Application of a DNA-DNA Hybridization Method for Detection of *Campylobacter jejuni* in Chicken Feces

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ABSTRACT. A direct colony hybridization method was used for the detection of *Campylobacter jejuni* in chicken feces. The biotin-labeled DNA prepared from the whole genome DNA of *C. jejuni* subsp. *jejuni* ATCC 33560 reacted well with homologous DNA and slightly with *C. coli* DNA. The method with the probe was found to be sensitive enough to detect a small number (10^2 CFU/g) of *C. jejuni* in chicken feces which contained a large number of background flora. It was suggested that this simple and sensitive method was useful for a wide survey of *C. jejuni*.—KEY WORDS: *Campylobacter jejuni*, chicken, DNA hybridization.

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Food-borne infection by campylobacters has been one of the major subjects to be investigated in public health. It has been suggested that chicken meat is one of the most important sources of the infection [2, 7–9, 18]. The presence of *Campylobacter jejuni* in poultry products is believed to be due to the contamination of meat by fecal matter [1, 11, 12]. In detecting *Campylobacter* species in fecal samples, the presence of enteric competing background flora is a problem. Moreover, there is no rapid and easy method to detect a small number of *Campylobacter*. These problems prevent the wide and reliable survey of *C. jejuni* and *C. coli* in animals and foods. Therefore, a simple, sensitive, and applicable method for the detection of *C. jejuni* and *C. coli* particularly in fecal samples has been hoped for.

Numerous reports on DNA-DNA hybridization methods with oligonucleotide probe [3, 16, 17] or nick-translated probe from whole genomic DNA [4–6, 10, 14, 15] for definite identification of *Campylobacter* species have been published. We applied a colony hybridization method for the detection of *C. jejuni* in chicken fecal samples.

Eight strains of *Campylobacter* species, 1 strain of *Escherichia coli*, and 1 strain of *Sallmonella* Enteritidis were used as references (Table 1). The culture medium for *Campylobacter* species was modified Skirrow agar consisting of Blood Agar Base No. 2 (Oxoid) with 5% sheep defibrinated blood and Campylobacter Supplement (10 mg vancomycin, 5 mg trimethoprim lactate, and 2500 I.U. polymyxin B; Oxoid). *C. jejuni*-negative fecal samples were collected from chickens in our laboratory. The culture was performed at 42°C for 48 hr under microaerophilic conditions.

Cells of *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 grown on a modified Skirrow agar plate were suspended in phosphate-buffered saline (pH 7.2). The suspension was centrifuged at 5,000 rpm for 15 min. The pellet was resuspended in 5.5 m*l* of a buffer solution (50 mM Tris-HCl and 20 mM EDTA, pH 8.0) containing SDS (2%) and pronase (100 μg/m*l*), incubated at 60°C for 1 hr, and stood on ice for 10 min. Two milliliters of saturated NaCl solution was added to the suspension and kept on ice for 5 min. The mixture was centrifuged at 5,000 rpm for 15 min and the supernatant was transferred into an appropriate test tube. After RNase was added (final solution: 20 μg/m*l*), the tube was incubated at 37°C for 15 min. Fifteen milliliters of ice-cold ethanol was added to the tube and chromosomal DNA was spooled out from the mixture. The DNA was rinsed with 70% ethanol, dried, and dissolved in 1 m*l* of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The concentration of the DNA was measured at OD 260 nm. Two micrograms of DNA was labeled with biotin-11-dUTP by a nick translation method according to the instructions of the manufacturer (non isotope probe labeling kit; Oncor). Labeled DNA was purified by Sephadex G-50 gel filtration and used as the probe.

Colonies of the reference bacteria were spotted onto the nylon membrane (Oncor). The membrane was soaked in alkaline solution (0.5 M NaOH and 1.5 M NaCl) for 15 min and twice in neutralizing solution (0.5 M Tris-HCl and 1.5 M NaCl, pH 7.4) for 5 min and then washed with 2 × SSC (0.15 M NaCl and 15 mM sodium citrate, pH 8.0). The membrane was air-dried, baked for 30 min at 80°C, and treated with pronase solution (1 mg/m*l* pronase, 15 mM sodium citrate, and 0.15 M NaCl, pH 7.0) at 37°C for 30 min. The membrane was prehybridized in Membrane Blocking Solution (Oncor) at 42°C for 30 min and hybridized at 42°C for 16 hr in Hybrisol III (Oncor)

Table 1. DNA-DNA hybridization between the probe derived from *C. jejuni* subsp. *jejuni* ATCC 33560 and 10 strains of bacteria

No.	Organism	Strain no.[a)]	Hybridization[b)]
1.	*C. jejuni* subsp. *jejuni*	ATCC 33560	++
2.	*C. jejuni* subsp. *doylei*	NCTC 11951	++
3.	*C. coli*	ATCC 33559	+
4.	*C. lari*	ATCC 35221	−
5.	*C. fetus* subsp. *fetus*	ATCC 27374	−
6.	*C. fetus* subsp. *venerealis*	ATCC 19438	−
7.	*C. hyointestinalis*	ATCC 35217	−
8.	*C. sputorum* biovar *fecalis*	ATCC 33709	−
9.	*Escherichia coli*	NIAH 1087	−
10.	*Salmonella* Enteritidis	NIAH 1206	−

a) ATCC, American Type Culture Collection, Rockville, MD. U.S.A.
NCTC, National Collection of Type Cultures, London, U.K.
NIAH, National Institute of Animal Health, Tsukuba, Japan.

b) ++, strongly positive.
+, positive.
−, negative.

containing the probe which had been denatured by heating at 100°C for 10 min. The optimal concentration of the probe was 10 *n*g/m*l*. The membrane was washed with 0.16 × SSC containing 0.1% SDS and 0.08% Washing Enhancer (Oncor) at 50°C for 30 min and then twice with 1 × SSC for 3 min at room temperature. The membrane was transferred in 1:1000-diluted Streptavidin Solution (Oncor) and kept for 10 min at room temperature. The membrane was washed 3 times with 1 × SSC for 5 min each time, soaked in 1:1000-diluted Biotin-labeled Alkaline Phosphatase Solution (Oncor) for 10 min, and then washed 3 times with 1 × SSC for 5 min each time. The membrane was immersed in 0.5 m*l*/cm^2 of staining buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5) with nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate at 37°C for 2 hr and the staining was stopped by washing with 75% ethanol. The membrane was air-dried and baked at 80°C for 30 min.

Table 1 shows the results of DNA-DNA hybridization between the probe and the 10 strains of bacteria. The reaction of *C. jejuni* subsp. *doylei* was at the same level as the homologous reaction. The reaction of *C. coli* was weaker than the homologous reaction. The other strains of bacteria did not react.

C. jejuni-negative and *C. jejuni*-inoculated chicken feces were cultured on the modified Skirrow agar plates and direct colony hybridization was then carried out. As shown in Fig. 1, no positive spot except the marker spot was detected in the case of *C. jejuni*-negative feces. On the other hand, the expected numbers of spots were detected in the case of *C. jejuni*-inoculated feces. This result was confirmed by repeated tests with more than 10 samples of feces from other chickens. It was revealed therefore that the probe did not cross-react with the competing background flora of chicken feces and that this method may detect *C. jejuni* in chicken feces reliably.

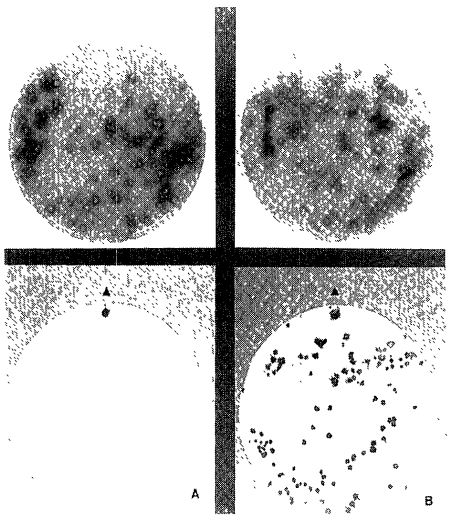

Fig. 1. Direct colony hybridization of *C. jejuni*. A, a membrane prepared from *C. jejuni*-negative fecal sample; B, a membrane prepared from *C. jejuni*-inoculated fecal sample. The membranes A and B were prepared directly from the modified Skirrow agars shown in the upper row.

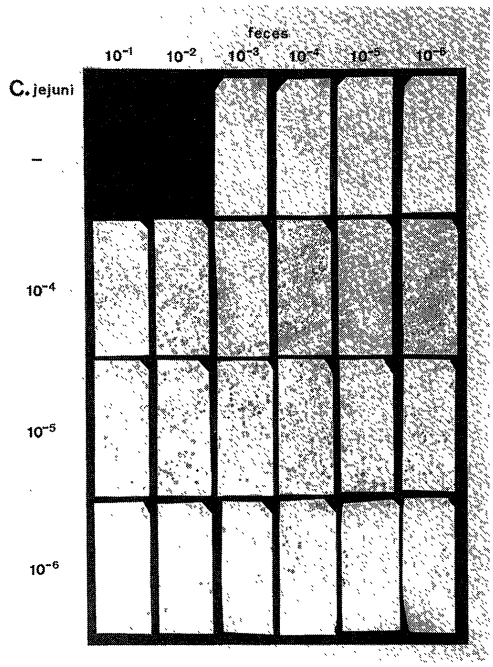

Fig. 2. Direct colony hybridization of *C. jejuni*. Dilutions of chicken feces and *C. jejuni* suspensions (10^9 CFU/m*l*) were mixed in the combinations shown in this figure and 0.1 m*l* of the mixture was cultured on the plates. A two fifth area of the hybridized membrane is shown. Anticipated numbers of *C. jejuni* spots on the membranes in the 10^{-4}, 10^{-5}, and 10^{-6} rows were approximately 400, 40, and 4, respectively.

Serial tenfold dilutions of the *C. jejuni*-negative chicken feces and a suspension of fresh culture of *C. jejuni* were prepared in the range from 10^{-1} to 10^{-6}. The number of *C. jejuni* cells in the original suspension was approximately 10^9 CFU/m*l*. The dilutions of the chicken feces and *C. jejuni* suspension were mixed in the ratio of 9 : 1 in the combinations indicated in Fig. 2. Each mixture (0.1 m*l*) was cultured on the modified Skirrow agar plate and the direct colony hybridization was performed. As shown in Fig. 2, anticipated numbers of spots were detected on all the membranes prepared from *C. jejuni*-inoculated plates including those with numerous colonies of other bacteria from fecal dilutions of 10^{-1} to 10^{-3}. No spot was detected on the membranes prepared from *C. jejuni*-not-inoculated plates. With this method, the detection limit of *C. jejuni* in chicken feces was approximately 10^2 CFU/g.

A small number of *C. jejuni* cannot be detected easily by the conventional culture method because of the influence of background flora. For this reason, an enriching culture is commonly carried out. Various types of enriching cultures have been used, and different results have been obtained [13]. The data presented in Fig. 2 reveal that the direct colony hybridization method was able to detect a small number of *C. jejuni* reliably without an enriching culture, even when the samples contained a

large number of background flora.

A detailed survey of *C. jejuni* contamination in chickens and other animals may be possible by this method.

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