Typing of *Campylobacter jejuni* isolated from poultry on the basis of *flaA*-RFLP by various restriction enzymes

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**A R T I C L E   I N F O**

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**A B S T R A C T**

RFLP analysis of the flagellin (*flaA*) gene was compared using three different restriction endonucleases i.e Ddel, HindIII and DpnII to determine the genetic diversity among 43 *Campylobacter jejuni* isolates of poultry origin from the same geographical area. *flaA* gene was amplified in all the isolates and RFLP analysis showed variations. DdeI-based RFLP was found most efficient in discriminating *C. jejuni* isolates by generating 15 different DdeI-RFLP patterns with discriminatory index (D.I) of 0.9258 whereas DpnII produced seven DpnII-RFLP patterns (D.I. = 0.8427). While HindIII enzyme produced only six HindIII-RFLP patterns (D.I. = 0.6977). The discrimination of Dpn-RFLP was comparable to discrimination given by Dde-RFLP analysis, which is generally used to study *flaA* gene RFLP.

1. Introduction

*Campylobacter* species are the second most emerging bacterial zoonotic pathogen after *Salmonella* causing gastroenteritis (Epps et al., 2013; Silva et al., 2011). Of the many *Campylobacter* species identified, *Campylobacter jejuni* (*C. jejuni*) is the most predominant pathogen implicated in food borne infections followed by *Campylobacter coli* (*C. coli*) (Biswas, Hannon, Townsend, Potter, & Allan, 2011; Bolton, 2015; Coward et al., 2008; Wieczorek & Osek, 2013). The caeca of the chickens is frequently colonized by this organism and consumption of broiler meat and its products contaminated during production and processing is considered to be the most frequent source of human infection (Aydin et al., 2007; Pearson et al., 1993; Wirz, Overesch, Kuhnert, & Korczak, 2010).

*Campylobacter* populations that infect broiler flocks can be complex, containing multiple genotypes, as *Campylobacter* species are associated with high frequency mutation in their surface antigen i.e. capsule, lipooligosaccharides and flagella (Hendrixson, 2006). A wide genetic diversity of *Campylobacter* populations in poultry sources has been reported in different studies and identification of *Campylobacter* at genus or species level does not help in understanding the epidemiology of the disease (Nachamkin, Bohachick, & Patton, 1993).

Many sub typing methods have been developed to differentiate *Campylobacter* strains for epidemiological purposes. These can be grouped into phenotypic methods and genotypic methods. Various phenotypic techniques used for characterization include serotyping (based on heat stable and heat labile antigens), biotyping (based on biochemical characters), phage typing and MEE (multi locus enzyme electrophoresis) (Dingle et al., 2001). Genotypic methods such as restriction fragment length polymorphism of the polymerase chain reaction products (PCR-RFLP) analysis based on the flagellin genes (*fla* typing), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), DNA microarray and amplified fragment length polymorphism (AFLP) analysis (Fitzgerald, Stanley, Andrew, & Jones, 2001; Gondo et al., 2006; Khoshbakht, Tabatabaei, Hosseinzadeh, Shekarforoush, & Aski, 2013; Vinueza-Burgos et al., 2017) are used in typing of *C. jejuni*. Although MLST and DNA microarray based techniques have been found to have high discriminatory index PCR-RFLP of flagellin gene using different restriction endonucleases is the preferred method in terms of handling, costs and time (Shi et al., 2002; El-Adawy et al., 2013).

Flagellin gene locus of *C. jejuni* contains two flagellin genes (*flaA* and *flaB*), which are highly conserved with variable regions interspersed between them (Khoshbakht, Tabatabaei, Hosseinzadeh, Aski, & Seifi, 2015; Thomrongsuwanakij, Blackall, & Chansiripornchai, 2017). This locus is suitable for restriction fragment length polymorphism (RFLP) analysis of PCR products. The conserved regions in this locus are also found in species other than *C. jejuni* making it suitable for typing other *Campylobacter* species. The level of discrimination in this technique is dependent on the restriction endonuclease chosen for the RFLP...
analysis (Owen & Leeton, 1999). Ddel restriction enzyme is the most preferred restriction enzyme for typing C. jejuni worldwide as it gives good discrimination for Campylobacter isolates within a given ecological niche (Ghorbanalizadgan, Bakhshi, & Peerayeh, 2016). The selection of a restriction enzyme for RFLP typing of any given fragment is based on the number of distinguishable fragments produced. Thus an average number of 4–8 fragments with recognizable band size are ideal for RFLP typing assay by any restriction enzyme. In silico analysis of the flaA gene led to selection of DpnII restriction enzyme as one of the suitable enzyme to study. The present study was aimed to determine the flaA gene RFLP patterns of DpnII enzyme and compare it with Ddel and HinfI for typing of C. jejuni isolates from poultry origin.

2. Material and methods

A total of 43 C. jejuni were isolated from poultry cloacal samples in and around Bikaner city (Rajasthan, India) and confirmed by amplification of genus specific 16S rRNA gene (Linton, Owen, & Stanley, 1996; Tang et al., 2009) and C. jejuni species specific hipO gene (Al-Amri, Senok, Ismaeel, Al-Mahmeed, & Botta, 2007; Linton, Lawson, Owen, & Stanley, 1997) as described previously by Yadav et al. (2016).

2.1. Amplification of flaA gene

Amplification of flaA gene was carried out as per method described by Nachamin et al. (1993) using forward primer F- GGATTTCGTATT AACACAAATGGTGCC and reverse primer R-CTGTAGTAATCTTAAAC ATTTTG. All PCR amplifications were performed in a mixture (25 µl) containing: 2.5 µl of the 10X PCR buffer, 2.5 µl of MgCl2 (25 mM), 0.5 µl of dNTPs (10 mM), 1 µl of each primer (100 µM), 0.5 µl (1 U) of the Taq DNA polymerase (Promega), 3 µl of the bacterial template DNA and 14 µl nuclease free water. The PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized under UVP gel documentation system (BioDoc-It Imaging System).

2.2. Restriction fragment length polymorphism (RFLP) of flaA gene

Restriction fragment length polymorphism of flaA PCR products was carried out using nucleotide site specific restriction endonuclease enzymes Ddel (3’..CTA..5’ and 5’ ATC..3’), HinfI (5’..G’ATC..3’ and 3’..CTA’G..5’) and DpnII (5’.. ‘GATC..3’ and 3’..CTAG’..5’). Selection of the novel restriction enzyme for flaA gene digestion, was based on the in silico analysis of flaA gene digestion by different restriction endonuclease enzymes (http://insilico.chez.eus/). DpnII restriction enzyme produced desired fragments (4–5) with different sizes ranging from 240 to 1500 bp with higher efficacy. Restriction enzymes were used as per the recommendation of the manufacturer (New England Biolabs). Briefly, to 10 µl of PCR product, 5 µl of nuclease free water, 2 µl of 10x buffer and 0.2 µl of restriction enzymes (Ddel, HinfI and DpnII) (0.2 µl = to 10 U/µl of Restriction enzyme) were added. The mixture was mixed gently and incubated in a water bath at 37°C for 3 h. The digest was resolved on 1.5% agarose gel and analyzed as described above. The images were analyzed with Pyelph application (Pavel & Vasile, 2012) and the resultant binary matrix was subjected to one of the agglomerative hierarchical clustering (UPGMA) method along with (Dice) similarity coefficient (Bikandi, San Millán, Rementeria, & Garaizar, 2004). Further the Discriminatory index of RFLP patterns were calculated using Discriminator power calculator tool (Bikandi et al., 2004).

3. Result and discussion

The flagellin gene locus of C. jejuni contains flaA gene which is arranged in tandem, is highly conserved and has short variable regions (Khoshbakht et al., 2013), therefore making it suitable for RFLP analysis. In the present study, PCR amplification of flaA gene sequence was performed for 43 isolates of C. jejuni. The amplicons were further subjected to restriction endonuclease digestion with Ddel, HinfI and DpnII.

Digestion by Ddel restriction enzyme produced 15 different Ddel-RFLP (Dde1 to Dde15) patterns with band size ranging from 200 to 1100 bp (Fig. 1) and discriminatory index of 0.9258. The discriminatory power of a tool giving discrimination above 0.5 is considered as a good method to discriminate isolates. Out of the total 15 Ddel-RFLP patterns, Dde9 was the most common pattern and found in 20.93% (9/43) isolates followed by Dde4 found in 9.30% (4/43) isolates while other remaining Dde types were found in less than four isolates (Table 1). During phylogenetic cluster analysis (on the basis of 80% genetic similarity) of C. jejuni, all 15 Ddel-RFLP patterns grouped in six clusters (Table 2). Cluster I and II had a single isolates having Dde1 and Dde15 patterns respectively. Highest numbers of 65.11% (28/43) isolates had seven Dde patterns (Dde6, Dde7, Dde8, Dde9, Dde10, Dde11 and Dde13) and were grouped in Cluster III followed by cluster...
Table 1
RFLP analysis of flaA gene sequence digested by Ddel, HinfI and DpnII restriction enzymes.

| Sr. No. | Patterns | Isolate ID (total 43 isolates) | Number of isolates (%) |
|---------|----------|--------------------------------|------------------------|
| 1       | Ddel1    | C5                             | 1 (2.32%)              |
| 2       | Ddel2    | C40,C43                        | 2 (4.65%)              |
| 3       | Ddel3    | C7,C35                         | 2 (4.65%)              |
| 4       | Ddel4    | C3                             | 3 (6.97%)              |
| 5       | Ddel5    | C6,C8,C9                       | 3 (6.97%)              |
| 6       | Ddel6    | C26,C27,C29,C31                | 4 (9.30%)              |
| 7       | Ddel7    | C33,C34                        | 2 (4.65%)              |
| 8       | Dde8     | C28,C30,C32                    | 3 (6.97%)              |
| 9       | Dde9     | C1,C4,C12,C13,C14,C17,C18,C20,C21 | 9 (20.93%)            |
| 10      | Dde10    | C10,C11,C15,C38                | 4 (9.30%)              |
| 11      | Dde11    | C22,C23,C24                    | 3 (6.97%)              |
| 12      | Dde12    | C16,C25,C39                    | 3 (6.97%)              |
| 13      | Dde13    | C2,C19,C36                     | 3 (6.97%)              |
| 14      | Dde14    | C41,C42                        | 2 (4.65%)              |
| 15      | Dde15    | C37                            | 1 (2.32%)              |

HinfI RFLP pattern

| Sr. No. | Patterns | Isolate ID (total 43 isolates) | Number of isolates (%) |
|---------|----------|--------------------------------|------------------------|
| 1       | Hinf1    | C5                             | 1 (2.32%)              |
| 2       | Hinf2    | C1,C4,C12,C13,C14,C17,C18,C20,C21 | 19 (44.18%)            |
| 3       | Hinf3    | C3,C6,C8,C9,C10,C11,C15,C16,C20,C21 | 12 (27.90%)           |
| 4       | Hinf4    | C2,C7,C19,C22,C23,C24,C25,C40,C41 | 9 (20.93%)             |
| 5       | Hinf5    | C37                            | 1 (2.32%)              |
| 6       | Hinf6    | C32                             | 1 (2.32%)              |

DpnII RFLP pattern

| Sr. No. | Patterns | Isolate ID (total 43 isolates) | Number of isolates (%) |
|---------|----------|--------------------------------|------------------------|
| 1       | Dpn1     | C4,C5,C10,C11,C15,C17,C19,C20 | 8 (18.60%)             |
| 2       | Dpn2     | C1,C12,C13,C18,C21,C23,C26,C28,C30,C33,C34,C37 | 12 (27.90%) |
| 3       | Dpn3     | C2,C7,C8,C22,C24,C25,C31       | 7 (16.27%)             |
| 4       | Dpn4     | C3,C9,C32,C40,C43              | 5 (11.62%)             |
| 5       | Dpn5     | C6,C14,C16,C41,C42             | 5 (11.62%)             |
| 6       | Dpn6     | C27,C29                        | 2 (4.65%)              |
| 7       | Dpn7     | C35,C36,C38,C39                | 4 (9.30%)              |

Table 2
Phylogenetic cluster analysis of flaA gene RFLP patterns obtained by digestion of Ddel, HinfI and DpnII restriction enzymes.

| S. No. | Clusters | flaA gene RFLP patterns | Number of isolates (%) |
|--------|----------|-------------------------|------------------------|
| 1       | Cluster I | Ddel1                   | 1 (2.32%)              |
| 2       | Cluster II| Ddel15                  | 1 (2.32%)              |
| 3       | Cluster III| Ddel6,Dde7,Dde8,       | 28 (65.11%)            |
| 4       | Cluster IV| Dde13,Dde12,Dde14      | 7 (16.27%)             |
| 5       | Cluster V | Dde2                    | 2 (4.65%)              |
| 6       | Cluster VI| Dde4,Dde5               | 4 (9.30%)              |

HinfI RFLP pattern

| S. No. | Clusters | flaA gene RFLP patterns | Number of isolates (%) |
|--------|----------|-------------------------|------------------------|
| 1       | Cluster I | Hinf1                   | 1 (2.32%)              |
| 2       | Cluster II| Hinf2, Hinf4, Hinf6    | 22 (51.16%)            |
| 3       | Cluster III| Hinf2, Hinf5          | 20 (46.51%)            |

DpnII RFLP pattern

| S. No. | Clusters | flaA gene RFLP patterns | Number of isolates (%) |
|--------|----------|-------------------------|------------------------|
| 1       | Cluster I | Dpn2                   | 12 (27.90%)            |
| 2       | Cluster II| Dpn4                   | 5 (11.62%)             |
| 3       | Cluster III| Dpn3,Dpn5,Dpn6       | 14 (32.55%)            |
| 4       | Cluster IV| Dpn1                   | 8 (18.60%)             |
| 5       | Cluster V | Dpn7                   | 4 (9.30%)              |

IV which consisted of 16.27% (7/43) isolates with three Dde patterns (Dde3, Dde12 and Dde14). Cluster V had 4.65% (2/43) isolates of Dde2 pattern and cluster VI possessed 9.30% (4/43) isolates with Dde4 and Dde5 patterns.

HinfI digested flaA gene produced only six (Hinf1 to Hinf6) different patterns (Fig. 2) and DpnII revealed seven (Dpn1 to Dpn7) different patterns (Fig. 3) with discriminatory index of 0.6977 and 0.8427 respectively. These were found less efficient than Ddel digested flaA gene RFLP patterns. Out of the total six Hinf-RFLP patterns obtained by digestion with HinfI enzyme, Hinf2 pattern was the most common and observed in 44.15% (19/43) isolates. Out of seven Dpn-RFLP patterns observed, Dpn2 was the most common pattern and comprised of 27.90% (12/43) isolates followed by Dpn1 found in 18.60% (8/43) isolates and Dpn3 in 16.27% (7/43) isolates (Table 1). Remaining Dpn patterns had less than five isolates. The number of bands varied from 3–6 with amplicon size ranging from 75 bp to 1300 bp with HinfI and DpnII restriction enzymes.

Hinf-RFLP based phylogenetic cluster analysis of C. jejuni revealed three clusters (on the basis of 80% genetic similarity). Cluster I had a single isolate i.e. C5 having Hinf1 pattern while maximum number of 51.16% (22/43) isolates grouped into cluster II and had three Hinf patterns (Hinf3, Hinf4 and Hinf6). Cluster III was found in 46.51% (20/43) isolates and had two Hinf patterns (Hinf2 and Hinf5) (Table 2).

DpnII-based RFLP patterns could be divided into five clusters on the basis of 80% genetic similarity. Cluster I had 27.90% (12/43) isolates with a single (Dpn2) pattern. Cluster II comprised of 11.62% (5/43) isolates and had one (Dpn4) pattern, cluster III had maximum 32.55% (14/43) isolates and had three Dpn patterns (Dpn3, Dpn5 and Dpn6), cluster IV possessed 18.60% (8/43) having Dpn1 pattern and cluster V comprised of 9.30% (4/43) isolates having Dpn7 pattern.

Ddel (D.I. – 0.9258) was found most efficient in discriminating C. jejuni isolates as has been reported by many workers (Hiett, Seal, & Siragusa, 2006; Khoshsakhht et al., 2015; Rajagunan et al., 2014; Vinueza-Burgos et al., 2017). The other enzymes i.e HinfI (D.I. – 0.6977) and DpnII (D.I. – 0.8427) were less discriminatory as compared to Ddel. Also, the numbers of patterns generated were highest when Ddel restriction enzyme was used as compared to that of HinfI and DpnII restriction enzyme.
4. Conclusion

*Campylobacter jejuni* typing using RFLP was carried out in the present study using different restriction endonucleases. *Dde*-based RFLP was found most efficient in discriminating *C. jejuni* isolates compared to *Hinf*-RFLP and *Dpn*-RFLP in terms of more pattern generated, higher typeability and greater discriminatory index value, however, *Dpn*II showed comparable discrimination of isolates as with *Dde*I. Thus, the present study suggests that *Dpn*-RFLP can be used as an efficient alternative for typing of *C. jejuni* isolates.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2018.06.003.

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