Bacteriological and Molecular Identification of *Campylobacter* Species in Chickens and Humans, at Zagazig City, Egypt

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A B S T R A C T

The genus Campylobacter is of great importance to public health because it includes several species that may cause diarrhea. Poultry and poultry products are known as important sources of human campylobacteriosis. A total of 533 samples from broiler chickens ;131 cloacal swabs, 39 chicken skin, 39 chicken cecal parts and 78 chicken meat thigh and breast meat (39 of each) were obtained from retail outlets, as well as, 246 stool swabs from persons attending the outpatient clinic of Al-Ahrar public hospital were examined. The isolation rate of Campylobacter species from chicken skin, thigh meat, breast meat, cecal parts, cloacal swabs and human stool samples were 30.8%, 38.5%, 30.8%, 41%, 35.1% and 5.3%, respectively. The conventional biochemical tests were used for discrimination between *C. jejuni* and other Campylobacter species based on standardized hippurate hydrolysis test. *C. jejuni* was isolated from cloacal swabs, skin, thigh meat, breast meat, cecal parts and human stool samples with the isolation rate of 45.7%, 50%, 46.7%, 41.7%, 81.3% and 76.9%, respectively. Real-Time PCR targeting *hip*O gene specific for *C. jejuni* was used for the confirmation of phenotypically identified 31 *C. jejuni* isolates. The results showed that the conventional culture methods and biochemical reactions were 100% in accordance with the results of PCR for identification and differentiation of *C. jejuni*.

Keywords: *C. jejuni, C. coli,* chickens, humans, RT-PCR, Egypt

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1. INTRODUCTION

Campylobacter species are Gram-negative bacteria within the family Campylobacteraceae that require microaerobic growth conditions. Thermophilic Campylobacters, including *C. jejuni* and *C. coli* that are the most frequent Campylobacter species isolated from patients with diarrhea in both industrial and developing countries (Rahimi and Ameri, 2011). Campylobacter species are widespread in nature prevailing mainly in the alimentary tract of wild and domesticated birds and mammals. Transmission to humans is most commonly through consumption and handling of chicken meat products contaminated with this zoonotic pathogen during slaughtering and carcass processing (EFSA, 2010).Conventional biochemical tests for discrimination between *C. jejuni* and *C. coli* depend mainly on hippurate hydrolysis which is the only phenotypic test for differentiation between the two species. However, both false positive and false negative results have been reported (Waino *et al.,* 2003). Therefore, molecular identification methods have been described as an alternative to the inaccurate, time consuming, biochemical phenotypic methods (LaGier *et al*., 2004). The aim of the current work was to investigate the contribution of chicken as a potential source of Campylobacter species particularly *C.*

jejuni infections in humans by using conventional and molecular tools.

2. MATERIAL AND METHODS

2.1. *Samples*

A total of 533 samples from broiler chickens at 6 weeks age; 131 cloacal swabs, 39 chicken skin, 39 cecal parts and 78 chicken meat (thigh and breast meat, 39 of each) were obtained from retail outlets at Zagazig, Egypt. Moreover, 246 stool swabs collected from persons attending the outpatient clinic of Al-Ahrar public hospital, Zagazig city, Sharkia Province, Egypt, were examined.

2.2. Samples preparation

2.2.1. Stool and cloacal swabs

Sterile swabs were inserted into the cloaca and voided human stool samples and then directly immersed into sterile Preston enrichment broth base containing Campylobacter growth supplement (Ellerbroek *et al.,* 2010).

2.2.2. Skin, cecal and meat samples

Twenty five grams from each incised skin, cecal parts and chicken meat (thigh and breast) were aseptically transferred to a sterile blender containing 225 ml of Preston enrichment broth for homogenization of the sample (Sallam, 2001).

2.3. Bacteriological examination

2.3.1. Isolation of Campylobacter species

The collected samples in Preston enrichment broth were incubated at 42ºC for 24-48 hours with less than 1 cm of headspace left in the culture vessel with tightly capped lids (Oxoid, 2006). After enrichment, 0.1 ml of the broth was streaked onto modified Campylobacter selective agar base Cefoperazone Charcoal Desoxycolate Agar (mCCDA) containing CCDA Selective Supplement. The plates were then incubated at 42ºC in darkness for 48 hours under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) (Vandepitte and Verhaegen, 2003).

2.3.2. Preliminary confirmation of thermophilic Campylobacter species

Thermophilic Campylobacter species were preliminary identified by their colonial morphology on mCCDA media. Suspected colonies were purified on blood agar plates and subjected to Gram staining, motility test, growth at 25ºC and 41.5ºC and oxidase test (ISO, 2006).

2.3.3. Biochemical identification of Campylobacters

The preliminary identified Campylobacter species were further subjected to catalase test, susceptibility to nalidixic acid and cephalothin and rapid hippurate hydrolysis test (Nachamkin, 1999).

2.4. Molecular identification of C. jejuni

2.4.1. DNA extraction

DNA extraction from the biochemically identified isolates was performed according to the manufacturer guidelines using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation, China).

2.4.2. Real-Time probe based PCR

A Real-Time probe based quantitative PCR (qPCR) reaction was used for the confirmation of 31 *C. jejuni* isolates. Species-specific primer and TaqMan probe sets targeting *hip*O gene specific for *C. jejuni* (LaGier *et al*., 2004) were synthesized (AlphaDNA, Canada). The sequences of primers and probe were Cj-F 5’- TGCTAGTGAGGTTGCAAAAGAA TT-3’, Cj-R 5’-TCATTTCGCAAA AAAATCCAAA-3’ and Cj-FAM 5’- ACGATGATTAAATTCACAATTTT TTTCGCC AAA-3’. Non-template DNA and positive controls of *C. jejuni, C. coli, E. coli, S. Typhimurium*, *Staph. aureus* and two biochemically identified Campylobacter isolates other than *C. jejuni* and *C. coli* were also run to determine the specificity of the reaction.

3. RESULTS

3.1. *Preliminary confirmation of thermophilic Campylobacter species*

Campylobacter species were preliminary identified by their colonial morphology on mCCDA and sheep blood agar. *C. jejuni* on mCCDA appeared as greyish, flat, moistened, with a tendency to spread colonies that may have a metal sheen. However, on 5-7% sheep blood agar *C. jejuni* had characteristic colonies of oil drop like appearance (translucent droplet-like colonies), slightly pink, round, convex, smooth and shiny, with a regular edge. Occasionally, *C. jejuni* showed greyish, flat, moistened, with a tendency to spread colonies on sheep blood agar. Campylobacter species were also confirmed by production of oxidase. The suspected Campylobacter organisms in freshly prepared cultures appeared as Gram negative (faint in color) curved, twisted bacilli. In old cultures, or when exposed to air for prolonged time, colonies transformed from spiral form to coccoid morphology. Examination of motility showed that Campylobacters are highly motile with characteristic corkscrew like motility, while in old cultures they were less motile. Moreover, thermophilic Campylobacters did not grow at 25°C in a microaerobic atmosphere or at 41.5°C aerobically for 48 hours.

3.2. *Identification of Campylobacter species*

For the identification of thermophilic Campylobacters to the species level, catalase test, susceptibility to nalidixic acid and cephalothin and rapid hippurate hydrolysis test were performed on 114 biochemically suspected isolates. The results showed that all Campylobacters were catalase positive, while, most of Campylobacter isolates were resistant to nalidixic acid; therefore, it was difficult to differentiate *C. lari* and *C. coli*. *C. jejuni* was differentiated by rapid Na hippurate hydrolysis test, formation of dark blue or purple color indicated a positive hippurate hydrolysis (Table 1).

3.3. *Confirmation of C. jejuni by Real-Time PCR*

qPCR targeting *hip*O gene specific for *C. jejuni* showed that 31 *C. jejuni* isolates were confirmed (Figure 1). The specificity of the reaction was characterized because primer and probe sets specific for *C. jejuni* did not amplify DNA from *C. coli* and other positive controls.

3.4. *Prevalence of Campylobacter species in different collected samples*

According to the phenotypic and biochemical identification, Campylobacter species were isolated from 21.4% of the examined samples. . The results indicated a high isolation rate of *Campylobacter* species from chicken (intestine (41%), thigh meat (38.5%), cloacal swabs (35.1%) and breast meat (30.8%) and neck skin (30.8%).

In humans, only 5.3% of the stool samples were positive for Campylobacter species (Table 2). Identification of the isolated Campylobacter species showed that *C. jejuni*, *C. coli / C. lari* and *C. hyointestinal* were identified in 54.4%, 42.1% and 3.5%, respectively. In chicken samples, *C. jejuni* were isolated from intestine, neck skin, thigh meat, cloacal swabs and breast meat with the isolation rates of 81.3%, 50%, 46.7%, 45.7% and 41.7% respectively.

However, the isolation rate of *C. coli*/*C. lari* from breast meat, thigh meat, neck skin, cloacal swabs and caecal parts was 58.3%, 53.3%, 50%, 47.8 and 12.5%. In humans, *C. jejuni* and *C. coli*/*C. lari* were identified from 76.9% and 23.1% out of the isolates respectively. *C. hyointestinal* was only identified with an incidence of 6.5% and 6.3% from cloacal swabs and intestine respectively.

4. DISCUSSION

Campylobacters are one of the most important food bacteria causing

Table (1): Results of thermophilic *Campylobacter* identification

Test / species	Catalase		Nalidixic acid		Cephalothin		Na hippurate	
	+	-	S	R	S	R	+	-
C. jejuni (62)	62	0	0	62	0	62	62	0
C. col i/ C. lari (48)	48	0	2	46	0	48	0	48
C. hyointestinal (4)	4	0	0	4	4	0	0	4

S: sensitive R: resistant

Figure (1): Amplification curve of biochemically suspected *C. jejuni* using probe bared qPCR (1:*C. jejuni* positive control, 2-12: biochemically suspected *C. jejuni* isolates, 13: Negative controls)

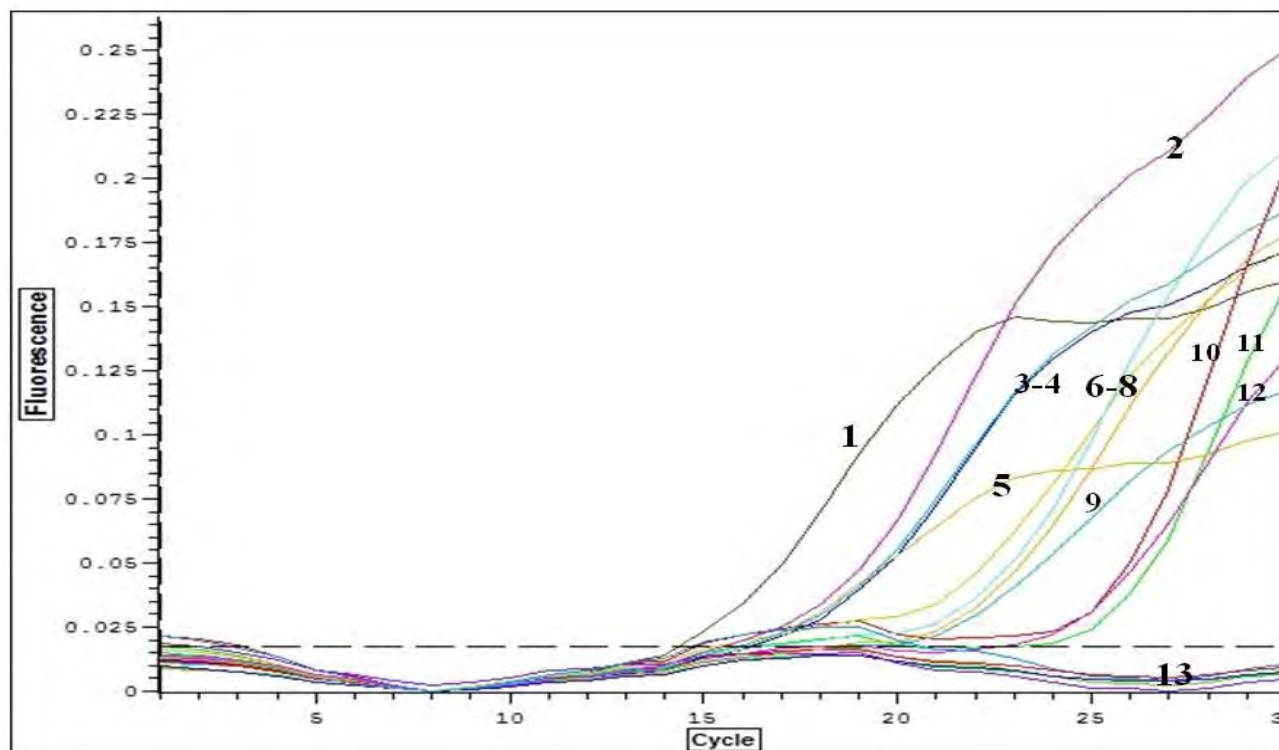

Table (2): Occurrence of different Campylobacter species in the examined samples [Number (proportion, 95% CI)]

Type of samples		Number examined	Campylobacter *species**	*C. jejuni***	*C. coli / C. lari***	*C. hyointestinal***
Chicken	Cloacal swabs	131	46 (35.1, 27-43.9)	21 (45.7, 30.9-61)	22 (47.8, 32.9-63.1)	3 (6.5, 1.4-17.9)
	Neck skin	39	12 (30.8, 17-47.6)	6 (50, 21.1-78.9)	6 (50, 21.1-78.9)	0 (0, 0-26.5)
	Breast meat	39	12 (30.8, 17-47.6)	5 (41.7, 15.2-72.3)	7 (58.3, 27.7-84.8)	0 (0, 0-26.5)
	Thigh meat	39	15 (38.5, 23.4-55.4)	7 (46.7, 21.3-73.4)	8 (53.3, 26.6-78.7)	0 (0, 0-21.8)
	Intestine	39	16 (41, 25.6-57.9)	13 (81.3, 54.5-96)	2 (12.5, 1.6-38.3)	1 (6.3, 0.2-30.2)
Humans	Stool	246	13 (5.3, 2.8-8.9)	10 (76.9, 46.2-95)	3 (23.1, 5-53.8)	0 (0, 0-24.7)
Total		533	114 (21.4, 18-25.1)	62 (54.4, 44.8-63.7)	48 (42.1, 32.9-51.7)	4 (3.5, 1-8.7)

* The isolation rate was calculated from the total number of the examined samples
** The isolation rate of each species was calculated from the total number of the isolated Campylobacters

gastroenteritis in humans in developed and developing countries (Rahimi and Ameri, 2011). Morethan 90% of the reported Campylobacter infections are caused by *C. jejuni* (NARMS, 2010). For the identification of thermophilic Campylobacter species, catalase test, susceptibility to nalidixic acid and cephalothin and rapid hippurate hydrolysis test were performed. The results showed that most of Campylobacter isolates were resistant to nalidixic acid (Table 1), so that the differentiation between *C. jejuni*, *C. lari* and *C. coli* based on the susceptibility to nalidixic acid was difficult. *C. jejuni* is differentiated by rapid Na hippurate hydrolysis test. However, this phenotypic distinction is not always accurate because other amino acids or peptides which are transported from the culture media or produced during the incubation can give false-positive results. The judgment on hippurate hydrolysis test is usually based on qualitative criteria which are not reliable and may lead to misinterpretation (Megraud, 1987). Therefore, standardization of hippurate hydrolysis test should be performed by optimizing the turbidity of cell suspension which was set between 0.8 (about MacFarland 6 turbidity) and 1.4 (at least 4 MacFarland) at 450 nm (Fitzgerald and Nachamkin, 2007). In order to confirm the identification and discrimination of *C. jejuni*, qPCR has been used targeting *hip*O (benzoglycine amidohydrolase) which is specific for the hippurate activity and discriminates *C. jejuni* from other Campylobacter species (Englen *et al*., 2003). The results in Table (1) showed that all 31 biochemically suspected *C. jejuni* isolates were confirmed by qPCR (Figure 1). The specificity of this reaction was characteristic because the primer and probe sets specific for *C. jejuni* did not amplify DNA from *C. coli* positive controls and other positive controls. Accordingly, the conventional culture methods and biochemical reactions were 100% in accordance with the results of PCR for identification and differentiation of *C. jejuni*. The same results were reported in New Zealand (Klena *et al.,* 2004) and in Egypt (Girgis *et al*., 2014). The Campylobacter species were isolated from 35.1% of the examined cloacal swabs. Similarly, the isolation rate of Campylobacter species from chicken cloacal swabs was (35.9%) that reported in Great Britain (Jorgensen *et al*., 2011). Nearly similar isolation rate was 39.2% in Estonia (Mäesaar *et al*., 2014) and 38.1% in Spain (Torralbo *et al*., 2014). Different studies reported higher prevalence rates of Campylobacter species in chickens (Ellerbroek *et al*., 2010). Such higher isolation rates could be attributed to the isolation of Campylobacter species from fresh fecal samples on the ground which is suspected to be highly contaminated with Campylobacter species from different sources such as wild birds, rodents and free living pets (Studer *et al*., 1999). Generally, the variation in Campylobacter species isolation rate between different studies could be attributed to different possible reasons, such as, type of examined samples, location, climate factors, hygienic measures and isolation as well as identification techniques (Jorgensen *et al*., 2011and Chatur *et al*., 2014). The prevalence of Campylobacter species in poultry is expected to be high in broilers slaughtered at 35–42 days, while in older chickens, the prevalence decreases reflecting acquired immunity (Kalupahana, *et al*., 2013). During the current study, the examined samples were collected from chickens at 6 weeks age, explaining the relatively high isolation rate of Campylobacters. Out of 46 Campylobacters isolated from cloacal swabs, 45.7% were identified as *C. jejuni* (Table 2). Nearly similar percentage of 44.4% was reported in Italy (Pezzotti *et al.,* 2003). Higher isolation rates of *C. jejuni* were also reported in different studies, in Nigeria (Salihu *et al*., 2012) and Malaysia (Mansouri-najand *et al*., 2012). However, lower prevalence rate of 31.4% was

obtained in Reunion Island (Henry *et al*., 2011). The Campylobacter species were isolated from 30.8% and 38.5% of the examined breast and thigh meat samples respectively. Comparable the occurrence of Campylobacter in chicken meat was reported in Bosnia (Uzunovic-Kamberovic *et al*., 2007) and Egypt (Saad, 2014). However, higher isolation rates were obtained by different studies in Iran (Zendehbad *et al*., 2013) and Poland (Wieczorek *et al*., 2013). Table (2) showed also that *C. jejuni* was isolated from 41.7% and 46.5% of breast and thigh meat samples respectively. The obtained results were lower than Sallam (2007) in Japan. However, Saad (2014) reported that the identification of *C. jejuni* was 6.9% in thigh meat samples. A large number of Campylobacter species are harbored by the intestinal tract of chicken, especially the ceca and colon. During processing activities, where the intestinal tract may leak or rupture, its contents would be transferred to the skin of carcasses. Chicken skin provides suitable microenvironment for the survival of Campylobacters due to accumulation of water which increases the surface area available for bacterial contamination (Chantarapanont *et al.,* 2003). The isolation rate of Campylobacters from skin samples was 30.8%, of which, 50% were identified as *C. jejuni* (Table 2). Different studies have also reported the isolation of Campylobacter species from chicken skin samples, for example, 47.5% in Egypt (Saad, 2014), 46.6% in North Germany (Garin *et al*., 2012) and 60% in Latvia (Kovalenko *et al*., 2013). Campylobacter species isolation rate was 41% from cecal parts (Table 2). Comparable results were also reported by Bester and Essack (2012) and Mäesaar *et al.* (2014). A previously conducted study reported that Campylobacter species were better detected by direct examination of the intestine than cloacal swabs (Bernadette *et al*., 2012). Such assumption was based on the fact that cecum is the main colonization site of Campylobacter species in chicken (Silva *et al*., 2011). Campylobacters remain highly important zoonotic pathogens worldwide. It has been estimated that as few as 500 cells of *C. jejuni* could cause human illness (Yang *et al*., 2003). For that reason, contamination of food with Campylobacters represents a potential health hazard. The occurrence of Campylobacter species in human stool samples was 5.3%. This result was nearly similar to 5.8% (Girgis *et al*., 2014) and 6.6% (Zaghloul *et al*., 2012) in Cairo. Moreover, in Alexandria, 6.4% (Pazzaglia *et al*., 1995). *C. jejuni* were identified in the current study from 76.9% of the examined human stool samples (Table 2). These results were nearly similar to 70.9% and 69.3% reported in Chile (Fernandez *et al*., 1994) and Romania (Sorokin *et al*., 2007) respectively.

In conclusion, the relatively high isolation rate of Campylobacters from chicken carcasses during the current study could be attributed to the fact that most of chickens are sold in pluck-shop markets that devoid hygienic measures leading to increase the contamination of slaughtered chicken carcasses with Campylobacters. In addition, the high proportion of chicken contaminated with Campylobacter species in different parts of the carcass pose risk for human Campylobacteriosis. Therefore, control of Campylobacter infection in poultry production is a major public health strategy for prevention of Campylobacteriosis.

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