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Prevalence of Pathogenic Genes in *Campylobacter jejuni* Isolated from Poultry and Human

Anjana Rizal[1], Ashok Kumar[2]*, Ambarish Sharan Vidyarthi[1]

[1]*Department of Biotechnology, BIT, Ranchi*
[2]*Department of Veterinary Public Health, IVRI, Bareily*

Abstract: *Campylobacter jejuni* is a major cause of human diarrhaeal disease. This study was conducted to know the occurrence of *C. jejuni* in diarrhoeal cases of human and poultry, and to determine the prevalence of different pathogenic genes in isolates recovered from chicken intestine and human stool samples in Meghalaya and Assam. A total of 560 samples comprising 350 chicken intestine and 210 human stool samples were processed for isolation of *C. jejuni*. Out of these, 60 chicken and 18 human samples were found positive for *C. jejuni*. The isolates were identified on the basis of morphological, biochemical and PCR based detection of 16S rRNA and *hip*O gene. The isolates were screened for the presence of 9 pathogenic genes namely *fla*A, *cad*F, *rac*R, *iam*, *vir*B11, *pld*A, *cdt*A, *cdt*B and *cdt*C, which are responsible for expression of adherence, invasion, colonization and cytotoxin production in *C. jejuni*. The isolates showed a wide variation for the presence of these genes. Six isolates were positive for all the 9 genes screened. All the isolates were positive for *fla*A gene. The *cad*F gene was detected in 88.33% chicken and 100% human isolates. Of the *C. jejuni* recovered from human, 5.5%, 77.77%, 27.77%, 55.55%, 50%, 27.77% and 27.77% were positive for *rac*R, *iam*, *vir*B11, *pld*A, *cdt*A, *cdt*B and *cdt*C, respectively. Out of the isolates from chicken, 18.33%, 60%, 11.66%, 26.66%, 13.33%, 20% and 35% isolates revealed the presence of *rac*R, *iam*, *vir*B11, *pld*A, *cdt*A, *cdt*B and *cdt*C, respectively. The results showed the importance of *C. jejuni* in the region and emphasize the need for detail study and proper preventive measures.

Key words: *Campylobacter jejuni*, pathogenic genes, diarrhoeal, human, poultry

* Corresponding author. Mailing address: Division of Veterinary Public Health, IVRI, Bareily, India, Tel:+91-5812301326 , Fax:+91-5812303284, E-mail: ashokakt@rediffmail.com

Introduction

Campylobacter, a motile, microaerophilic, gram negative organism has been long associated with the cause of veterinary diseases. The organism gained more importance particularly during the last 30 years as it has also been recognized as a major cause of human illnesses ranging from gastroenteritis to Guillain-Barre Syndrome (Khanna, *et al*., 1996, Tauxe, 2001, Moore *et al*., 2005). Amongst *Campylobacter*s, mainly thermophilic *Campylobacter* species have been implicated in food borne infections and *Campylobacter jejuni* has now become the most frequently reported organism from cases of bacterial gastroenteritis in humans in many countries (Crushell *et al* ., 2004, Iovine, 2008). *Campylobacter* species are widely distributed in nature. Most of them are adapted to the intestinal tract of warm-blooded animals and birds. *C. jejuni* is particularly adapted to poultry, which probably forms the largest reservoirs of the species. Contaminated water and foods of animal origin, especially improperly processed chicken have been implicated as vehicles for the transmission of campylobacteriosis. The information on epidemiology of *Campylobacter* is sketchy in developing countries including India and more research work is required for formulation of strategies for its proper control and prevention.

The pathogenesis of *C. jejuni* is poorly understood as compared to other enteric pathogens. Various pathogenic mechanisms like production of cholera like enterotoxin and cytotoxin and ability to adhere and invade epithelial cells have been proposed to play the role in cases of enteritis (Crushell *et al*., 2004). Several genes have now been identified as virulent, pathogenic and important for adherence and toxin production. The *cdt*A, *cdt*B and *cdt*C genes have been identified for expression of cytotoxin production (Purdy *et al* ., 2000, Lara – Tejero and Galan, 2001). The *fla*A (Nuijten *et al*., 2000), *cad*F (*Campylobacter* Adhesion to Fibronectin F), (Ziprine *et al*., 2001) and *rac*R (Reduce Ability to Colonize R) (Bras *et al*., 1999) genes have been identified to be responsible for expression of adherence and colonization. The products of *vir*B11 (Bacon *et al*., 2000) and *pld*A (Phospholipase A) (Ziprin *et al*., 2001) genes help in invasion. Carvalho *et al*.

(2001) showed the presence of 1.6 kb genetic marker of *Campylobacter* strains associated with adherence and invasion of HEP – 2 cells in vitro and named it as invasion-associated marker (IAM) but Al-Mahmeed *et al* . (2006) discussed the absence of this marker in the isolates from clinical cases in the children but not in older patients and suggested more research on the prevalence of this marker.

Keeping in view the paucity of information on *Campylobacter* in this part of the world, we undertook this study for isolation of *C. jejuni* from human and poultry and screening the isolates for presence of various *vir*ulence and toxin genes.

Materials and Methods

Isolation and identification of *C. jejuni*: *C. jejuni* strains were isolated from chicken intestine collected at the time of slaughter and human stool samples from hospitals located in Meghalaya and Assam. Isolation was performed by directly streaking the samples onto modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with CCDA supplement (Oxoid) and incubated at 42°C under microaerobic conditions for 48 h. The isolates were identified as *C. jejuni* based on their morphological, biochemical tests including catalase, oxidase, H_2S (TSI), hippurate hydrolysis, resistance to nalidixic acid and sensitivity towards cephalothin.

PCR based confirmation and detection of *vir*ulence and toxin genes: The isolates were confirmed by PCR (polymerase chain reaction) based detection of 16S rRNA and *hip*O gene using published primers (Linton *et al.*, 1997). The confirmed *C. jejuni* isolates were screened for the presence of 9 pathogenic genes namely *fla*A, *cad*F, *rac*R, *iam*, *vir*B11, *pld*A, *cdt*A, *cdt*B and *cdt*C. The Template DNAs for PCR was extracted by using wizard® Genomic DNA purification kit (Promega). The primer sequences of all the genes, size of PCR amplicons and PCR conditions used in this study are presented in table 1. All PCR amplifications were performed in a mixture (50µl) containing 1X PCR buffer [50mM Tris/HCl, 10mM KCl, 5mM $(NH_4)2SO_4$, pH 8.3], 2.6 mM $MgCl_2$, 0.2mM each of dATP, dGTP, dCTP and dTTP, 1.25 U Taq Polymerase, 5 µl ext*rac*ted template DNA and 0.25 µl of a 100mM solution of each primer. Nuclease free water was added to make the final volume to 50µl and subjected to 30 cycles for amplification in a thermal cycler (Biorad). The cyclic conditions were as follows: denaturation at 94^{0}C for 1 min, annealing at a temperature specific to the primer pair with slight modification in the temperature for 16S rRNA, *hip*O, *fla*A and *cad*F genes (as given in table 1) for 1 min and extension at 72^{0}C for 1 min. The PCR product was analyzed by electrophoresis on 1.5% agarose gel (Merck). DNA bands were stained with ethidium bromide and visualized.

Name of the gene	Sequence (5'⟶3')	Product (bp)	Annealing temperature	Reference
16S rRNA	F-AAT CTA ATG GCT TAA CCA TTA R-GTA ACT AGT TTA GTA TTC CGG	854	55^{0}C	Linton *et al* ., 1997
*hip*O	F-GAA GAG GGT TTG GGT GGT R-AGC TAG CTT CGC ATA ATA ACT TG	735	56.2^{0}C	Linton *et al* ., 1997
*fla*A	F-ATG GGA TTT CGT ATT TG R-GTC AAA CGG GTA GCA ATA CC	450	46.5^{0}C	Oyofo *et al*., 1992
*vir*B11	F-TCT TGT GAG TTG CCT TAC CCC TTT T R-CCT GCG TGT CCT GTG TTA TTT ACC C	494	53^{0}C	Datta *et al*., 2003
*rac*R	F-GAT GAT CCT GAC TTT G R-TCT CCT ATT TTT ACC C	584	45^{0}C	Datta *et al*., 2003
*cdt*A	F-CCT TGT GAT GCA AGC AAT C R-ACA CTC CAT TTG CTT TCT G	370	49^{0}C	Eyigor *et al.*, 1999
*cdt*B	F-GTT AAA ATC CCC TGC TAT CAA CCA R-GTT GGC ACT TGG AAT TTG CAA GGC	495	51^{0}C	Bang *et al*., 2003
*cdt*C	F-CGA TGA GTT AAA ACA AAA AGA TA R-TTG GCA TTA TAG AAA ATA CAG TT	182	47^{0}C	Eyigor *et al*., 1999
*cad*F	F-TTG AAG GTA ATT TAG ATA TG R-CTA ATA CCT AAA GTT GAA AC	400	47^{0}C	Konkel *et al*., 1999
iam	F-GCG CAA AAT ATT ATC ACC C R-TTC ACG ACT ACT ATG CGG	518	52^{0}C	Carvalho *et al*.,2001

Table1: PCR Primers & annealing temperatures

Source	No. of samples	No. of *C. jejuni* isolates	Genes detected – number of positive isolates (% positive)										
			16S rRNA	*hip*O	*fla*A	*cad*F	*rac*R	*iam*	*vir*B11	*pld*A	*cdt*A	*cdt*B	*cdt*C
Humans	210	18	18 (100)	18 (100)	18 (100)	18 (100)	1 (5.5)	14 (77.77)	5 (27.77)	10 (55.55)	9 (50)	5 (27.77)	5 (27.77)
Chicken intestine	350	60	60 (100)	60 (100)	60 (100)	53 (88.33)	11 (18.33)	36 (60)	7 (11.66)	16 (26.66)	8 (13.33)	12 (20)	21 (25)

Table 2: Detection of different genes in *Campylobacter jejuni* by PCR

Results and Discussions

Out of total 560 samples screened, 78 (13.92%) revealed the presence of *C. jejuni* (Table 2). All the isolates were confirmed by PCR based detection of 16S rRNA and *hip*O gene (fig. 1 and 2). Because of strong conservation of regions in the rRNA genes (5S, 16S and 23S rRNA), these genes are suitable targets for PCR based identification of *Campylobacter*. The hippuricase gene (*hip*O) is specific for *C. jejuni* and it is not detected in any other *Campylobacter* species (Kolackova and Karpiskova, 2005). The analyses of results indicated that 17.14% of the chicken samples and 8.57% of human samples were positive for *C. jejuni*. The human isolates were from patients suffering from acute gastroenteritis and below the age of 6 years except one sample. The varying isolation rates of *C. jejuni* from human and chicken faecal samples have been recorded in different studies (Nadeau *et al.*, 2002, Baserisalehi *et al.*, 2005). Eyigor *et al*. (1999) also emphasized that the isolation rates may vary, depending on the year, sampling procedure, isolation methodology, and whether the sample is fresh or frozen.

The findings for the prevalence of different pathogenic genes are summarized in table 2. All the isolates from chicken and human origin had *fla*A gene (fig.3). The *cad*F gene (fig.4) was present in 88.33% isolates from chicken and all the isolates from human. The findings are in agreement with earlier observations regarding the presence of *fla*A and *cad*F genes in *C. jejuni* species isolated from human as well as chicken (Konkel *et al* ., 1999a, Dorrell *et al* ., 2001, Bang *et al* ., 2003, Datta *et al* ., 2003, Rozynek *et al* ., 2005). The products of these genes are responsible for the expression of adherence and colonization (Nuijten *et al*., 2000, Ziprin *et al.*, 2001). The prevalence of *fla*A gene in all the isolates indicates pathogenic potential since the *fla*A gene plays an important role in *Campylobacter* pathogenesis. The high prevalence of the *cad*F gene encoding *Campylobacter* adhesion to fibrionectin, an important *vir*ulence factor further prove the pathogenic potential of isolates as it was found in 100% isolates originating from clinical cases of human. Involvement of the *cad*F gene products in *Campylobacter* colonization has clearly been shown by *in vivo* colonization using a chicken model by Ziprin *et al.* (1999). The *iam* gene (fig.5) that encodes for Invasion associated marker (IAM)) was detected in 77.77% chicken and 60% human isolates. Rozynek *et al*. (2005) described and detected this marker in 50% of the *Campylobacter* isolates from chicken where as Carvalho *et al*. (2001) detected it in 85% human isolates. However, some workers suggest that it is not a universal marker due to its low prevalence among *Campylobacter* isolates from human (Rozynek *et al*., 2005). The contradictory opinions necessitate more research work to know the specific role of this marker in the isolates from different sources.

Bacon *et al*. (2000) investigated the possible involvement of a plasmid in virulence of *C. jejuni*. A plasmid (p*Vir*) contains four open reading frames and mutation in any of the four genes - the *vir*B11 homologue gene - results in reduction in adherence and invasion. The presence of *vir*B11 (fig.6) in 11.66% and 22.77% chicken and human isolates, respectively during the present study are almost in agreement with the results of earlier studies (Bacon *et al* ., 2000, Datta *et al* ., 2003).

Another marker analyzed was the cytotoxin encoding gene cluster. The cytopathic effect of the cytotoxin is associated with damage to nuclear DNA, resulting in the inhibition of the cell cycle in G2 or M phase (Whitehouse *et al*., 1998). The prevalence rates for *cdt*A, *cdt*B and *cdt*C (fig.7, 8 and 9) were found as 13.33%, 20%, 35% in chicken and 50%, 27.77%, 22.77% in human isolates. The percent occurrence of *rac*R (fig.10) and *pld*A (fig.11) in chicken and human isolates was 55.55, 26.66 and 5.5, 18.33, respectively. High prevalence of *cdt*, *pld*A, *rac*R genes has been detected in previous studies (Bang *et al*., 2000, Datta *et al*., 2003, Al-Mahmeed *et al*., 2006). The reason for low percentage of occurrence of these genes in

the present study could not be ascertained, however, may be due to genetic reason or variation in the isolates from different geographic area. The low percent of *cdt* genes needs to be compared with CDT production and test on different cell lines to study the phenotypic cha*rac*ters of the isolates.

The occurrence *C. jejuni* in chicken intestinal samples indicate the public health hazard due to this emerging food borne organism in the region. The contamination of carcass may take place from intestinal contents during slaughtering and/or post slaughtering processes. The isolates showed a wide variation for the presence of pathogenic genes; however, presence of many important virulence genes revealed the pathogenic potential of the isolates. The findings on isolation of *C. jejuni* 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.

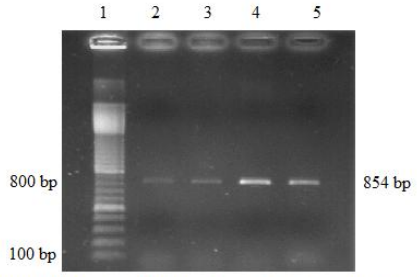

Fig.1: PCR detection of 16S rRNA gene (854bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-5 test organisms

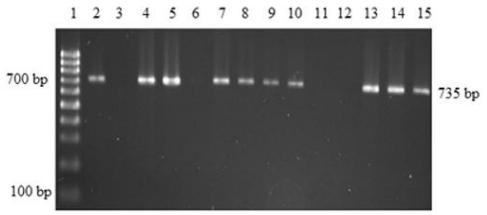

Fig.2: PCR detection of *hip*O gene (735bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-15 test organisms

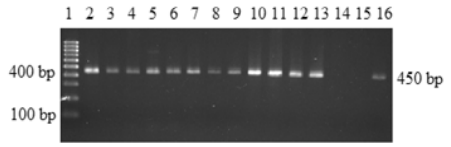

Fig.3: PCR detection of *fla*A gene (450bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-16 test organisms

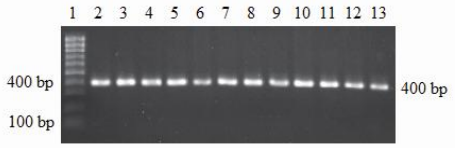

Fig.4: PCR detection of *cad*F gene (400bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-13 test organisms

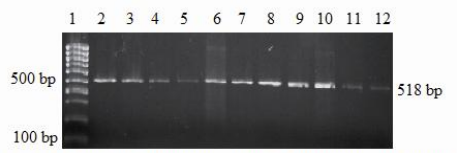

Fig.5: PCR detection of *iam* gene (518bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-12 test organisms

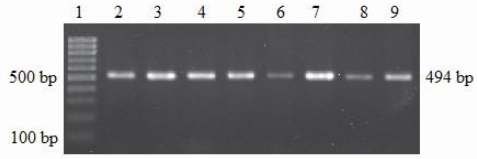

Fig.6: PCR detection of *vir*B11 gene (494bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-9 test organisms

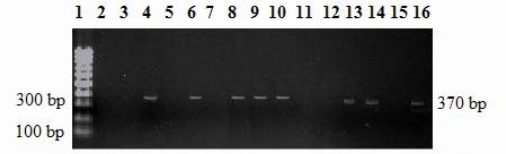

Fig.7: PCR detection of *cdt*A gene (370bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-16 test organisms

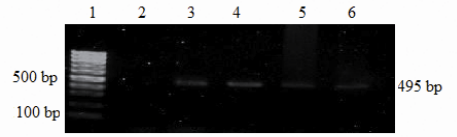

Fig.8: PCR detection of *cdt*B gene (495bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-6 test organisms

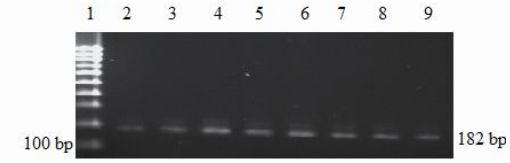

Fig.9: PCR detection of *cdt*C gene (182bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-9 test organisms

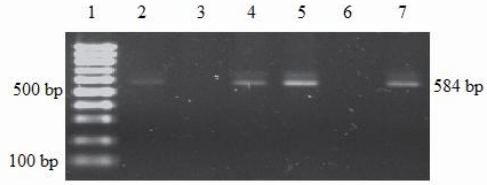

Fig.10: PCR detection of *rac*R gene (584bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-7 test organisms

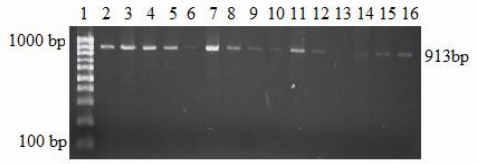

Fig.11: PCR detection of *pld*A gene (913bp) of *Campylobacter jejuni*. Lane 1- Marker, Lane 2-16 test organisms

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