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Original Research Article

Prevalence of *Campylobacter* species in milk and some dairy products

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ABSTRACT

Campylobacteriosis is assumed to be mainly a food-borne disease. Also the importance of milk as a source of human Campylobacter enteritis was confirmed by the European Union summary report on food-borne disease outbreaks. Therefore, the present study was undertaken to detect the prevalence of Campylobacters in milk and milk products. A total of 250 samples (100 milk, 50 Domiati cheese, 50 kareish cheese and 50 ice-cream) were collected from the different collection points in El-Minia and Beni-Suef Governorates. The samples were examined by microbiological culture method, and presumptive isolates were further confirmed by genetic amplification (PCR) using specific primers of hippuricase gene.The overall prevalence of *Campylobacter species* were 13% in raw milk, 52% in kareish cheese, 18% in Domiati cheese and 6% in ice-cream. PCR amplification of *hipO* gene of isolated *C. jejuni* from the milk and milk products samples had been shown identical fingerprints with human isolates at 323bp, which indicates the zoonotic hazards of *Campylobacter jejuni* in Egypt.

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1. Introduction

Milk is a basic food in human diet either in its original form or in a various dairy products, as it contains high quality of animal protein and fats as well as vitamins and minerals which are important nutrients either for young, adult or elderly people. On the other hand, milk has a high water activity (a_w=0.99) and slight acidic pH (ICMSF, 2005 and Roos, 2011). Because of this, milk is an excellent substrate for the growth of microorganisms and raw milk acts as the main source for various pathogens such as Campylobacter (Leedom, 2006), which is the leading cause of zoonotic infections in many countries, and the public health burden of campylobacteriosis is increasing day to day (Horrocks et al., 2009).

Generally, campylobacteriosis is assumed to be mainly a food-borne disease (Man, 2011). Moreover the main source of Campylobacter infection is probably raw milk and milk products which are the most commonly implicated vehicles in food-borne outbreaks of campylobacter enteritis (Richter et al., 1992 and Bean et al., 1996). C*ampylobacter jejuni* and *Campylobacter coli* are the most important from a food safety point of view (CDC, 2005). Other species such as *C. upsaliensis*, *C. fetus*, *C.*

hyointestinalis, *C. laridis* and *C. ureolyticus* have occasionally been reported as causing human illness (Yan et al., 2005). In the United States, 2.4 million campylobacteriosis cases estimated to occur per year (Schielke et al., 2014). Cattle frequently harbor Campylobacter as commensal in their gastrointestinal tract and Campylobacters in raw milk most commonly derived from secondary fecal contamination during the milking process (Oliver et al., 2005). Also udder excretion in addition to fecal matter may be a route of bulk milk contamination (Bianchini et al., 2014).

Dairy products are liable to contamination with different types of microorganisms from different sources during production, processing and handling, which lead them to be unfit for consumption and constitute a public health hazard (Todaro et al., 2013). Cheeses are ready-to-eat food products that do not undergo any further treatment to ensure their safety before consumption, contamination of cheese with foodborne pathogens may occur at several stages. Pathogens may also enter cheese during processing, if hygienic and process controls are inadequate (Fernandes, 2008). On the other hand, manufacture of kareish cheese from raw milk is still primitive and unhygienic, a fact that may expose the product to serious contamination. Environmental conditions prevailing during processing and storage, combined with the composition of the cheese often, reduce considerably its quality (Reps et al., 2002).

The microbial content of ice-cream as well as individual pathogens like Campylobacter largely reflects the quality and safety of ingredients used for its manufacture. The fluid, dry components as well as addition of flavors, coloring agents, fruits, nuts and chocolate chips to the mix after pasteurization can be a source of contamination. In addition poorly cleaned equipment, air incorporation, poor use of product rerun and personnel are considering post-pasteurization contamination sources (Goff, 1988). *Campylobacter* could be detected at different percentage in ice-cream (Nasr et al., 2004 and Rahimi et al., 2013). The incubation period of *Campylobacter jejuni* microorganism typically varies from one to seven days (Butzler, 2004). The infective dose of *C. jejuni* ranges from 500 to 10.000 cells, depending on the virulence of strain, damage of cells from environmental stresses and the susceptibility of the host (Snyder and Poland, 1990; Doyle, 1991; Reed, 1994 and Philips, 1995). The prevalence of *Campylobacter* spp. may vary in different dairy products, it has been shown that Campylobacter isolates can be found more frequently in raw milk samples and soft cheeses (Hussain et al., 2007; El-Sharoud, 2009; Salihu et al., 2010). As *Campylobacter* spp. continues to be highly important human pathogens, and the public health burden of campylobacteriosis is increasing day to day the present work was planned to study the prevalence of *Campylobacter spp*. in milk, Domiati cheese, kareish cheese and ice-cream, identifying and characterizing of the prevalent Campylobacter by using the culturing method and PCR technique and finally to know the public health risk from *Campylobacter* spp. in milk and dairy products with its control.

2. Materials and methods

2.1. Collection of samples

A total of 250 random samples including raw milk (100), soft cheeses (Domiati and kareish, 50 samples of each) and small scale ice-cream(50 samples)were collected from different retail shops and vendors in El-Minia and Beni-Suef Governorates (equal number of samples of each governorate). All samples were collected in sterilized bottles and transported to the laboratory in an insulated ice box at 4˚C within 1-2 h of collection and analyzed immediately upon arrival.

2.2. Isolation, Purification and Identification

Samples were examined for the presence of *Campylobacter* spp. using selective enrichment and isolation protocol recommended by Roberts and Greenwood (2003).One ml. of the homogenized samples was aseptically inoculated into sterile screw capped tube, containing 9ml of Bolton broth (Oxoid Ltd, Basingstoke, Hampshire, England) containing 5% laked horse blood and Bolton broth selective supplement which incubated under appropriate microaerophilic conditions in anaerobic jar by using the Gas Pack System BBL (5% O_2, 10% CO_2 and 85% N_2) at 37˚C for about 4 hours prior to increasing the temperature to 41.5˚C for the remainder of the 48 hours of the incubation time for

resuscitation. Loopful of the incubated broth was plated onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, Oxoid) with CCDA selective supplement and the plates were incubated for 48 hours at 41.5˚C under appropriate microaerophilic conditions, suspected colonies were selected and isolated. Presumptive colonies of *Campylobacter* spp. were subjected to standard biochemical tests, including oxidase test, catalase production test, nitrate reduction test, hydrogen sulphide production using lead acetate paper, glycine tolerance test, NaCl 3.5% tolerance test, sensitivity to Nalidixic acid and Cephalothin and Hippurate hydrolysis test. Biochemically identified *C. jejuni* colonieswere stored at -70 °C in nutrient broths with 15% glycerol until subjected to molecular PCR identification.

2.3. Molecular characterization of *C. jejuni*

2.3.1. DNA amplification reaction

PCR mix contained 6ul template DNA and 20 pmol of each hipO primer (Wang et al., 2002), hipO gene (F, 5'ACTTCTTTATTGCTTGCTGC3' and R, 5'GCCACAACAAGTAAAGAAGC3') was performed in a total reaction volume of 25 µL containing PCR Master Mix. Thermo cycler conditions were 94˚C for 6 min, followed by 35 cycles of 95˚C for 30 s, 55˚C for 30 s and 72˚C for 30 s and finally 72˚C for 10 min. Negative controls (PCR-grade H2O 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 Gel Pilot 100 bp ladder (molecular weight marker) supplied from QIAGEN (USA). The gel was photographed by a gel documentation system and the data was analyzed through computer software. The positive results were indicative at 323bp.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
HipO	94˚C 6 min.	95˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.

Cycling conditions of the different primersduring cPCR

Primer	Target agent	Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
CJF	*C. jejuni*	*hipO*	ACTTCTTTATTGCTTGCTGC	323 bp	Wang*et al.*, 2002
CJR			GCCACAACAAGTAAAGAAGC		

Oligonucleotide primers sequences.

3. Results

Table 1. Incidence of isolated *Campylobacter* spp. in the examined milk and milk products samples.

Examined samples	No. of samples	No. of *Campylobacter* spp.	%	Identified isolates: No. of *C. jejuni*	%	No. of *C. coli*	%	No. of *C. laridis*	%
Raw milk	100	13	13%	2	2%	8	8%	3	3%
Domiati cheese	50	9	18%	3	6%	1	2%	5	10%
kareish cheese	50	26	52%	7	14%	4	8%	15	30%
Ice-cream	50	3	6%	3	6%	-	-	-	-
Total	250	51	20.4%	15	6%	13	5.2%	23	9.2%

Table 2. Incidence of different *Campylobacter* spp. in the examined milk and milk products samples collected from different sources.

Sources of milk and milk products samples	No. of samples	No. of *Campylobacter* spp.	%
Minia	125	15	12%
Beni-suef	125	36	28.8%
Total	250	51	20.4%

Table 3. Incidence of *Campylobacter jejuni* in the examined milk and milk products samples according to the biochemical tests and PCR assay.

Examined samples	No. of examined samples	Biochemical tests		PCR assay	
		No. of *C. jejuni*	%	No. of *C. jejuni*	%
Raw milk	100	2	2%	1	1%
Domiati cheese	50	3	6%	2	4%
Kareish cheese	50	7	14%	2	4%
Ice-cream	50	3	6%	1	2%
Total	250	15	6%	6	2.4%

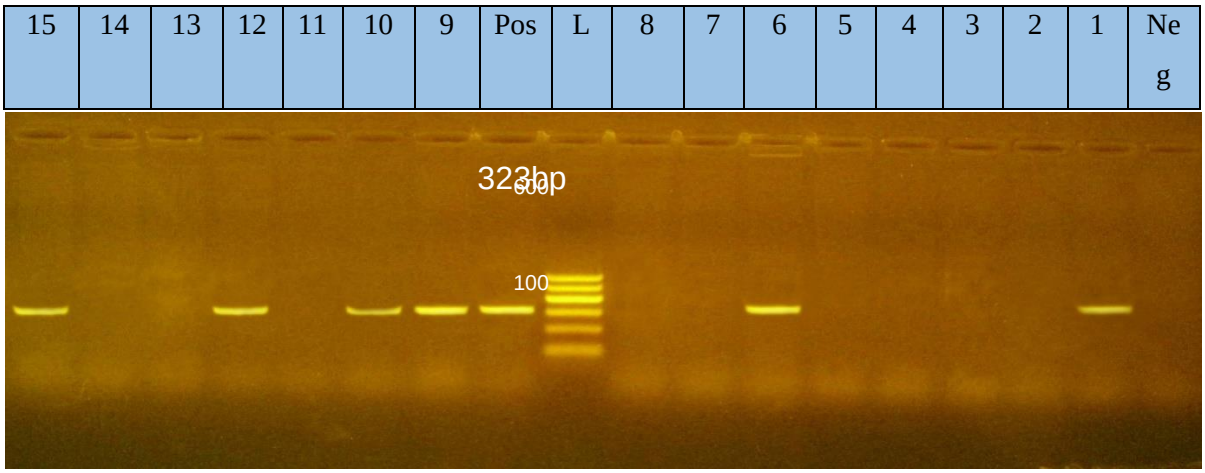

Fig. 1. Result of PCR technique for identification of *Campylobacter jejuni*. Lane Neg.: Control negative. Lane 1: Positive strain isolated from raw milk. Lanes 2, 3, 4, 5: Negative strains. Lane 6: Positive strain isolated from ice-cream. Lanes 7, 8: Negative strains. Lane L: 100 bp ladder as molecular size DNA marker. Lane Pos.: Control positive *Campylobacter jejuni* for hipO gene. Lane 9, 10: Positive strains isolated from Domiati cheese. Lane 11, 13, 14: Negative strains. Lane 12, 15: Positive strains isolated from kareish cheese.

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4. Discussion

Acute gastroenteritis, though common, can be lethal and therefore constitutes one of the most common challenges faced by medical practitioners in the developing countries. Etiological agents can be viral, bacterial, or protozoan. Bacterial agents can be either enteropathogenic, toxigenic, or both. Among the bacterial agents, thermotolerant *Campylobacter* is the most frequent cause of intestinal infections worldwide (Salehi et al., 2014).

Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health

importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli* (Nachamkin and Blaser, 2000). The results reported in Table (1) showed that *Campylobacter* spp. were isolated from 13 (13%) out of 100 examined raw milk samples. These results were nearly in agreement with those obtained by Khanzadi et al. (2010) and Giacometti et al. (2012). Lower percentages were recorded by Barakat et al. (2015) and Modi et al. (2015). Discrepantly, higher percentages were recorded by Wicker et al. (2001); Martin et al. (2007) and Mabotel et al. (2011). However, some investigators failed to detect *Campylobacter* spp. in the examined milk samples Singh et al. (2009).

The discrepancy between the various studies and the present study could be attributed to multitude factors of which level of contamination of milk with Campylobacters, type and condition of *Campylobacter* strain present in milk, storage temperature as the survival of Campylobacter in milk will decrease with increasing storage temperature and methods of isolation, variety of enrichment broth systems, high sensitivity of the organism to normal atmospheric concentration of oxygen and adverse conditions resulting from acid development in raw milk that represent stress factor on the organism resulting in failure of cultural trials even from contaminated samples (Ray and Johnson, 1984). On the other hand some authors suggested that the presence of natural bactericidal compounds or systems in milk such as lactoperoxidase (Lps) affect the viability of the organism in milk (Abdel-Hakiem, 1994) whereas the pH developed by the lactics in raw milk (5 or less) leading to activate this system in milk, whereby the bacteria is destroyed (Barrell, 1981). Moreover, it has been recorded that lactic acid is an inhibitory to *C. jejuni* as pH 5 completely destroys the organism, the existence of an injured, viable but non culturable form of the bacterium (Abdel-Hady, 1993) and the competing microorganisms which might produce toxic metabolites to *Campylobacter* and growth of these organisms may lower the pH of the milk resulting in a higher rate of *Campylobacter* inactivation during a cold storage period (Boer et al., 1984).

On the other hand, Table (1) showed that different Campylobacter organisms could be isolated in variant percentages from the examined raw milk samples, as 13 isolates recovered were identified as *C. jejuni* (2 isolates), *C. coli* (8 isolates), *C. laridis* (3 isolates). Most human infections, about 85% to 95%, involve *C. jejuni*, while *C. coli* is responsible for the majority of the remainder (Lansing et al, 2005). Although the majority of documented *Campylobacter* infections are caused by *C. jejuni*, *C. coli*, and *C. fetus*, other species are being increasingly recognized as human pathogens. *Campylobacter laridis* is infrequently isolated from humans, but has been associated with enteritis, bacteremia, permanent pacemaker infection, purulent pleurisy and urinary tract infection (Matsuda and Moore, 2011).

In Egypt, Domiati cheese represents a major sector of the dairy industry, and it is estimated that 36% of total milk production is utilized in the manufacture of these products (Anonymous, 2002). A variable prevalence rate of Campylobacter in cheese was recorded (Jain and Shrivastava, 2012 and Giacometti et al., 2013). As recorded in Table (1) it was apparent that out of 50 examined samples of Domiati cheese, Campylobacter isolated from 9 samples (18%). This result was close to that obtained by Hussain et al. (2007) and El-Sharoud (2009) while lower percentages were recorded by El-Nokrashy et al. (1998), Nasr et al. (2004) and Rahimi et al. (2013). Several investigators failed to isolate *Campylobacter spp*. from the examined cheese samples (Whyte et al., 2004 and Modi et al., 2015).

On the other hand, from the results presented in Table (1) it was evident that different Campylobacter organisms could be isolated in a variant percentages from the examined Domiati cheese samples, as the 9 isolates were identified as 3 (6%) *C. jejuni*, 1 (2%) *C. coli* and 5 (10%) *C. laridis*.

The higher percentage of Campylobacter could be attributed to inadequate pasteurization of milk or post pasteurization contamination, starter failure, poor control of salt addition, persistence of pathogens in the biofilms, lack of general food hygiene related knowledge and infrastructure of marketing could be the sources of contamination (Truzyan, 2003; Nasr et al., 2004 and Latorre et al., 2010). Another possibility to explain the results

obtained is Campylobacter's capacity of generating viable non-culturable forms (VNC) in adverse environment, which are viable but not culturable (Trachoo et al., 2002) and the VNC form in *Campylobacter* spp. is induced by factors such as stress due to scarcity of nutrients and represents the organism's survival strategy in the natural environment (Rowe et al., 1998).

Kareish cheese is the most popular soft cheese in Egypt due to its remarkable health quality as only known relatively fat free cheese, an excellent source of protein, calcium, phosphorus and many micronutrients and its cheap price. It comprises about 50% of white soft cheese produced in Egypt (Hegazy et al., 2012).

The results in Table 1 revealed that *Campylobacter* spp. were isolated from 26 (52%) of 50 examined kareish cheese samples. According to the results represented in Table (1), it was evident that different *Campylobacter* organisms could be isolated in a variant percentage from the examined samples and 26 isolates were identified as 7 (14%) *C. jejuni*, 4 (8%) *C. coli* and 15 (30%) *C. laridis*. The recorded results were higher than those reported by Nasr et al. (2004), while lower percentages were reported by Barakat et al. (2015) and Omara et al. (2015) but some investigators failed to isolate *Campylobacter spp*. from Kareish cheese (Abdel-Hady 1993; Federighi et al., 1999; Whyte et al., 2004 and Modi et al., 2015).

Consumption of unpasteurized milk and milk products has been implicated in infections of 23% human cases with campylobacteriosis in Egypt (Wang et al., 2013). The occurrence of *Campylobacter* species in traditional dairy products could be due to environmental contamination with infected animal wastes or unsanitary food production and storage practices (Rahimi et al., 2013). The obtained data indicate how is the inferior quality and risky hazardous as food as white soft Egyptian cheese with different varieties which might be an etiology for foodborne illness.

The microbiological quality of ice cream can be low, as it is a good growth-medium for microbes due to its nutrients (lactose, proteins, etc.) and to its almost neutral pH of 6–7and long storage duration (Kanbakan et al., 2004). The data presented in Table (1) showed that out of 50 examined samples of small scale ice-cream 3 samples (6%) contained *Campylobacter* spp. which represented by *C. jejuni*.

These results were agreement with those reported by Nasr et al. (2004), however lower incidence was reported by Rahimi et al. (2013). Also some investigators failed to isolate *Campylobacter spp*. from ice-cream (Miljkovic and Katic, 1989 and Abdel-Hady, 1993).

The results presented in Table 2 summarized that the prevalence of *Campylobacter* spp. was 12% in El-Minia Governorate and 28.8% in Beni-Suef Governorate. The differentiation of *C. jejuni* from *C. coli* relies on the ability of *C. jejuni* to hydrolyze hippurate (Roop et al., 1984), but certain atypical *C. jejuni* strains fail to do so (Roop et al., 1984; Nicholson and Patton, 1993), these limitations might in principle be overcame by the use of PCR-based genotypic methods. The PCR is a method which is definitive, reliable, easy to use and is required to facilitate rapid identification of *C. jejuni* (Linton et al., 1997). The hipO gene is specific for *C. jejuni* strains (Sinha et al., 2004). The used PCR protocol based on the design of a primer pair that targets a 323bp fragment of the hipO gene (F, 5'ACTTCTTTATTGCTTGCTGC3' and R, 5'GCCACAACAAGTAAAGAAGC3').

It is clearly obvious from finding presented in Photo (3) that, lanes 1, 6, 9, 10, 12 and 15 were positive for *C. jejuni* at 323 bp while lanes from 2-5, 7, 8, 11, 13 and 14 indicated negative results for *C. jejuni* strains. PCR amplification of hipO gene of *C. jejuni* isolated from the examined samples have shown identical fingerprints with human isolates at 323bp in accordance with Wang et al. (2002), while Khalifa et al. (2013) shown that PCR amplification of hipO gene of *C. jejuni* isolated from the examined samples have shown identical fingerprints with human isolates at 344bp.

It is evident from the results in Table 3 that Considerable variability was observed in the frequency of isolation of *Campylobacter jejuni* by biochemical test and PCR as 15 (6%) and 6 (2.4%) *Campylobacter* isolates were recovered from milk and some milk products samples were identified as *C. jejuni* by biochemical tests and PCR assay, respectively. Furthermore, Table (3) shown that the

incidence of *C. jejuni* in the examined milk samples was 1%. Whereas, in Domiati cheese and kareish cheese was isolated in percentage of 4% of each of them and Ice-cream was 2% by PCR assay.

This study showed that the PCR based on the hippuricase (hipO) gene provided a tool for specific identification and isolation of *C. jejuni*. The 9 bacterial isolates not confirmed as *C. jejuni* could probably be explained by difficulties in identifying the correct colony or over growth from neighboring colonies (Jensen et al., 2005) Meanwhile, Campylobacters are a diverse and fastidious group of bacteria that may form spherical or coccoid bodies if exposed to air for prolonged periods which is a degenerated form that has lost its motility and is difficult to subculture (Buck et al., 1983). As *Campylobacter* are fragile organisms, they susceptible to a number of environmental conditions such as the presence of oxygen, temperature, pH, UV and humidity (Isohanni and Lyhs, 2009). The microaerophilic nature of the Campylobacters may be related to their sensitivity to toxic reduced forms of oxygen, such as superoxide radicles and hydrogen peroxide which lead to the damage in DNA and protein structures (Park, 2002). On the other hand, mutation in hipO gene has previously been identified as a source of failure for the PCR assay targeting that gene (On and Jordan, 2003). Both physicochemical relating to the PCR and biological relating to the diversity of *Campylobacter* factors account for this variability.

5. Conclusion

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 contaminated milk and dairy product incriminated in the high infection rate among people and highlights on the epidemiology of the disease in Egypt and provide the background for the design of cost efficient control strategies must be taken in consideration.

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