [23] and 0.05 µM universal primers (16S- rDNA gene) [22] 16S-F(5'-GGAGGCGACGACTAGGGAATA) and 16S-R (5'-TGACGCGGCGGTGAGTACAAG) 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.

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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 H₂S 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.

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 previously were 5% [33], 27.9%
C. coli and 23.4% [35]. These differences in the prevalence of being

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Campylobacter isolate</th>
<th>C. jejuni</th>
<th>C. coli</th>
<th>Mixed infection</th>
<th>C. upsaliensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1-Apparently healthy cattle</td>
<td>50</td>
<td>17</td>
<td>34</td>
<td>11</td>
<td>64.70</td>
</tr>
<tr>
<td>2-Beef meat</td>
<td>30</td>
<td>5</td>
<td>16.66</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>3-Beef liver</td>
<td>30</td>
<td>8</td>
<td>26.66</td>
<td>5</td>
<td>62.5</td>
</tr>
<tr>
<td>4-Children with diarrhea</td>
<td>50</td>
<td>13</td>
<td>26</td>
<td>26</td>
<td>60.46</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>43</td>
<td>26.87</td>
<td>26</td>
<td>60.46</td>
</tr>
</tbody>
</table>

[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 [38], 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 than 78% [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 \textit{C. coli} and \textit{C. jejuni} strains resulted in the expected amplicons, except for two strains were identified as \textit{C. jejuni} and \textit{C. coli} by biochemically and then they were found to be \textit{C. coli} and \textit{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 \textit{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 \textit{C. jejuni} and asp- gene of \textit{C. coli} isolated from cattle, beef and liver have shown identical fingerprints with human \textit{C. jejuni} and \textit{C. coli} at 344bp and 500bp respectively. The biochemically identified \textit{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 \textit{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 \textit{C. jejuni} strains that were hippuricase negative by phenotypic methods and therefore difficult to differentiate from \textit{C. coli} [48]. In addition to clinical use, the method has potential as a diagnostic kit for detecting thermophilic \textit{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 \textit{C. jejuni} and \textit{C. coli} [49, 50].

We can conclude from our study that healthy cattle consider as reservoir for a number of thermophilic \textit{Campylobacter} species, highlighting the importance of non-poultry farms as possible sources of Campylobacter infection. The high prevalence of \textit{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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**REFERENCES**


