Original Research

Virulence Characterization of *Campylobacter jejuni* Isolated from Poultry in India

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Rec. Date:	Jan 17, 2020 04:34
Accept Date:	Feb 22, 2020 06:45
DOI	10.5455/ijlr.20200117043429

Abstract

The present study was conducted for detection of various virulence factor (adherence, invasion, colonization, motility, lipo-oligosacchrides) and cytolethal distending toxin genes along antibiotic resistance determination among Campylobacter jejuni isolates of poultry origin in India. Adherence and lipo-oligosacchrides genes i.e. cadF, porA, jlpA, dnaJ, wlaN, waaC and capA were detected in 97.67%, 93.02%, 90.69%, 88.37%, 88.37%, 65.11% and 51.16% of the isolates respectively. Cytolethal distending toxin genes i.e. cdtC, cdtA and cdtB, were detected in higher proportions (86.04% to 97.67%) followed by flagellin gene i.e flaAflaBflgR (69.76% to 100%) and invasion genes i.e. iamAB, pldAand ciaB (34.88% to 88.37%) isolates. Further, phylogenetic analysis of iamAB gene revealed their unique genetic makeup and didn't found homology from Indian origin.

Key words: *Campylobacter jejuni*, Poultry, Virulence Genes

How to cite: Yadav, R., & Maherchandani, S. (2020). Virulence Characterization of *Campylobacter jejuni* Isolated from Poultry in India. International Journal of Livestock Research, 10(3), 132-140. doi: 10.5455/ijlr.20200117043429

Introduction

Campylobacter had received serious attention as causative agent of diarrhoea only since 1973 among human population (Butzler *et al.,* 1973). *C. jejuni* is responsible for the majority (80-90%) of these infections and is now a second most emerging food borne zoonotic pathogen after *Salmonella* (Andrzejewska *et al.,* 2011; Epps *et al.,* 2013). Poultry are considered to be its major reservoir as bacterium efficiently colonizes into caecal mucosal crypts of the gastrointestinal tract (Moore *et al.,* 2005; Bolton, 2015; Mani *et al.,* 2018). These microaerophillic gram-negative rods possess several virulence factors associated with their survival and pathogenicity, however pathogenesis of infections is still not clearly

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understood (Lluque *et al.,* 2017). The major virulence attribute of *C. jejuni* are adhesion, invasion, presence of lipo-oligosacchrides responsible for evading host defense mechanism and production of cytotoxins (Datta *et al.,* 2003; Fouts *et al.,* 2005; Bolton, 2015). Adherance is governed by *Campylobacter* adhesion protein A (*cap*A) a autotransporter responsible for initial step of interaction (Flanagan *et al.,* 2009), factor for *Campylobacter* adhesion to fibronectin (*cad*F) (Rizal *et al.,* 2010; Mahmoodipour *et al.,* 2017), heat shock proteins (*dna*J), thermoregulation protein (Rozynek *et al.,* 2005), major outer membrane protein (MOMP) also called *por*A (Flanagan *et al.,* 2009) and a surface expressed lipoprotein loosely attached to the bacterial cell surface (*jlp*A) (Jin *et al.,* 2003). Lipo-oligosacchrides (LOS) (different from LPS) lack an O-polysaccharide chain and plays a crucial role in immune avoidance, serum resistance, adherence and invasion of intestinal epithelial cell (Javed *et al.,* 2012; Yang *et al.,* 2014). The two locomotors flagellar filament *fla*A (major flagellin) and *fla*B (minor flagellin) govern motility, initial interaction to host, antigenic phage variation, invasion and colonization of gastrointestinal tract (Casabonne *et al.,* 2016). Their expression is regulated by response regulator *flg*R gene. Colonization leads to production of bacterial tripartitecytolethal distending toxins (CDT) encoded by three linked genes *i.e. cdt*A, *cdt*B and *cdt*C, causing severe enteritis including severe abdominal cramps, diarrhea with blood or mucus and fever (Lee and Newell, 2006; Gargi, 2013; Zhang *et al.,* 2016). Invasion associated marker (*iam*) is responsible for invasion and colonization of multiple hosts (Jribi *et al.,* 2017).

Wide strain variations have been reported detection of virulence associated genes from different geographical locations (Gonzalez-Hein *et al.,* 2013) all over the world but there is lack of documentation from Indian origin *C. jejuni* isolates. Therefore, present study was carried out for detection of virulence and toxin associated genes from poultry origin in India.

Material and Methods

A total of 43 *C. jejuni* isolated during 2014-2016 from local poultry farms in and around Bikaner, India in previous study (Yadav *et al.,* 2016) were subjected for determination of virulence associated genes.

Amplification of Virulence Associated Genes

Detection of various virulence factors was done using primer sets as reported earlier or designed for the present study (Table 1). The DNA extraction was carried out as described in previous study (Ertas *et al.,* 2004; Yadav *et al.,* 2016). All PCR amplifications were performed in a mixture (25 µl) containing: 2.5µl of the 10X PCR buffer, 2.5µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 1 µl of each primer (100 µM), 0.5 µl (1U) of the *Taq* DNA polymerase (Promega), 3 µl (50-100 ng) of the bacterial template DNA and 14 µl nuclease free water. The PCR products were analyzed by electrophoresis on 1.5% agarose gel for 1 h at 100V. The gel was then visualized under UVP gel documentation system (BioDoc-It Imaging System).

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 DOI 10.5455/ijlr.20200117043429

Table 1: PCR primers and conditions for detection of virulence associated genes

S.no	Virulence factors	Gene name	Primer sequence	A. temp (°C)	Size (bp)	References
1	Adherence	*cad*F	F- TTGAAGGTAATTTAGATATG R- CTAATACCTAAAGTTGAAAC	45	400	Konkel *et al.* (1999)
2		*cap*A	F-TGAATCGAAGTGGAAAAATAGAAG R- CCCATTTTTGTATCTTCATAACCT	60	1351	Flanagan *et al.* (2009)
3		*jlp*A	F- TCTCAGGACTCTGGAATAAAGATTG R-GTGTGCTATAGTCACTAACAGGGATG	60	868	Flanagan *et al.* (2009)
4		*por*A	F- CAATTTGACTATAATGCTGCTGATG R- ATGCTGAGAAGTTAAGTTTTGGAGA	50	932	Chae *et al.* (2012)
5		*dna*J	F- AAGGCTTTGGCTCATC R- CTTTTTGTTCATCGTT	46	720	Datta *et al.* (2003)
6	Lipooligosachrides	LOS-*wla*N	F- TGCTGGGTATACAAAGGTTGTG R- AATTTTGGATATGGGTGGGG	60	330	Muller *et al.* (2006)
7		*waa*C	F- TAATGAAAATAGCAATTGTTCGT R-GATACAAAAATCACTTTTATCGA	42	1029	Khoshbakht *et al.* (2013)
8	Motility	*fla*A	F- GGATTTCGTATTAACACAAATGGTGC R- CTGTAGTAATCTTAAAACATTTTG	52	1725	Nachamkin *et al.* (1993)
9		*fla*B	F- ATAAACACCAACATCGGTGCA R- GTTACGTTGACTCATAGCATA	50	1670	Chae *et al.* (2012)
10		*flg*R	F- GAGCGTTTAGAATGGGTGTG R- GCCAGGAATTGATGGCATAG	54	390	Wilson *et al.* (2010)
11	Invasion	*iam*AB	F- CGACTACTATGCGGATCAAG R- TTGTAAATGCTATATTTTGGG	53	601	This study
12		*cia*B	F- TTTTTATCAGTCCTTA R- TTTCGGTATCATTAGC	42	986	Datta *et al.* (2003)
13		*pld*A	F- AAGCTTATGCGTTTTT R- TATAAGGCTTTCTCCA	45	913	Datta *et al.* (2003)
14	Cytolethal distending toxins	*cdt*A	F- CCTTGTGATGCAAGCAATC R-ACACTCCATTTGCTTTCTG	49	370	Talukder *et al.* (2008)
15		*cdt*B	F- CAGAAAGCAAATGGAGTGTT R- AGCTAAAAGCGGTGGAGTAT	51	620	Talukder *et al.* (2008)
16		*cdt*C	F- CGATGAGTTAAAACAAAAAGATA R- TTGGCATTATAGAAAATACAGTT	47	182	Talukder *et al.* (2008)

Sequence Analysis of *iam*AB Gene

The primers were designed by primer 3 tool of NCBI for *iam*AB gene from genebank at NCBI (Accession no. AF023133). PCR products from three isolates were sequenced (DNA Sequencing Facility, Delhi University). The sequences obtained were subjected to nucleotide BLAST (Basic Local Alignment Search

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tool) to determine the similarity with the already prevalent gene sequences and were published with accession numbers KX840464, KX840465 and KX840466 respectively in NCBI gene bank database. The sequences were also aligned using Bio edit and MEGA6 software to study the variations in the nucleotide sequences and their phylogenetic cluster analysis (Bikandi *et al.,* 2004).

Results and Discussion

Campylobacter jejuni are blessed with some adherence, invasion and some cell surface expressive virulent factors responsible for its high prevalence and pathogenicity as compared to other enteric bacteria (Biswas *et al.,* 2011). All the 43 isolates of *C. jejuni* were subjected to the PCR detection for 16 virulence genes associated with adherence, invasion, flagellin, Lipo-oligosacchrides and toxin production (Fig.1).

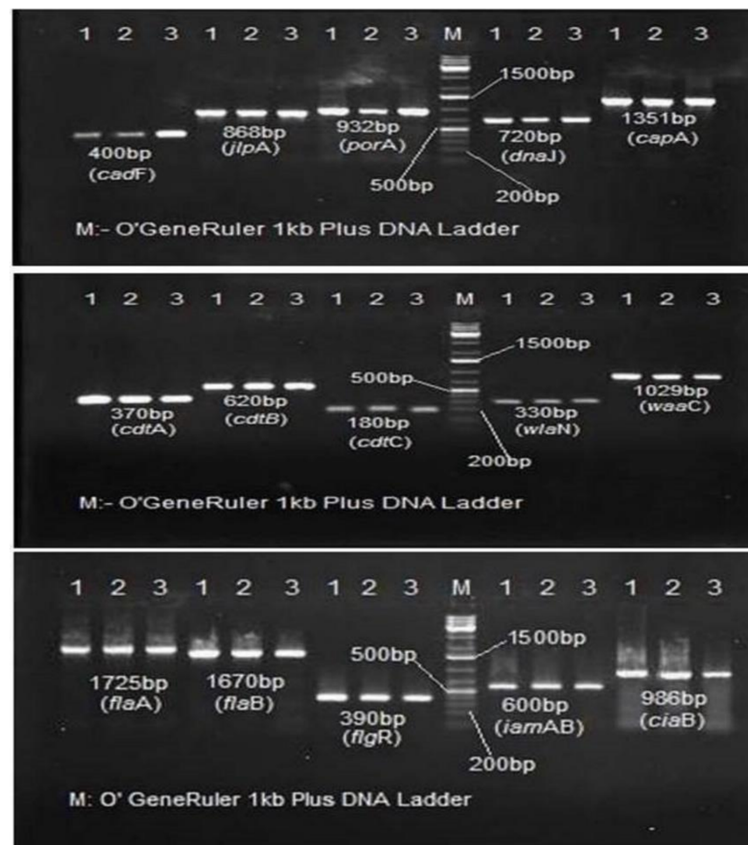

Fig. 1: Agarose gel electrophoresis image of various virulence associated genes of *C. jejuni*

Flagellin gene, *fla*A was detected in all the isolates followed by *fla*B gene and *flg*R gene in 72.09% and 69.76 % of the isolates. The *fla*A gene also has role in type III secretion apparatus for the *Campylobacter* invasion antigens (*Cia* proteins) important for *in vitro* cell invasion (Konkel *et al.,* 2004; Khoshbakht *et al.,* 2013) and chick colonization (Ziprin *et al.,* 2001). Chickens exposed to the *flg*R (*fla*A and *fla*B expression regulator) mutants showed delayed colonization (Hermans *et al.,* 2011).

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Adherence associated genes namely; *cad*F, *cap*A, *jlp*A, *por*A and *dna*J were found to be present in 97.67%, 51.36%, 90.69%, 93.02% and 83.37% of the isolates respectively. There were 44.18% isolates having all the five adherence associated genes. The active role of adherence associated genes in pathogenesis and colonization of *C. jejuni* has been reported previously (Negretti*et al.,* 2017). *cad*F deletion mutants of *C. jejuni* had 50-60% reduction in binding to immobilized fibronectin, intestinal human cells (INT 407) and caecum epithelial cells (Chansiripornchai and Sasipreeyajan 2009). Likewise, *cap*A deletion mutant exhibited 47% reduction in binding of *Campylobacter* to chicken LMH epithelial cells in comparison to wild-type isolate (Fouts *et al.,* 2005). The *jlp*A (Jejuni lipoprotein A) was identified in having role in binding to HEp-2 cells (Flanagan *et al.,* 2009). The *jlp*A null mutant reduced binding of such *Campylobacter* by 18-19.4% when compared to wild-type *C. jejuni*; however, no difference in invasion was observed (Jin *et al.,* 2003). Both the lipo-oligosaccharides genes namely *wla*N and *waa*C were found in 88.37% and 65.11% of the isolates and in total 60.46% of the isolates had both the genes. The *wla*N gene has a role in biosynthesis of lipo-oligosacchrides molecules and regulation of protein glycosylation whereas *waa*C encodes for heptosyltransferase I and attaches the first heptose (HEp-I) to *Kdo* (Karlyshev *et al.,* 2005).

The *C. jejuni* isolates from present study were found highly toxic as all three cytolethal distending toxin linked genes i.e. *cdt*A, *cdt*B, and *cdt*C genes were found in 93.02%, 86.04% and 97.67% of the isolates. Cytolethal distending toxin (CDT) is a complex acting together to block cell division by performing cell cycle arrest (Ge *et al.,* 2008). The *cdt*B is the active holotoxin and causes cell cycle arrest by cleaving dsDNA molecules during the G1 and G2 phase (Ramachandran *et al.,* 2017). The genes *cdt*A and *cdt*C usually bind to the cell surface and help in the delivery of active subunit *Cdt*B to cause DNA damage (Ghorbanalizadgan *et al.,* 2014). Invasion associated genes *iam*AB, *pld*A and *cia*B genes were detected in 88.37%, 46.51% and 34.88% of the isolates.Invasion associated marker (*iam*) is 1.6 kb genetic marker having ABC transporter (*iam*A) gene and integral membrane protein (*iam*B) gene and have been found to be associated with adherence and invasion of Hep-2 cells *in vitro* (Carvalho *et al.,* 2001).

Sequence Analysis of *iam*AB Gene

On comparison of partial sequences of *iam*AB gene namely KX840464 of C4 isolate, KX840465 of C22 isolate and KX840466 of C23 isolate, five nucleotide variations were observed in KX840464 as compared to KX840465 and KX840466 *viz.* A166G (Met to Ile), C255T (Phe to Ser), C261T (Leu to Ser), A385G (Ser to Ser), and G426T (Phe to Cys). KX840465 (C22) and KX840466 (C23) were identical. For phylogenetic analysis, in addition to three isolates from the current study, we selected 15 sequences (from across the world) of *iam*AB gene from the public domain. The phylogenetic tree constructed using these 18 sequences revealed three major clusters (Fig. 2).

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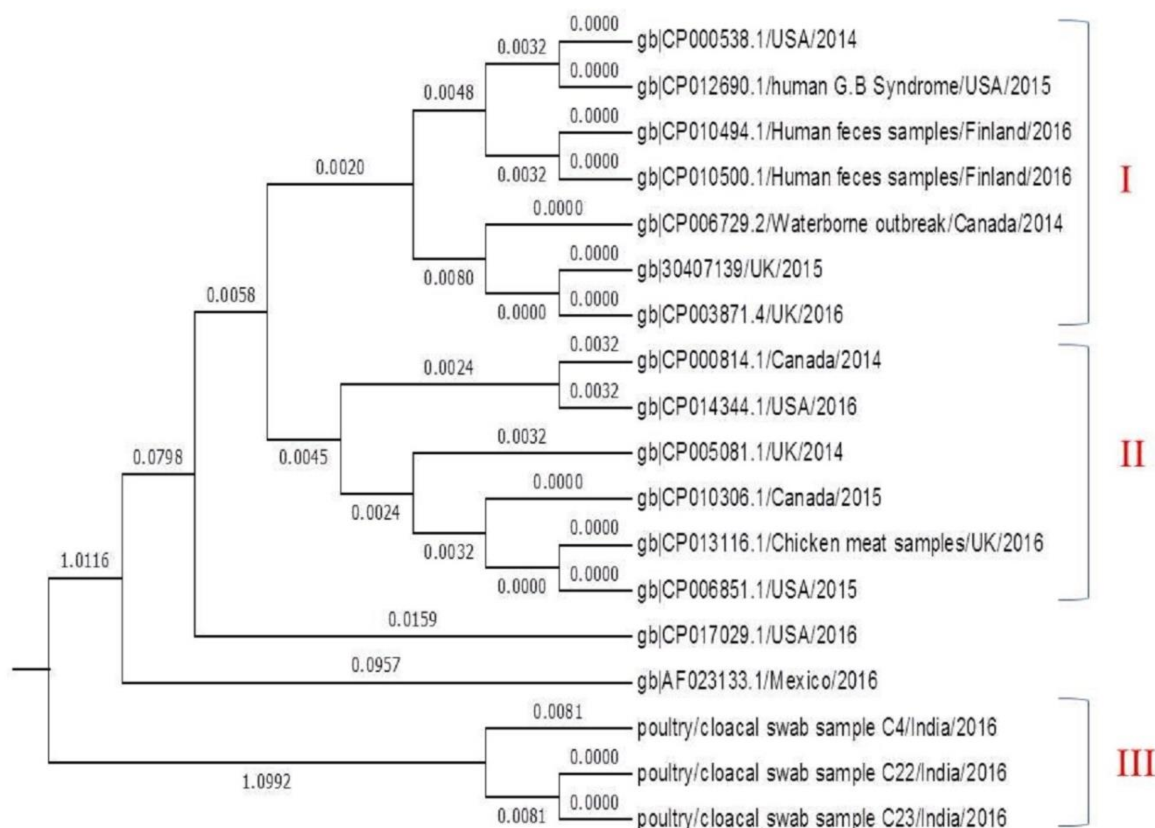

Fig. 2: Phylogenetic analysis of *iam*AB gene sequences

All three isolates under study grouped under a separate cluster (cluster III). Though, sequences of *iam*AB gene for other isolates from India are unavailable in the public domain but separation of the isolates into an entirely different cluster suggests their unique genetic character. The only single poultry isolate (originating from UK) available in the public domain did not cluster together with the isolates under study; rather it grouped under a separate cluster (cluster II). Cluster I and cluster II represented *C. jejuni* isolates from US, Canada, Finland, UK and most of them belonged to humans. Taken together, we didn't found any *iam*AB gene sequence of *C. jejuni* isolates originating from India in public domain. Thus, the phylogenetic analysis of *iam*AB gene sequences from present study isolates suggested their unique genetic makeup.

Conclusion

C. jejuni isolates from present study were detected as potentially pathogenic by presence of number of virulence genes in high proportions. The isolates had potential to produce cytolethal distending toxin due to high presence of tripartite *cdt* gene complex. To our best knowledge, we didn't find any *iam*AB partial gene cds of Indian origin *C. jejuni* isolates published in public domain. The phylogenetic analysis of *iam*AB gene sequences revealed their unique genetic identity to the sequence taken from rest parts of the world.

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Acknowledgment

The study was funded by Rajasthan University of Veterinary and Animal Sciences University (RAJUVAS), Bikaner as part of Ph.D dissertation of the first author Dr. Rahul Yadav under supervision of major advisor Prof. Sunil Maherchandani.

Conflict of Interest

Authors don't have any conflict of interest.

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DOI 10.5455/ijlr.20200117043429