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Characterization of *Campylobacter jejuni* An Important Zoonotic Food Borne Pathogen from Mastitis Milk and Raw Milk Samples

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ABSTRACT

Background: *Campylobacter* has emerged as an important zoonotic food borne pathogen of human and animals worldwide. *Campylobacter* is one of the most common bacterial enteropathogens of food borne origin in industrialized countries with *C. jejuni* being the most common species followed by *C. coli.* There are very few cases reported from mastitis therefore this study was aimed to determine the incidence of *Campylobacter jejuni* from from mastitis milk and raw milk samples.

Methods: Total of 72 milk samples comprising mastitis milk (20) and raw milk (52) were collected. The samples were subjected to cultural examination, biochemical as well as molecular identification. The isolates were further subjected to phenotypic characterization by biochemical test and genotypic characterization by polymerase chain reaction. The isolates were subjected to PCR targeting *hip* O and *MAP* A genes.

Result: The 52 samples showed growth on modified Blood Free Charcoal Cefoperazone Deoxycholate agar media and 18 (34.61%) samples showed typical morphological characteristics. The result revealed that 10 (19.23%) isolates were positive by phenotypic characteristic and 7(70%) by Polymerase chain reaction for *C. jejuni.* The outcome result showed that importance of *Campylobacter jejuni* in cattle, especially raw milk and milk from mastitis cows, as a potential source for transmission of Campylobacteriosis in human and dairy farm environment. This can cause acute bacterial gastroenteritis in humans and associated with foodborn infection, food safety and a serious public health threat.

Key words: CCDA, *C. jejuni, Hip O*, Hippurate, *MAP*.

INTRODUCTION

Campylobacteriosis is the most frequently reported zoonosis and the main bacterial foodborne disease in humans (Zendehbad *et al*., 2015). *Campylobacter* is a major medical and economic problem worldwide, with numbers of cases continuing to increase (Kaakoush *et al*., 2015). *Campylobacter* is 1 of 4 key global causes of diarrheal diseases considered to be the most common bacterial cause of human gastroenteritis in the world (WHO, 2018). *Campylobacter jejuni* is a leading cause of bacterial diarrheal disease worldwide and it is responsible for zoonoses (Facciola *et al*., 2017). The pathogenesis of *C. jejuni* is poorly understood as compared to other enteric pathogens (Rizal *et al*., 2010). *Campylobacter* spp. can be transferred from animals to humans by contaminated food of animal origin, especially undercooked poultry meat and unpasteurized milk/dairy products (Luangtongkum *et al.,* 2012).

Campylobacter jejuni can cause mastitis in cow and the bovine udder is potential source of *C. jejuni* in raw milk (Lander and Gill, 1980). Poor pretreatment of the teats with disinfectant or contact of the milking cluster with the parlor floor result into higher levels of fecal *Campylobacter* contamination (Beumer *et al*., 1988). *Campylobacter* generally colonize in the lower gastrointestinal tract where there is a lower pH environment in the rumen. The stratified epithelium of the rumen lacks the necessary receptors to sustain a persistent high level of *Campylobacter* colonization (Grahamm *et al*., 2005). The common vehicles for *Campylobacter* foodborne disease transmission are unpasteurized bovine milk and milk products and *Campylobacter* spp. in raw milk derive most commonly from secondary fecal contamination during the milking process (Oliver *et al*., 2005).

Campylobacter is one of the most common pathogen-related causes of diarrheal illnesses globally and has been recognized as a significant factor of human disease for more than three decades (Magana *et al*., 2017). In humans, clinical signs of Campylobacteriosis include diarrhea, abdominal pain, fever, headache, nausea and vomiting. Most of *Campylobacter* are sporadic and self-limiting, but there are post-infection complications, for example, Guillain-Barres syndrome (Hansson *et al*., 2018).

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Campylobacter jejuni is difficult to grow and identify and common milk contaminant due to fastidious growth requirement. Hence this study was attempted to isolate and identify the presence of *C. jejuni* using cultural, biochemical and PCR technique and compare these techniques for detection of *C. jejuni* in milk samples.

MATERIALS AND METHODS

Collection of samples

The present study was conducted at Department of Animal Biotechnology, Madras Veterinary College, Chennai, Tamil Nadu in the year 2018-19. A total of 72 samples comprising Mastitis milk sample (20) were collected from Department of Clinics, Madras Veterinary College, Chennai and raw milk samples (52) from cattle farms. All the samples were collected using sterile containers and transported immediately to the laboratory under cold conditions for microbiological analysis.

Processing of samples

The isolation was performed according to Man (2011) and the isolates were identified by biochemical tests as described by (Fitzgerald and Nachamkin, 2007 and Lastovica and Allos, 2008). The *Campylobacter jejuni* (ATCC 33291) was used as standard and reference strain.

Phenotypic characterization

Cultural examination

Samples were enriched in Blood free modified Charcoal Cefoperazone Deoxycholate (mCCDA) broth (Hutchinson and Bolton, 1984) with CCDA supplement under microaerophillic conditions (candle jar method) by using internal gas generation system (Microaerophilic gas pack CampyPack-BD oxoid). On selective agar colonies were grey/white or creamy grey in colour, smooth, glistening and convex with entire edges and moist in appearance, dew drop with the tendency to spread. *Campylobacter* species are gram negative rods, 0.5-8 µm long and 0.2-0.5 µm wide with characteristically curved, spiral, or S-shaped cells.

Biochemical test

The isolates were identified as *C. jejuni* based on their morphological and biochemical tests. Suspected colonies were sub-cultured and confirmed by biochemical tests as catalase, oxidase, nitrate and hippurate hydrolysis, Ninhydrin test, H_2S production.

Molecular confirmation of *Campylobacter jejuni*

The biochemically identified isolates were further employed for molecular confirmation as *C. jejuni* by polymerase chain reaction amplifying specific target gene using species-specific oligonucleotide primers. DNA were extracted by Phenol-Chloroform extraction method and the DNA concentration was quantified by nanodrop and stored at -20°C until further processing.

Genotypic confirmation of isolates by polymerase chain reaction for *Hip O* gene and *MAP A* gene

Polymerase chain reaction was carried out using primers for species specific genes. The PCR was performed in a thermal cycler (Applied Biosystem). The *hipO* gene region is the hippuricase gene, specific for *C. jejuni*. Primers for specific identification were designed using the *hipO* gene sequences of *C. jejuni* based on the sequences available in the GenBank.

The isolates were confirmed by PCR using designed primers in the study as forward primer (5-TTCCATGACC ACCTCTTCC-3) and reverse primer (5-CTACTTCTTTATT GCTTGCTGC-3).

The primers used for amplification of *MAP A* gene were forward primer (5-CTATTTTATTTTTGAGTGCTTGTG-3) and reverse primers (5-GCTTTATTTGCCATTTGTTTTATTA-3) (Khoshbakht *et al*., 2015).

The PCR reactions were performed in 25 µl reaction mixture, containing 12.5 µl PCR master mix (2X-Ampliqon), 1 µl of each primer of a 10 µM primer concentration, 1 µl $MgCl_2$ (25 mM), 3 µl template DNA and 6.5 µl nuclease-free water making a total volume of 25 µl. The amplification conditions were initial denaturation at 94°C for 3 min, 35 cycles with denaturation at 94°C for 1 min, annealing at 53°C for *HipO* gene for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min respectively (Al Amri *et al*., 2007). The annealing temperature for *Map* A gene was optimized as 52°C for 1 min (Khoshbakht *et al*., 2015). The DNA from *C. jejuni* (ATCC 33291) was included as positive control for PCR identification of the isolates and the master mix without sample DNA used as negative control. The amplified products were observed and photographed using gel documentation System (Applied Biosystems).

RESULTS AND DISCUSSION

Campylobacter spp. is a major cause of gastroenteritis, there is an urgent need to control these pathogens with zoonotic and public health point of view. The current study total 52 samples studied for presence of *Campylobacter* from raw milk and milk from mastitis cases. The *Campylobacter* species are difficult to isolate but the results from inoculation studies showed that plates with either blood or charcoal had a better recovery rate than other media used for isolation. Modified blood free Charcoal Cefoperazone Deoxycholate agar is commonly used worldwide (Bolton *et al*., 1984; Hutchinson and Bolton, 1984). In current study a total of 72 samples were processed for isolation of *Campylobacter* and 52 isolates showed growth on mCCDA agar plates. The isolates showed typical grey/white or creamy grey in colour and moist spreading type colonies with sticky nature were confirmed phenotypically as *Campylobacter*. The suspected colonies were examined for morphological characteristics, motility, Gram's staining. The overall incidence of *Campylobacter* was found to be 18 (34.61%) by cultural examination.

Biochemical characterization

The isolates were processed for phenotypic characterization and identified by biochemical tests, *viz.* oxidase, catalase, indoxyl acetate hydrolysis tests and H_2S production in triple sugar iron test. The test for hippurate hydrolysis is critical for separation of *Campylobacter jejuni* strains. Glycine and benzoic acid are formed when hippurate is hydrolyzed by *C. jejuni* (Morris *et al*., 1985). In present study 10 (19.23%) isolates, 4 from mastitis milk samples and 6 from raw milk samples were positive for catalase, oxidase, nitrate and hippurate hydrolysis, Ninhydrin test. *C. jejuni* biotype 2 strains are H_2S positive, whereas *C. jejuni* biotype 1 strains are H_2S negative (Penner, 1988). Two samples from mastitis milk were positive for H_2S production belongs to biotyope 2 (Table 1). The most of the samples were negative for H_2S production categorized as biotype 1 of *C. jejuni*. The hippuricase enzyme hydrolyzes hippurate and this test is used routinely in the clinical laboratory. (Malik *et al*., 2014) studied hundred chicken caecal samples and reported hippurate hydrolysis test positive for two isolates, categorized as *C. jejuni* and negative for 30 isolates. In current study all the samples found to be positive for ninhydrin test confirmed as *C. jejuni*.

Genotypic characterization

The isolates were confirmed by polymerase chain with species specific primers for *Hip* O and *MAP* A gene. The size of PCR product for *Hip* O gene was 270 bp and the size of the PCR product for *MAP* A gene was 589 bp (Fig 1). The incidence of *C. jejuni* using three different screening tests was given in Table (2) Fig (2).

Raw milk samples are among the potentially important sources of *Campylobacter* spp. A total of 100 raw milk samples were screened and revealed a prevalence of 3% (Kumar *et al*., 2015).

A lower prevalence rate of 1.41% was observed by (Elango *et al*., 2010) in Chennai, India. A total of 60 milk samples collected from different milk vendors of different areas of the Faizabad district out of which *Campylobacter jejuni* (4) were recovered from 60 milk samples in accordance with this study (Shekhar *et al*., 2010). *C. jejuni* was detected in 34 (12%) bulk tank milk by specific PCR (Bianchini *et al*., 2014).

Table 1: Comparative results of biochemical test of *C. jejuni* isolated from milk samples.

Samples/source	Samples examined	Isolates showed growth on mCCDA agar	Biochemical test						
			Catalse	oxidase	nitrate	Glysin	Ninhydrin	H_2S production	
								Positive	Negative
Mastitis milk	20	13	4	4	4	4	4	2	2
Raw milk	52	39	6	6	6	6	6	0	6
Total	72	52	10	10	10	10	10	2	8

ML: Mastitis milk, RM: Raw milk.

Table 2: Comparative results of morphological, biochemical and genotypic characterization of *C. jejuni* isolates.

Samples/source	Cultural examination	Phenotypic characterization	Genotypic characterization
Mastitis milk	11 (84.61%)	4 (30.76%)	3 (75%)
Raw milk	7 (17.94%)	6 (15.38%)	4 (66.66%)
Total	18 (34.61%)	10 (19.23%)	7 (70%)

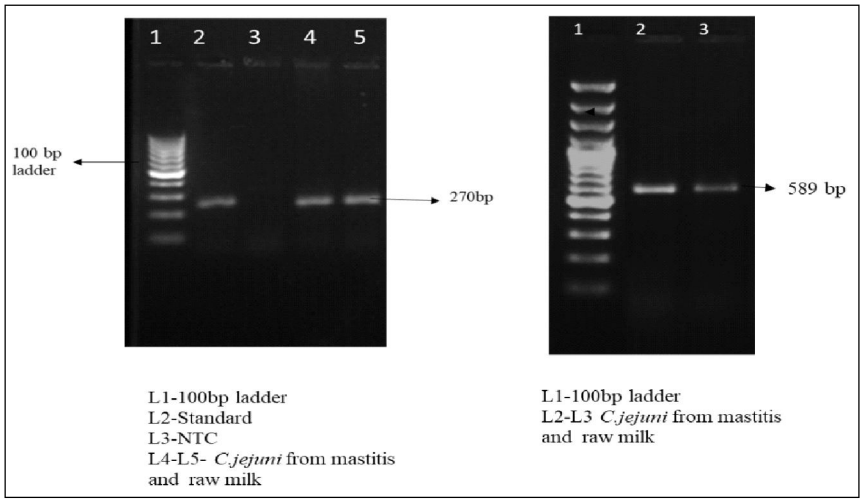

Fig 1: PCR based amplification product of *Hip* O gene and *MAP* A from mastitis and raw milk samples (270 bp and 589 bp respectively).

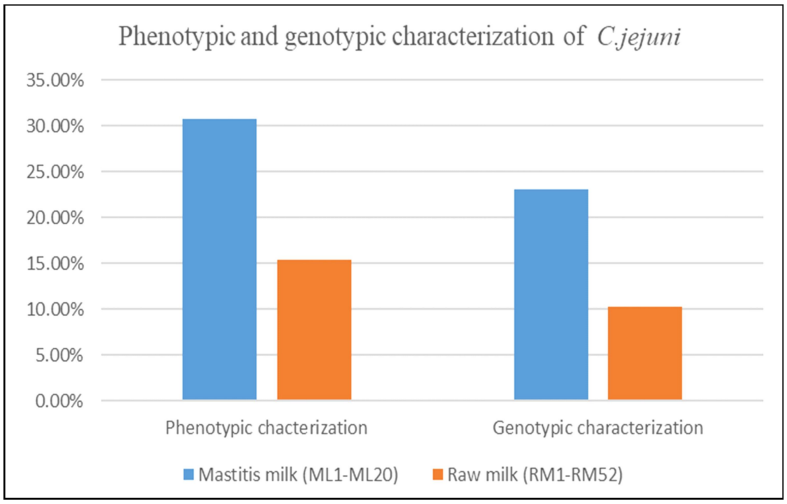

Fig 2: Comparative result of phenotypic and genotypic study of *C. jejuni.*

A total of 42 *C. jejuni* isolates were obtained with a prevalence rate of 1.36% (Elango *et al*., 2012). *Campylobacter* species were detected in 7 (2.91%) raw milk samples and all the isolate identified were *Campylobacter jejuni* whereas none of the milk product was positive. (Modi *et al*., 2015), while in this study 3 isolates from mastitis milk and 4 isolates from raw milk samples found to be positive for *C. jejuni. Campylobacter* spp. was isolated from 16 (5.3%) samples by conventional method, the isolates were identified by biochemical analyses and the polymerase chain reaction which revealed that 12 (75%) samples were *Campylobacter jejuni* (Kilicaltun *et al*., 2014) in accordance with our study.

CONCLUSION

The result from our study showed that milk samples are the main source transmission of *Campylobacter* and related Food infections. The presence of *C. jejuni* in milk can also be due to contaminated sources in the dairy farm environment and direct contact as a possible reservoir for *Campylobacter* transmission to livestock. Possibly as a result of contact with bovine feces, contaminated water or direct contamination in dairy farm environment can be responsible for mastitis in cattle. Consumption of raw milk can be as a potential source of transmission of *Campylobacter* to human. The human consumption of unpasteurized or improperly pasturized milk can be result into Campylobacteriosis and gastroenteritis.

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