Zoonotic Hazards of Campylobacteriosis in some areas in Egypt

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Abstract: A total of 2130 samples collected from diarrhea chicken, raw milk, milk products and stool of patient with diarrhea from Menia, Fayoum, Cairo and Qaluobya in Egypt. Samples were subjected to standard phenotypic identification of *C.jejuni*, and subsequently immunofluorescent technique (IFT) identification and genetic amplification by PCR using specific primers of hippuricase gene. The overall prevalence of *Campylobacter jejuni* in intestine and liver of chicken were 40.4 % and 37.5 % respectively, 30% tape water, 4.44% raw milk, Karish cheese and yoghurt 6.66% and 13.33% respectively and 70 (35%) children stool. The positive results of *C.jejuni* were detected by IFT expressed by green fluorescence staining. PCR amplification of *hipO* gene of *C. jejuni* isolated from the clinically diseased chicken and the environmental samples have shown identical fingerprints with human isolates at 344bp, indicating the zoonotic hazards of *Campylobacter jejuni* in Egypt**.**
[Barakat A. M. A.; Mona M. Sobhy; El Fadaly, H. A. A.; Nagwa S. Rabie; Nashwa O. Khalifa; Eman S. Ramadan; Kotb, M. H. R.; Zeinab M. S. Amin Girh; Dalia M. Sedik and Mona S. Zaki. **Zoonotic Hazards of Campylobacteriosis in some areas in Egypt.** *Life Sci J* 2015;12(7):9-14]. (ISSN:1097-8135). http://www.lifesciencesite.com. 2

Keywords: *Campylobacter*, milk product, fluorescence, prevalence, PCR**.**

1. Introduction

Food borne illness is any illness resulting from the consumption of contaminated food. There are two types of food poisoning: infectious agent and toxic agent. Food infection refers to the presence of bacteria or other microbes which infect the body after consumption. In spite of the common term food poisoning, most cases are caused by a variety of pathogenic bacteria, viruses, or parasites that contaminate food rather than chemical or natural toxins **(Food Standards Agency, 2007).** Food borne illness usually arises from improper handling, preparation, or food storage. The action of monitoring food to ensure that it will not cause food borne illness is known as food safety. **(World Health Organization, 2007).**

A diarrheal disease survey in Cairo, Egypt determined the prevalence, seasonality, and household risk factors for Campylobacter-associated diarrhea in young children. Among cases showed that Campylobacter spp. isolations were more prevalent during the rainy season (p=0.001) and positively associated with keeping fowl in the home (p=0.003) or having an outdoor source of drinking water (p=0.029) (**Pazzaglia *et al.*, 1993**).

Bacteria are a common cause of food borne illness. The individual bacteria involved were as follows: *Campylobacter jejuni* 77.3%, *Salmonella* 20.9%, *Escherichia coli O157:H7* 1.4%, and all others less than 0.1%. *Campylobacter* organism is one of the most common causes of human bacterial gastroenteritis. For instance, an estimated 2 million cases of *Campylobacter* enteritis occur annually in the U.S., accounting for 5-7% of cases of gastroenteritis **Doyle and Erickson (2007).** About 15 of every 100,000 people are diagnosed with Campylobacteriosis every year, and with many cases going unreported, up to 0.5% of the general population may unknowingly harbor *Campylobacter* in their gut **(Marler, 2015**).

USDA researchers have noted that most retail chicken is contaminated with *C. jejuni*; reported an isolation rate of 98% for retail chicken meat. *C. jejuni* counts often exceed 103 per 100 g. Skin and giblets have particularly high levels of contamination. 12% of raw milk samples from dairy farms were contaminated with *C. jejuni*. Raw milk is presumed to be contaminated by bovine feces; however, direct contamination of milk as a consequence of mastitis also occurs (**USDA, 2008**).

Most cases of Campylobacteriosis are sporadic or involve many people have been traced to contaminated water or milk. Other sources of *Campylobacter* include children and intimate contact with other infected individuals. *C. jejuni* is commonly present in the gastrointestinal tract of healthy cattle,

pigs, chickens, turkeys, ducks, and geese, and direct animal exposure can lead to infection. Pets that may carry *Campylobacter* include birds, cats, dogs, hamsters, and turtles. The organism is also occasionally isolated from streams, lakes and ponds (**Marler, 2005**). *Campylobacter* survival in surface water in a Mediterranean area (**Rodríguez and Araujo, 2012**). In Egypt *Campylobacter* infections detected in children exposed to infected backyard poultry (**El-Tras *et al*., 2015**).

A large animal reservoir is present as well, with up to 100% of poultry, including chickens, turkeys, and waterfowl, having asymptomatic infections in their intestinal tracts. Infected chicken feces may contain up to 10^9 bacteria per 25 grams, and due to the installations, the bacteria are rapidly spread to other chickens. This vastly exceeds the infectious dose of 1000-10,000 bacteria for humans (**Humphrey, *et al.*, 2007**). In 2013, the UK's Food Standards Agency warned that two-thirds of all raw chicken bought from UK shops was contaminated with campylobacter, affecting an estimated half a million people annually and killing approximately 100.

The instances of Campylobacter have increased in the past decade, according to the study, most frequently because of the "improper handling of foods by consumers and food service workers. **Wagenaar *et al.* (2013)** found that 23%of infected human cases with campylobacteriosis were associated with the consumption of unpasteurized milk and milk products in Egypt. Milk and dairy products were a major causative agent of intestinal disease *C. jejuni* it was maintained it's viable for a long period and survival under food preservation conditions (**Wang, *et al.*, 2013**).

Culture-based methods are time consuming and expensive, requiring filtration, selective enrichment, isolation and biochemical confirmation (9 days to report). The application of molecular tools, such as PCR, may help to circumvent some of the limitations of current methods **(King and Adams, 2008).** The *hipO* gene is specific for *C. jejuni* strains (**Sinh *et al.*, 2004).** Previously we used *hipO* gene for identification *C. jejuni* in clinically diarrheic chicken, dairy cattle and human **(Khalifa *et al*., 2013).**

This study was aimed to investigate the zoonotic hazards of *C.jejuni* isolated from clinical and environmental samples.

The aim of this work was to reduce the serious of Campylobacter jejuni as a foodborne disease worldwide. We describe the phenotypic and genetic characteristics of *C. jejuni* isolated from both clinical and environmental sources aiming to define their public health importance in Egypt.

2. Materials and Methods

4.1. Phase1-collection of samples from:

1.a. Chicken: We collected 880 samples from chicken (680 intestinal content, 200 liver samples from diseased, dead chicken and chicken meat from market under raw chicken) from different localities in Menia, Fayoum, and Cairo and Qaluobya governorates in Egypt**.** All samples transfer quickly to Lab (**Table, 1**).

1.b. Milk and milk products:

We collected 1050 specimens from milk and milk products (450 samples from raw milk and 300 samples karish cheeses and 300 specimens of yoghourt) were purchased from different stores in the same locality**.** All samples are collected on thioglucolate broth (**Table, 1**).

1.c. Children:

Stool samples were collected from (200) children (up to 14 years old) suffer from diarrhea and admitted to the governmental hospitals in the same governorates mentioned above **(Table, 1**). 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*.

Table (1): Samples collected from different localities in Egypt

Site of samples	No. of samples	Chicken samples		Milk&milk products			Human stools
		Intestine	Liver	Milk	Cheese	Yogurt	
Menia	580	240	80	120	60	60	20
Fayoum	510	170	20	100	70	70	80
Cairo	450	120	20	120	50	100	40
Qaluobya	590	150	80	110	80	70	100
Total	2130	680	200	450	300	300	200

4.2. Phase2- Isolation, purification and Identification

About 10 g of each sample were homogenized in sterile 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). A loopful of enrichment broth was plated on semisolid thioglycolate broth (Oxoid) and incubated in microaerophilic atmosphere at 25° C, 37°c and 42 °C for 48 -72hrs. Microscopic examination for the

incubated samples for detection of *Campylobacter* microorganisms identified under phase contrast microscope using (4 00 x) magnification power as cited by **(Smibert,1984)** for detection of characteristic motility **(Figure, 1) and** deep stab growth, typical growth ring test **(Figure,2**). According to **Holt *et al*. (1994**) suspected colonies plated onto blood agar plates **(Figure, 3)**. *Campylobacter* isolates were subculture and identified by biochemical tests including catalase production test, nitrate reduction test, hydrogen sulphide production using lead acetate paper, glycine tolerance test, sodium chloride (NaCl) 3.5% tolerance test, Hippurate hydrolysis test and sensitivity to nalidixic acid and cephalothin**.** Identified colonies were stored at -70 °C in nutrient broths with 15% glycerol until subjected to molecular identification **Sheppard and Dallas (2009**).

4.3. Phase3:

3.a. Indirect Fluorescent Antibody Techniques:

Immunofluorescent identification of *Campylobacter jejuni:*

The identification of *Campylobacter jejuni* was carried out according to Harlow **and Lane (1988)**. A volume of 20 µl is applied in duplicate to microscopic slides and prepared for immunofluorescence technique according to **Mellick *et al*. (1965).** The glass slides were fixed in ethanol at 18 - 25^{o}C for 30 minutes , air dried and antibody for *C. jejuni was added(* it was prepared by intramuscular injection in rabbits with 2 ml of 10^{11} organisms/ml of a *C. jejuni* as cited by **(Brooks *et al*., 2002)**. Sample slide carried out in a humid chamber at 37°C for 30 minutes in incubator. Subsequently, the slides are washed two times for 10 minutes in PBS and one time for 10 min. in distal water. Then added Antirabbit fluorescein isothiocyanate (FITC). Staining is carried out in a humid chamber at 37°C for 30 minutes in incubator. Then, the slides were washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in an epifluorescent microscope. Samples that show green fluorescent typical morphology of *C. jejuni* are considered positive **(Figure, 4**).

3. A. Molecular characterization of *Campylobacter jejuni*:

Isolation of DNA: DNA extracts were prepared for each isolate by 8 minutes boiling of 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 (**Iroala *et al*., 2012**).

DNA amplification reaction:

PCR mix contained 5ul template DNA and 0.2 µM *hipO* primers (**Persson and Olsen, 2005**), *hipO* – F (5` -GACT TCGT GCAG ATAT GGAT GCTT) and hipO-R (5`-GCTA TAAC TATC CGAA GAAG CCATCA) was performed in a total reaction volume of 25 µL containing PCR Master Mix (Jena Bioscience Co. Jena, Germany). Thermo cycler conditions were 94 ^{o}C for 6 min, followed by 35 cycles of 94 ^{o}C for 50 s, 57 ^{o}C for 40 s and 72 ^{o}C for 50 s and finally 72 ^{o}C for 3 min. Negative controls (PCR-grade H_2O without template) was incorporated with each set of test samples and subjected to PCR assays. The PCR amplified products were loaded onto gels of 1.5% agarose gel and stained with ethidium bromide, electrophoresis was carried out and visualized under UV rays against GeneRuler 100 bp plus DNA ladder (molecular weight marker) ready to use (Fermentas, Canada).The positive results were indicative at 344bp.

3. Results

In this investigation samples collected from Menia, Fayoum, Cairo and Qaluobya in Egypt for isolation of *Campylobacter jejuni* from chicken, milk, milk products and children **Tables (2, 3 & 4). Identification** of *C. jejuni* carried out by demonstration of characteristic motility (**Figure, 1) and** deep stab growth, typical growth ring test on semisolid thioglycolate broth **(Figure, 2**). Growth colonies observed onto blood agar plates **(Figure, 3)** and green fluorescence staining by IFT shown in Fig. **(4).**

The prevalence of *Campylobacter jejuni* was 275 (40.4%) intestinal contents, 75 (37.5%) liver from diseased chicken, (30%) , 4.44% raw milk, 6.66% karish cheese and13.33% yoghurt and 70 (35%) children stool (**Table, 2**).

Table (2): Incidence of *Campylobacter jejuni* from different cases

Type of samples	No. of samples	Positive samples	%
Diseased Chickens	880	340	**38.64%**
Milk &milk products	1050	80	**7.62%**
Human stool	200	80	**40.00%**
Total number	**2130**	**500**	**23.47%**

Table (3): Incidence of *Campylobacter jejuni isolated* from chicken samples.

Type of samples	No. of samples	Positive samples	%
Intestine of diseased chickens	**680**	**260**	**38.2%**
Liver of diseased chickens	200	80	40%
Total	880	340	37.75%

Table (4): Incidence of *Campylobacter jejuni isolated* from milk and milk products.

Type of samples	No. of samples	Positive Samples	%
Raw milk	450	20	4.4%
Cheese	300	20	6.7%
Yoghurt	**300**	**40**	**13.4**%
Total	**1050**	**80**	***7.6%***

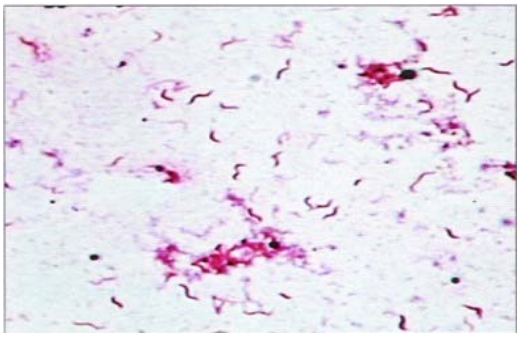


Figure (1): *Campylobacter* organisms from 48 hrs. semisolid thiol medium stained by Grams stain showing comma, S-shape, and Spiral forms (Magnification 1000X).

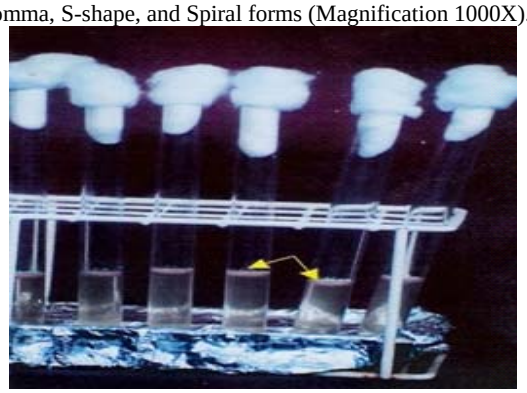


Figure (2): Growth in Semisolid thiol medium after 48 hrs. showing the characterstic ring form of *Campylobacter* species.

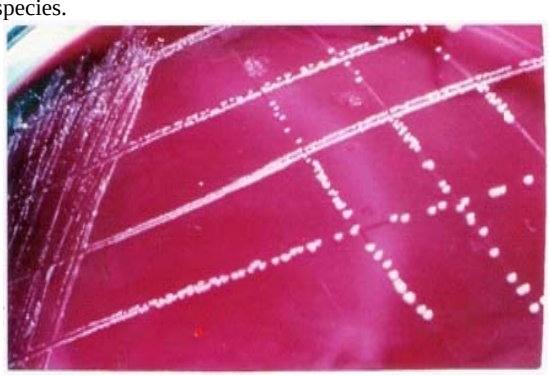
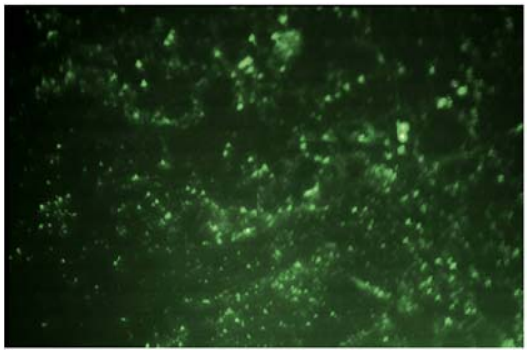


Figure (3): *Campylobacter* colonies after 48 hrs. on blood agar plates.



Figer (4): positive results of *Campylobacterjejuni* by IFT

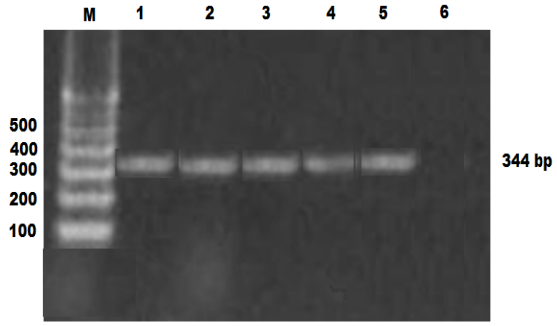


Figure (5*):* PCR amplification of the 344 bp product of the DNA extracted from *Campylobacter jejuni*. Lane M: a 100 bp molecular size marker. Lanes 1, 2, 3 and 4 and 5: *Campylobacter jejuni* isolated from diseased chicken, water, milk, milk products and human respectively. Lane 6: negative control.

4. Discussion:

Campylobacter is one of the most common causes of diarrheal illness in the world. Active surveillance through the Foodborne Diseases indicates that about 14 cases are diagnosed each year for each 100,000 persons in the population. Many more cases go undiagnosed or unreported, and campylobacteriosis is estimated to affect over 1.3 million persons every year. Campylobacteriosis occurs much more frequently in the summer months

than in the winter. The organism is isolated from infants and young adults more frequently than from persons in other age groups and from males more frequently than females (**CDC, 2014**).

Campylobacter jejuni is one of the most zoonotic pathogens between animal and humans. Human illness due to *C. jejuni* infection is closely associated with consumption of poultry products. Tables (2, 3 & 4) illustrate the prevalence of C. jejuni from different samples (23.47%)**.** These results were agreed with **Vandamme *et al.* (2010) and Anonymous (2010).** Chickens have been considered as a reservoir and a main source of human campylobacteriosis. Furthermore, poultry, contamination levels peak during the summer months and this seasonal pattern is reflected in the number of reported *Campylobacter* infections (**Vandamme *et al.*, 2010**).

The prevalence of *Campylobacter jejuni* was found to be 275 (40.4%) intestinal contents and 75 (37.5%) liver of diseased chicken. Our result is higher than *C. jejuni* isolated from 36% in chicken with diarrhea **(Khalifa, *et al.*, 2013)** and 23·5% of poultry **(El-Tras, *et al.*, 2015)** in Egypt**.** These differences in the prevalence of chicken associated *Campylobacter* can be attributed to several factors, including isolation methods, sample size and type seasonal variations children and geographical location **(Allos, 2001).** Although all commercial poultry species can carry *Campylobacters*, the risk is greater from chicken because of the high levels of consumption (**Humphrey, *et al.*, 2007**).

It is noticed that *C. jejuni* isolated from 4.44% raw milk, 6.66% karish cheese and 13.33% yoghurt. An observation in agreement with **Saad *et al.* (2007**) who isolated *C. jejuni* from raw milk and milk products in Assiut, Governorate. It's clear from our findings that the incidence of C. Jejuni is lower than 12% in raw milk samples collected from dairy farms. Raw milk is presumed to be contaminated by bovine feces; however, direct contamination of milk as a consequence of mastitis also occurs (**USDA, 2009**).

The prevalence of *Campylobacter jejuni* in children with diarrhea was 70 (35%). This finding is higher than that has been cited in our previous work (26%) *in stool samples collected from* children in Toukh, Kaliobia of the attributed to the high infection in chicken and milk and milk product in the same locality of children inhabitants mentioned above. As human *C. jejuni* infections occur mainly from contaminated poultry or raw milk and milk products (**Solomon and Hoover, 1999**). Animal food products were most commonly contaminated by this pathogen during slaughter and carcass dressing (**Berndtson *et al.*, 1996).** Moreover, consumption of unpasteurized milk and milk products had been implicated in infection of 23% human cases with campylobacteriosis in Egypt (**Wang, *et al.*, 2013).**

In our study PCR amplification of the 344 bp product of the DNA extracted from *C. jejuni* isolated from chicken , milk and milk product showed identical fingerprints with human isolates, these compatibility of the obtained DNA bands based on hippuricase gene amplified at 344bp is in accordance with **Person and Olsen (2005).** A finding substantiates our previous uses of *hipO* gene in molecular study of isolated C. jejuni strains from chicken, dairy cattle and human to determine their zoonotic importance **(Iroala, *et al.*, 2012 and Khalifa *et al.*, 2013).**

The results from this study further highlight the importance of *Campylobacter jejuni* in public health and underscore the need for enhanced efforts in the surveillance and investigation of sources for better control of the zoonotic transmission of *Campylobacter* species. We can conclude from our study that the high prevalence of *C. jejuni* in clinically diseased chicken and contaminated milk and dairy product incriminated in the high infection rate among children. Highlighted on the epidemiology of the disease in Egypt and provide the background for the design of cost efficient control strategies.

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