Comparison of Multilocus Sequence Typing (MLST) and Repetitive Sequence-Based PCR (rep-PCR) Fingerprinting for Differentiation of Campylobacter jejuni Isolated from Broiler in Chiang Mai, Thailand

Patchanee, Prapas¹, Chomporn Chokboonmongkol², Karl-Hans Zessin³, Thomas Alter⁴, Sarinya Pornaem⁵, and Nipa Chokesajjawatee⁵*

¹Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Thailand
²Animal Health and Technical Service Office, Bangkok Agro-Industrial Products Public CO., LTD, Bangkok, Thailand
³Department Panel, Veterinary Public Health, Free University Berlin, Germany
⁴Institute of Food Hygiene, Free University Berlin, Germany
⁵National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, Thailand

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We compared rapid fingerprinting using repetitive sequence-based PCR (rep-PCR) for subtyping Campylobacter jejuni isolates to the widely used multilocus sequence typing (MLST). Representative C. jejuni isolates (n = 16) from broilers were analyzed using MLST and rep-PCR. Both techniques demonstrated an equal discriminatory power of 0.8917, and 9 subgroups were identified. Clonal identification of all 16 isolates was identical for both techniques. The rep-PCR as described in this study may be used as a rapid and cost-effective alternative for subtyping of C. jejuni isolates, or as an effective screening tool in large epidemiological studies.

Keywords: MLST, rep-PCR, Campylobacter, broiler

Campylobacter jejuni is one of the most common causes of foodborne human enteritis worldwide. It is also a major concern in veterinary public health, as consumption of poultry products is an important risk factor for human campylobacteriosis. Many molecular methods for clonal identification of C. jejuni strains have been reported, such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and multilocus sequence typing (MLST) [2, 5, 11, 12]. MLST is widely used in epidemiological studies for several foodborne pathogens. The MLST database for Campylobacter spp. is well established and can be accessed worldwide through a Web access (http://pubmlst.org/campylobacter/). MLST is accepted as a suitable technique for bacterial typing because of its high discriminatory power, reproducibility, and data portability, providing that a sufficient number of suitable genes are analyzed [13].

However, the time, cost, and labor associated with the MLST technique may hinder its use in large epidemiologic studies, or in time-sensitive or resource-constrained situations. In this study, we evaluated the ability of repetitive sequence-based PCR (rep-PCR) to sub-type C. jejuni strains as a potential rapid, high throughput, and more cost-efficient alternative. This technique, described by Versalovic et al. [14], involves PCR-mediated genomic fingerprinting with the use of interspersed repetitive sequences.

Sixteen C. jejuni strains were isolated from cecum and skin samples of broilers from a slaughterhouse in Chiang Mai, Thailand. The Campylobacter isolation was done according to the standard method ISO 10272-1:2006 (E) Part 1 [8]. The presumptive Campylobacter isolates were subcultured into 2 ml of Brucella broth and incubated in a microaerobic atmosphere at 42°C for 24 h. The cell pellets were collected and the DNA was extracted using a DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA). Species of the Campylobacter isolates were identified using multiplex PCR according to the protocols described by Lund et al. [9] and Wang et al. [15].

Resistance to the antimicrobial drugs erythromycin (ery), ciprofloxacin (cip), tetracycline (tet), gentamicin (gen), and
ampicillin (amp) were tested by a disk diffusion technique (Kirby Bauer method) using commercially prepared antimicrobial disks (Oxoid, Hampshire, England). The majority of the isolates (75%) were resistant to at least one of the 5 antimicrobial drugs. Only 3 isolates (18.75%) were sensitive to all drugs tested (Fig. 1).

The MLST genotyping was done following a published protocol and using primers described by Dingle et al. [2] and http://pubmlst.org. The system is based on PCR amplification of seven housekeeping genes: \( \text{aspA} \) (aspartase A), \( \text{glnA} \) (glutamine synthetase), \( \text{gltA} \) (citrate synthase), \( \text{glyA} \) (serine hydroxymethyltransferase), \( \text{pgm} \) (phosphoglucomutase), \( \text{tkt} \) (transketolase), and \( \text{uncA} \) (ATP synthase \( \alpha \) subunit), followed by DNA sequencing. All sequences were compared with existing alleles in the MLST \( \text{C. jejuni} \) database to determine allele numbers and sequence types (STs) (http://pubmlst.org). Phylogenetic relationships among sequence types and allelic profiles were investigated by running PHYLIP analysis on all STs in the database and grouping isolates in clonal complexes (CCs). The MLST analysis result is shown in Fig. 1. All allelic sequences were matched with the existing database and 9 STs were identified. Among these, 3 STs (ST-305, ST-5213, and ST-1075) have never been reported before in Thailand. ST-2274 was the most frequent ST in this study, found in five isolates from four different farms. In all cases, isolates from broiler cecum and skin of the same slaughter lot clustered together, demonstrating a clonal relationship of these isolates. The data suggested that contamination of chicken carcasses in the slaughterhouse is possibly due to \( \text{C. jejuni} \) in the intestine of the birds.

For rep-PCR fingerprinting, two types of fingerprints were generated using primers ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA TAA GTG ACT GGG GTG AGC G-3') in the first reaction, and a single primer (GTG), (5'-GTG GTG GTG GTG GTG GTG GTG-3'), in the second reaction. The reactions were carried out in 25 µl reaction volumes, using 0.625 units of Ex Taq DNA polymerase and buffer system (Takara Bio Inc., Shiga, Japan), 200 µM of each deoxynucleoside triphosphate, 800 nM of the corresponding primer, and 240 ng of the DNA template. The amplification cycles were initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing (at 52°C for ERIC-PCR and 40°C for GTG-PCR) for 1 min, and extension at 65°C for 10 min, and an additional final extension at 65°C for 20 min [1]. Five microliter samples of the amplified products were analyzed by 1% agarose gel electrophoresis. The gel images were captured using the image scanner Typhoon 9410 (Amersham Pharmacia Biotech Inc., New Jersey, USA). The fingerprint patterns were analyzed using GelCompar II, ver. 5.0 (Applied Maths BVBA, Kortrijk, Belgium). Similarities between isolates were calculated using Pearson’s product moment correlation. A range between ca. 100 and 20,000 bp and 1% optimization were used for the similarity calculation. Composite data based on both ERIC and GTG fingerprints were calculated using the similarity average from experiments with equal weight assignment.

**Fig. 1.** Phylogenetic analysis of \( \text{C. jejuni} \) isolates based on MLST analysis.
The dendogram was generated using the unweighted pair group method using average linkages (UPGMA).

Fig. 2 shows the cluster analysis of the composite data from ERIC- and GTG- fingerprint patterns. The ERIC fingerprints were able to differentiate most isolates with different STs, except for ST-2274 and ST-2276, which yielded identical ERIC patterns. The GTG fingerprints had less discriminatory power because many strains with different STs (ST-2274, 50, 51, 305, and 574) yielded identical GTG fingerprints. However, it generated distinct patterns differentiating ST-2274 from ST-2276, and can therefore be used to supplement the ERIC fingerprints to increase the discriminatory power of the analysis. With a similarity cut-off value at 0.98, the composite fingerprints identified a total of 9 distinct profiles among the 16 C. jejuni isolates. All isolates with the same STs, identified by MLST, demonstrated identical rep-PCR fingerprints, whereas the isolates with different STs yielded different patterns.

The discriminatory power using the Simpson’s diversity index [6] of each subtyping method was calculated using a Web-based calculator accessed at http://insilico.ehu.es/mini_tools/discriminatory_power/. An equal discriminatory power of 0.8917 was obtained from both MLST and rep-PCR fingerprinting.

In this study, we found no relationship between the resistance profiles and the genomic profile generated by either MLST or rep-PCR. One possible explanation is that the resistance determinants may be encoded in mobile genetic elements, or may be encoded in the regions outside the primers’ detection range, or may be caused by mutations that do not produce significant differences in the amplified fragments.

The use of ERIC primers in Campylobacter spp. fingerprinting has been reported by several investigators [4, 7, 10]. However, low-stringency annealing temperatures were used (40°C to 25°C), making the reproducibility of the generated patterns questionable. In this study, long-range amplification with a high-stringency annealing temperature specific for the ERIC primers (52°C) was used with satisfactory result, and the amplified fragments larger than 1,500 bp were consistently amplified.

From the MLST dendrogram (Fig. 1) and rep-PCR dendrogram (Fig. 2), we found some discordance of phylogenetic relationship among some isolates (ST5213-ST51-ST50). The MLST dendrogram was generated based on a matrix of pairwise differences in the allelic profiles (7 loci) of the isolates. The dendrogram can be used effectively in identifying identical or closely related isolates, but the topology of the dendrogram in resolving the relationship of further related strains can be somewhat arbitrary [3]. The true relationship among these isolates should be further investigated with a more reliable method such as whole genome hybridization.

Although data portability of the rep-PCR is cumbersome, the technique provides good discriminatory power in a rapid and cost-efficient manner. It can be used as an effective screening tool for large numbers of isolates, as well as in more limited short-term epidemiological studies.
With high throughput capability, a single investigator could analyze hundreds of samples and have results available on the following day.

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REFERENCES