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

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Summary


In this study, 28 poultry-originating Campylobacter jejuni isolates were typed by fla typing. The restriction enzymes of AluI, DdeI, HinfI, EcoRI, and PstI were used alone or in combination. Six different profiles were obtained with AluI, DdeI and HinfI+DdeI combination. Seven, five and two different profiles were obtained with PstI, HinfI and EcoRI digestion, respectively. The PstI 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 During the past decade, traditional typing methods, such as bacteriophage typing and serotyping, have been replaced by newer techniques, such as plasmid fingerprinting, ribotyping, restriction fragment length polymorphism of the polymerase chain reaction products (PCR-RFLP)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 flaA genes, such as DdeI (Nachamkin et al., 1996), Hinfl (Santesteban et al., 1996) and EcoRI/PstI (Alm et al., 1993). Low discrimination by Hinfl has been reported previously (Owen et al. 1994; Ayling et al., 1996; Santesteban et al., 1996). When a single enzyme is considered, DdeI has repeatedly been confirmed as more discriminatory than Hinfl, PstI or EcoRI; while AluI has been found to produce too many small bands to be practical for analysis (Was-senaar & Newell, 2000; Petersen & Newell, 2001). Therefore, this enzyme makes less useful for interlaboratory standardization. Nevertheless differences in the AluI profiles of the flaA 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 AluI, DdeI, Hinfl, EcoRI and PstI 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 randomly selected and examined for RFLP analysis.

DNA extraction

Isolates were cultivated at 42 °C for 24–48 h on blood agar containing 7% defbrinated 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 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 (NH₄)SO₄, 0.1% Tween 20), MgCl₂ (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 initial 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 AluI, Ddel, HinfI, EcoRI PstI and HinfI+Ddel 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: AluI, Ddel, HinfI, EcoRI and PstI. 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 AluI, Ddel and HinfI+Ddel combination, six different profiles were obtained. After digestion by PstI, HinfI and EcoRI, seven, five and two different profiles were obtained, res-

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<th>Band profiles</th>
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<th>HinfI (n=5)</th>
<th>EcoRI (n=2)</th>
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Fig. 1. Agarose gel electrophoresis showing the PCR-RFLP profiles of the 28 randomly selected C. jejuni isolates from broilers using the restriction endonuclease PstI (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 PstI.

respectively. The PstI was observed to provide the best discrimination level when compared to other enzymes (Fig. 1).

DISCUSSION

In the present study, we used AluI, DdeI, HinfI, EcoRI and PstI 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 DdeI 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 DdeI 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 DdeI with HinfI patterns and detected that HinfI alone was not very discriminatory. Aydin et al. (2007) reported that fewer RFLP profiles were observed using the HinfI enzyme compared with the DdeI enzyme.

Owen & Leeton (1999) used combined EcoRI/PstI RFLP analysis of flaA because both enzymes can be included in a single digest, and are significantly cheaper than separate digests of HinfI and DdeI. Same authors found that EcoRI and PstI double digests of the flaA 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 DdeI and AluI, and detected 40 different band profiles and emphasized that the combined use of DdeI and AluI enhanced the discriminatory power of fla-RFLP typing. In all but one case, the AluI profiles that were associated with the same DdeI 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 flaA 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 (DdeI profile type and AluI profile type) were identified among the 120 isolates from wildlife, humans and broiler flocks.

Five different restriction enzymes (AluI, DdeI, HinfI, EcoRI and PstI) were used alone or in combination in this study. In contrast to previous reports (Wassenaar & Newell, 2000; Petersen & Newell, 2001) in which DdeI has been confirmed as more discriminatory, in this study PstI 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 PstI 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 DdeI. 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 HinfI 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. PstI appeared to the provide the best discrimination for poultry-originating C. jejuni isolates. This study is the first to announce the use of PstI for discrimination of poultry-originating C. jejuni isolates in Turkey. Further studies should focus on the use of PstI enzyme to determine the different C. jejuni types among the chicken population.

REFERENCES


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