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GENOTYPING OF *CAMPYLOBACTER JEJUNI* ISOLATES OF POULTRY ORIGIN WITH DIFFERENT RESTRICTION ENZYMES

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Summary

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In this study, 28 poultry-originating *Campylobacter jejuni* isolates were typed by *fla* typing. The restriction enzymes of *Alu*I, *Dde*I, *Hinf*I, *EcoR*I, and *Pst*I were used alone or in combination. Six different profiles were obtained with *Alu*I, *Dde*I and *Hinf*I+*Dde*I combination. Seven, five and two different profiles were obtained with *Pst*I, *Hinf*I and *EcoR*I digestion, respectively. The *Pst*I was found most discriminative for typing poultry-originated isolates.

Key words: *Campylobacter jejuni, fla* typing, poultry

INTRODUCTION

Campylobacter jejuni is one of the leading causes of bacterial food-borne infections in the world today (Conlan *et al.,* 2007). Spread by the faeco-oral route, *C. jejuni* can colonize the intestinal mucosa of most warm-blooded animals (Newell & Fearnley, 2003).

C. jejuni is known for its high genetic heterogeneity. A considerable variability is observed among *C. jejuni* isolated from poultry at flock level (Wittwer *et al.,* 2005). Even infection of individual birds by multiple *Campylobacter* strains has been reported (Schouls *et al.,* 2003), indicating different potential infection sources for flocks (Petersen *et al.,* 2001; Wittwer *et al.,* 2005).

The tracing of diverse microorganisms like campylobacters through the food chain requires appropriate methods for strain differentiation (Takahashi *et al.,* 2006). During the past decade, traditional typing methods, such as bacteriophage typing and serotyping, have been replaced by newer techniques, such as plasmid fingerprinting, ribotyping, restriction fragment length polymorphism of the polymerase chain reaction products (PCR-RFLP) analysis based on the flagellin genes (*fla* typing), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) analysis (Tenover *et al.,* 1995; Rivoal *et al.,* 1999, Alter *et al.,* 2005; Borck & Pedersen, 2005). PFGE is a highly discriminatory and reproducible method for the typing of *C. jejuni* (Suzuki *et al.,* 1993; 1994; Hanninen *et al.,* 2001), but it is labour- and time-consuming. Among these, *fla* typing has the advantages of

low levels of nontypeability, acceptable levels of discriminatory power, and cost-effectiveness. In addition, *fla* typing has emerged as a suitable target for rapid investigation of a large number of isolates, and it is recommended for typing campylobacteria (Nachamkin *et al.,* 1996; Nielsen *et al.,* 2000; Petersen & On, 2000).

The level of discrimination depends principally on the restriction endonuclease chosen for the RFLP analysis (Owen & Leeton, 1999). Various restriction enzymes have been widely used for the RFLP analysis of the *fla*A genes, such as *Dde*I (Nachamkin *et al.,* 1996), *Hinf*I (Santesteban *et al.,* 1996) and *Eco*RI/*Pst*I (Alm *et al.,* 1993). Low discrimination by *Hinf*I has been reported previously (Owen *et al.* 1994; Ayling *et al.,* 1996; Santesteban *et al.,* 1996). When a single enzyme is considered, *Dde*I has repeatedly been confirmed as more discriminatory than *Hinf*I, *Pst*I *or Eco*RI; while *Alu*I has been found to produce too many small bands to be practical for analysis (Wassenaar & Newell, 2000; Petersen & Newell, 2001). Therefore, this enzyme makes less useful for interlaboratory standardization. Nevertheless differences in the *Alu*I profiles of the *fla*A genes between these isolates appear to be epidemiologically important (Nielsen *et al.,* 2000).

This study aimed at determining the genetic variability of poultry-originating *C. jejuni* isolates with restriction enzymes of *Alu*I, *Dde*I, *Hinf*I, *Eco*RI and *Pst*I alone or in combination and detection of the discriminatory power of these enzymes.

MATERIALS AND METHODS

Isolates

Faecal samples were collected from 200 broilers at a local poultry abattoir in eastern Turkey during five months period. The broilers, selected randomly, were produced in 20 different flocks in the region. There is only one company that is involved in poultry production in the region and all flocks (250 flocks in total) in the area belong to this company. *Campylobacter*-suspected colonies were observed in 102 samples collected from 20 broiler flocks. Isolates were identified as *C. jejuni* based on biochemical tests. Of these 102 *C. jejuni* isolates, 28 were ramdomly selected and examined for RFLP analysis.

DNA extraction

Isolates were cultivated at 42 °C for 24–48 h on blood agar containing 7% defibrinated sheep blood under microaerophilic conditions obtained by a gas generating kit (BR 056A; Oxoid, UK). A loopful of colonies was transferred into an Eppendorf tube containing 400 mL phosphate-buffered saline (PBS). The tubes were vortexed and centrifuged at 11,600×*g* for 5 min. The supernatant was discarded and the pellet was resuspended in 375 µL Salt–Tris EDTA (STE) buffer (100 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5 mL of 20 mg/mL proteinase K, and 20 mL 10% SDS). The suspension was incubated at 55 °C for 4 h, vortexing every 30 min. An equal volume of phenol was added to the suspension, and the Eppendorf tube was shaken vigorously by hand for 5 min and then centrifuged at 11,600×*g* for 10 min. The upper phase was transferred to a new Eppendorf tube. Genomic DNA was precipitated with 99% ethanol and 0.3 M sodium acetate at −20°C for 1 h. After the precipitation, the mixture was centrifuged at 11,600×*g* for 10 min and the upper phase was discarded. The pellet was washed twice with 90% and 70% ethanol, respectively,

each step followed by 5 min centrifugation. The pellet was dried, gently resuspended in 200 µL sterile distilled water and used as a target DNA in PCR (Valentine *et al.,* 1991).

fla typing

Flagellin gene typing was performed as described by Nachamkin *et al.* (1996). A PCR reaction volume of 50 µL containing 5 µL of purified DNA solution, 10× PCR buffer (750 mM Tris–HCl, 200 mM $(NH_4)SO_4$, 0.1% Tween 20), $MgCl_2$ (2.5 mM), 1 µM forward and reverse primers (FLA1 5′-ATG GGA TTT CGT ATT AAC AC -3′, FLA2 5′-CTG TAG TAA TCT TAA AAC ATT TTG -3′), deoxynucleoside triphosphates (200 µM), and Taq DNA polymerase (1.25 U; 5 U/mL, Fermentas, Lithuania). This solution was subjected to PCR with the following times and temperatures: an inital denaturation step at 94 °C for 1 min, then 34 cycles of denaturation at 94 °C for 15 s, annealing at 45 °C for 45 s and extension at 72 °C for 1.45 min followed by a final extension step at 72 °C for 5 min. A 10 µL sample of each reaction was analysed on a 1% agarose gel containing 0.5 µg/mL ethidium bromide. The DNA fragments were visualised by UV illumination. If a strong band was obtained, PCR amplicons (10 µL) were digested with 1 U of the restriction enzymes *Alu*I, *Dde*I, *Hinf*I, *Eco*RI *Pst*I and *Hinf*I+*Dde*I combination (Promega, Madison, USA) and kept at 37 °C for overnight according to the manufacturer's instructions. The enzyme-digested products were analysed by gel electrophoresis using 2% agarose in 0.5×Tris borate EDTA (TBE) buffer. The gels were stained with ethidium bromide (0.5 µg/mL) and photographed. Size of the RFLP bands were determined by comparison with a 100-bp DNA ladder (Promega).

RESULTS

A flagellin gene fragment of 1.7 kb was amplified from all *C. jejuni* isolates using a *fla* gene specific PCR. Digestion with five different restriction endonucleases gave several profiles of fragments: *Alu*I, *Dde*I, *Hinf*I, *Eco*RI and *Pst*I. The restriction profiles of each isolate for each used enzyme are shown in Table 1. In the agarose gel electrophoresis of the PCR products digested with the *Alu*I, *Dde*I and *Hinf*I+*Dde*I combination, six different profiles were obtained. After digestion by *Pst*I, *Hinf*I and *Eco*RI, seven, five and two different profiles were obtained, res-

Table 1. Distribution of restriction profiles amongst 28 C.*jejuni* isolates, randomly selected out of 102 positive samples (n=number of restriction profiles)

Band profiles	*Pst*I (n=7)	*Alu*I (n=6)	*Dde*I (n=6)	*Hinf*I (n=5)	*EcoR*I (n=2)	*Hinf*I+*Dde*I (n=6)
A	14	18	7	12	26	12
B	5	5	7	12	2	8
C	3	2	6	2	–	3
D	2	1	4	1	–	3
E	2	1	2	1	–	1
F	1	1	2	–	–	1
G	1	–	–	–	–	–
Total	28	28	28	28	28	28

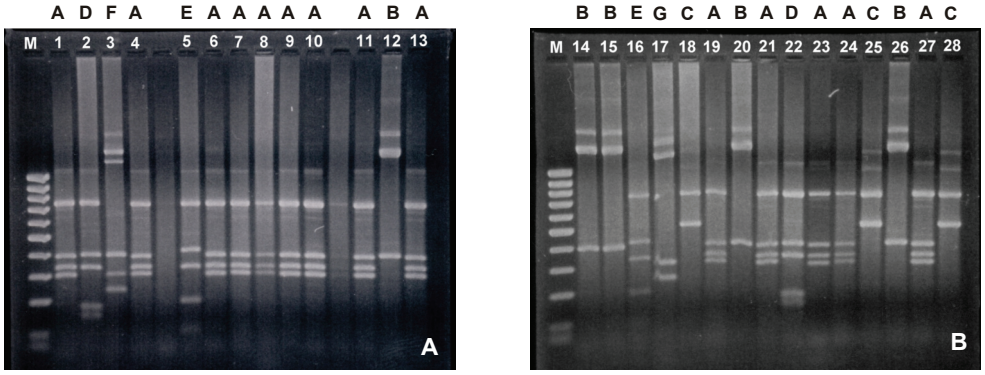

Fig. 1. Agarose gel electrophoresis showing the PCR-RFLP profiles of the 28 ramdomly selected *C. jejuni* isolates from broilers using the restriction endonuclease *Pst*I (13 isolates on Fig. 1A and 15 isolates on Fig. 1B). M: 100-bp DNA ladder; 1 through 28: isolate number; A through G: profiles obtained after digestion with *Pst*I.

pectively. The *Pst*I was observed to provide the best discrimination level when compared to other enzymes (Fig. 1).

DISCUSSION

In the present study, we used *Alu*I, *Dde*I, *Hinf*I, *Eco*RI and *Pst*I enzymes to determine the genetic heterogeneity of *C. jejuni* strains isolated from poultry.

Madden *et al.* (1998) performed PCR-RFLP analysis with the restriction enzyme *Dde*I digestion on *C. jejuni* isolates of poultry origin and obtained three different band profiles. In a study carried out by PCR-RFLP analysis with the restriction enzyme *Dde*I of *Campylobacter* spp. isolates collected from a poultry slaughterhouse, *C. jejuni* isolates yielded eleven distinct profiles (Rivoal *et al.,* 1999). Harrington *et al.* (2003) reported that the best discrimination level can be enhanced by combining *Dde*I with *Hinf*I patterns and detected that *Hinf*I alone was not very discriminatory. Aydin *et al.* (2007) reported that fewer RFLP profiles were observed using the *Hinf*I enzyme compared with the *Dde*I enzyme.

Owen & Leeton (1999) used combined *Eco*RI/*Pst*I RFLP analysis of *fla*A because both enzymes can be included in a single digest, and are significantly cheaper than separate digests of *Hinf*I and *Dde*I. Same authors found that *Eco*RI and *Pst*I double digests of the *fla*A PCR product from each strain gave a total of fourteen different RFLP patterns for human, other mammalian and poultry strains (Owen & Leeton, 1999). Nielsen *et al.* (2000) typed 80 *C. jejuni* strains (isolated from humans, cattle and chickens) using six different genotyping methods, including PCR-RFLP with *Dde*I and *Alu*I, and detected 40 different band profiles and emphasized that the combined use of *Dde*I and *Alu*I enhanced the discriminatory power of *fla*-RFLP typing. In all but one case, the *Alu*I profiles that were associated with the same *Dde*I profile were highly similar, distinguished by one or two band differences. Provided that it is unknown whether they are caused by major or minor sequence differences between the *fla*A genes in question, it is not meaningful to interpret similarity between profiles as a close interstrain relationship

but it is reasonable to regard each *fla*-RFLP type combination as a separate type (Nielsen *et al.,* 2000). Petersen *et al.* (2001) reported that a total of 32 different *fla* types (*Dde*I profile type and *Alu*I profile type) were identified among the 120 isolates from wildlife, humans and broiler flocks.

Five different restriction enzymes (*Alu*I, *Dde*I, *Hinf*I, *Eco*RI and *Pst*I) were used alone or in combination in this study. In contrast to previous reports (Wassenaar & Newell, 2000; Petersen & Newell, 2001) in which *Dde*I has been confirmed as more discriminatory, in this study *Pst*I was found to be best discriminative enzyme for poultry-originated *C. jejuni* isolates tested. Previous studies were performed at different times and geographically distinct countries, which may be the reason why *Pst*I was seen to be best discriminative enzyme among our isolates. It is also possible that *Campylobacter* infections could undergo genetic variations within this time. However, further studies are needed to reveal this possibility.

Our results are in agreement with those of Ertas *et al.* (2004) and Aydin *et al.* (2007) which reported the presence of seven and six profiles, respectively among poultry isolates of *C. jejuni* by PCR-RFLP analysis using restriction enzyme *Dde*I. Although these studies were performed at different times, the observed similar results may suggest about a common profile of *C. jejuni* in Turkey. Our results are in contrary to those of Owen *et al.* (1994), Ayling *et al.* (1996), Santesteban *et al.* (1996), Harrington *et al.* (2003) and Aydin *et al*. (2007) who found that digestion with *Hinf*I alone was not sufficiently discriminatory for *fla*-typing of *C. jejuni* isolates.

Although we typed a small number of *C. jejuni* isolates by PCR-RFLP assay in this study, the results showed a high genetic diversity among *C. jejuni* isolates. *Pst*I appeared to the provide the best discrimination for poultry-originating *C. jejuni* isolates. This study is the first to announce the use of *Pst*I for discrimination of poultry-originating *C. jejuni* isolates in Turkey. Further studies should focus on the use of *Pst*I enzyme to determine the different *C. jejuni* types among the chicken population.

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