Characterization of Campylobacters Newly Isolated from Swine Gastric Mucosa

LEE, YEONHEE*, JINHUI LEE, HYUNJOO CHO, ENJOO SHIN, JONGHWAN PARK[1], AND JAE HAK PARK[1]

Department of Biology, Seoul Women's University, Seoul 139-774, Korea
[1]Department of Laboratory Animal Science, College of Veterinary Medicine, Seoul National University, Suwon 441-744, Korea

Abstract *Campylobacter* is a pathogen for both humans and animals that can be transferred from animals to humans. Four isolates, which grew under 5–10% CO_2 and had small and translucent colonies, were obtained from swine gastric mucosa and characterized using various methods. These bacteria were gram negative, spirally shaped with round ends. One or two non-sheathed polar flagella were observed under electron microscopy. A PCR with species-specific protein (SSP) primers for 16S rRNA gene in *Campylobacter* produced a typical 462 bp fragment. The isolates had various biochemical and molecular characteristics which differentiated them from other Campylobacters. The isolates were catalase and oxidase positive, urease (rapid) negative, nitrate reduction positive, indoxyl acetate hydrolysis positive, γ-glutamyl transpeptidase negative, and alkaline phosphatase negative. All four isolates showed growth at 37°C and 42°C but not at 25°C, were resistant to cephalotin and cefoperazone, and susceptible to carbenicillin. The isolates showed various results in the reduction of chloride to triphenyl tetrazolium (TTC) and a susceptibility to nalidixic acid. Western blot analysis of these isolates with antiserum raised against one isolate showed different patterns from those of reference strains. A dendrogram drawn with the RAPD results showed that these isolates belonged to a new *Campylobacter* spp. group different from those of *C. jejuni*, *C. doylei*, *C. lari*, and *C. coli*.

Key words: *Campylobacter*, gastric mucosa, swine

Campylobacter jejuni/coli is the most common food-borne bacterial pathogen and the leading cause of food-borne disease in humans in industrialized nations [3]. Furthermore, it is an important enteric pathogen in infants and clinical symptoms of infection include diarrhea, fever, vomiting, and abdominal pain. Illness is frequently self-limited and

prognosis is favorable in most cases with symptomatic treatment. Although the majority of cases consist of limited diarrheal illness, severe sequelae can affect a small portion of patients with campylobacteriosis and can result in reactive arthritis and Guillain-Barré syndrome, the most common cause of acute paralysis in both children and adults [11]. Substantial evidence has established that the route for human infection is through the ingestion of adulterated food and drink [2]. Various pathogenic bacteria including *Aeromonas hydrophila*, *Campylobacter coli/jejuni*, *Listeria monocytogens*, *Salmonella* spp., *Staphylococcus aureus*, and *Yersinia enterocolitica* are prevalent in pigs [14]. Some slaughtered animals harbor these potential pathogens in their intestinal flora and, consequently, these organisms are transferred to carcasses and resulting meat products [5]. Pathogen reduction during meat processing and safe handling of raw meat in the kitchen are necessary to prevent illness [2, 5].

In hostile environments, *Campylobacter* may adapt its physiology to prolongation of survival, potentially including adoption of a viable, non-culturable form and a change to coccal cell morphology [7, 9]. Because of these properties, it is very difficult to isolate and subculture Campylobacters. Recently, new Campylobacters have been identified in various animals and characterized owing to the development of new detection and culture methods [1, 4, 13].

In this study, four *Campylobacter*-like strains were isolated from swine gastric mucosa. Biochemical, phenotypic, immunochemical, and molecular data showed that these bacteria belonged to a new *Campylobacter* spp.

MATERIALS AND METHODS

Reagents

The Bacto transport medium, Stuart and Brucella medium, were purchased from Difco (Detroit, U.S.A.). The GC medium base, isovitalex, and horse serum were purchased

*Corresponding author
Phone: 82-2-970-5664; Fax: 82-2-970-5669;
E-mail: yhlee@swu.ac.kr

from BBL (Cockeyswille, U.S.A.). Fungizone, amphotericin B, polymyxin B, vancomycin, and trimethoprim were purchased from Sigma (St. Louis, U.S.A.) and Taq polymerase from Takara (Shiga, Japan).

Isolation and Culture Conditions

From freshly pooled stomachs, the parts with gastritis or other unhealthy symptoms like keratinization were dissected, washed in a phosphate buffered saline (PBS, pH 7.0), dipped into the transport medium, and kept in ice for transportation to the laboratory 2 h away. In the laboratory, the samples were cut into small pieces with a sterile razor blade and streaked onto the Brucella medium [Brucella broth, fungizone (2.5 g/ml amphotericin B), Skirrow's supplement (0.016 mg/ml polymyxin B, 0.5 mg/ml vancomycin, 0.25 mg/ml trimethoprim)] supplemented with 10% horse serum and a modified chocholate agar medium (GC medium base, 1% hemoglobin, fungizone, skirrow's supplement, 1% isovitalex). The bacteria were cultured under 5-10% CO_2 or anaerobically in an anaerobic jar at 37°C for 4-7 days. Colonies with a small and translucent appearance [12] were inoculated by streaking onto the medium to obtain a pure isolate. C. jejuni subsp. jenuni (LMG 8841^T), C. jejuni subsp. doylei (LMG 8843^T), C. lari (LMG 8846^T), and C. coli (LMG 6640^T) used in this study were obtained via KCTC (Korea Collection of Type Culture) from BCCM/LMG (Laboratorium voor Microbiologie Universiteit Gent).

PCR with Primers Specific to Campylobacter

The genomic DNA was isolated using a Genomic DNA isolation kit (Promega, Madison, U.S.A.). A total 50 µl volume of the PCR mixture contained 2 µg of genomic DNA, 2.5 µl of 20 µM primer 1 (5'-GGAGGATGACAC-TTTTCGGAGC-3') and 2.5 µl of primer 2 (5'-ATTACTG-AGATGACTAGCACCCC-3') specific to 16S rRNA gene of Campylobacter, both designed by Giesendorf [6], and 1 unit of Taq polymerase. The PCR was constituted with one cycle at 94°C for 5 min, 50°C for 1 min, and 72°C for 1 min, 39 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and 1 cycle at 94°C for 1 min, 50°C for 1 min and 72°C for 5 min. The PCR products were electrophoresed in a 0.8% agarose gel and visualized with ethidium bromide.

Electron Microscopy (TEM)

The bacterial cells were prepared as described above and observed under an electron microscope (JEM 100CX II, JEOL, Japan) after negative staining.

Biochemical Characterization

Motility, gram staining, oxidase, catalase, urease, esterase, nitrate reduction, hippurate hydrolysis, indoxyl acetate hydrolysis, γ-glutamyl transpeptidase, reduction of chloride

to triphenyl tetrazolium (TTC), pyrrolidonyl arylamidase, L-arginine arylamidase, L-asparate arylamidase, alkaline phosphatase, H_2S production, glucose assimilation, succinate assimilation, acetate assimilation, propionate assimilation, malate assimilation, and citrate assimilation assays were performed according to the procedures as described in Bergy's Manual [8] and API CAMPY (API, Marcy-I'Etoile, France). Susceptibilities to nalidixic acid, cephalotin, cefoperazone, and carbenicillin were determined with a disk test following the instructions of NCCLS [12].

Random Amplified Polymorphic DNA (RAPD)

A 25 µl total volume of the PCR mixture contained 60 ng of genomic DNA, 4 µl of a 5 pmole/µl RAPD analysis primer (Amersham Pharmacia, Uppsala, Sweden) and 1 unit of Taq polymerase. The RAPD primers were RP3 (5'-GTAGAC-CCGT-3'), RP4 (5'-AAGAGCCCGT-3'), and RP5 (5'-AAC-GCGAAC-3'). The PCR was constituted with three cycles at 94°C for 5 min, 34°C for 5 min, and 72°C for 5 min, 29 cycles at 94°C for 1 min, 34°C for 1 min, and 72°C for 2 min, and a final cycle at 72°C for 10 min. The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. RAPD results were analyzed with a Bio-profile image analysis system (Vilber Lourmat, France).

Preparation of Antiserum Against an Isolate

Bacterial cells were sonicated and total proteins were fixed with 10% formalin. Proteins (1 mg/kg) were intramuscularly injected into rabbits (New Zealand White Rabbit, male) three times in 4-week intervals. The animals were sedated with sodium pentobarbitone and the total blood was obtained from the carotid artery. The antiserum was then divided into aliquots and stored at -20°C.

Western Blot Analysis

Bacterial cells were prepared as described above and proteins were extracted from cells by boiling in 2% SDS. Then, solubilized proteins were electrophoresed in a 10% SDS denaturing gel and blotted onto a nitrocellulose membrane. After blocking with 10% BSA in PBS, antiserum was added to the membrane. The antibody was detected with a second antibody conjugated with alkaline phosphatase and a chromogenic reaction by adding 5-bromo-4-chloro-3-indolyl phosphate disodium salt/nitroblue tetrazolium chloride (BCIP/NBT).

RESULTS AND DISCUSSION

Four strains with small and transparent colonies were successfully subcultured from swine gastric mucosa. When the PCR was performed with primers specific to the 16S rRNA gene in Campylobacter spp., all four isolates produced 462 bp fragments (Fig. 1) [6]. All isolates were
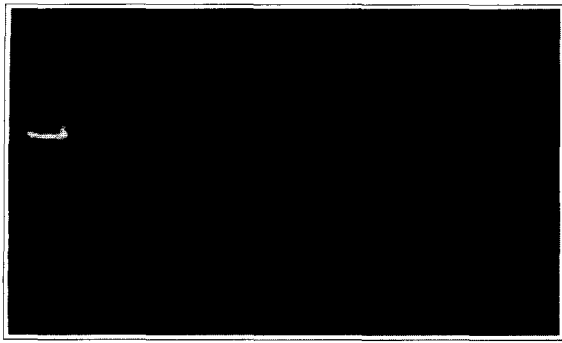
Fig. 1. A PCR with SSP primers for *Campylobacter.*

PCR was performed with SSP primers for *Campylobacter* and visualized with ethidium bromide as described in Materials and Methods. (M, 1 kb DNA ladder; a, *C. jejuni* subsp. *jejuni*; b, *C. jejuni* subsp. *doylei*; c, *C. lari*; d, *C. coli*; e, No. 21-1; f, No. 21-2; g, No. 31; h, No. 48; i, *H. pylori*).

samples, there was a ruffle around the bacteria as often observed in old cultures [9]. All these phenotypic characteristics coincided with the characteristics of Campylobacters reported by others [7].

All isolates were microaerophilic and asaccharolytic; that is, they did not ferment glucose, maltose, mannitol, lactose, ribose, and D-xylose. Key phenotypic traits that differentiate these strains from other closely related bacteria, especially from other Campylobacters, are shown in Table 1. These include their ability to reduce nitrate to nitrite, the non-existence of alkaline phosphatase, resistance to cephalotin, and their growth at 42°C. All four strains grew at both 37°C and 42°C, but not at 25°C. Oxidase and catalase activities were positive, while strong urease activities were negative. Hippurate was not hydrolyzed and γ-glutamyl transpeptidase activity was negative. Indoxyl acetate hydrolysis was positive. The four isolates exhibited different results in their esterase activity, TTC reduction, succinate, acetate, propionate, and citrate assimilations, and susceptibility to nalidixic acid. Based on these biochemical and physiological data, the four isolates had very similar characteristics to *C. coli* except for the resistance to nalidixic acid of isolates 21-1 and 21-2. The key differences between *C. jejuni* and *C. coli* were hippurate hydrolysis and resistance to tetrazolium chloride and
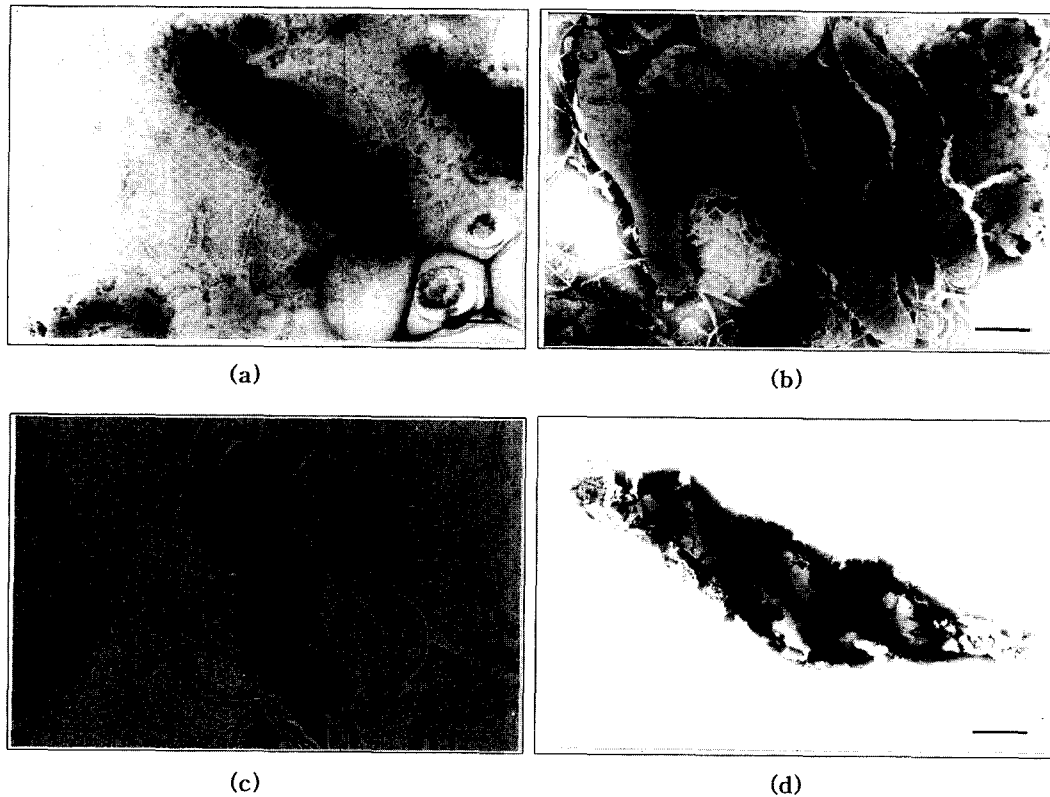
motile, gram-negative, and spirally shaped (Fig. 2). Each bacterium had one or two non-sheathed flagella, one at each side, and the tips of the flagella were not round. Isolate Nos. 21-1, 21-2, and 31 were 2.5-3 μm long and 5-6 μm wide with 2-4 twists. Isolate 48 was 3-4 μm long and 2 μm wide with 4-6 twists. Spherical forms were observed in old cultures. No endospores were produced. In some

(a)

(b)

(c)

(d)

Fig. 2. Electron micrographs of the isolates.
(a), No. 21-1 (×20,000, Bar: 0.5 um); (b), No. 21-2 (×20,000, Bar: 0.5 um); (c), No. 31 (×17,500, Bar: 0.55 um); (d), No. 48 (×17,500, Bar: 0.55 um).

Table 1. Characterization of Campylobacters isolated from swine gastric mucosa.

	21-1	21-2	31	48	C. jejuni subsp. jejuni	C. jejuni subsp. doylei	C. lari	C. coli
α-Hemolysis	+	+	+	+	+	+	+	+
Pitting on BA	-	-	-	-	-	-	-	-
Oxidase activity	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+
Urease activity (rapid)	-	-	-	-	-	-	+	-
Nitrate reduction	+	+	+	+	+	-	-	+
Hippurate hydrolysis	-	-	-	-	+	+	-	-
Indoxyl acetate hydroysis	+	+	+	+	+	+	-	+
γ-Glutamyl transpeptidase activity	-	-	-	-	-	-	+	-
TTC	-	+	+	-	-	+	-	+
Growth at								
25°C	-	-	-	-	-	-	-	-
37°C	+	+	+	+	+	+	+	+
42°C	+	+	+	+	+	+	+	+
Susceptibility to[1]								
Nalidixic acid (30 μg)	R	R	S	S	S	S	S	S
Cephalotin (30 μ)	R	R	R	R	R	S	R	R
Cefoperazone (75 μ)	R	R	R	R	R	MS	R	R
Carbenicillin (100 μ)	S	S	S	S	S	S	I	S

[1]Susceptibiltiy was determined by the size of the clear zone around each disk containing the following antibiomicrobial agents as described in NCCLS: Nalidixic acid (30 μg), ≤13 (R), 14-18 (I), ≥19 (S); cephalothin (30 μg), ≤14 (R), 15-17 (I), - (MS), ≥18 (S); carbenicillin (100), ≤17 (R), 18-22 (I), - (MS), ≥23 (S); cefoperazone (75), ≤15 (R), - (I), 16-20 (MS), ≥21 (S).

nalidixic acid [8]. *Campylobacter jejuni* was hippurate positive, whereas all other strains of *Campylobcter* species and some *Campylobacter*-like organisms were hippurate negative. Since the four isolates were hippurate negative, they would seem to belong to *C. coli*. However, the isolates Nos. 21-1 and 48 were negative to TTC whereas *C. coli* was positive. When resistance to nalidixic acid was checked, the isolates Nos. 21-1 and 21-2 were resistant, whereas *C. jejuni* and *C. coli* were susceptible. Accordingly,

the biochemical and physiological data showed that these four isolates were different from both *C. jejuni* and *C. coli*. Western blot analysis with antiserum raised against the isolate No. 48 showed the differences among the isolates and the reference strains (Fig. 3). As a dendrogram drawn with the Western blot showed, the four isolates are more closely related to the reference strains (Fig. 4).

To confirm the difference among the isolates and the reference strains, RAPDs were performed with various random primers (Fig. 5). RAPD has been successfully used for sub-typing various types of bacteria including animal and human *Campylobacter* spp. [10], and RAD with RP3 produced the same pattern of *C. coli* in all four isolates. However, the RAPD patterns with RP4 and RP5 were different from those of *C. coli*, as shown in Fig. 5. When a dendrogram was drawn, the newly isolated strains were similar to each other forming a group while their similarities to other reference strains were small (Fig. 6).
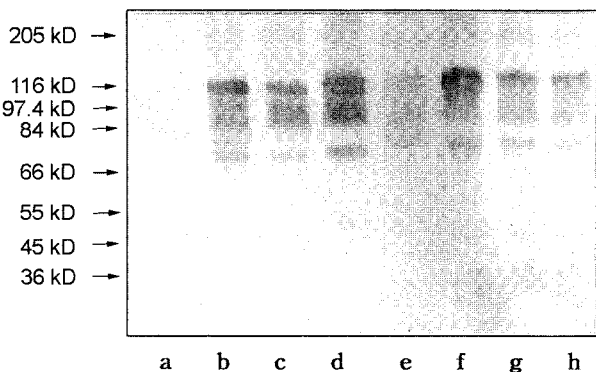

205 kD →
116 kD →
97.4 kD →
84 kD →
66 kD →
55 kD →
45 kD →
36 kD →

a   b   c   d   e   f   g   h

Fig. 3. Western blot analysis of the isolates.
Total proteins extracted from each isolate were electrophoresed using a 10% SDS denaturing gel and blotted onto a nitrocellulose membrane. Proteins bound with antiserum raised against isolate No. 48 were visualized as described in Materials and Methods. a, *C. lari*; b, *C. coli*; c, *C. jejuni* subsp. *doylei*; d, *C. jejuni* subsp. *jejuni*; e, No. 31; f, No. 21-2; g, No. 48; h, No. 21-1.
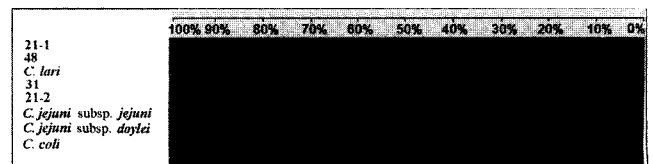

100% 90%   80%   70%   60%   50%   40%   30%   20%   10%   0%
21-1
48
C. lari
31
21-2
C. jejuni subsp. jejuni
C. jejuni subsp. doylei
C. coli

Fig. 4. Dendrogram of the Western blot analysis.
The Western blot analysis was analyzed with a Bio-profile image analysis system.

(a)   (b)   (c)

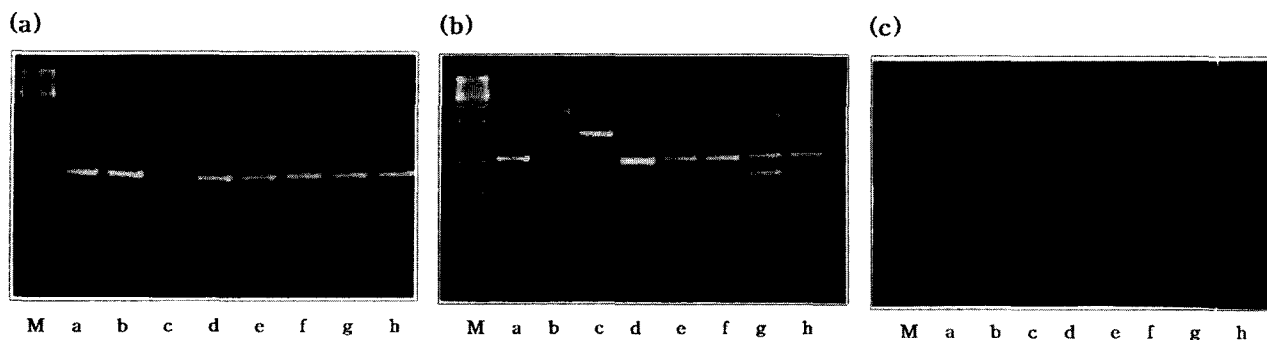M a b c d e f g h   M a b c d e f g h   M a b c d e f g h
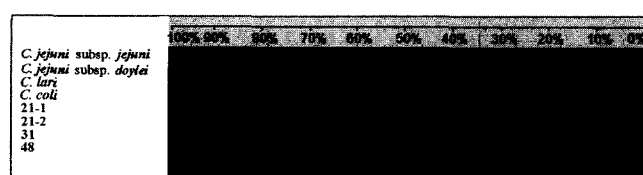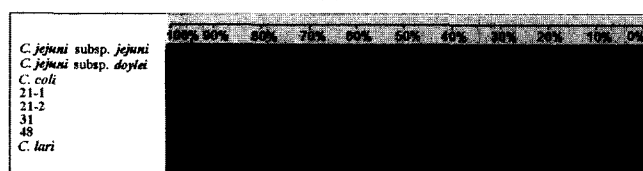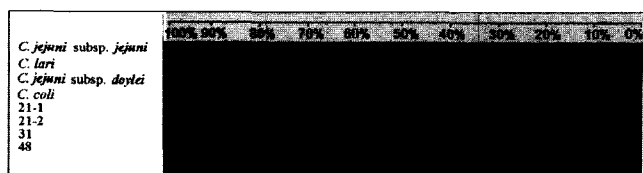
Fig. 5. RAPD of the isolates.
RAPD was performed with RP3 (a), RP4 (b), and RP5 (c) and the products were visualized with ethidium bromide as described in Materials and Methods.
M, 1 kb DNA ladder; a, *C. jejuni* subsp. *jejuni*; b, *C. jejuni* subsp. *doylei*; c, *C. lari*; d, *C. coli*; e, No. 21-1; f, No. 21-2; g, No. 31; h, No. 48.

(a)

(b)

(c)

Fig. 6. Dendrogram of the RAPD results.
The RAPD results were analyzed with a Bio-profile image analysis system.

To further characterize these isolates, total 16S rRNA sequencing is in progress, and a preliminary result (data not shown) shows that these isolates are closely related to *C. coli* but do not belong to the same group as *C. coli*.

Based on these phenotypic, biochemical, and RAPD results, and Western blot analysis, the four isolates could be differentiated from each other and also from other known Campylobacters including *C. jejuni*, *C. doley*, *C. lari*, and *C. coli*, thus belonging to a new group.

Ackowledgments

REFERENCES

1. Alderton, M. R., V. Korolik, P. J. Coloe, F. E. Dewhirst, and B. J. Paster. 1995. *Campylobacter hyoilei* sp. *nov.* associated with porcine proliferative enteritis. *Int. J. Syst. Bacteriol.* **45**: 61-66.
2. Borch, E., T. Nesbakken, and H. Christensen. 1996. Hazard identification in swine slaughter with respect to foodborne bacteria. *Int. J. Food Microbiol.* **30**: 9-25.
3. Butzler, J. P. and J. Oosterom. 1991. Campylobacter: Pathogenicity and significance in foods. *Int. J. Food Microbiol.* **12**: 1-8.
4. Delong, W. J., M. D. Jaworski, and A. C. Ward. 1996. Antigenic and restriction enzyme analysis of *Campylobacter* spp. associated with abortion in sheep. *Am. J. Vet. Res.* **57**: 163-167.
5. Fransen, N. G., A. M. van den Elzen, B. A. Urlings, and P. G. Bijker. 1996. Pathogenic microorganism in slaughterhouse sludge. *Int. J. Food Microbiol.* **33**: 2-3.
6. Giesendorf, B. A. J., W. G. V. Quint, M. G. V. Henkens, H. Stegeman, F. A. Huf, and H. G. M. Niesters. 1992. Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **58**: 3804-3808.
7. Harvey, P. and S. Leach. 1998. Analysis of coccal cell formation by *Campylobacter jejuni* using continuous culture techniques, and the importance of oxidative stress. *J. Appl. Microbiol.* **85**: 398-404.
8. Hebert G. A., D. G. Hollis, R. E. Weaver, M. A. Lambert, M. J. Blaser, and C. W. Moss. 1982. 30 Years of Campylobacters: Biochemical characteristics and a biotyping proposal for *Campylobacter jejuni*. *J. Clin. Microbiol.* **15**: 1065-1073.
9. Krieg, N. R. and J. G. Holt. 1984. *Bergey's Manual of Systematic Bacteriology*. 1984. Vol 1. Williams & Wilkins, Baltimore, MA, U.S.A.

10. Madden, R. H., L. Moran, and P. Scates. 1996. Sub-typing of animal and human *Campylobacter* spp. using RAPD. *Lett. Appl. Microbiol.* **23**: 167– 170.

11. Nachamkin, I., B. M. Allos, and T. Ho. 1998. *Campylobacter* species and Guillain-Barr syndrome. *Clin. Microbiol. Rev.* **11**: 555– 567.

12. National Committee for Clinical Laboratory Standards. 1998. *Performance standards for antimicrobial testing; eight informational supplement.* NCCLS document M100-S8. National Committee for Clinical Laboratory Standards, Wayne, PA, U.S.A.

13. On, S. L. W. 1996. Identification methods for *Campylobacter, Helicobacter,* and related organisms. *Clin. Microbiol. Rev.* **9**: 405– 422.

14. Weijtens, M. J., R. D. Teinders, H. A. Urlings, and J. Van der Plas. 1999. *Campylobacter* infections in fattening pigs; excretion pattern and genetic diversity. *J. Appl. Microbiol.* **86**: 63– 70.