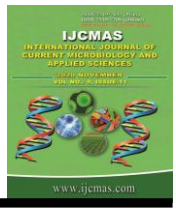
Int.J.Curr.Microbiol.App.Sci (2020) *9*(11): 1884-1892

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 9 Number 11 (2020)**
Journal homepage: http://www.ijcmas.com



Original Research Article https://doi.org/10.20546/ijcmas.2020.911.223

Isolation, Identification and Molecular Detection of Zoonotic *Campylobacter jejuni* Isolated from Mutton and Beef Samples

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Keywords

CCDA, Hip O, MAP, Hippurate, *C. jejuni*

Article Info

Accepted:
12 October 2020
Available Online:
10 November 2020

A B S T R A C T

Campylobacter species are a leading cause of food-borne disease and *C. jejuni* highlight the most potential public health impact of Campylobacter contamination by pathogens originating from animals or animal products.. The total of 33meat samples comprising 8 from sheep (5) and goat (3) also 25 beef samples were screened by morphological, biochemical and molecular technique. The isolates were subjected to phenotypic characterization using biochemical test and genotypic characterization. The isolates from chevon (3 out of 3) and mutton (2 out of 5) were positive for morphological and biochemical examination. The 20 (80%) beef samples were found to be positive by morphological examination and 12(48%) isolates showed biochemical reactions positive for *C.jejuni*. The isolates were subjected to PCR targeting hip O and MAP A genes. The result showed 66.66 % from chevon, 20% mutton and 20% isolates from beef samples were found to be positive for *C.jejuni*. These findings suggest that PCR should be the preferred diagnostic method for detection of *Campylobacter* in livestock. The good hygienic and manufacturing practices must be followed in the entire food chain to prevent the contamination of food due to microbe which can cause Campylobacteriosis among the consumers.

Introduction

The pathogenesis of *C. jejuni* is poorly understood as compared to other enteric pathogens (Rizal *et al*., 2010). Cattle are a major source of food and the cattle industry engages people from farms to processing plants and meat markets, it is plausible that beef-products contaminated with Campylobacter spp. would pose a significant public health concern (Sanad *et al*., 2011). The sudden onset of fever, abdominal cramps, and diarrhoea with blood and leukocytes are characteristics of *C. jejuni* infection (Kim *et al.*, 2015). Campylobacter spp. can be transferred from animals to humans by contaminated food of animal origin. Chicken has been recognized as a major source for

human infection, whereas cattle might also contribute to a lesser extent. Cattle is the second major reservoir for *C. jejuni* (Jonas *et al*., 2015). The consumption of contaminated meat and meat products are responsible for more than 90% of human infections caused by *Campylobacter jejuni* (Mikulic *et al*., 2016). Campylobacteris considered as a principal cause of most important zoonotic food-borne disease in humans for approximately 166 million diarrheal cases and globally 37,600 deaths per year (Oh *et al*., 2018).

In humans, clinical signs of Campylobacteriosis include diarrhea, abdominal pain, fever, headache, nausea and vomiting. Most of Campylobacter are sporadic and self-limiting, The main recognized sequelae are Guillain-Barré Syndrome (GBS), the Reactive Arthritis (REA) and irritable bowel syndrome (IBS). Thermo tolerant Campylobacter which has a clinical significance due to the consumption of meat and meat products are *C. jejuni* and its closely connected (Mikulic *et al*., 2016). For more than three decades Campylobacter is pathogen-related causes and significant factor of diarrheal illnesses in human (Magana *et al*., 2017). This zoonotic infection is of great public health concern, with meats known as the major risk factor (Carron *et al*., 2018). *Campylobacter* spp. is a zoonotic bacterium and cause of human gastroenteritis worldwide and main symptom is diarrhea (Hlashwayo *et al*., 2020).

Campylobacter is difficult to isolate, grow and identify. Only *Campylobacter jejuni* can be routinely identified with phenotypic markers, and commercial systems may misidentify non-jejuni species (Fitzgerald *et al*., 2016). Campylobacter poses an important risk for humans through shedding of the pathogen in livestock waste and contamination of water sources, environment, and food by colonization of different animal reservoirs (Gahamanyi *et al*., 2020). The reservoir and source of human campylobacteriosis is primarily considered to be poultry, but also other such as ruminants, pets and environmental sources are related with infection burden (Maesaar *et al*., 2020).There is a high incidence of Campylobacter species in meat carcasses, suggesting these to be a reservoir of Campylobacteriosis agents, and consumption of undercooked meats is a potential health risk to consumers (Igwaran and Okoh, 2020).

The major transmission routes of Campylobacteriosis in humans are consumption of contaminated or undercooked meat. Despite the size of the livestock and meat industry in India, little is known about the Campylobacteriosis as zoonotic foodborne pathogen. Hence this study was attempted to detect the presence of *C. jejuni* using morphological, biochemical and PCR technique and compare these techniques for detection among different sources from animal origin.

Materials and Methods

Collection of samples

A total of (8) meat sample of Sheep (5) and Goat (3) meat collected from retail outlet and beef (25) samples from slaughter house 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 reference strain *Campylobacter jejuni* (ATCC33291) was used as standard for PCR.

Phenotypic characterization

Morphological examination

Sample was enriched in modified Charcoal Cefoperazone Deoxycholate (mCCDA) broth (Hutchinson and Bolton, 1984) with CCDA supplement (FD 135) under microaerophillic conditions (candle jar method) by using internal gas generation system using (Microaerophilic gas pack CampyPack-BD oxoid).

Biochemical test

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

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 was 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 hipO gene specific identification were designed using the 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 for hipO gene as forward primer (5-TTCCATGACCACCTCTTCC-3) and reverse primer (5-CTACTTCTTTATTGCTTGCTGC -3).

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

The PCR reactions were performed in 25 µl reaction mixture, containing 12.5 µl PCR master mix (2X-Ampliqon), 1µl of each primer of a 10 µM primer concentration,1µl $MgCl_2$ (25mM), 3µl template DNA and 6.5 µl nuclease-free water making a total volume of 25 µl. The amplification conditions consisted of 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 Campylobacter species are difficult to isolate but the results from inoculation studies

showed that plates with 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 all samples showed growth on mCCDA agar plates. On selective agar, Blood free modified charcoalcefoperazone deoxycholate (mCCDA), colonies were found to be typical 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 with sticky nature were confirmed phenotypically as *Campylobacter*. The suspected colonies were examined for morphological characteristics, motility, Gram's staining. Campylobacter species are Gram negative rods with characteristically curved, spiral, or S-shaped cells. The overall incidence of *Campylobacter* was found to be (3 out of 3) in chevon and (2 out of 5) mutton also 20 (80%) in beef by morphological examination (Table 1 and 2).

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. In current investigation of eight samples from mutton and chevon processed five isolates showed positive reaction for all biochemical test. The Twelve isolates from beef sampled showed positive reaction for biochemical tests and tentatively confirmed as Campylobacter. The test for hippurate hydrolysis is critical for separation of *Campylobacter jejuni* and *C. coli* strains. Glycine and benzoic acid are formed when hippurate is hydrolyzed by *C. jejuni* (Morris *et al*., 1985). Out of the 46 isolates screened, 33 were found positive for hippurate hydrolysis and were classified as *C. jejuni* (Kumar *et al*., 2015). In current study 5 isolates from chevon and mutton and 12 isolates from beef were confirmed as *C.jejuni* on basis of hippurate hydrolysis test.

Two samples from chevon and one from beef were positive for H_2S production. The most of the samples were negative for H_2S production *C. jejuni* biotype 2 strains are H_2S positive, whereas *C. jejuni* biotype 1 strains are H_2S negative (Penner, 1988).In this studythree isolates were positive for H_2S production belong to biotyope 2 while 14belong to biotype 1 of *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. Three isolates (two from chevon and one from mutton) as well as five from beef samples showed specific amplification and confirmed as *C.jejuni*.

Incidence of *Campylobacter jejuni* in chevon mutton and beef

Among meat samples processed, the prevalence of Campylobacter was recorded in raw beef (10.9%) and raw mutton (5.1%). The study reported that the prevalence of Campylobacter spp. was significantly higher in the food commodities, which included raw/undercooked ingredients (Hussai *et al*., 2007). A total of 183chevon, and 42 carabeef were processed and samples showed characteristic colonies on mCCDA plates. The prevalence rate of 7.6% was recorded in chevon. None of the isolates were recovered from beef samples. Most of the obtained isolates were classified as *C. jejuni* indicating that the *C. jejuni* was the most commonly found species while in current study five beef samples were confirmed as *C. jejuni*. The 183

chevon samples processed, 14 (7.6%) were reported as Campylobacter 10 were identified to be *C. jejuni* through molecular means (Monika *et al*., 2016), while in our study, 5 (62.5%) from muton and chevon samples were found to be positive by molecular identification using species specific primer. Among the 853 livestock faecal samples, Campylobacter were detected by culture in 106 samples (12%); 72 samples (68%) tested positive for *C. jejuni* (Osbjer *et al*., 2016). A total of Mutton (n=100) samples were collected from different open markets of Kolkata cityCampylobacter spp. was detected 64% of mutton meat samples. The most prevalent species recovered from samples was *Campylobacter jejuni* with 58.8% of the isolates confirmed (Sharma *et al*., 2016) while lower incidence was recorded from this study.

Rahimi *et al*., (2010) conducted a study to determine the prevalence of Campylobacter spp. isolated from retail raw meats in Iran. A total of (n = 190) beef, (n = 225) lamb, and (n = 180) goat raw meat samples were purchased from randomly selected retail outlets and were evaluated for the presence of Campylobacter spp. The highest prevalence of Campylobacter spp. was found in lamb meat (12.0%), followed by goat meat (9.4%), beef meat (2.4%). The most prevalent Campylobacter spp. isolated from the meat samples was *Campylobacter jejuni* (84.0%) in accordance with current study. A total of 200 samples consisting of 100 meat and 100 liver surface swabs were collected from 47 lamb and 53 goat kid carcasses at 23 retail markets in Northern Greece and 125 Campylobacter isolates were recovered from 32 meat surfaces (32%) and 44 liver surfaces (44%) and *C. jejuni* (40.8%) detected species by multiplex polymerase chain reaction (Lazou *et al*., 2014).The overall prevalence of Campylobacter in different sample groups was 41.2%, 37.2%,23.7%, and 35.1% for goat meat, goat stomachs, RTE goat skewers, and goat faecal samples, respectively. *C. jejuni* was isolated in 10.1% samples (Mpalang *et al*., 2014). Pallavi and Kumar (2014) studied the prevalence of Campylobacter species in foods of animal origin. A total of 50 chevon were collected from retail meat markets, slaughter houses and analyzed for isolation biochemical characterization and confirmed by polymerase chain reaction. The prevalence of Campylobacter spp. in chevon 6% was observed while in current study highest incidence rate was observed.

The study to isolate and detect Campylobacter species in meat samples, including mutton offals, beef, beef offals the samples were subjected to both traditional culture on modified charcoal cefoperazone deoxycholate agar (mCCDA) plates and PCR techniques. From culture, a total of 845 presumptive isolates were obtained, of which 28.40% (208/845) were identified as 32.5% (208/640) were obtained from retail markets, 15.17% (22/145) from butcheries, and 16.67% (10/60) from open markets. Campylobacter presumptive isolates from mutton sample 4 (44.44%) and 30 (33.71%) from beef were identified as genus Campylobacter. These were then characterised into species level, of which the prevalence rate of *C. jejuni* was observed (16.66%) (Igwaran and Okoh, 2020). Campylobacter detection and prevalence calculations estimate in 17.8% (95% CI 12.6-24.5) of 2907 goat samples; 12.6% (95% CI 8.4-18.5) of 2382 sheep samples; and 12.3% (95% CI 9.5-15.8) of 6545 cattle samples suggested that meat and organs were significantly less likely to be contaminated than gut samples (Thomas *et al*., 2020).The overall prevalence of Campylobacter for ovine trim based on PCR-detection was 33% (39 out of 120 samples) with prevalence for hogget, lamb and mutton carcass trim of 56% (28out of 50), 11% (4 out of 35) and 20% (7 out of 35), respectively (Rivas *et al*., 2020) in conformity with our study (Fig. 1).

Table.1 Result of biochemical test of *C. jejuni* isolated from different sources

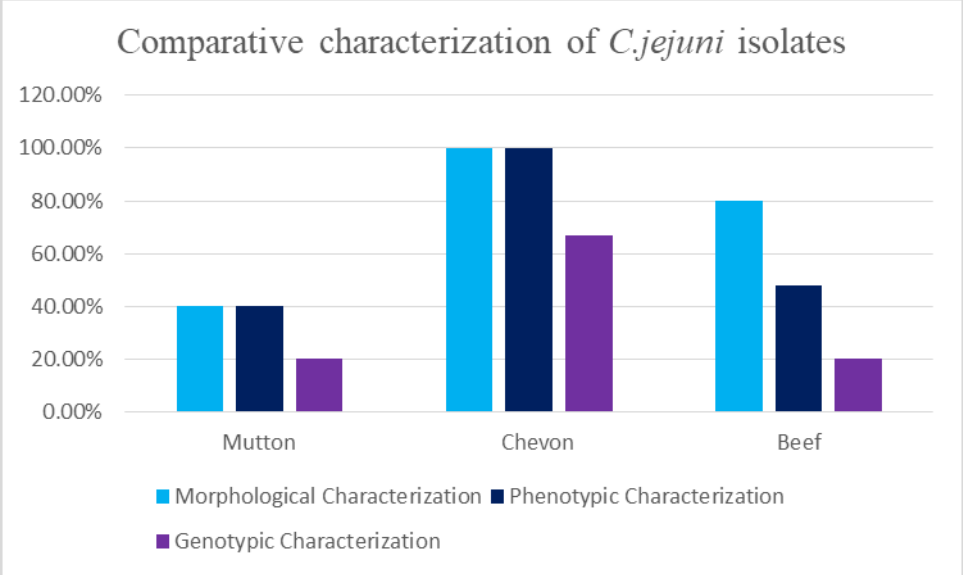
Sr. No.	Samples/source	Samples Examined	Isolates showed growth on mCCDA agar	Biochemical test						
				Catalse	oxidase	nitrate	Glysin	Ninhydrin	H_2S production	
									Positive	Negative
1	Mutton (M1-M5)	5	5	2	2	2	2	2	0	2
2	Chevon (M6-M8)	3	3	3	3	3	3	3	2	1
3	Beef (BF1-BF25)	25	25	12	12	12	12	12	1	11

M-sheep and goat meat BF-beef samples

Table.2 Morphological, phenotypic and genotypic characterization of *C. jejuni* isolates

Sr.No.	Samples/source	Morphological Characterization	Phenotypic Characterization	Genotypic Characterization
1	**Mutton**	**2(40%)**	**2(40%)**	**1(20%)**
2	**Chevon**	**3(100%)**	**3(100%)**	**2(66.66%)**
3	**Beef**	**20 (80%)**	**12(48%)**	**5(20%)**

Figure.1 Comparative result of phenotypic and genotypic study of *C. jejuni*



It is concluded in this study, we were able to isolates, identify and study incidence of *Campylobacter jejuni* in samples obtained from retail market from chevon and meat and beef with high incidence rate. The current study reveals that consumption of

undercooked meat products and cross-contamination during food handling and preparation must be avoided to ensure food safety at home and in the food service industry. The role of animal sources should not be overlooked with regard to public health, and animal products and consumer markets should be frequently inspected to minimize the risk of infection. As microbial food safety is an essential public health issue, the sincere efforts are needed to create awareness among the people about the hazards of foodborne diseases. Incidence data from the current study will be helpful to public health professionals to identify high risk food items sold in the shops.

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How to cite this article:

Sumedha Bobade, K. Vijayarani, K.G. Tirumurugaan, A. Thangavelu and Vairamuthu, S. 2020. Isolation, Identification and Molecular Detection of Zoonotic *C.jejuni* Isolated from Mutton and Beef Samples. *Int.J.Curr.Microbiol.App.Sci*. 9(11): 1884-1892.
doi: https://doi.org/10.20546/ijcmas.2020.911.223