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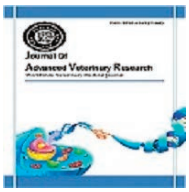

Bacteriological and Molecular Identification of Thermophilic *Campylobacters* of Animal and Human Origins in Beni-Suef Governorate, Egypt

Sherin R. Rouby[1], Gihan K. Abdel-Latef[2], Sahar Abdel Aleem Abdel Aziz[2*]

[1]*Department of Veterinary Medicine, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt.*
[2]*Department of Hygiene, Zoonoses and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511,* Egypt.

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ABSTRACT

Thermophilic species of the genus *Campylobacter* are generally considered commensals of livestock and the leading cause of bacterial food-borne zoonoses. The present study was delineated to clarify the role of *Campylobacter* species as a diarrheagenic pathogen in animals and man and to investigate the fecal carriage rate of *Campylobacters* in animals and in-contact humans. A total number of 78 fecal samples were collected from diarrheic and non-diarrheic cattle (*n*=26), sheep (*n*=28) and humans (*n*=24). Samples were enriched in Preston broth, followed by streaking on selective *Campylobacter* agar base medium. The suspected colonies were tested morphologically and biochemically. *Campylobacter* spp. was recovered from 29 (37.17%) out of 78 fecal samples (34.61%, 42.85% and 33.33%) for cattle, sheep and humans, respectively. Positive correlation between the occurrence of diarrhea and the isolation of *Campylobacters* was observed in samples of human origin while in adult ruminants particularly sheep, high fecal carriage rate was observed in non-diarrheic animals. The isolates were identified to genus and species levels by polymerase chain reaction targeting the 16S *rRNA* gene, the *mapA* gene and the *ceuE* gene which revealed that all of isolates were *Campylobacter jejuni*. These findings pose a significant epidemiological implication where cattle and sheep act as vehicles of, and excrete *Campylobacter jejuni* which is capable of causing disease in the local community in the area of investigation.

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Introduction

From the beginning of the last century, *Campylobacter* was recognized as an animal pathogen. Currently the genus *Campylobacter* counts 27 species, 9 subspecies and 3 biovars, out of them 19 species are considered to be pathogenic for humans and 9 for the animals (Ngulukun, 2017) with the thermotolerant *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) represent the most important species in terms of the food safety point and public health potentials (Foster *et al*., 2004). Thermotolerant *Campylobacters* are frequent colonizers of the intestine of livestock such as cattle and sheep (Leuchtefeld and Wang, 1982), which represent potential reservoirs for humans. High fecal carriage rates of *C. jejuni/coli* have been reported in young animals than in older animals. In the latter, the organisms can be occasionally detected in feces due to low numbers or due to intermittent shedding. Contact with infected animals, consumption of contaminated water or raw milk and travelling in high prevalence areas constitute the major risk factors in human infections (Friedman *et al*., 2000).

Campylobacter jejuni/coli are the leading cause of food-borne bacterial gastroenteritis worldwide (Moore *et al*., 2005; Behringer *et al*., 2011; Mdegela *et al*., 2011; Silva *et al*., 2011). *Campylobacter*iosis is associated with 400–500 million cases of diarrhea per year; this number of cases often exceeds those recorded for salmonellosis and shigellosis (Ruiz-Palacios, 2007). In developing countries, the disease remains underreported due to the lack of regular surveillance programs (Coker *et al*., 2002; Workman *et al*., 2006).

Human infection is characterized by self-limiting diarrhea, acute dysentery, abdominal cramps lasting for 7 days and fever (Skirrow and Blaser, 2000; Moore *et al*., 2005; Messens *et al*., 2009, Blanco and pall, 2018) however, some individuals develop sequelae after the acute phase, *C. jejuni* infection may lead to autoimmune conditions known as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome. They have also been reported to be involved in extra-gastrointestinal manifestations, including bacteremia, lung infections, brain abscesses, meningitis, and reactive arthritis (Man, 2011). More recent studies suggested that *C. jejuni* infections can lead to inflam-

*Corresponding author: Sahar Abdel Aleem Abdel Aziz
E-mail address: abdelaziz.sahar@yahoo.com

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matory bowel disease such as Crohn's disease as well as abortion (Horrocks *et al*., 2009).

Diagnosis of campylobacteriosis relies on isolation and identification of the causative organism using a battery of biochemical tests. *Campylobacters* require microaerobic conditions and grow optimally at 42°C; they neither ferment nor oxidize carbohydrates. The close relationship between the species makes the tests unreliable in distinction between *Campylobacter* species (Presson and Olsen, 2005). Using molecular techniques, *Campylobacter* can be easily identified not only to genus level but also to species level (Moore *et al*., 2005; Silva *et al*., 2011).

The objective of the present study was directed to clarify the role of *Campylobacter* species as a diarrheagenic pathogen in animals and man employing different bacteriological, biochemical and molecular tools.

Materials and methods

Area of study

The present study was performed on cattle, sheep and in-contact humans located in Beni-Suef Governorate (coordinates: 29°04'N31°05'E), Egypt from June 2016 till May 2017.

Animals and clinical samples

A total number of 54 animals consisting of 26 cattle (six calves of 1-8 weeks and 20 adults) and 28 sheep (five newly born lambs, 12 sheep of 6-12 months and 11 >one years old) were examined for signs of enteritis in terms of fecal consistency, color and odor.

Amongst 54 animals, five cattle (four calves and one adult) and four sheep (one newly born lamb and three sheep of 6-12 months) were suffered clinically from enteritis manifested by diarrhea. Fecal samples of these animals were subjected for exhaustive bacteriological examination for isolation of *Campylobacter* spp.

Human samples

Diarrheic (*n*=7) and non-diarrheic (*n*=17) humans in actual contact with the above mentioned examined animals were included in this study. Stool samples of humans were subjected for exhaustive bacteriological examination for isolation of *Campylobacter* spp.

Samples were kept at 4°C and processed for isolation of *Campylobacter* spp. within one hour of collection. Data of animal and human samples are illustrated in Table 1.

The present study was approved by the institutional animal care and use committee of Beni-Suef University (BSU-IACUC) and IRB (Faculty of Medicine, Beni-Suef University).

Isolation and phenotypic identification of Campylobacter species

For selective isolation of *Campylobacter* spp., approximately one gram from each fecal sample was inoculated into a tube containing 9 ml of Preston *Campylobacter* selective enrichment broth (prepared by adding 12.5 g of Nutrient Broth No. 2 in 475 ml of distilled water and sterilized by autoclaving followed by cooling at 50°C then adding aseptically 25 ml of lysed horse blood, and one vial of Preston *Campylobacter* Selective Supplement (Oxoid Ltd, Basingstoke, Hampshire, England). Broth was incubated at 42 °C for 24 hrs. in anaerobic jar using commercial Gas Packs System BBL (5.0% O_2, 10.0% CO_2 and 85.0% N_2, Oxoid, Unipath, Basingstoke, Hampshire, England) (Bolton and Robertson, 1982).

A loopful from each broth was streaked on the surface of *Campylobacter* selective agar base containing an antibiotic supplement for the selective isolation of *Campylobacter* species (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood. All inoculated plates were incubated for 24-48 hrs. at 42°C under the above mentioned conditions (Roberts and Greenwood, 2003).

Suspected colonies that appeared as translucent white, moist and glistening were picked and re-streaked onto selective media and incubated at 41.5°C into gas pack apparatus under microaerophilic condition for 24 hrs. for purification. Phenotypic identification of presumptive *Campylobacter* species was done using standard biochemical and microbiological techniques according to On (1996).

DNA extraction

Pure colonies on selective media plates were picked and suspended in sterile deionized distilled water then boiled in a water bath for 10 minutes. The samples were cooled immediately for 5-10 minutes on ice and centrifuged at 10000 × g for 10 min. The supernatants were used as DNA templates for the molecular identification (Van Eys *et al*., 1989).

DNA amplification

The 16S *rRNA* gene (Linton *et al*., 1997) was amplified to detect *Campylobacter* on the genus level while; the *mapA* gene (Stucki *et al*., 1995) and the *ceuE* gene (Gonzalez *et al*., 1997) were selected to detect *Campylobacters* on species level (*C. jejuni* and *C. coli*, respectively). Primer sequences and origin are illustrated in Table 2.

Amplification reactions were carried out using a DNA thermal cycler (Labnet® Multigen Gradient thermal cycler, Catalog TC9600-G- 230V (Labnet international, Inc. Edison, NJ, USA) with the following program: one cycle of 5 min at 95°C, 35 cycles each consisting of 45 s at 94°C, 45 s at 59°C, 1 min. at 72°C and a final extension step of 10 min. at 72°C.

Table 1. Data of animal and human samples and results of isolation

Species/ Number	Age	No.	Results of isolation						Total
			Diarrheic			Non- diarrheic			
			No.	Result	%	No.	Result	%	
Animals (*n*=54)									
Cattle (*n*=26)	Calves under 2 months old	6	4	4	100	2	0	0	9 (34.61%)
	Adults	20	1	1	100	19	4	21.05	
Sheep (*n*=28)	Newly born lambs	5	1	1	100	4	0	0	
	6:12 months old	12	3	0	0	9	4	44.44	12 (42.85%)
	Above one year	11	0	0	0	11	7	63.63	
Humans (*n*=24)	Adults	24	7	5	71.43	17	3	17.64	8 (33.33%)
Total (*n*= 78)	29 (37.17%)								

DNA electrophoresis

The PCR amplicons were analyzed by running 15 µl of the PCR products in 1.5% agarose gel stained with ethidium bromide (0.5µg/ml) and visualized under ultra-violet (UV) light using gel documentation and analysis system

Results

Out of 78 fecal samples screened for the existence of *Campylobacters*, 29 samples generated typical bacterial colonies (Table 1). Colonies appeared as translucent white, moist and glistening. The highest isolation rates of *Campylobacter* species were found in sheep (42.85%) followed by cattle (34.61%) and then humans (33.33%). *Campylobacters* were isolated from diarrheic and non-diarrheic fecal samples; however, in humans and young animal samples there was a strong association between the isolation of *Campylobacters* and the occurrence of diarrhea (71.43% and 100%, respectively) (Table 1).

The 16S *rRNA* gene based PCR on cultures collected from selective agar plates generated PCR products with a length of 857 bp (Fig. 1a, b) and the *mapA* gene based PCR generated PCR products with a length of 589 bp in all isolates (Fig. 1c, d), while no results could be obtained using the *ceuE* gene based PCR, thereby, all isolates were identified as *C. jejuni.*

Discussion

Thermotolerant *Campylobacter* infections constitute a zoonotic and public health problem. In the present study, a total of 54 domestic animals consisted of cattle (n=26) and sheep (n=28) were surveyed for fecal carriage of *Campylobacter* spp. beside 24 human stool samples from livestock contacts throughout the period between June 2016 and May 2017 in Beni-Suef, Egypt.

Out of 78 fecal samples, 29 samples (37.17%) yielded typical bacterial colonies of *Campylobacter* spp. In the current study, the enrichment step was employed prior to the inoculation and culture technique in order to enhance the isolation rate. Several authors confirmed that the average number of *Campylobacter* in intestinal samples collected from adult ruminants is low (Stanley *et al*., 1998; Nielsen, 2002), thereby an enrichment step is necessary to increase the recovery of *Campylobacter* from bovine and ovine samples.

The highest isolation rates were observed in sheep (42.85%), followed by cattle (34.61%). Thermophilic campyobacters are readily colonize the intestinal tract of ruminants (Stanley and Jones, 2003) however isolation rates vary between herds and flocks as a result of several factors such as

Table 2. Primer sequences specific for *Campylobacter* organisms

Gene name	Sequence	Amplification/ bp
16S rRNA	5́ ATC TAA TGG CTT AAC CAT TAA AC 3́ 5́ GGA CGG TAA CTA GTT TAG TAT T 3́	857 bp for *Campylobacter* genus
mapA	5́ CTATTTTATTTTTGAGTGCTTGTG 3́ 5́ GCTTTATTTGCCATTTGTTTTATTA 3́	589 bp for *Campylobacter jejuni*
ceuE	5́ AATTGAAAATTGCTCCAACTATG 3́ 5́ TGATTTTATTATTTGTAGCAGCG 3́	462 bp for *Campylobacter coli*

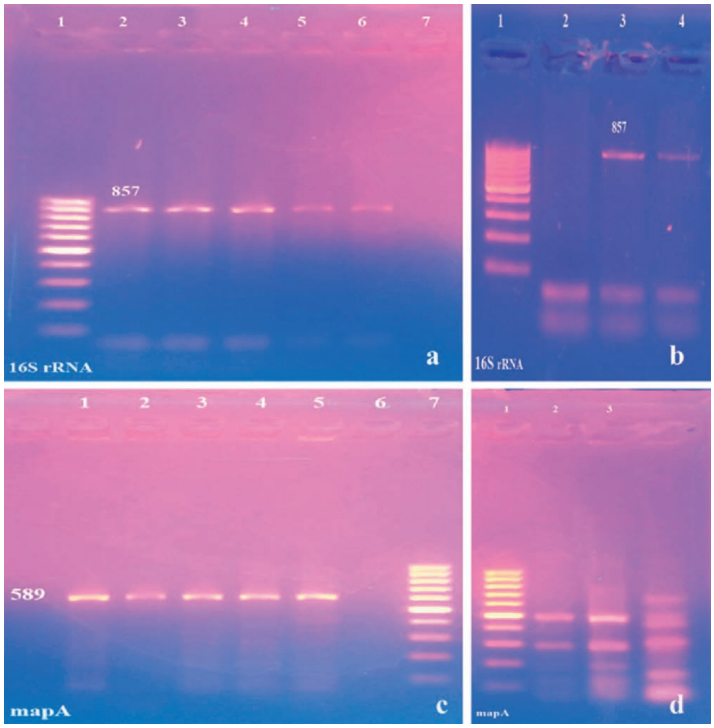

Fig. 1. Gel electrophoresis of PCR product using 16S *rRNA* gene specific primer (a, b) and *mapA* gene specific primer for *Campylobacter* organisms (c, d). a: Lane 1: Ladder 100 bp, Lanes 2, 3, 4: Sheep isolates, Lanes 5, 6: Cattle isolates and Lane 7: control negative. b: Lane 1: Ladder 100 bp, Lane 2: control negative and Lanes 3, 4: Human isolates. c: Lanes 1, 2, 3: Sheep isolates, Lanes 4,5: Cattle isolates and Lane 6: control negative Lane 7: Ladder 100 bp. d: Lane 1: Ladder 100 bp and Lanes 2, 3, 4: Human isolates.

husbandry practices, stocking density, age of animal and the housing method (Stanley and Jones, 2003). In the current study high isolation rate was observed in young animals suffered from diarrhea (100%) while in adult animals particularly sheep high isolation rate (63.63) was observed among apparently health animals. Newborn animals acquire *Campylobacters* horizontally from the farm environment components; these bacteria easily colonize the host due to the underdevelopment of their gastrointestinal tract (Stanley *et al*., 1998).

Association of *Campylobacter* infection with diarrhea can be explained on the basis of invasion of *C. jejuni* to the intestine that causes cellular inflammation resulting from the production of cytotoxins followed by the reduction of the absorptive capacity of the intestine as reported by Van Deun *et al*. (2007) who believed that the ability of this pathogen to reach the intestinal tract is in part due to its resistance to gastric acids and also to bile salts.

The obtained results indicate that *Campylobacters* is frequently isolated from the intestine of healthy and diarrheic animals and considered to be part of the normal intestinal flora, hence domestic ruminants play an integral role in the ecology of *C. jejuni* and may serve as source of infection resulting in outbreaks of disease or sporadic cases in humans as explained by Di Giannatale *et al*. (2014). Under such situation of the frequent isolation of *C. jejuni* as substantiated in this study, it is of at most importance to take in consideration that although *C. jejuni* mainly colonizes the gastrointestinal tract in animals, it may cross the intestinal epithelial barrier leading to bacteremia that occasionally cause mastitis in cattle and may reach the gravid uterus, resulting in subsequent placentitis, fetal infection and abortion as reported by Di Giannatale *et al*. (2014).

Regarding humans, the highest isolation rate of *Campylobacter* shedding was obtained from diarrheic patients (71.43%). The obtained results indicate that *C. jejuni* is one of the most important bacterial causes of diarrhea among people leading to inflammatory enteritis and acute dysentery with severe abdominal pain and fever as the commonest manifestations as reported by Blanco and Pall (2018). The most commonly reported symptoms of patients with laboratory-confirmed infections include diarrhea, fever, and abdominal cramping (Blaser *et al*., 1983) Less frequently, *C. jejuni* infections produce bacteremia, septic arthritis, and other extra-intestinal symptoms (Peterson, 1994).

Campylobacter is the most common bacterium inducing gastroenteritis in human beings globally in developed and developing countries especially in Africa, Asia and Middle East (Blaser, 1997; Allos, 2001; Dasti *et al*., 2010; Kaakoush *et al*., 2015) that may lead to fatal consequence especially in very young children, geriatric people and immune compromised patients (WHO, 2017). According to the bacteriological findings obtained in this study, *Campylobacter jejuni* is shed in the feces of both diarrheic and healthy animals and humans. However fecal shedding by healthy animals is intermittent and only few organisms are shed (Di Giannatale *et al*., 2014).

Campylobacter isolates isolated in this study could be identified by PCR on both genus and species levels. Employing PCR, all the 29 *Campylobacter* isolates yielded 857 bp amplicon specific for 16S *rRNA* gene, and showed the specific 589 bp in *mapA* gene typical for *C. jejuni*.

C. jejuni had been reported in Egypt as 8.4% (Shimaa *et al*., 2015 in Cairo, Fayoum Minya and El-Qalubia governorates), 5.2% (Girgis *et al*., 2014 in Ain Shams University hospital) and 27.5% (Moustafa *et al*., 2018 in Assiut Unit hospital).

Although sodium hippurate hydrolysis reaction is the only biochemical test used to differentiate between *C. jejuni* and *C. coli*, the test is time consuming and sometimes difficult to interpret when the enzymatic activity is impaired under the methodological condition (Rautelin *et al*., 1999). PCR is a recommended molecular technique for a decisive diagnosis and distinction between *Campylobacter* species (Oyofo *et al*., 1992; Comi *et al*., 1995; Sails *et al*., 1998) and offers an effective alternative to traditional biochemical methods for field studies.

The current study elucidates the important role that cattle and sheep play in the dissemination of *Campylobacters*. Apparently healthy animals shedding *Campylobacters* in their feces may contribute in the spread of infection amongst the herd and pose a high zoonotic risk to in-contact humans through contamination of milk at the farm level, the carcasses at slaughter house and surface water during removal of abattoir effluents to land (Gannon, 1999).

Conclusion

Domestic ruminants play an integral role in the ecology of *C. jejuni* and may serve as source of infection resulting in outbreaks of disease or sporadic cases in humans. PCR is a highly recommended molecular technique for a decisive diagnosis and distinction between different *Campylobacter* species recovered from animals and humans.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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