Molecular Characterization of *Campylobacter jejuni* Isolates of Poultry Origin

M. Suman Kumar*, Ashok Kumar, A. Rizal1, K.N. Bhilegaonkar, R.S. Rathore, Lokesh K.M and R.K. Agarwal2

Division of Veterinary Public Health
Indian Veterinary Research Institute, Izatnagar - 243 122

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**ABSTRACT**

*Campylobacter jejuni* is one of the leading causes of bacterial foodborne infections in the world today. Several case control studies have suggested the handling and consumption of contaminated poultry meat as one of the important routes of transmission to humans. The present study was conducted to investigate the genetic diversity among 29 *Campylobacter jejuni* isolates of chicken origin using two PCR-based genotyping techniques. The *fla*-typing method using restriction enzyme *HinfI* revealed a total of 18 *fla*-types, with all the isolates under study found typeable. Randomly amplified polymorphic assay using a universal primer (HLWL85) clustered the isolates into 24 types, with one isolate found untypeable. The isolates under study revealed significant molecular heterogeneity and *fla*-typing proved to be a useful, relatively simple and reliable technique for subtyping *C. jejuni* among the two methods.

**Keywords:** Genotyping, *fla*-typing, molecular heterogeneity, RAPD

**Introduction**

*Campylobacter* is one of the leading causes of enteritis amongst the food-borne zoonotic pathogens in both the developed, as well as the developing countries (Kirkpatrick and Tribble, 2011). The total illness due to *Campylobacter* spp. is estimated to be more than three times the number of cases caused by *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* combined (CDC, 2008). The most important pathogenic strains reported from food-borne illness belong to the group of thermotolerant campylobacters, notably *C. jejuni*, *C. coli* and to a lesser extent, *C. lari* (Griffiths and Park, 1990). Domestic animals and poultry act as source of human infections. The ingestion of faecal contaminated chicken meat or poor food handling practices associated with raw chicken represents the primary route of its transmission to humans and is considered as the most consistent risk factor (Zorman *et al.*, 2006; OIE Terrestrial Manual, 2008). Handling, preparation and consumption of broiler meat may account for 20–30% of human cases of campylobacteriosis, while 50–80% may be attributed to the chicken reservoir as a whole (EFSA, 2010). With the increase in poultry meat consumption, the dynamics of animal production and consumer exposure have changed, leading to new challenges in limiting poultry-borne zoonoses like campylobacteriosis and salmonellosis.

The diarrhoegenic potential of thermophilic campylobacters and its prevalence in India is firmly established (Gugnani, 1999). However, the figures for actual prevalence and economic losses incurred in India are unfortunately not available. The sporadic studies conducted in different parts of the country have revealed the presence of *Campylobacter* organisms in various species of animals and birds.
(Pillai, 1986), foods of animal origin (Khanna et al., 1996), foods of plant origin (Kumar et al., 2001) and from cases of human diarrhea. Common biotypes and serotypes have been isolated from human cases and birds (Kumar, 1989; Khanna et al., 1996). There is an immediate need for systematic epidemiological study on Campylobacter infections in this part of the world.

Campylobacters are fastidious in nature and easily suppressed by other enteropathogens during culture and isolation. Also, the specific detection of Campylobacter at species and subspecies level becomes more difficult and tedious due to the inert nature of these organisms to most of the traditional biochemical tests used for characterization (On, 2001). These limitations have resulted in efforts to develop more rapid, sensitive and reliable procedures for detection and typing of Campylobacter spp. With upcoming of the newer molecular techniques, the burden has been eased to a certain extent with several molecular typing methods being used to support studies on the epidemiology of Campylobacter infections. The traditionally used phenotypic procedures like serotyping have some drawbacks such as technical difficulties and time consumption. Also, the high cost involved in production of quality control antiserum and the presence of a significant number of untypeable strains have restricted the use of serotyping techniques for large-scale epidemiological studies (Wassenaar and Newell, 2000). In recent years, molecular methods have been proposed as an alternative to serologic methods for typing Campylobacter strains.

Restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products belonging to the fla gene (fla-typing), has emerged as a suitable target for rapidly investigating large number of isolates. The presence of highly conserved and variable regions in the flaA gene deems this locus appropriate for RFLP analysis of PCR products (Meinersmann et al., 1997). Different primers and restriction enzymes have been used for fla-typing of C. jejuni. The enzymes Alu 1, Dde 1, Hinf 1, EcoR 1, and Pst 1 are all currently used in various combinations or alone (Nachamkin et al., 1996; Aquino et al., 2010; Muller et al., 2011).

Random amplified polymorphic DNA (RAPD) based methods employing various primers and reaction conditions have also been developed for Campylobacter spp. This method has been found highly discriminatory in a number of studies on establishing genetic diversity among C. jejuni and C. coli isolates from different sources (Hilton et al., 1997). Ten-mer primers have been frequently used for RAPD analysis of C. jejuni from diverse sources (Madden et al., 1996; Misawa et al., 2000; Wieczorek, 2009; Adzitey et al., 2012).

In this study, a molecular epidemiological investigation using PCR-based DNA fingerprinting of C. jejuni isolates from chicken intestine and meat has been described. The genetic variation among these isolates was deduced by the application of fla-typing and RAPD. The discriminatory capacity of these methods for genotyping was also compared.

Materials and Methods

The C. jejuni isolates analyzed in the present study were collected from different slaughter houses in Meghalaya and Assam and were available in freeze-dried form at the Division of VPH, IVRI, Izatnagar. A total of 29 isolates, comprising chicken meat (9) and chicken intestine (20), were revived using brain heart infusion broth (Titan Biotech, India) with campylobacter growth supplement (FD 009), as the enrichment medium. A loopful of freeze-dried culture was suspended in BHI broth and incubated at 42°C for 48 h under microaerophilic condition. Tubes showing turbidity were streaked onto blood-based Brucella agar media (HiMedia, India) and incubated under suitable conditions. The colonies were confirmed by Gram’s staining to visualize Gram negative, non spore-forming, comma, short spirally curved or “S” shaped rods.

DNA was extracted directly from the colonies on blood-agar plates using DNasy Blood and Tissue Kit (QIAGEN, USA) following the manufacturer’s protocol. Flagellin gene typing (fla-typing) was performed as per the protocol of CAMPYNET (2001) with necessary modifications. The flaA gene was amplified in a reaction mixture (50 µl) comprising 5 µl of 10 x Taq buffer, 5 µl of 2 mM concentration of each dNTP, 50 pmol of each primer (Fla-F 5’-GGA
TTT CGT ATT AAC ACA AAT GGT GC-3' and Fla-R 5'-CTG TAG TAA TCT TAA AAC ATT TTG-3'), 1.25 U of Taq polymerase, 5 µl of DNA and nuclease free water to make up to 50 µl. The amplification was carried out in a thermal cycler (Corbett Research, Australia) with the following cycling conditions: denaturation temperature of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s; annealing at 45°C for 45 s and extension at 72°C for 105 s. The final extension step was done at 72°C for 5 min. The amplification product was confirmed for the presence and purity of flaA gene product by electrophoresis on a 1.5% agarose and suitable products were subjected to restriction enzyme digestion with HinfI (Fermentas, Canada). The digestion mixture (30 µl) comprised of 3µl of 10 x buffer, 0.2 µl (2 U) of the enzyme, 21.8 µl of nuclease-free water and 5 µl of PCR product each. The reaction tubes were incubated at 37°C overnight and stored at 4°C till further examination. The restriction digestion products were electrophoresed in a 2% agarose gel (Gonitec, USA) at 80 V for 75 min and stained with ethidium bromide (0.5 µg/ml). Approximately 16 µl of the digested product was loaded along with a 100 bp plus ladder (GeneRuler 100 bp Plus DNA Ladder, Fermentas, Canada) as the molecular marker. The band patterns obtained were documented and analyzed using Alphalmager analysis tool.

DNA templates prepared as described above were used for RAPD analysis according to the method described by Mazurier et al. (1992). The reaction was carried out in a PCR mixture (25 µl) consisting of 2.5 µl of 10 x Taq buffer, 2.5 µl of 2 mM concentration of each dNTP, 25 pmol of the primer, 1µ of Taq polymerase, 3 µl of template DNA and nuclease-free water to 25 µl. The amplification cycles included initial denaturation at 94°C for 5 min followed by 45 repeats of denaturation at 94°C for 1 min; annealing at 36°C for 1 min and extension at 72°C for 1 min. Final extension was carried out at 72°C for 7 min. The universal primer HLWL85 (ACA ACT GCT C) was employed in the present study. The PCR products were characterized by gel electrophoresis on 1.5% agarose gel at 75 V for 3 h with 100 bp plus DNA ladder (Fermentas, Canada) used as molecular weight marker.

The interpretation of results was done by pairwise binary band matching (Tenover et al., 1995).

Only the distinct bands were considered for analysis by binary scoring pattern, wherein a score of 1 for the presence and 0 for absence of a band was assigned. The dendrogram was constructed using the software TREECON for Windows v1.3b. Bioinformatics and Evolutionary Genomics, Belgium (Van de Peer and De Wachter, 1994). Similarity index was analyzed by Dice coefficient and clustering by unweighted pair group method with arithmetic mean (UPGMA) method to determine the relatedness of the isolates. Numerical index of discrimination was calculated by Simpson’s index of diversity (Hunter and Gaston, 1988).

Results and Discussion

Molecular typing has enhanced the reliability and efficacy of epidemiological studies with the identification of devastating food-borne outbreaks and has thus enabled effective disease containment (Rangel et al., 2005). It provides necessary information for infection control and contributes to the risk assessment of transmission of food-borne pathogens. Further, the confusing epidemiological evidence of Campylobacter spp. partly due to the sporadic nature of the disease, along with the organism’s wide distribution, and high levels of genetic and antigenic diversity (Zorman et al., 2006), call for the use of genotyping techniques with high reproducibility and discriminatory ability. In the present study, PCR-based RFLP and RAPD techniques were evaluated for genotyping Campylobacter spp. with the aim of knowing the molecular heterogeneity of the isolates from chicken origin. A total of 29 C. jejuni isolates were subjected to flaA gene PCR to produce a ~1725 bp amplicon (Fig. 1). The restriction enzyme digestion of the amplified product with HinfI revealed a total of 18 fla-types with a D value (discriminatory power) of 0.9507. The number of bands in each profile varied between 2 to 4 and were spread across the molecular weight range of 380 to 1150 bp (Fig. 2). Genotyping with the RAPD primer HLWL85 revealed 25 types out of the 28 isolates, with one isolate found untypeable. The number of bands ranged from 2 to 8 across the range of 150 to 2850 bp (Fig. 3). One C. jejuni isolate was untypeable, giving a typeability of 98.4%. The D value of the primer was found to be 0.9977.
The occurrence of *flaA* gene in all the isolates screened has been reported by other workers (Bang *et al.*, 2003; Datta *et al.*, 2003, Wieczorek and Osek, 2008). The *flaA* gene plays an important role in pathogenesis, and the highly conserved and variable regions present in the locus make the gene a suitable candidate for RFLP studies (Meinersmann *et al.*, 1997). Variability within the *fla*-coding sequence has been used as the basis for RFLP studies for subtyping *C. jejuni* successfully, as is evident from the present study.

The *fla*-typing of chicken origin isolates revealed high genetic diversity. Such high degree of variation among *Campylobacter* isolates have also been reported by Hannon *et al.* (2009) and Koene *et al.* (2009). The dominant *fla*-type was shared by five isolates, and four additional groups containing two isolates were observed. Although variations observed in *fla*-typing do not allow a direct comparison of results attained in different laboratories, this technique has been proven to be useful, relatively simple and reliable in subtyping *C. jejuni* (Djordjevic *et al.*, 2007). The overall discriminatory power and typeability of PCR-RFLP method was satisfactory and in agreement with reports of various other workers.

$D$ values of 0.973 and 0.970 were observed by Nachamkin *et al.* (1993, 1996) and Santesteban *et al.* (1996), respectively. Nielsen *et al.* (2000) reported $D$ value of 0.96, while Sails *et al.* (2000) reported a value of 0.923. The $D$ value of *Hinfl* used

**Fig. 1.** Agarose gel showing *flaA* amplification product
Lane M : 100 bp plus ladder; Lane 1-4 : *flaA* PCR product (1725 bp)

**Fig. 2.** *fla*-types of *C. jejuni* using restriction endonuclease *Hinfl*
Lane M : 100 bp plus ladder; Lane 1 : C 59; Lane 2 : C 52; Lane 3 : C 140; Lane 4 : C 149; Lane 5 : C 168; Lane 6 : C 155; Lane 7 : C 159; Lane 8 : I 14; Lane 9 : I 52; Lane 10 : I 59; Lane 11 : I 60; Lane 12 : I 67; Lane 13 : I 73; Lane 14 : I 80; Lane 15 : I 82; Lane 16 : I 90; Lane 17 : I 102; Lane 18 : I 107; Lane 19 : I 140
Fig. 3. RAPD profiles of *C. jejuni* isolates using HLWL85 primer

Lane M: 100 bp plus ladder; Lane 1: C 52; Lane 2: C 140; Lane 3: C 149; Lane 4: C 168; Lane 5: C 155; Lane 6: C 159; Lane 7: I 14; Lane 8: I 23; Lane 9: I 52; Lane 10: I 59; Lane 11: I 60; Lane 12: I 67; Lane 13: I 73; Lane 14: I 80; Lane 15: I 82; Lane 16: I 90; Lane 17: I 91; Lane 18: I 102; Lane 19: I 107; Lane 20: I 140

in the present study is in agreement with the above reports. The typeability was observed to be 100% and supports the findings of Wassenaar and Newell (2000). This technique is not only fast, easy and cheap, but also has a sufficient discriminatory power (Acke et al., 2010) as is also evident from the findings in the present study. *Fla*-typing has also been proven to be an alternative to conventional methods like serotyping, with Nachamkin et al. (1996) reporting a 100% correlation between the two methods.

Random amplified polymorphic DNA methods have been found highly discriminatory in a number of studies on establishing the genetic diversity among *Campylobacter* isolates (Ertas et al., 2004; Miwa et al., 2003). The primer HLWL85 used in the study typed 28 of the 29 isolates with a typeability of 98.4%. Fayos et al. (1992) reported a typeability percentage of 87, while Madden et al. (1996) reported 100% typeability for this technique. Hilton et al. (1997) and Nielsen et al. (2000) reported D values of 0.999 and 0.984, respectively.

The results of this study showed extensive genomic diversity among the *C. jejuni* isolates in both the genotyping methods. Earlier investigations have also reported high heterogeneity among the *Campylobacter* isolates (Madden et al., 2007; Ridley et al., 2008). Although this organism has a natural ability for transformation, genomic rearrangements most likely explain this high genetic diversity (Ridley et al., 2008). Both the PCR-based methods used in the present study are characterized by a relatively high discriminatory power (D=0.95) and they were also easy and rapid to perform as well as had a low cost, which make them attractive options for complementing or replacing PFGE as the primary typing tool for *Campylobacter* strains. Similar findings were reported by Wieczorek (2009). Among the two methods, RAPD was found more discriminatory on comparing the D values. These findings are in agreement with the reports of Madden et al. (1996) and Nielsen et al. (2000), who compared the two techniques and observed higher discriminatory ability of RAPD typing. Contradictory to this observation, Ertas et al. (2004) reported that *fla*-typing was the better method amongst the two. The high discriminatory potential of RAPD can be attributed to its ability to determine polymorphism in the entire genome (Wassenaar and Newell, 2000).
However, disadvantages such as minor differences and weak band patterns may lead to subjective interpretation of results, thus leading to a somewhat poor discriminatory capacity (Nielsen et al., 2000). Further, it was observed that the RAPD technique sometimes required a second PCR assay as it gave negative results in the first trial or yielded weak bands, an observation which is in agreement with the above workers. Also, this technique is largely affected by genetic instability (Newell et al., 2000) owing to aging of the cultures or other stress conditions which may influence the overall results. Taking into consideration the latter facts, fla-typing seems to be a better suited method for genotyping Campylobacter isolates as evident by its ability to type all the isolates under study and also to give an acceptable level of discriminatory index.

A comparison of genotypes from food animals and foods of animal origin with human isolates gives a fair estimate of the number of human cases attributable to animal sources. Standardization and harmonization of typing methods between laboratories involved in such studies along with the construction of databases will allow for a comparison of data and define outbreaks. Further studies using isolates of diverse origin will expand our understanding of this food-borne pathogen and give a wider epidemiological picture, an immediate requirement that needs to be addressed in this part of the world.

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