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Prevalence of *Campylobacter* spp. Isolated from Poultry, Human and Environment in Junagadh District of Gujarat, India

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A B S T R A C T

A total of 500 samples, comprising 150 poultry meat, 150 intestinal (caeca), 50 water, 50 equipment swab and 50 workers' hand swabs from retail poultry meat outlets and 50 diarrhoeic stool samples from children under-5 years of age from children hospitals in Junagadh city were screened. The samples were subjected to isolation followed by confirmation using a multiplex PCR assay. Out of samples processed, highest isolation rate was observed in poultry intestinal contents (38.67% each), followed by poultry meat (16%), equipment swab (10%), worker hand washing swab (6%), human stools (4%) and none of the isolate present in water samples. A total of 92 campylobacters (18.4%) were isolated comprising 64 *C. coli* (12.8%) and 28 *C. jejuni* (5.6%). It may be concluded from the present study that *Campylobacter* spp. is highly prevalent in poultry, human and environment in Junagadh district of Gujarat region. The occurrence *Campylobacter* spp. in poultry intestinal contents, equipment swab and worker hands washing swab samples which indicate contamination of carcass may take place during slaughtering and/or post slaughtering processes. The findings on isolation of *Campylobacter* spp. from clinical cases of children further prove the importance of infection that necessitates the need for proper preventive measures to control the infection in food production and consumption.

Introduction

Campylobacter are microaerophilic Gram-negative bacteria causes gastroenteritis in humans and is responsible for 400–500 million cases of infection each year worldwide (Ruiz-Palacios, 2007). *Campylobacter jejuni* and *C. coli*are considered as potential agents responsible for several undiagnosed cases of diarrhea among children especially in developing countries including India (Silva *et al.*, 2011; Ghorbanalizadgan *et al.*, 2014). Incidence rate of 4.5% was observed in the southern India (Rajendran *et al.*, 2012) and 10.28-13.5% from diarrheic cases in North India (Ghosh *et* *al.*, 2013).*Campylobacter* spp. have also been isolated from meat of different species, milk/milk products, vegetables, fruits, sewage, human stool and faecal samples of different animals (Kumar *et al.*, 2001; Datta *et al.*, 2003; Pallavi *et al.*, 2015). These organisms are responsible for bacterial foodborne illness in European Union (Anonymous, 2005; Behringer *et al.*, 2011), about 0.8 million cases in USA (Scallan *et al.*, 2011) and 0.5 million cases in UK each year (Tam *et al.*, 2011). The total number of cases reported to be caused by *Campylobacter* spp. was more than thrice the cases caused by *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* altogether (CDC, 2008). Poultry act as the reservoir *Campylobacter* spp. and are the main source of infection to humans (Silva *et al.*, 2011; Suman *et al.*, 2012; Pallavi *et al.*, 2015). Therefore, the present study was under taken with the objective to determine the prevalence and isolate *Campylobacter* spp. from variety of samples collected from Junagadh district, Gujarat State of India.

Materials and Methods

Sample collection

A total of 450 samples, comprised of 150 poultry meat, 150 intestinal (caeca) samples, 50 water, 50 equipment swab and 50 hand washing swab of workers from retail poultry meat shops from Junagadh district, Gujarat State were collected aseptically. In addition, 50 faecal samples were collected from children under 5 years of age with diarrhoea from human hospitals in Junagadh. All these samples were collected in sterilized polythene bags and transported to the laboratory in an icebox for microbiological analysis.

Isolation of organisms

These samples were enriched into blood free campylobacter selectivity broth and Preston broth with CCDA and campylobacter growth supplements under microaerophilic condition (85% N_2, 5% O_2 and 10% CO_2) at 42°C for 48 hours. Swabs were inoculated on blood free campylobacter selective agar with modified CCDA.

Biochemical tests

Oxidase, catalase, H_2S production on Triple sugar iron agar, indoxyl acetate hydrolysis and hippurate hydrolysis tests were performed to differentiate isolates at genus and species level as per methods described in the Burgey's Manual of Systemic Bacteriology.

Polymerase Chain Reaction

The presumptive *Campylobacter* isolates were further confirmed at genus and species level by polymerase chain reaction assay. DNA was extracted using a loopful of overnight grown culture of *Campylobacter* spp. suspended in100µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA). After proper mixing, the tubes were kept in boiling water bath at 100°C for 10 min. Then, cell debris was removed by centrifugation and the tube was immediately transferred to ice and supernatant was used as DNA template for PCR assay. The oligonucleotide primers targeting lipid gene '*lpx*A' were synthesized from Eurofins Genomics India Pvt. Ltd., India (Table 1). Species specific PCR performed to confirm *Campylobacter* spp. as described by Klenaet al.2004 with modifications. PCR was performed in a total reaction volume of25µl containing 2X PCR Master Mix (Thermo Cat. No. K0171) with 2.5 µl of 10X dream *Taq*buffer, 2.5 µl of 2 mM of each dNTP, 15 pmol of each primer, 1 U dream *Taq* polymerase with 2 µl of bacterial DNA template extracted using DNeasy blood and tissue kit and nuclease-free water up to 25 µl. The mPCR amplification was performed in a thermal cycler with initial denaturation at

95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30s and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The amplified products were electrophoresed in 2.0% agarose gel with ethidium bromide (0.5 µg/ml) and image was taken in a gel documentation system (Vilberlourmat)

Results and Discussion

On cultural isolation, *Campylobacter* spp. showed a characteristic small (1-2 mm), circular, flat to slightly raised, grey coloured colonies having a spreading or watery nature on Blood Free Campylobacter Selectivity Agar plates after incubation for 48 hrs. (Fig. 1). The colonies turned grayish and sticky when left for longer time on the agar plates. Gram negative, non-spore forming, mostly comma, short spirally curved, arc or “S” shaped rods were seen on Gram’s staining (Fig. 2). Hanging drop preparation of 48 h incubated culture from agar plates showed typical cork screw darting type of motility. None of the isolates grew under aerobic condition at 36ºC.

All these isolates were positive for oxidase, catalase and indoxyl acetate hydrolysis tests and negative for H_2S production on Triple sugar iron agar. The presumptive isolates showing positive hippurate hydrolysis test, positive indoxyl acetate hydrolysis test, resistance to cephalothin and sensitivity to nalidixic acid were considered as *C. jejuni* whereas isolates showing negative hippurate hydrolysis test, positive indoxyl acetate hydrolysis test, resistance to cephalothin and sensitivity to nalidixic acid were considered as *C. coli*.

Out of 500 samples processed for isolation, the highest no. of isolates was recovered in poultry intestinal contents (38.67 %), followed by poultry meat (16.00%), equipment swab (10.00%), worker hand washing swab (6.00%), human stools (4.00%). None of the isolate was present in water samples. A total of 92 campylobacters (18.40%) were isolated comprising 64 *C. coli* (12.80%) and 28 C.*jejuni* (5.60%) (Table 2).

Table.1 Primers used to identify *Campylobacter* spp. isolates

Species	Primer sequence (5’-3’)	Product size
C. jejuni	F-ACAACTTGGTGACGATGTTGTA	331 bp
	R-CAATCATGDGCDATATGASAATAHGCCAT	
C. coli	F- AGACAAATAAGAGAGAATCAG	391 bp
	R-CAATCATGDGCDATATGASAATAHGCCAT	

(F) = Forward primer; (R) = Reverse primer

Table.2 *Campylobacters* spp. isolated from poultry, human and environmental samples

Type of samples	Samples	Positive *Campylobacter* spp.(%)	Positive *C. coli* (%)	Positive *C. jejuni* (%)
Poultry meats	150	24(16.00)	16(10.61)	8(5.30)
Intestinal contents	150	58(38.67)	42(28.00)	16(10.67)
Equipment swabs	50	5 (10.00)	3(6.00)	2(4.00)
Hand washing swabs	50	3(6.00)	2(4.00)	1(2.00)
Human faeces	50	2(4.00)	1(2.00)	1(2.00)
Water	50	-	-	-
Total	**500**	**92(18.40)**	**64(12.80)**	**28(5.60)**

Fig.1 *Campylobacter* colonies on mCCD agar

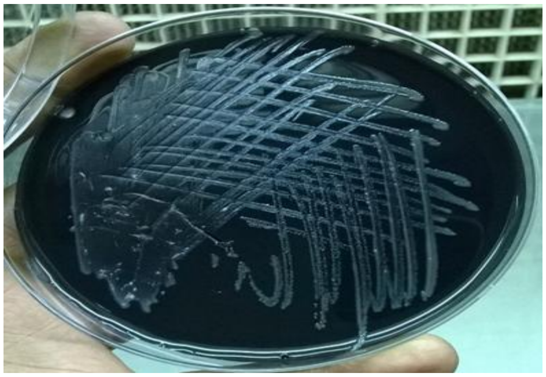


Fig.2 Gram's staining of *Campylobacter* organisms

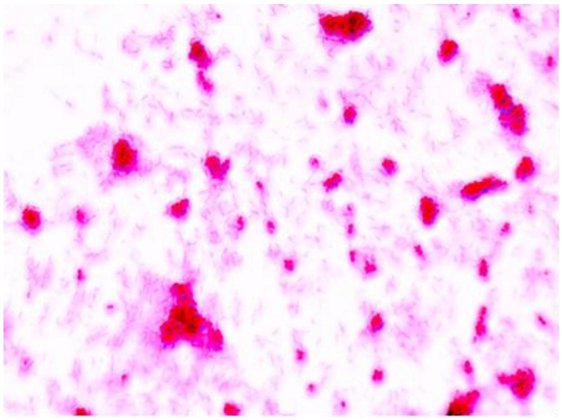
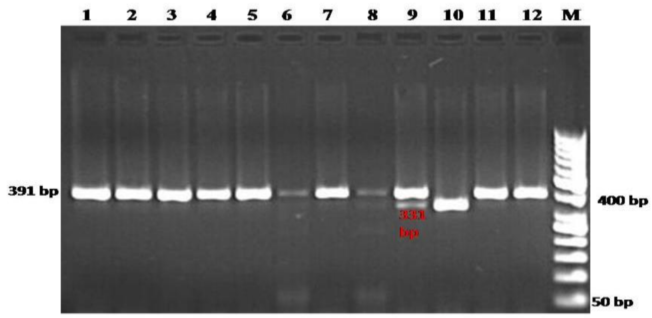


Fig.3 Confirmation of *Campylobacter* spp. by PCR targeting lpxA gene



Lane 1-8 and 11-12 (*C. coli* - 391bp), Lane 9 (*C. jejuni* and *C. coli* mixed culture- 331bp and 391bp), Lane 10 (*C. jejuni* - 331bp), M= Molecular marker 50bp

A multiplex PCR assay was employed for identification / confirmation of 2 species of *Campylobacter* namely, *C. coli*, and *C. jejuni*. The lipid gene, *lpx*A was targeted in this assay which yielded discriminatory band sizes for speciation. Out of the 92 isolates obtained in this study, 28 yielded preferred amplicon of 331 bp for *C. jejuni* and the rest 64 isolates gave amplicon of 391 bp for *C. coli* (Fig. 3).

Blood-free campylobacter agar was used as the primary isolation medium during the present study. In the past, various researchers have used blood-free media for isolation studies of campylobacters with good efficiency (Merino *et al.,* 1986; Oyarzabal *et al.,* 2005). Typical colony morphology was found in all the culture positive plates. The cultures showed characteristic spiral or S-shaped cell morphology on crystal violet staining and were Gram negative on Gram staining studies.

A multiplex PCR assay targeting the *lpx*A gene as described by Klena *et al*., (2004) was performed to identify the species of *Campylobacter*. Only two of the four species targeted in the study were isolated in the present study and Kumar (2011) with slight modifications. The *Campylobacter jejuni* isolates amplified a 331 bp product, while *Campylobacter coli* isolates amplified a 391 bp product, thus differentiating into the respective species. Muller *et al.,* (2006) and Eyles *et al.*, (2006) have reported specificity in the identification of *Campylobacter* at species level using this assay. The mPCR based assay has been reported to be in agreement with the results of hippurate hydrolysis test for species level identification of campylobacters.

The overall prevalence (18.40 %) of *Campylobacter* spp. in present study correlates with the observation of Rajkumar *et al.*, (2010) who was reported prevalence of *C.jejuni* and *C. coli* from poultry skin samples in small scale poultry dressing units of Northern India, as 18% from unorganized and 12% from organized farms. Similarly, Rizal *et al.*, (2010) recorded 17.14% of the chicken samples and 8.57% of human samples were positive for *C. jejuni*.

Singh *et al.,* (2009) and Sumankumar *et al.,* (2012) also reported an overall prevalence of 12.7% and 10.67%, respectively, from poultry meat and carcass collected from local poultry farms and retail markets of Bareilly which is lower as compared to present study. Suzuki and Yamamoto (2009) summarized the papers describing *Campylobacter* contamination of retail poultry meats and by-products in Japan and reported an average occurrence of approximately 60% which is higher compared to present study. Similarly, Tayde and Brahmbhatt (2012) also reported an overall prevalence of 34.66% from poultry meat and caecal contents collected from local retail markets of Anand, Gujarat which is higher compared to present study.

This variation in findings during the time of over 25 years may be due to variation in prevalence rate from time to time, disparity in hygienic and sanitary conditions of the poultry houses or use of different materials and methodology in different studies.

This study showed that out of a total of 92 *Campylobacter* isolates recovered, 64 (69.56%) were confirmed as *C. coli* and 28 (30.44%) as *C. jejuni* among poultry, human and environmental samples which was in concordance with the finding of Prasanna (2013) and Rajagunalan *et al.,* (2014) at Pantnagar and Bareilly regions. Similar findings were also reported by other workers (Zorman *et al.,* 2006 and Henry *et al.,* 2011). In contrast to the findings of the present study, many of the workers reported a higher prevalence of *C. jejuni* in poultry than *C. coli* (Sumankumar *et al.,* 2012; Tayde and Brahmbhatt, 2012). The reason for this difference in prevalence rates of *C. jejuni* and *C. coli* among poultry is unknown; however, impact of differences in the isolation procedures and geographic differences has been suggested by Zorman *et al.*, 2006.

Although there is significant variation in prevalence of *Campylobacter* spp. in different species of animals as reported by different workers, the organism was found to be predominant in poultry meat. Campylobacter is normal inhabitant of intestinal tract of most of the animals. However faulty handling and processing with improper storage are responsible for presence of the organism in the meat. Besides, Campylobacter poses a potential risk for consumers especially in establishment lacking adequate sanitary measures to prevent cross contamination.

In conclusion the occurrence *Campylobacte*r spp. in poultry intestinal contents, equipment swab and worker hands washing swab samples which indicate contamination of carcass may take place during slaughtering and/or post slaughtering processes. The isolation of Campylobacter spp. from clinical cases of children further prove the importance of infection that necessitates the need for proper preventive measures to control the infection in food production and consumption.

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