Identification of Campylobacter jejuni Isolates from Cloacal and Carcass Swabs of Chickens in Thailand by a 5’ Nuclease Fluorogenic Polymerase Chain Reaction Assay

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ABSTRACT

A rapid 5’ nuclease fluorogenic polymerase chain reaction (PCR) assay for identifying Campylobacter jejuni was applied to Campylobacter isolates from chicken cloacal and carcass swabs collected from three chicken farms and a slaughterhouse in Thailand. The primers and the probe were based on the sequence of the gyrA gene in C. jejuni. C. jejuni isolates were identified by fluorogenic PCR assay of bacterial cells directly from Campylobacter-selective agar medium. This assay allowed the identification of C. jejuni within 1 day after colonies appeared on selective media. The fluorogenic PCR assay yielded results comparable to those of the conventional test kit (kappa = 0.76) but required less time. When the two methods disagreed with regard to species identification, results were confirmed by PCR restriction fragment length polymorphism of 23S rRNA genes. In these instances, the fluorogenic PCR assay correctly identified more isolates of C. jejuni than did the conventional test kit (six of seven isolates were unidentifiable by the conventional test kit). The fluorogenic PCR assay is a rapid and specific method that outperforms the conventional test kit in the identification of C. jejuni from environmental samples.

Campylobacter has been implicated as a major cause of foodborne disease in many countries, including Thailand (20, 32). In particular, Campylobacter jejuni has been cited as the most frequent cause of foodborne disease (1) and has been associated with Guillain-Barré syndrome, a human neurodegenerative disease (2). Campylobacter is frequently isolated from poultry meat products (3, 24, 31), which have frequently been identified as sources of sporadic cases of campylobacteriosis (25). Contamination of poultry products can be traced back to farms (5), where chickens are commonly found to harbor Campylobacter (9). In addition to its presence in meat products, Campylobacter has been shown to rapidly develop resistance to clinically relevant antimicrobial agents such as fluoroquinolones (13). Since 1970, there has been substantial improvement in techniques for the isolation and identification of Campylobacter. Common techniques employed to isolate Campylobacter include culturing on selective growth media and filtration methods (22). There are numerous culture media available for the isolation of Campylobacter (6), but the antibiotics included in these media as selective agents to control competing flora may also inhibit some strains of campylobacteriosis (25). Procedures are time-consuming because of the slow growth rate of Campylobacter in culture. Polymerase chain reaction (PCR) techniques for the detection and identification of Campylobacter have been developed and successfully applied to samples from foods (30), fecal samples (18), and poultry products (33). PCR-based assays offer a rapid alternative for identifying Campylobacter without sacrificing specificity (23).

A recently developed 5’ nuclease fluorogenic PCR assay (11) can be used to rapidly identify specific target genes or discriminate between different alleles of the same target gene (17). This fluorogenic PCR assay uses a nontargetable oligonucleotide hybridization probe that contains a fluorescent reporter dye and a quencher dye. During PCR cycling, the probe first specifically hybridizes to the corresponding template but is digested by the exonuclease activity of Taq DNA polymerase as it moves along the template strand. This cleavage results in an increase in fluorescence emission reporter dye, which can be measured by fluorescence spectrometry. The use of an internal probe carrying a signal-generating system in combination with target-specific primers increases the specificity of the PCR reaction. The level of fluorescence measured at the end of the PCR cycle provides qualitative information on the presence or absence of the nucleic acid target (19). A fluorogenic PCR assay using primers and probes specific for the gyrA gene was recently developed in our laboratory. This assay successfully identified and enumerated a variety of laboratory and clinical C. jejuni isolates (34). The present
study was conducted to evaluate the performance of this fluorogenic PCR assay in the identification of C. jejuni in samples derived from poultry farms and slaughterhouses and to compare its performance with that of a widely accepted conventional test kit for species identification of Campylobacter spp.

MATERIALS AND METHODS

Sample collection and primary isolation. This study was conducted as part of a 3-year epidemiological study designed to determine the prevalence and antimicrobial susceptibility profiles of Escherichia coli, Salmonella, and Campylobacter in food animal and meat products in Thailand. Samples were collected during the rainy season, from May to July 2000. One hundred fifty-five 5- to 6-week-old chickens were randomly selected for sample collection from three convenience-sampled chicken farms in Thailand. Farms were selected according to their willingness to participate and their possession of chickens at market age (6 to 7 weeks) at the time of sample collection. Each farm had 5,000 to 8,000 chickens and was within 20 km of the laboratory facilities at Chiang Mai University. One hundred three chickens from a slaughterhouse were systematically selected for sample collection after defeathering by selecting 2 birds from every batch of 10 chickens processed on a single day.

Cloacal swab samples were collected by swabbing the inside of the cloacal area of each chicken with a sterile cotton swab, which was then placed in Stuart's transport medium (RCM Supply, Bangkok, Thailand). At the slaughterhouse, samples were collected from the uneviscerated chicken carcasses after killing and defeathering and before immersion in the chilling tank. Cloacal swabs were collected, along with a swab of the area surrounding the cloaca and under the wing of each bird at the slaughterhouse, with sterile cotton swabs that were placed in Stuart's transport medium. Samples were kept on ice during transport and refrigerated until processing (within 12 h after collection). Swabs were streaked directly onto Karmali (KSA) agar plates (Oxoid, Basingstoke, UK) and incubated in plastic bags at 42°C for up to 48 h under microaerobic conditions (5% O2, 10% CO2) with the Anaerocult C system (Merck, Whitehouse Station, N.J.). Suspected Campylobacter colonies were confirmed by oxidase testing, catalase testing, and Gram staining. Gram-negative spiral rods that were oxidase- and catalase-positive were identified as C. jejuni, C. coli, C. lari, or C. concisus. For species confirmation, the API CAMPY test strips (BioMerieux, Marcy l’Etoile, France) were used as recommended by the manufacturer. Bacterial cells from BASB agar plates were used to inoculate both portions of the strip. The first half of the test strip, which was incubated at 37°C under aerobic conditions for 24 h, included tests for urease, reduction of nitrate, esterase, hippurate, gamma glutamyl transferase, reduction of chloride to triphenyl tetrazolium, pyrrolidonyl arylamidase, and alkaline phosphatase. The second half of the strip, including tests for production of H2S; for glucose, succinate, acetate, propionate, malate, and citrate assimilation; and for susceptibility to nalidixic acid, cefazoline, and erythromycin, was incubated at 37°C with 5% CO2 for up to 48 h. The results of each test were read as specified by the manufacturer, and the final identification was obtained by referring to the analytical profile index (20890) provided by the manufacturer. C. jejuni 43429 was used as a positive control.

Conventional test kit. The API CAMPY test strips (BioMerieux, Marcy l’Etoile, France) were recommended by the manufacturer for identifying C. jejuni. Conventional test kits were used to inoculate both portions of the strip. The first half of the test strip, which was incubated at 37°C under aerobic conditions for 24 h, included tests for urease, reduction of nitrate, esterase, hippurate, gamma glutamyl transferase, reduction of chloride to triphenyl tetrazolium, pyrrolidonyl arylamidase, and alkaline phosphatase. The second half of the strip, including tests for production of H2S; for glucose, succinate, acetate, propionate, malate, and citrate assimilation; and for susceptibility to nalidixic acid, cefazoline, and erythromycin, was incubated at 37°C with 5% CO2 for up to 48 h. The results of each test were read as specified by the manufacturer, and the final identification was obtained by referring to the analytical profile index (20890) provided by the manufacturer. C. jejuni 43429 was used as a positive control.

DNA extraction for PCR-RFLP. Bacterial cells scraped from BASB agar plates inoculated 24 h earlier were pelleted, and DNA was extracted by standard methods (4). Briefly, cells were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and lysed with 0.5% sodium dodecyl sulfate in the presence of 100 mg of proteinase K per ml. Cellular debris was removed by complexing with hexadecytrimethyl ammonium bromide followed by phenol-chloroform extraction and RNase treatment. DNA was precipitated with isopropanol and resuspended in TE, and its concentration was determined with a DU530 spectrophotometer (Beckman Instruments, Schaumburg, Ill.).

PCR-RFLP of the 23S rRNA. PCR-RFLP of the 23S rRNA was used to confirm the identity of C. jejuni isolates. The protocol described by Ferrer and Engvall (10) was used. A primer pair consisting of THERM1 (5′-ATG TCG TCA ACC AAC ATT AGT-3′) and THERM4 (5′-TTG CGG TAA TGC TAA CCC-3′) was used to amplify 491 bp of a highly polymorphic region of the 23S rRNA gene; RFLP banding patterns of this region were previously shown to be specific for all species of therophilic Campylobacter (10). The PCR reaction mix contained 0.25 mM each primer per ml, 1× PCR buffer, 1.5 mM MgCl2, 0.1 mM each dNTP, 0.05 U of AmpliTaq Gold polymerase (Perkin-Elmer) per ml, 0.01 U of AmpErase UNG (Perkin-Elmer) per ml, 4.5 mM MgCl2, 0.05% gelatin, and 0.01% Tween 20. A sterile toothpick was used to transfer bacteria from a single colony on BASB or KSA agar medium to the fluorogenic PCR reaction mix. Prior to initial PCR denaturation, all fluorogenic PCR reaction mixtures were incubated at 50°C for 2 min in the presence of AmpErase UNG to prevent PCR product carryover. With 50-µl PCR reactions, initial denaturation was conducted at 95°C for 10 min; the annealing and polymerization steps were combined at 60°C for 1 min and followed by denaturation at 95°C for 30 s. This process was repeated 40 times. Fluorescence emissions were monitored with an ABI Prism 7700 sequence detection system (Perkin-Elmer). The assay was performed on each sample twice. If the fluorogenic PCR assay results from identical samples were not consistent, or if they were in conflict with conventional test kit results, species identification was confirmed by PCR restriction fragment length polymorphism (PCR-RFLP) of the 23S rRNA gene as described below. C. jejuni 43429 and Campylobacter coli 1777208 were used as positive and negative controls, respectively.

PCR-RFLP of the 23S rRNA. PCR-RFLP of the 23S rRNA was used to confirm the identity of C. jejuni isolates. The protocol described by Ferrer and Engvall (10) was used. A primer pair consisting of THERM1 (5′-ATG TCG TCA ACC AAC ATT AGT-3′) and THERM4 (5′-TTG CGG TAA TGC TAA CCC-3′) was used to amplify 491 bp of a highly polymorphic region of the 23S rRNA gene; RFLP banding patterns of this region were previously shown to be specific for all species of therophilic Campylobacter (10). The PCR reaction mix contained 0.25 mM each primer per ml, 1× PCR buffer, 1.5 mM MgCl2, 0.1 mM each dNTP, 0.05 U of AmpliTaq (PE Applied Biosystem) per ml, and 1.6 ng of DNA template per ml. The PTC-100 thermocycler (MJ Research, Watertown, Mass.) was used as follows. The cycling protocol was preceded by a 12-min incubation at 94°C and consisted of initial denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. The 50-µl PCR sample was cycled 45 times, and then a final extension at 72°C for 5 min was carried out. PCR products were visualized by elec-
TABLE 1. Proportions of farm and slaughterhouse samples yielding Campylobacter

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples yielding Campylobacter/no. of chickens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chicken farms</strong></td>
<td></td>
</tr>
<tr>
<td>Farm A</td>
<td>38/55 (69)</td>
</tr>
<tr>
<td>Farm B</td>
<td>17/50 (34)</td>
</tr>
<tr>
<td>Farm C</td>
<td>42/50 (84)</td>
</tr>
<tr>
<td><strong>Total from chicken farms</strong></td>
<td>97/155 (63)</td>
</tr>
<tr>
<td><strong>Slaughterhouse</strong></td>
<td></td>
</tr>
<tr>
<td>Cloacal swab</td>
<td>42/103 (41)</td>
</tr>
<tr>
<td>Under wing swab</td>
<td>4/103 (4)</td>
</tr>
<tr>
<td><strong>Total from slaughterhouse</strong></td>
<td>46/206 (22)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>143/361 (40)</td>
</tr>
</tbody>
</table>

**RESULTS**

Of a total of 361 samples collected from chickens on three farms and at one slaughterhouse, 143 (40%) yielded presumptive Campylobacter after primary isolation and biochemical analyses (Table 1). The overall prevalence of Campylobacter from chicken cloacal swab samples from the three farms was found to be 62%, while the Campylobacter prevalence for the same type samples from the slaughterhouse was 41%. However, the Campylobacter prevalence for the finished carcasses was only 3.9%.

Of the 143 isolates, 86 were transported back to the United States in dry ice and were subsequently kept at −80°C in the lab at Michigan State University. Of the 86 isolates that were transported and frozen, 79 were recovered (92%) from frozen stocks and were analyzed further by the fluorogenic PCR assay. However, because resources were limited, only 59 of these isolates were subjected to testing with conventional test kit for species identification (Table 2). The remaining isolates were not successfully recovered from frozen stocks. In the 59-isolate subset, good agreement was observed between results obtained with the fluorogenic PCR assay and those obtained with the conventional test kit for the identification of C. jejuni (kappa = 0.76). Of seven isolates that produced discordant test results, one was identified as C. jejuni by the conventional test kit but was determined not to be C. jejuni by the fluorogenic PCR assay. The remaining six isolates were identified as C. jejuni by the fluorogenic PCR assay but were unidentifiable by the conventional test kit.

**DISCUSSION**

The proportion of cloacal swab samples yielding Campylobacter isolates from the slaughterhouse (41%) in this study was higher than levels previously observed in America (20%) (14) for similar types of samples. However, the prevalence of Campylobacter on chicken farms was comparable to that previously found in China (61%) (35) but lower than that found in Britain (90%) (9). The lower proportion of isolates found in chicken carcasses was possibly due to the incubation temperature. The selective plates were incubated only at 42°C, eliminating the possibility of detecting nonthermophilic Campylobacter, which may have contributed to the low levels detected.
comparable results; hence, the fluorogenic PCR assay offers some flexibility in the choice of growth media. Additionally, this assay could be performed as soon as colonies appeared on selective media. The fluorogenic assay eliminated the need to extract DNA or visualize results by gel electrophoresis, which reduced the required amount of time and labor considerably. Finally, this assay showed a higher success rate in detecting C. jejuni than did the conventional test kit because of a high initial number of templates. We are working on methods to allow us to simultaneously identify and quantify the numbers of cells in environmental samples without isolating colonies on selective medium. One method under study, immunomagnetic separation (16), has been used successfully in combination with other techniques to detect Campylobacter (15) and other foodborne bacteria from various sources (7, 29). Immunomagnetic separation was shown to improve the detectability level, particularly for environmental samples (8).

In conclusion, the fluorogenic PCR assay is a sensitive and accurate method for identifying C. jejuni directly from a colony on selective medium without extracting DNA or performing gel electrophoresis. It can be applied for the detection of other species of Campylobacter and is a promising technique for use in combination with cell separation techniques to provide a rapid assay for the identification and enumeration of Campylobacter in food and environmental samples.

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REFERENCES


