Direct Detection of *Campylobacter jejuni* in Chicken Cecal Contents by PCR

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**ABSTRACT.** *Campylobacter jejuni* in chicken feces was detected by PCR and Southern blot hybridization (SBH). The detection limits of *C. jejuni* in chicken feces were 34,000 cells by PCR and 340 cells by SBH. Some cecal contents of chickens up to 3 weeks old were *C. jejuni* positive by SBH whereas all of them were negative by PCR. Two of 51 cecal contents of 18-day-old chicken embryos were *C. jejuni* positive by PCR and SBH; but, *C. jejuni* were not isolated from the samples by conventional culture with selective enrichment. — **KEY WORDS:** *Campylobacter*, chicken, PCR.

*Campylobacter jejuni* has been identified as one of the major causes of diarrheal disease in humans throughout the world [3, 16, 23]. One of the possible vehicles of human infection with *Campylobacter* spp. is poultry [1, 8, 10, 18]. Many surveys have been carried out to determine the source of *C. jejuni* contamination of poultry [2, 10, 17, 21]; however, the source of the contamination remains unclear.

For the detection of *C. jejuni*, culture with selective enrichment has been used [18, 22, 24], but this method may lose sensitivity because of nonoptimal growth conditions [25]. Transformation to a nonculturable coccoid form has previously been reported for cells of *C. jejuni* [19], though, the role of this coccoid form in *C. jejuni* contamination is still unknown.

In recent years, the polymerase chain reaction (PCR) has been successfully applied to identify *Campylobacter* spp. in water [12, 15], in some dairy products [7, 9, 24, 25] and in chicken litter [11]. This technique allows the detection not only of viable bacteria but also the nonculturable form of *C. jejuni* [9, 25]. Prior to this, a direct colony hybridization method had been developed for detection of *C. jejuni* in chicken feces [4]. This method was sufficiently sensitive to detect small numbers of *C. jejuni* in chicken feces despite the background flora. According to a survey of *C. jejuni* in broilers from assignment to slaughter using this method, chickens became positive at 1 to 3 weeks of age and some newly introduced chickens were also found to be *C. jejuni* positive [5]. However, *C. jejuni* were not isolated from the cecal contents of the chickens by culture with selective enrichment.

In the present study, the detection limits of *C. jejuni* in chicken feces by PCR and Southern blot hybridization (SBH) were estimated, and the existence of *C. jejuni* in the cecal contents of chickens and embryos as a possible source of contamination of broilers with *C. jejuni* was investigated.

*Campylobacter jejuni* subsp. *jejuni* ATCC 33560 was used as the standard strain. It was cultured on modified Skirrow agar plates containing 5% defibrinated sheep blood and Skirrow’s selective supplement SR069 (Oxoid, England) at 42°C under microaerophilic conditions. For the experiment of the detection limit of PCR and SBH, serial 10-fold dilutions of *C. jejuni* (3.4 × 10¹⁰ – 3.4 × 10⁶ cfu/ml) suspended in PBS were mixed with *C. jejuni*-free chicken feces. The mixtures were centrifuged at 400 × g for 15 min at 4°C and the supernatants were recentrifuged at 3,000 × g for 20 min at 4°C. The pellets were suspended in 300 µl lysing solution (2% SDS, 100 µg/ml pronase, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0). The lysing mixtures were incubated at 60°C for 1 hr and held on ice for 10 min. One hundred and thirty microliters of saturated NaCl solution was added to the suspension and held on ice for 5 min. The mixtures were centrifuged at 12,000 × g for 10 min and the supernatants were extracted twice with equal volumes of phenol-chloroform-isoamylalcohol (25:24:1), and purified with a Sep-Pak C-18 chromatography cartridge (Waters, Millford, Mass.). After vacuum drying, the samples were resuspended in 100 µl of double distilled water and used as the template DNA for PCR. Two synthetic oligonucleotides previously reported by Oyofo et al. [14] were used. The primers pg3 and pg50 generate a 456-bp fragment from the *C. jejuni* Flagellin A gene in the PCR. The PCR mixture (50 µl) contained 10 µl of the template DNA solution, 10 mM Tris-HCl (pH 8.9), 80 mM KCl, 1.5 mM MgCl₂, 500 µg/ml bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X. 0.2 mM each deoxyribonucleoside triphosphate, 10 pmol of each primer, and 1 Unit of Taq DNA polymerase (Toyobo Co. Ltd., Tokyo). This solution was covered with mineral oil to prevent evaporation and subjected to 35 cycles of amplification in a PCR reactor (MJ Research, Watertown, Mass.). Each cycle consisted of 1 min at 94°C, 1 min at 37°C, and 1 min at 74°C. The PCR products were analyzed by electrophoresis on 1% agarose gels with ethidium bromide staining. For Southern blotting, the gel was denatured in 0.2 N NaOH/0.6M NaCl, and transferred to a nylon membrane (Oncor Inc., Gaithersburg, Md.) with 25 mM phosphate buffer (pH 7.2). The preparation of the probe has been described previously [4]. In brief, the probe was biotinylated by nick-translation from DNA of *C. jejuni* ATCC 33560. The membrane was air-dried, baked for 30 min at 80°C, prehybridized in the membrane blocking solution (Oncor) at 42°C for 30 min and hybridized at 42°C for 16 hr in Hybrisol III (Oncor) containing a denatured probe that was heated at 100°C for 10 min. The membrane was washed with 0.16 × SSC (0.15 M NaCl and 15 mM sodium citrate, pH 8.0) containing 0.1% SDS and 0.08% Washing Enhancer (Oncor) at 50°C for 30 min and twice with 1 × SSC for 3 min at room temperature. The hybridized membrane was dipped in a blocking buffer (Oncor) at 42°C.
for 30 min. The membrane was transferred into 1:1,000 diluted streptavidine solution (Oncor) and held for 10 min at room temperature and washed 3 times with 1×SSC for 5 min. It was then washed in 1:1,000 diluted biotin-labeled alkaline phosphatase solution (Oncor) for 10 min and washed 3 times with the buffer as described above. Finally, it was immersed in a staining buffer (Oncor) with substrates (nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate) at 37°C for 2 hr. The reaction was stopped by adding 75% ethanol and baking the membrane at 80°C for 30 min.

The sensitivity of the detection of *C. jejuni* by PCR was less than 10 cells (data not shown), when we used a sample of *C. jejuni* ATCC 33560 alone, revealing that PCR is sensitive enough to detect small numbers of *C. jejuni*. Although, as shown in Fig. 1, the sensitivity decreased to 34,000 cells, of *C. jejuni* (Lane C) by PCR, when DNA was extracted from chicken feces. This may have been due to the presence of nonspecific inhibitors of PCR in the fecal samples [20]. The detection limit of SBH was 340 cells (Lane E); one hundred times greater than PCR.

It was so sensitive that there might be some extra bands on the membrane which were not detected on the gel by PCR (Fig. 1).

Chickens (0 to 3-week-old) were obtained from a growing farm. Newly hatched chickens and 18-day-old fertile eggs were transported from a commercial hatchery to our laboratory. The fertile eggs were sacrificed by being dipped into 0.1% benzalkonium chloride solution for 1 hr. After the shells were aseptically removed, the ceca were collected. The cecal contents were suspended in 1 ml of phosphate buffered saline (PBS, pH 7.2), and were used for the extraction of DNA for PCR as described above. Samples of the cecal contents were enriched in 10 ml of Preston broth containing 7% defibrinated sheep blood and Preston’s selective supplement SR117 (Oxoid), and incubated microaerobically at 42°C for 24 hr. One hundred microliters of the culture was plated on Butzler agar containing 5% defibrinated sheep blood and Butzler’s selective supplement SR085 (Oxoid), and incubated for 48 hr under the same conditions as described above.

As shown in Table 1, all samples from the 0 to 3-week-old chickens were negative by PCR. According to SBH, the number of positive samples obtained from 0, 1, 2, and 3-week-old chickens were 2, 1, 5, and 2 samples, respectively. No *C. jejuni* were isolated from these cecal contents by conventional culture with selective enrichment. It is suggested that only DNA of *C. jejuni* existed in the cecal contents of some of the 0 to 3-week-old chickens. It was not revealed whether that *C. jejuni* was dead or in the nonculturable stage in the present study. All of the 40 samples from cecal contents of newly hatched chickens were negative by PCR, but 3 of the 40 samples were positive by SBH. Two of 51 samples from cecal contents of chicken embryos were positive by PCR. The same samples were also positive by SBH. However, *C. jejuni* was not isolated from them by conventional culture with selective enrichment.

Maruyama and Katsube [13] reported that *C. jejuni* were recovered from eggs laid by experimentally infected Japanese quail (*Coturnix coturnix japonica*). Whereas, Doyle [6] has reported that *C. jejuni* were not recovered from the egg contents but only from 2 of 226 shells of eggs laid by hens that were excreting *C. jejuni* in their feces. In the present study, *C. jejuni* DNA was detected in the cecal contents of newly hatched chickens and 18-day-old chicken embryos by PCR and/or SBH (Table 1), but no live *C. jejuni* were isolated by conventional culture with selective enrichment. It is therefore suggested that some chicken embryos were invaded by *C. jejuni*, however, it is not clear that these *C. jejuni*, detected by PCR and/or SBH, were the source of the spread in the broilers because the bacteria were not culturable.

In conclusion, *C. jejuni* DNA were detected from the cecal contents of newly hatched chickens and 18-day-old embryos by PCR and SBH, but *C. jejuni* were not detected by the conventional culture method. It is still difficult to explain the existence of infectious *C. jejuni* in cecal contents of newly hatched chickens and embryos, and further

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**Fig. 1.** Detection limit of *C. jejuni* in chicken fecal samples by PCR and SBH. Number of the bacteria: Lane A, 3,400,000 cells; B, 340,000 cells; C, 34,000 cells; D, 3,400 cells; E, 340 cells; F, 34 cells; G, λ Hind III marker.

**Table 1.** Detection of *C. jejuni* in cecal contents of 18-day-old chicken embryos, newly hatched chickens, and chickens obtained from a farm, by PCR, SBH and conventional culture method

<table>
<thead>
<tr>
<th>18-day-old embryo</th>
<th>Newly hatched chicken</th>
<th>Age of chickens (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR 2/50*</td>
<td>0/40</td>
<td>0/10 0/6 0/6 0/6</td>
</tr>
<tr>
<td>SBH 2/51</td>
<td>3/40</td>
<td>2/10 1/6 5/6 2/6</td>
</tr>
<tr>
<td>Culture 0/51</td>
<td>0/40</td>
<td>0/10 0/6 0/6 0/6</td>
</tr>
</tbody>
</table>

* Positive/samples.
investigations will be necessary to define the origin of \textit{C. jejuni} contamination of broilers.

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