THESIS

RAPID DETECTION AND MOLECULAR SUBTYPING OF *CAMPYLOBACTER* IN POULTRY SAMPLES

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THESIS

RAPID DETECTION AND MOLECULAR SUBTYPING OF *CAMPYLOBACTER* IN POULTRY SAMPLES

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Two alternative methods, multiplex PCR (mPCR) and immunomagnetic separation (IMS) followed by plating to CCDA agar, were compared for their suitability to detect *Campylobacter jejuni* and *C. coli* in chicken meat. IMS followed by plating could detect *C. jejuni* and *C. coli* inoculated at 10^0 cfu/g in meat after 12 h of incubation whereas the mPCR method could detect both species at the same inoculation level after 16 h of incubation. However, the total analytical time to identify *C. jejuni* and *C. coli* in chicken meat using IMS followed by plating was 72-96 h while the time used by mPCR was only 22 h. Thus, the mPCR method for the detection of *C. jejuni* and *C. coli* in chicken meat could be performed with less total analytical time than IMS followed by plating.

Additional study was conducted to further investigate *C. jejuni* and *C. coli* isolated from seven commercial poultry farms and two slaughterhouses by molecular epidemiological analysis using high-resolution genotyping method of amplified fragment length polymorphism (AFLP). AFLP analysis of 314 *Campylobacter* isolates revealed 48 AFLP strains of *C. jejuni* and 95 AFLP strains of *C. coli*. The intralinkage homologies of the AFLP patterns among all *C. jejuni* isolates were 65%. The intralinkage homologies of the AFLP patterns among all *C. coli* isolates were 73%. The AFLP banding patterns of *C. coli* strains contained many closely distributed bands, which were more homologous than the patterns for *C. jejuni*. The *C. coli* strains from broilers and slaughtering process seem to be more closely related to each other than *C. jejuni* strains. This finding suggests that *C. coli* strains are more clonal than *C. jejuni* strains.

In most farms, broad diversity of *C. jejuni* and *C. coli* strains were found and AFLP type distribution changed during the slaughter line. Some genotypes of both species were found in chicken intestine and may be the source of contamination of chicken meat during slaughtering and cutting process in slaughterhouse. Contamination of *C. jejuni* and *C. coli* in chicken meat occur directly from intestinal content and feces or indirectly from bird to bird and environment in slaughterhouse. AFLP fingerprint is the effective method to discriminate between *C. jejuni* and *C. coli* strains in which the interlinkage homology of the AFLP pattern is only 35 to 42%. In addition, it can distinguish genetically unrelated- from related-strains. Therefore, AFLP analysis is a suitable epidemiological tool for investigations of *Campylobacter*.

Student's signature

Thesis Advisor's signature

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Chalermkiat Saengthongpinit

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LIST OF ABBREVIATIONS

DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxynucleotide
PCR	=	Polymerase Chain Reaction
UV	=	ultraviolet
V	=	volt
rpm	=	round per minute
°C	=	Degree Celsius
h	=	hour
min	=	minute
s	=	second
mm	=	millimeter
µm	=	micrometer
l	=	liter
µl	=	microliter
ml	=	milliter
g	=	gram
µg	=	microgram
mg	=	milligram
µM	=	micromolar
mM	=	millimolar
M	=	molar
U	=	unit
cfu	=	colony forming unit
bp	=	base pair
A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine

RAPID DETECTION AND MOLECULAR SUBTYPING OF *CAMPYLOBACTER* IN POULTRY SAMPLES

INTRODUCTION

Campylobacter is one of the most common food-borne pathogens in both developed and developing countries, causing gastroenteritis characterized by watery and/or bloody diarrhea. It can be associated with Guillain-Barré syndrome (GBS), reactive arthritis, Reiter's syndrome and haemolytic uremic (HUS) syndromes (Tauxe, 2000; Food Safety Authority of Ireland [FSAI], 2002; Lake *et al*., 2003). During the past 20 years, the infection rate of *Campylobacter* has continued to increase in many developed countries, part of this increase may be due to the improvement of detection and reporting (Tauxe, 2000). The infective dose of *Campylobacter* is considered to be low, ranging from 500 to 10,000 cells (FSAI, 2002). Only three *Campylobacter* species including *C. jejuni*, *C. coli* and *C. lari* already represent approximately 90% of all human campylobacteriosis cases (Stern and Line, 2000). In the European Union, approximately 0.2 million human cases of campylobacteriosis are reported annually (European Food Safety Authority [EFSA], 2006a). Wild birds and domestic animals are common reservoirs for *Campylobacter*. Poultry products are important vehicles for the bacteria to infect humans. Epidemiological studies show that consumption or handling of poultry meat should be considered as a major risk factor for human infection with *C. jejuni* or *C. coli* (Coker, 2000; FSAI, 2002).

Traditional analytical methods for determination of *Campylobacter* are based on phenotyping which require four to five days. Due to the perishable nature of food products, this time-consuming method is unacceptable. Advanced analytical assays, *i.e.* rapid, sensitive and specific methods, are urgently needed to support veterinary intervention programs. For this reason, immunomagnetic separation (IMS) using magnetic beads coated with immunoglobulins is exploited to isolate and concentrate *C. jejuni* from sample tested leaving cells viable. It also reduces the time of the pre-

enrichment step. A short pre-enrichment step is needed to improve the sensitivity and overcome the problems of inhibitors from the food sources (Yu *et al*., 2001).

Polymerase chain reaction (PCR) assay is a relatively rapid, sensitive and specific method to detect *Campylobacter* in food samples. Multiplex PCR (mPCR) may not only identify *Campylobacter*, but also simultaneously discriminate between different species of *Campylobacter*, including *C. jejuni* and *C. coli* (Denis *et al*., 1999). However, one disadvantage of PCR assay is the presence of inhibitors of amplification in food that require effective clean-up methods to overcome possible interference (Denis *et al*., 2001).

The national and global epidemiological studies of classical serotyping have been restricted because of a high level of nontypeability and time-consuming. Therefore, molecular subtyping that can solve these problems was developed for universal use. For subtyping of *Campylobacter*, amplified fragment length polymorphism (AFLP) is performed. AFLP is a method that is based on complete digestion of the whole bacterial gene with two restriction enzymes. PCR amplification of the digestion products are designed by adapter-specific primers that have an extension of one to three nucleotides at their 3' ends running into the unknown chromosomal restriction fragment. The PCR primer that spans the average frequency restriction site is labeled with fluorescent. The resultant labeled PCR products are analyzed on denaturing polyacrylamide gel. After polyacrylamide gel electrophoresis, a highly informative pattern of 50 to 500 bands is obtained. The number of bands generated typically 80 to 100 bands can be reduced by incorporating one or more specific nucleotides in the PCR primers adjacent to the restriction site. This polymorphic pattern can differentiate strains of *Campylobacter* spp. (Savelkoul *et al*., 1999; Wassennaar and Newell, 2000).

The advantage of AFLP technique is that a random portion of the whole genome is sampled. AFLP is the most promising method for providing reproducible profiles and appears to be insensitive to the genetic instability which makes it less complicated than other molecular methods. Although major capital investment may

restrict the use of this method in research laboratories, it seems like that this technique will be used more widely for global epidemiological studies (Newell *et al*., 2000; Wassennaar and Newell, 2000).

OBJECTIVES

1. To evaluate the suitability of the detection of *C. jejuni* and *C. coli* in pure culture and chicken meat samples with different incubation times (0-21 h) using IMS followed by plating to charcoal cefoperazone-deoxycholate agar (CCDA) or multiplex polymerase chain reaction (mPCR).

2. To investigate *C. jejuni* and *C. coli* isolated from seven commercial poultry farms and two slaughterhouses by molecular epidemiological analysis using high-resolution genotyping method of amplified fragment length polymorphism (AFLP).

LITERATURE REVIEW

1. History of the genus *Campylobacter*

Campylobacter spp. was not recognized as human pathogens until the 1970s but has undoubtedly caused enteritis for many years. In 1886, Theodor Escherich published the articles which he described spiral bacteria in the colons of dead children that he called "cholera infantum". Unfortunately, these articles published in German until Kist reported Escherich's findings at the Third International *Campylobacter* workshop in Ottawa in 1985 (Kist, 1985). An organism called *Campylobacter fetus* was isolated by McFadyean and Stockman in aborted sheep in 1913 (McFadyean and Stockman, 1913). Five years later, Smith discovered the same spiral bacteria in aborted ovine fetuses. He and Taylor confirmed and proposed the name as "*Vibrio fetus*" (Smith and Taylor, 1919). Eighteen years later, in 1931, Jones found *Vibrio* which caused winter dysentery in calves and called it, "*Vibrio jejuni*" (Jones *et al*., 1931). Doyle described another *Vibrio* associated with swine dysentery and classified them as *Vibrio coli* in 1944 (Doyle, 1944). In 1957, King described the first case of vibriosis in human with the name "related *Vibrio*" because of the different biochemical and antigenic characteristics from *Vibrio* (King, 1957, 1962). Until 1963, *Vibrio fetus* and *Vibrio bubulus* were transferred to the genus *Campylobacter* by Sebald and Véron. Ten years later, Véron and Chatelain published the study on the taxonomy of the microaerophilic *Vibrio* like organism and separated four species in the genus *Campylobacter*: *Campylobacter jejuni*, *Campylobacter coli, Campylobacter fetus* and *Campylobacter sputorum* (Véron and Chatelain, 1973).

2. Taxonomy and characteristics of the organism

2.1 The family *Campylobacteraceae*

The genera *Campylobacter*, *Acrobacter, Sulfurospirillum* and the generically misclassified *Bacteroides urelyticus* are in the family of gram negative, nonsaccharolytic bacteria with a low G + C content and microaerobic growth

requirements. They occur primarily as commensals or parasites in domestic animals and humans. The characteristics of members of this family have following general characteristics; Cells are curve, S-shape, or spiral rods and are 0.2 to 0.8 μm wide and 0.5 to 5 μm long. They are nonsporeforming bacteria and typically motile with a corkscrew-like motion. There is a single polar unsheathed flagellum at one or both ends of the cells. Cells grow under microaerobic conditions but some also grow under aerobic or anaerobic conditions. The optimum growth temperature is 30 to 37°C. In general, biochemical characteristics are negative methyl red reaction, acetoin production, indole production and reduction of fumarate to succinate. Most species in this family are presence of oxidase activity, reduction of nitrate and absence of hippurate hydrolysis (Vandamme, 2000).

2.2 The genus *Campylobacter*

Campylobacter is a spiral shape bacilli with one or two flagella at the pole and is highly motile. It has a "gull-winged" morphology following cell division. *Campylobacter* grows at optimum temperature of 42°C, with a range between 30.5°C and 45°C, optimum growth at 10% carbon dioxide, 5-6% oxygen, and 85% nitrogen. *Campylobacter* cells in old cultures may form coccoid bodies which are considered degenerative forms (Hazeleger *et al*., 1994). Viable but nonculturable stage may be found in starvation condition (Rollins and Colwell, 1986). Biochemical characteristics of several species require fumarate with formate or hydrogen for growth under microaerobic conditions. This genus can reduce nitrate to nitrite. Tyrosine, casein, gelatin and starch are not hydrolyzed. There is no lipase or lecithinase activity but oxidase activity is present in every species except *C. gralcilis*. Most enteropathogenic campylobacters are catalase-positive. The G + C content of the DNA is 29 to 47 mol%. Many species are pathogenic in animals and humans which are found in oral cavity, stomach, intestine, placenta and blood (Stern and Line, 2000; Vandamme, 2000).

Table 1 Differential characteristics between *Campylobacter* species[a].

Species	Alpha hemolysis	Catalase	Hippurate hydrolysis	Urease	Nitrate reduction	Selenite reduction	H_2S/ TSI	Indoxyl acetate hydrolysis	Growth:					Resistance to:	
									25°C	42°C	Minimal medium	Glycine 1%	NaCl 4%	Nalidixic acid	Cephalothin
C. coli	V	+	–	–	+	+	V	+	–	+	+	+	–	–	+
C. concisus	V	–	–	–	V	V	–	–	–	V	–	V	–	V	–
C. curvus	V	–	V	–	+	–	V	V	–	V	V[b]	+	–	+	–
C. fetus subsp. fetus	–	+	–	–	+	V[b]	–	–	+	V[b]	V	+	–	+	–
C. fetus subsp. venerealis	V	V[b]	–	–	+	–	–	–	+	–	V[b]	–	–	V	–
C. gracilis	–	V	–	–	V[b]	–	–	V	–	V	V	+	–	V	–
C. helveticus	+	–	–	–	+	–	–	+	–	+	–	V	–	–	–

Table 1 (Continued)

Species	Alpha hemolysis	Catalase	Hippurate hydrolysis	Urease	Nitrate reduction	Selenite reduction	H_2S/TSI	Indoxyl acetate hydrolysis	Growth:					Resistance to:	
									25°C	42°C	Minimal medium	Glycine 1%	NaCl 4%	Nalidixic acid	Cephalothin
C. hyointestinalis subsp. hyointestinalis	V	+	–	–	+	+	+	–	V	+	V	+	–	+	V
C. hyointestinalis subsp. lawsonii	V	+	–	+	+	+	+	–	–	+	V	V	–	+	–
C. jejuni subsp. doylei	+	V	+	–	–	–	–	+	–	–	–	V	–	–	–
C. jejuni subsp. jejuni	+	+	+	–	+	V	–	+	–	+	–	+	–	–	+
C. lari	V	+	–	V	+	V	–	–	–	+	–	+	–	V	+
C. mucosalis	–	–	–	–	–	–	+	–	–	+	–	V	–	V[b]	–

Table 1 (Continued)

Species	Alpha hemolysis	Catalase	Hippurate hydrolysis	Urease	Nitrate reduction	Selenite reduction	H_2S/ TSI	Indoxyl acetate hydrolysis	Growth:					Resistance to:	
									25°C	42°C	Minimal medium	Glycine 1%	NaCl 4%	Nalidixic acid	Cephalothin
C. rectus	+	V	–	–	+	–	–	+	–	V	–	+	–	V[b]	–
C. showae	+	+	–	–	+	–	V	V	–	V	V	V	–	–	–
C. sputorum	+	V	–	V	+	V	+	–	–	+	V	+	V	V	–
C. upsaliensis	+	–	–	–	+	+	–	+	–	V[b]	–	+	–	–	V

[a] +, characteristic present in over 90% of the strains examined;
–, characteristic present in less than 11% of the strains examined;
V, strain-depedent reaction
[b] At least 80% of the strains examined contained this characteristic
Source: Adapted from Vandamme (2000)

2.3 *C. jejuni* and *C. coli*

Both *C. jejuni* and *C. coli* are the most important enteropathogens in human. *C. jejuni* is associated with Guillain-Barré syndrome (GBS) and can lead to reactive arthritis (Caughey, 1984; Nachamkin, *et al*., 1998). *C. jejuni* is divided in two subspecies: *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. The different of biochemical characteristic of these subspecies are the nitrate reduction and catalase activity (Table 1). The separation of *C. coli* and *C. jejuni* subsp. *jejuni* could cause taxanomic problem. Normally, hippurate hydrolysis in *C. coli* is negative and *C. jejuni* subsp. *jejuni* is positive, however, some strains of *C. jejuni* subsp. *jejuni* also give negative result. The other additional tests such as hydrogen sulfide production in triple-sugar-iron agar, growth on the minimal medium (On *et al*., 1996) and utilization of propionate (Occhialini *et al*., 1996) can be used to separate the two species. The different characteristics of *Campylobacter* species are shown in Table 1.

2.4 The viable but non-culturable state

C. jejuni can sometimes form so-called viable but non-culturable (VBNC) state when found in a moribund condition. Morphology of them is coccoid shape but spiral VBNC can occur as well (Lazaro *et al*., 1999). Perhaps the coccoid VBNC transform to the spiral form but reversion of coccoid cells is not easy and need very specific conditions. Even though, the coccoid cells are metabolically active and show signs of respiratory activity, they cannot resuscitate by normal conventional culturing method. This state is adapted for survival and cells become progressively debilitated until they finally die. VBNC state could be induced by exposure to the sublethal adverse environmental conditions, *i.e.* prolonged exposure to water or freeze-thaw injury or recovery after passage of the cells through a susceptible host. It seems that VBNC state is the mechanism to survive of bacteria in aquatic environment and low temperature which enhance the spread of *Campylobacter*. Therefore, the impact of VBNC state could not be ignored in human and animal epidemiology of campylobacteriosis (McClure *et al*., 2002; Ziprin, 2004).

3. Characteristics of the disease

The incubation period after ingestion of *Campylobacter* is between 2 to 5 days. The symptom of campylobacteriosis in human is watery and/or bloody diarrhea, abdominal pain and cramps, fever and headache. Diarrhea caused by these organisms can be mild like merely loose to profuse stools, which may contain blood, slimy and extremely foul smelling. The duration of symptom is less than 7 days and show severe clinical sign in 2 to 5 days (Stern and Line, 2000). The infective dose for humans may be as few as 500 – 10,000 cells depending on vehicle of ingested materials and the susceptibility of the individual. Actually, campylobacteriosis is a self-limiting disease. Recurrent infections occur in 10 to 20% of patients but frequently less severe than the original symptom (FSAI, 2002). Treatment by antibiotic is used only in the complications and recurring episode cases. Erythromycin is a highly effective treatment. Fluoroquinolone is used to be effective antimicrobial for *Campylobacter*. However, the emergence of fluoroquinolone-resistant strains of *C. jejuni* was reported in Western Europe in 1990, after the veterinary use of this drug was approved (Altekruse and Swerdlow, 2002). Hospitalization from campylobacteriosis may account for 10% of total cases and death is rare (Stern and Line, 2000).

Many reports of this disease can be found in association with other syndromes such as reactive arthropathies, Reiter's syndrome, GBS and associating neurological disorders. GBS is an acute inflammatory demyelinating neuropathy which affects sensory and motor fibers. Inflammatory neuropathy is caused by immunological reaction to *C. jejuni* provoking an autoimmune-mediated attack on neural tissue that mimic structure like *Campylobacter* lipopolysaccharides (Ziprin, 2004). The GBS is defined as a clinical entity that is characterized by rapidly symmetrical limb weakness, loss of tendon reflexes, absent of sensory signs, and autonomic dysfunctions (Hahn, 1998; Lake *et al*., 2003). Reactive arthritis is from the inflammation of joints at a location distant from the enteric site of infection. The other extraintestinal infections by *Campylobacter* and post infection sequelae are found but rare such as bacteremia, hepatitis, cholecystitis, pancreatitis, renal infection

abortion, stillbirth, bacteremia in newborn infant and maternal death (Ziprin, 2004). Because *Campylobacter* contaminated foods from animal origin very often cause human gastroenteritis, vaccinating animal may be the way to reduce carriage of the organism in their gastrointestinal tract and vaccinating human would be advantage to prevent campylobacteriosis as well. Unfortunately, the development of a *Campylobacter* vaccine is still unsuccessful, although there have been a few good attempts, but with little success (Ziprin, 2004).

4. Mechanism of pathogenicity and virulence factors

The high titers of humoral IgG, IgM and IgA in human campylobacteriosis indicate that bacterial cellular constituents pass from intestinal tract to epithelium. The antigens on the serosal side are presented to the immune system receptors following by cellular and humoral constituents are able to respond. Poor antibody response to *Campylobacter* is associated with patients who have prolonged or intermittent enteritis. The mechanism of pathogenesis is from the attachment, invasion and toxin formation to epithelium of the host. The outer membrane protein (OMP) of enteropathogenic *Campylobacter* which adheres to epithelial cells is a major virulence to host membrane. There is no remarkable difference in the expression of pathogenic genes responsible for the expression of cytotoxin, adherence, invasion and colonization which are found in *Campylobacter* isolated from a variety of sources (Stern and Line, 2000; Boxall, 2005). Pathogen-specific factors in disease causation are also iron acquisition, host cell invasion, toxin production, inflammation, active serosal fluid secretion and epithelial disruption with leakage of serosal fluid. Major factors of the disease are the health of host and host immunity, virulence of bacteria and environment factor (Altekruse and Swerdlow, 2002).

4.1 Motility

Motility is conferred by the polar flagellum and the corkscrew motion allows *Campylobacter* to go through the mucus layer of intestine. The flagellum composes of two closely related proteins, the major subunit Fla A and the minor

subunit Fla B. Mutants which do not express subunit A produce truncated, stubby flagella and completely non-motile. Mutants which cannot express subunit B are still capable movement but decreased motility. Nonflagellated mutant strains adhere much less efficiently to host cells than the flagellated parent strain. A non-motile bacterium with paralyzed flagella was discovered when the gene *pfl*A was mutated. Motility is required for optimal *Campylobacter* adherence to host cells and for invasion ability. The polar flagellum and the spiral shape of *Campylobacter* confer a distinctive motility which is particularly effective in a viscous matrix. This motility may allow *Campylobacter* to penetrate the mucus layer and seek host cell receptors involved in colonization or invasion of the intestinal mucosa (Hu and Kopecko, 2000; Boxall, 2005).

4.2 Chemotaxis

The ability to detect, move up and move down in chemical gradients has been shown as vital to *Campylobacter* colonization. Non-chemotaxic mutants were incapable of colonizing the intestines. The organisms reside into the deep crypts of Lieberkuhn lining the intestinal tract. They can easily move through the mucin layer of the gut surface into crypt with the benefit of spiral shape and high motility. Gastric mucin is the chemotactic attractants for bacteria and flagella and is served as an important role for colonization in gut. The motility of *Campylobacter* move through the thick layers of mucus attracted by two chemotractants: the glycoprotein component of mucus and a terminal sugar of mucin (L-fucose) (Stern and Line, 2000; Boxall, 2005).

4.3 Adherence and colonization

Bacterial adherence is due to a particular interaction between molecules on the bacterial surface (adhesions) and molecules on the host surface (receptors). Pili and afimbrial adhesions are used by bacteria to attach to host cells. Pili are hair-like, rod-shaped protein structures which extend out from the bacterial surface and afimbrial adhesions are bacterial surface proteins which are not organized in a rodlike

structure but mediate tight binding between bacteria and the host cell. The ability of *C. jejuni* to colonize the gastrointestinal tract by binding the epithelial cells has been proposed to be essential for disease production. *C. jejuni* isolated from patients with fever and diarrhea showed much greater binding to epithelial cells than did strain isolated from asymptomatic patients (Hu and Kopecko, 2000).

The requirements for colonization of *Campylobacter* to mucus barrier are motility and chemotaxis. Nevertheless, specific adherence to or degradation of mucus components may be involved. In addition to bacterial colonization of mucus layer and adherence to the mucosal cell surface, invasive bacteria appear to bind transiently to the host cell before internalization. Mutations in the virulence factor genes *cad*F, *dna*J, *pld*A and *cia*B impair the ability of *C. jejuni* to colonize the cecum of chicks. These genes may influence the virulence of strain (Hu and Kopecko, 2000; Boxall, 2005).

4.4 Toxin

In many studies, toxins which produced from *Campylobacter* are enterotoxin and cytotoxin. Their toxic activities affect various culture cell lines and some studies have included assays in animal models. Ruiz-palacios *et al*. (1983) reported that enterotoxin production of *Campylobacter* is associated with illness of patient but could not find an association between diarrhea and cytotoxin production. Cytolethal distending toxin (CDT) is the one of *Campylobacter* cytotoxin which causes cells to become slowly distended and leads to cell death. Most of *C. jejuni* and *C. coli* strains carry the *cdt* gene but there are differences in the amount of CDT produced. It is still not clear why *C. jejuni* produces high titers of the toxin, while *C. coli* produces less. Nonetheless, some *cdt*-negative strains can exhibit cytotoxic effect. Other alternative toxigenic genes and *Campylobacter* toxins is still rater limited, and perharp the next few years will bring a significant improvement in our understanding of their roles (Hu and Kopecko, 2000; Boxall, 2005).

5. *Campylobacter* in foods and the environment

5.1 Poultry and poultry products

Many species of poultry are associated with high rates of colonization by *C. jejuni* such as chickens, turkeys, domestic ducks, ostrich, pheasants, emu and guinea fowl. In birds, *i.e.* hawks, parrots and pigeons were isolated *Campylobacter* as well. However, the most important species of avain to infect human is chickens (Stern and Line, 2000). Data of sporadic cases of *Campylobacter* infection based on case-control studies showed that at least 50% are relevant with handling or consumption of chicken meat. Some reports indicated that the contaminated chicken meat with these bacteria contains serotypes as same as in human cases (Newell and Wagenaar, 2000). It has been recognized that three out of four of live broilers and more than 80% of retail poultry meat is contaminated (Jacobs-Reitsma, 2000; Hiett *et al*., 2002).

C. jejuni is usually not isolated from the hatchery but typically isolated from the farmland environment during the first two weeks when chicks are placed. After the third or fourth week of production, most members of the flocks are contaminated with the organism. The appearance of *Campylobacter* in production facilities in farm comes from the poor farm management, inadequate biosecurity, poor insect or rodent control, the droppings of wild birds and contaminated feed and water (Ziprin, 2004). Usually, there are 10^7 cfu/g *C. jejuni* in the intestinal tract of chickens but not apparent clinical illness of disease. When processing in slaughterhouse, the releasing of intestinal contents may contaminate most raw poultry products and become the source of human infection after being contacted with them (Stern and Line, 2000).

Colonized birds enter the slaughterhouses with *Campylobacter* organism not only in their feathers and skin but also predominatly in intestinal tract. These bacterial sources lead to contamination of working surface, equipment, water and air in plant. Especially water used during processing of chicken contributes to spreading

Table 2 Presence of thermotolerant *Campylobacter* in poultry products.

Product	Stage of process	Sample type	Total Number of samples	% of positive samples	Country	Year
Chicken carcasses	After chilling	Carcass rinse	80	86	USA	1995
Chicken breasts	After processing	Meat (10g)	156	68	Japan	1991
Chicken breasts	At retail	Meat (25g)	32	38	Italy	1996
Chicken breasts	At retail	Swabs	616	58	France	1996
Chicken breasts	At retail	Meat (25g)	2,016	28	Germany	1998
Chicken meat	At retail	Not specified	676	33	Denmark	1997
Chicken carcasses	At retail	Carcass rinse	330	69	USA	1997
Chicken products	At retail	Meat (25g)	1,165	37	The Netherlands	1997
Chicken products	At retail	Meat (10g)	120	38	N. Ireland	1998
Chicken carcasses	At retail, frozen	Carcass rinse	199	14	Finland	1989
Chicken livers	At retail, frozen	Eluded liquid	126	93	Chile	1996
Chicken carcasses	After storage[a]	Carcass rinse	80	28	USA	1995
Turkey carcasses	Before chilling	Swabs	236	3	USA	1990

Table 2 (Continued)

Product	Stage of process	Sample type	Total Number of samples	% of positive samples	Country	Year
Turkey breasts	At retail	Meat (25g)	30	20	Italy	1996
Turkey meat	At retail	Not specified	311	25	Denmark	1997
Duck carcasses	Before chilling	Swabs	200	48	USA	1990
Goose carcasses	Before chilling	Swabs	200	38	USA	1990
Poultry[b] meat	At retail	Not specified	285	26	Denmark	1997

Source: Adapted from Jacobs-Reitsma (2000)
[a]Storage at 4°C for 10 days
[b]Ducks, pigeons, quails and ostriches

of *Campylobacter* and complicates the in-plant control. Scalding step, necessary to facilitate defeathering, at 55 to 60°C for several minutes helps to reduce the amount of *Campylobacter* numbers on skin surface. On the other hand, high temperature in scalding step affects the skin surface to attach firmly of bacteria. The step of evisceration frequently initiates some leakage of intestinal contents and followed with contamination of carcasses and offal. Although the next step of washing and chilling tend to reduce some amount of bacteria but it cannot eliminate them. Freezing of the products reduce the level of contamination, but some bacteria can survive at –20°C for three months. Overall slaughtering process can reduce approximately of contamination 1000 fold (Jacobs-Reitsma, 2000).

Table 2 summarizes the prevalence of *Campylobacter* in poultry products in many studies from 1989-1998. Each study is different in method and sampling procedure which may be effected with the isolation percentage but most of the results show the high percentages of *Campylobacter* contamination in poultry. This is helpful to realize the high risk for the handling, preparing and consumption of them.

5.2 Cattle, pigs and sheep

Cattle have been implicated in many outbreaks of *Campylobacter* in human via consumption of unpasteurized milk, meat and offal. Unpasteurized milk has frequently caused many outbreaks of campylobacteriosis. *Campylobacter* can be isolated from feces of healthy cows which is present in the intestinal tract and living like commensal organism. In general, numbers of thermotolerant *Campylobacter* organisms in cattle were about 10^2 cfu/g of fresh feces and this is much lower than in poultry which is 10^7- 10^9 cfu/g. The colonization in dairy herd has been associated with unchlorinated drinking water. The colonization of *Campylobacter* in young animals are found more than older animals and grazing animals carry bacteria less often than feedlot cattle (Jacobs-Reitsma, 2000; McClure *et al*., 2002).

Milk is contaminated by exposure to fecal material or mastitis infection. Unpasteurized goats' milk may transmit *Campylobacter* infection to humans as well as raw cows' milk. In general, milk-borne infection can be controlled by proper pasteurization and prevent recontamination after heat treatment. Cattle as a meat source are not a major part of transmission of *Campylobacter* to human. Even though *C. jejuni* can be excreted from feces of cattle, followed with contamination on carcasses during processing in slaughterhouse but the contaminants are normally low (Stern and Line, 2000). Offal of pigs, cow and sheep in UK from table 3 show the high percentages of *Campylobacter* (47%) which are the source for campylobacteriosis in human particularly in countries where people prefer to eat them. *Campylobacter* does not cause problems in food with high solute concentrations such as cheese and also fermented milk products, for instance, yogurt

because of low pH and the sensitivity of the organisms to lactic acid (Jacobs-Reitsma, 2000).

Pigs are frequently colonized with *C. coli* and without evidence of disease. In one study, *Campylobacter* spp. can be isolated from 70 to 100% of pigs on a pig farm which predominantly with *C. coli* over *C. jejuni.* Both species have concentration ranging from 10^3 to 10^7 cfu/g of caecal content (Harvey *et al*., 1999). Serotypes of *C. coli* isolation from swine are not always the same serotype in humans. The slaughtering process of swine, cattle and sheep can be contaminated from intestinal content to meat but less frequent than in poultry. As the evisceration process in poultry slaughtering is often in mechanical and proceeds at a very high turn over rate. In spite of the high percentage of *Campylobacter* in cattle, sheep and pigs after slaughtering but overnight forced-air chilling of the carcasses causes the reduction of the number of *Campylobacter* (Jacobs-Reitsma, 2000; Altekruse and Swerdlow, 2002).

Table 3 summarizes the results of many studies on the presence of *Campylobacter* in meat and milk products. Due to different methods used which may contribute to the differences in isolation rate in various studies, these data should be interpreted with care. Cooked meat products and pork sausages usually does not detect *Campylobacter* if using the sufficient heating of raw material. However, small number of *Campylobacter* contamination may be from cross-contamination from raw-meat products. Dogs and cats which harbor *Campylobacter* in their intestinal tracts may serve as important reservoirs for human infection. Moreno *et al*. (1993) reported the isolated *Campylobacter* 34% from healthy dogs and 66% from healthy cats. *Campylobacter* are isolated from kennel population higher than from household pets and isolated more frequent in young puppies and young kitten than in mature dogs and cats (Stern and Line, 2000). *C. upsaliensis*, *C. jejuni* and *C. coli* have been detected from fecal samples of domestic and stray dogs in Australia at a rate 34%, 7% and 2% respectively (Baker *et al*., 1999). *C. jejuni* were isolated 13% from scouring dogs in Hungary and also isolated 27% *Campylobacter* spp. from dogs with diarrhea in Norway (Varga *et al*., 1990; Sandberg *et al*., 2002).

Table 3 Presence of thermotolerant *Campylobacter* in meat and milk products.

Product	**Stage of process/ Description**	**Total Number of samples**	**% of positive samples**	**Country**	**Year**
Beef carcasses	After chilling	62	10	Belgium	1998
Beef carcasses	After overnight chilling	657	0.3	Australia	1998
Beef	After processing	100	0	N. Ireland	1998
Beef meat	At retail	127	23.6	UK	1989
Pork diaphragm muscles	Before chilling	200	23.5	Canada	1989
Pork carcasses	After chilling	49[a]	2	Belgium	1998
Pork	At retail	158	18.4	UK	1989
Pig livers	At slaughter	400	6	N. Ireland	1998
Pork sausages	At retail	42	2.4	Italy	1996
Lamb	At retail	103	15.5	UK	1989
Sheep	After chilling	465	3	Australia	1999
Offal[b]	At retail	689	47	UK	1989
Raw meat products[c]	At retail	2,330	0.6	UK	1998
Cooked meats	At retail	86	2.3	UK	1989

Table 3 (Continued)

Product	Stage of process/ Description	Total Number of samples	% of positive samples	Country	Year
Raw cows' milk	292 farm bulk tanks	292	12.3	USA	1992
Raw cows' milk	Dairy bulk tanks	130	1.5	Poland	1996
Raw cows' milk	At retail	985	5.9	UK	1988
Raw goats' milk	Various sources	2,477	0.04	UK	1985

[a]Each sample was a composite of gauze swab samplings of 5 half carcasses
[b]Liver, kidney and heart from pigs, cows, or sheep
[c]Sausages and hamburgers
Source: Adapted from Jacobs-Reitsma (2000)

5.3 Pets and other animals

Exotic pets such as golden Syrian hamster, wild rodent and numerous zoo animals including ungulate, felines and primates may harbor *Campylobacter*. *C. jejuni* was isolated from intestinal content of rabbit with watery diarrhea and without fever. Domestic ferrets and young mice are also susceptible to infection with *C. jejuni*. Ferrets develop disease like human and mice infection results in reduction of carbohydrate absorption but no symptom of illness. Wild rodent and flies which carry *Campylobacter* could be important vectors in contamination of poultry house (Stern and Line, 2000; Boxall, 2005).

5.4 Water and seafood

Campylobacter in surface water may be a source of disease outbreak in human and farm animal contamination. The organism could be isolated from streams, seawater and other recreational water. Generally, this water is not for human consumption but sometimes accidental ingestion of a significant amount of water could occur as they can mix with community water system which is already treated with chlorine. Contaminations of *Campylobacter* in surface water are caused by fecal contamination of wild birds or domestic animals or from sewage effluent (Jacobs-Reitsma, 2000). Contaminated drinking water of VBNC forms of *Campylobacter* is a possible role in the large number of waterborne gastroenteritis outbreaks. Differences in the survival of *C. jejuni, C. lari* and *C. coli* in water and their differing incidence in the source of water contamination (*e.g.* birds, sewage effluent) are reflected in the isolation rate from surface water (McClure *et al*., 2002).

Due to the existence of *Campylobacter* in marine environment, many species of marine animals may become contaminated by the organism. Consumption of raw clams has been described as a cause of human campylobacteriosis and different types of seafood could detect *Campylobacter*. Gulls are often colonized with *C. lari* which are from the seafood product contamination (Jacobs-Reitsma, 2000). In a Dutch study, *Campylobacter* was isolated 69% from mussels and 27% from oysters while in British study, *Campylobacter* was found in 47% cockles, mussels and scallops (Wilson and Moore, 1996; Endtz *et al*., 1997). Shellfish beds located near sewage effluents, farmland runoffs and waterfowl reservoirs were reported to be a health risk to persons consuming raw oysters. Furthermore, depuration of oysters cannot fully eliminate *Campylobacter*, therefore, thermal processing of shellfish is generally recommended (Jacobs-Reitsma, 2000).

6. Epidemiology

Campylobacter is one of the most commonly reported causes of human bacterial gastroenteritis in many countries around the world. Variation in incidence rates in many countries may be due to differences in infection rate of animal, different patterns of food consumption and differences in food production system. Furthermore, variations in incidence rates can occur from differences in diagnosis, reporting systems and cases definition of surveillance systems in each country (Friedman *et al*., 2000). The patterns of human campylobacteriosis epidemiology are numerous sporadic, individual infections and occasional large outbreaks. Many studies have shown the close relation of *C. jejuni* serogroup isolated from humans and poultry sources. Some studies in the U.S. reported the risk factor of this disease which is from eating undercooked or raw chicken, handling raw chicken and poor kitchen hygiene. *Campylobacter* from swine which typically *C. coli* do not belong to the serogroup from humans. For that reason, pigs are not the major source of the disease in human. However, cattle are an important source of milkborne campylobacteriosis in human but their meat products are not strongly concerned (Stern and Line, 2000).

In many developed countries, including the U.S., the UK, the Netherlands, France, Sweden and Australia, *Campylobacter* is the major cause of acute infectious diarrhea. In 2005, a total of 200,122 cases of campylobacteriosis were reported from 22 EU member states and two non-member states. The overall incidence was 51.6 per 100,000 population. The overall EU incidence in 2005 represents an increase by 7.8% when compared to 2004 (EFSA, 2006b). The estimated incidence rates in many developed countries are shown in Table 4. Nevertheless, the true incidence of the disease should have been higher in general population due to small proportion of people with gastroenteritis request to consult with physician. *Campylobacter* affects all age groups but in bimodal age distribution, the first peak in children below 5 years and the second peak in young adult between 15 to 44 years. The indcidence is 1.2 to 1.5 times higher in males than in females. In developing countries *Campylobacter*

Table 4 Comparison of reported campylobacteriosis incidences between countries.

Country	**Period**	**Rate/100,000**	**Reference**
UK	2005	88.5	EFSA, 2006b
Germany	2005	75.3	EFSA, 2006b
Czech Republic	2005	302.7	EFSA, 2006b
The Netherlands	2005	46.2	EFSA, 2006b
France	2005	3.3	EFSA, 2006b
Sweden	2005	66.2	EFSA, 2006b
USA	2004	12.8	CDC, 2006
Canada	2000	40.1	Lake *et al*., 2007
Australia	2003	116.5	Lake *et al*., 2007
New Zealand	2005	370.3	Lake *et al*., 2007

infection mostly occurs in children less than 5 years of age and the prevalence of infection is greater than in developed countries (Friedman *et al*., 2000; Stern and Line, 2000).

The outbreaks of campylobacteriosis mostly occur from consumption of raw unpasteurized milk or contaminated milk after pasteurization. However, the vast majority of the disease is not related to outbreak but happens as sporadic individual infections. Most sporadic cases of *Campylobacter* infections are associated with consumption foods of animal origin especially poultry and high incidence in a summer time. The sources of sporadic infections and individual cases are transmitted from pets and other animals and contaminated drinking water. Person-to-person transmission of *Campylobacter* was demonstrated that transmission from ill children to family members occurred frequently. Perinatal transmission is also possible but rare. Campylobacteriosis in pregnant women with bacteremia may pass on a severe

systemic infection to fetus (Friedman *et al*., 2000; Stern and Line, 2000). In Switzerland, a case of maternal sepsis from *C. jejuni* was found the organism on placental tissues by using molecular methods (Meyer *et al*., 1997).

Direct animal contact for transmission of *Campylobacter* in humans can occur among people living in farming community and those employed in poultry and red meat plants. There are many reports in high incidence of campylobacteriosis in humans who have occupational exposure in farm animals such as poultry and cattle. Serological surveys demonstrated people work in poultry and red meat slaughter houses have a higher rate of seropositivity to *Campylobacter* antigen than rural field laborers. Similar bio- or serotypes of *Campylobacter* are frequenltly isolated from pet owners and their pets such as dogs and cats. Puppies and kitten are commonly a higher risk factor than adult animals (Stern and Line, 2000).

From the end of nineteen century, antimicrobial resistance to *Campylobacter* infections in humans has been reported in Europe and Asia. Fluoroquinolones, such as ciprofloxacin, are frequently used for treatment of campylobacteriosis and fluoroquinolone-resistant *Campylobacter* found in many countries, *i.e.* the U.S, Europe. During 1989 to 1990, a survey in 19 U.S. countries was reported no fluoroquinolone-resistant *C. jejuni* or *C. coli* strains (Friedman *et al*., 2000). Until 1997, 217 samples of *Campylobacter* from five states in the U.S. were tested for antimicrobial susceptibility. The isolated 86% were resistant to one or more antimicrobial agents, 50% were resistant to two or more antimicrobial and 37% were resistant to two quinolone antibiotics (Centers for Disease Control and Prevention [CDC], 1997). In Europe, there are reports suggesting the fluoroquinolone-resistant *Campylobacter* in human is associated with fluoroquinolone used in poultry. Veterinary use of fluoroquinolones in poultry in the U.S. since 1995 may explain about fluoroquinolone-resistant *Campylobacter* in human in recent years. On the other hand, the use of fluoroquinolone is prohibited in Australia and a study reported in 1997 revealed that none of 98 *C. jejuni* or *C. coli* resistant to fluoroquinolones (Huysmans and Turnidge, 1997).

7. Detection, isolation and identification

Campylobacter is typically fragile bacteria that are difficult to culture and maintain in laboratory. Therefore, food samples must be held under suitable condition by using enrichment methods, microaerophilic condition and proper incubation time. Media originally used for the isolation of the organism from feces were used and subsequent modification have been required to isolate from meat, food and water from environment. Direct plating method can be used for poultry samples which may harbor sufficiently large numbers of bacteria but most food and environmental samples still need enrichment broth for detecting small numbers of *Campylobacter*. Some of them need particular conditions for their recovery. The food samples should be covered for exposure to air, transported under temperature 0-4°C and analyzed immediately after the collection of samples. *Campylobacter* is preferred in microaerophilic condition at 5% O_2, 10% CO_2 and 85% N_2.

There are many different enrichment broths and selective plating media for *Campylobacter*. This media usually use a basal medium consisting of beef extract, yeast extract, peptone and NaCl which addition with lysed or laked horse or sheep blood for protecting the toxic effect of oxygen derivatives. Some media have charcoal instead of blood. The other essential chemicals for aerotolerance of bacteria in food samples are supplements with ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP supplement). Media commonly used with antibiotics including cefoperazone, amphotericin B, polymixin B, cycloheximide, rifampicin, trimethoprim lactate and vancomycin (Stern and Line, 2000; McClure *et al*., 2002).

Pre-enrichment could overcome damage to *Campylobacter* cell caused by drying, starvation, heating and freezing and/or oxygen radicals. Many approaches have been taken to avoid the toxic components in the media on sublethally injured cells by a delay in the addition of antibiotics and preliminary period of incubation at reduced temperature. The 4 h pre-enrichment time at 37°C rather than 42°C could help the recovery of low levels of *C. jejuni* and prevention of the outgrowth of competitive flora (Uyttendaele and Debevere, 1996). A delay of 4-8 h before adding

antibiotic to broth could increase the isolation rate of *Campylobacter* in contaminated river water sample compare with direct culture in selective broth (Mason *et al*., 1999). There are many pre-enrichment broths for *Campylobacter* but five broths in particular seem to be in common use: Preston broth, Exeter broth, Bolton broth, CEB broth (*Campylobacter* enrichment broth) and Park & Sanders broth (Donnison, 2003). Several agars have been developed for the isolation of *Campylobacter*. Three of the most commonly used agars are CCDA, Preston agar and Butzler agar. In comparison, the detection of *Campylobacter* in manually shelled egg samples and raw meat samples were seen no substantial difference among these agars (Zanetti *et al*., 1996).

There is an international standard method for detection of thermotolerant *Campylobacter* in food and animal feeding stuffs (International Organization for Standardization [ISO], 1995). This method is recommended for enrichment in either Preston broth or Park and Sanders broth. The incubation time at 42°C in a microaerophilic atmosphere for 18h is used for Preston broth. For Park and Sanders broth, the initial suspension is incubated at 32°C for 4h, then added with antibiotic solution and incubated at 37°C for 2h followed by 42°C for 40-42h in a microaerophilic atmosphere. The enrichment broth cultured are streaked on Karmali agar and a second selective agar from one of these following Preston agar, Skirrow agar, Butzler agar and CCDA. Agar plates are incubated at 42°C for 24-48h in a microaerophilic atmosphere and confirmatory tests on characteristic colonies. Typical colonies on selective agar media are smooth, convex and glistening with a distinct edge or flat, translucent, shiny, and spreading with an irregular edge. They are colorless to light cream or grayish with diameter range from pinpoint to 5 mm.

Presumptive *Campylobacter* isolated can be observed using phase-contrast or dark-field microscopy for characteristic morphology and motility. However, cells from culture more than 24 hours may appear in coccidal form and nonmotile (Stern and Line, 2000). Biochemical and growth parameter test (Table1) are used to identify *Campylobacter* species. A commercial kit (API campy) is available for differentiation of *Campylobacter* spp. by using standard biochemical tests but it can

be problematic in the variability and atypical reactions of some strains (Phillips, 2001).

Many rapid methods have been developed for isolation and detection of *Campylobacter* in recent years. Some of them have been evaluated for using in foods but very few of these methods were developed for commercial products. Due to the fact that the food industry is not doing a lot of *Campylobacter* testing, the market of test kits is still small. The explanation for the lack of testing by the food industry may be from following reasons: the organism does not grow in food under normal storage condition; it does not survive well and easily controlled in processed foods; it is prevalent in raw foods which must control by consumer preparation; and the organism is fastidious and difficult for the detection and maintenance in laboratory (McClure *et al*., 2002). Nevertheless, there are numerous methods which are available and developing for detection and identification of *Campylobacter* such as latex agglutination test, polymerase chain reaction (PCR) technique, enzyme-linked immunosorbent assay (ELISA) and immunomagnetic separation (IMS) method.

7.1 Latex agglutination test

Latex agglutination tests for *Campylobacter* have been available for many years. The tests are used for confirmation of presumptive isolated of *Campylobacter* with convenience and ease of observing the result of serological agglutination. The concentrations of cells ranging from 10^6 to 10^8 cfu/ml are needed for agglutination and can detect non-cultural coccoid forms of *Campylobacter* in a similar sensitivity. Latex agglutination tests have been applied to pre-enrichment of samples in broth culture before doing agglutination for improving the speed of this method. The Microscreen® *Campylobacter* latex kit has been used by filtration and pre-enrichment with charcoal cefoperazone desoxycholate (CCD) broth for detection in fresh, frozen raw meat and water samples (McClure *et al*., 2002).

7.2 PCR assays

The PCR method is a rapid method for detection and identification of many bacterial pathogens. PCR assay is a relatively rapid, sensitive and specific method to determine *Campylobacter*. In the so-called multiplex mode, PCR may not only identify *Campylobacter*, but may also discriminate simultaneously between different species of *Campylobacter*, including *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* subsp. *fetus* and *C. upsaliensis* (Wang *et al*., 2002).

The gene targets such as *23S* rRNA gene sequences, *16S* rRNA gene, the *ceuE* gene, the *mapA* gene, the GTPase gene and the flagellin (*flaA* and *flaB*) gene have been described in several assays for *Campylobacter* identification. Linton *et al*. (1997) used PCR detection of *C. jejuni* and *C. coli* direct from stool by designing three sets of primers; *16S* rRNA, hippuricase gene sequence and aspartokinase gene. GTPase gene has been used for discrimination among four thermophilic *Campylobacter* spp.; *C. jejuni*, *C. coli, C. lari* and *C. upsaliensis* (van Doorn *et al*., 1997). *23S* rRNA and *hipO* were selected for identification and differentiation of *C. jejuni*, *C. coli, C. lari*, *C. fetus* subsp. *fetus* and *C. upsaliensis* from clinical and environmental samples and the sensitivity range of the colony mPCR in number of cfu/ml was 10^8 to 10^{13} for *C. jejuni*, 10^6 to 10^{13} for *C. coli* and *C. upsaliensis* 10^7 to 10^{13} for *C. lari* and 10^2 to 10^{13} for *C. fetus* subsp. *fetus* (Wang *et al*., 2002). Two *Campylobacter* flagellin genes, *flaA* and *flaB*, were performed in the seminested PCR for detection of small numbers of *C. jejuni* and *C. coli* in environmental and food samples. The assay was detected *C. jejuni* 3 to 15 cfu per 100 ml in water samples and also detected ≤ 3 cfu/g of food with overnight enrichment (Waage *et al*., 1999). Denis *et al*. (1999) developed three set of primers; *16S* rRNA, *ceuE* gene and *mapA* gene for simultaneous identification of *C. jejuni* and *C. coli* in poultry fecal samples. The efficiency of the identification of both species by the mPCR assay is 100% efficiency compared to 34% with biochemical test.

The PCR assay was used to determine *Campylobacter* contamination in chicken production from farm to consumers. In France, Denis *et al*. (2001) used a

PCR assay for detection of *C. jejuni* and *C. coli* from bird dropping in poultry houses, neck skins, livers, hearts, gizzards, wings, legs and escalope from slaughterhouses and supermarkets. *Campylobacter* was detected in 79.2% of poultry houses. Form 303 samples, 201 (66.3%) were *Campylobacter* positive which included 17.5% supermarket samples, 5.6% slaughterhouses and 43.2% fecal samples. The sensitivity was 5 cfu/g of samples and 1.5×10^3 cfu/ml of Preston pre-enrichment broth.

Mateo *et al*. (2005) also used three sets of primers; *16S* rRNA, *ceuE* gene and *mapA* gene for simultaneous identification of *C. jejuni* and *C. coli* which developed by Denis *et al*. (1999) in retail poultry products. Seventy-three samples were examined using both conventional culture and molecular methods. The total of 54 samples were *Campylobacter* positive by PCR but 50 samples were positive by conventional method. Two of 64 concordant result samples were considered to be false-negatives by conventional method because retesting by PCR were *Campylobacter* positive. The detection limit of the PCR method was that 5 CFUs corresponded to 0.2 CFUs per 5 µl PCR mixture.

The disadvantage of PCR assay is the presence of inhibitors of amplification in food that require effective clean-up methods to overcome possible interference (Denis *et al*., 2001). Many of food samples and enrichment media such as charcoal and iron are inhibition of PCR and decreasing its capacity as well. Some preparation methods have been used to overcome this inhibition, *e.g.* heat treatment at 96°C, buoyant density centrifugation but some method using Oxyrase™ to enhance the growth of *C. jejuni* (Sails *et al*., 2001; McClure *et al*., 2002).

7.3 ELISA

ELISA, usually combined with PCR method, has been developed to reduce the time required for detection and identification of *Campylobacter*. The method could facilitate specific and sensitive detection of PCR amplification products by increasing throughput. Sails *et al*. (2001) investigated five biotin-labelled probes targeted to detect digoxygenin-labelled PCR products from *C. jejuni* and *C. coli* by

using the PCR ELISA assay. The sensitivity was demonstrated to be 10-100 fold more sensitive than a gel-based PCR method and can be completed in 7 h.

Another research to compare the automated ELISA and PCR method which included a sample preparation method based on Buoyant Density Centrifugation (BDC) was evaluated for detection and identification of *C. jejuni* and *C. coli* in poultry products. The automated ELISA could easily be repeatable and reliable method and take minimum 2.5 working days, while BDC-PCR method requires 3 working days (Lilga and Hanninen, 2001).

7.4 IMS

IMS is widely used to separate and concentrate target bacteria from food sample by removing components of food matrix which may interfere with analysis (Brovko *et al*., 2004). For *Campylobacter*, IMS could reduce pre-enrichment time and may overcome the problems of inhibitors from food sources and therefore increase the sensitivity of downstream detection methods including polymerase chain reaction, ELISAs and electrochemiluminescence. IMS using magnetic beads coated with immunoglobulins was exploited to isolate and concentrate *C. jejuni* from poultry which leaves cells viable, and reduces the time of the pre-enrichment step. A short pre-enrichment step is needed to improve the sensitivity and overcome the problems of inhibitors from the food sources (Yu *et al*., 2001).

Many researches were used IMS combination with PCR for detection *Campylobacter* in milk, chicken, feces and food. An IMS and PCR assay was developed for the detection of *C. jejuni* in milk and chicken products. The bacteria were captured from the food samples by magnetic particles coated with specific immunoglobulins and the bound bacteria were lysed and detected by PCR. For chicken meat, this method could detect 420 cfu/g after 18 h enrichment, 42 cfu/g after 24 h and 4.2 cfu/g after 36 h. For contaminated milk, 63 cfu/g could be detected after 18 h enrichment and 6.3 cfu/g after 36h (Docherty *et al*., 1996). Without an enrichment step, an immunocapture PCR method for detection of *C. jejuni* in foods

can be performed in 8 h and one cell of the organism in a milliliter sample can be detected (Waller and Ogata, 2000).

Immunomagnetic capture-fluorescent PCR assay for *C. jejuni* in foods and water samples without pre-enrichment step can be performd in 8 h with a detection limit of 10 cfu/ml. This method also could detect *C. jejuni* of VBNC state (Liu *et al*., 2006). Lund *et al*. (2003) isolated *Campylobacter* spp. from chicken feces by using magnetic beads followed by PCR and the result could be obtained in <6 h. The detection limits was 36 cfu/ml and the diagnostic specificity was 0.99.

Some researchers used IMS and coupled with non-PCR method for detection *Campylobacter* such as IMS followed by plating and IMS in combination with immunoassay. Yu *et al*. (2001) used IMS procedures followed by plating to Karmali agar to concentrate *C. jejuni* from poultry meat. Without pre-enrichment step, this approach could detect 10^4 cfu/g in poultry meats. The detection limit of tosylactivated and streptavidin magnetic beads in pure culture *Campylobacter* suspension was 10^4 cfu/ml and 10^3 cfu/ml, respectively. IMS in combination with enzyme-linked immunoassay coupled with enzyme electrode to detect *C. jejuni* had a detection limit of 2.1×10^4 cfu/ml in pure culture samples and chicken carcass samples. All steps of this method could be completed within 2.5 h. Three blocking reagents (Bovine serum albumin, Casein and Tween-20) were tested to minimize non-specific binding. Bovine serum albumin showed the best blocking capacity in the range of 10^3-10^5 cfu/ml *C. jejuni* contamination (Che *et al*., 2001).

8. Molecular subtyping

The subtyping of *Campylobacter* spp. is an important requirement for epidemiological studies to trace back the source and route of transmission. It also identify and monitor the temporally and geographically strains of the organism with important phenotypic characteristics as well as to develop strategies to control *Campylobacter* in the food chain. In the previous case, the phenotypic methods, such as phage typing and serotyping are the chosen methods. However, the major

disadvantages of phenotyping are the high number of untypeable strains, time-consuming and technically demanding requirements of the techniques.

The genetically base methods called genotyping have been developed for enhancing sensitivity and discriminatory power and improving availability. The major advantage of genotyping techniques is universally available and some techniques have been used in some laboratories such as ribotyping, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), PCR-RFLP (restriction fragment length polymorphism) and flagellin typing (*fla* typing) (Wassennaar and Newell, 2000). Although, many genotyping techniques of *Campylobacter* spp. are currently available, they are different in discriminatory power, typeability, reproducibility, sensitivity to genetic instability, time, cost and availability (Table 5).

Genotyping techniques are more useful for investigating sources of infection and transmission route of animal and humans. Most methods of genotyping can reveal the enormous diversity of *C. jejuni* and *C. coli* in host and environment such as human campylobacteriosis, pig, poultry, slaughterhouse, seawater and sewage plants. In the future, genotyping will be used for identifying lineages strains which are more or less virulent or correlation with hosts (Wassenaar and Newell, 2000).

8.1 Serotyping

Serotyping for *Campylobacter* was developed in Canada in 1980s. There are two generally accepted and well-evaluated serotyping schemes. First, the Penner scheme is based on using heat-stable (HS) antigens by a passive hemagglutination technique using soluble antigen extracts of isolates and specific antisera raised to the antigens of *Campylobacter*. Second, the Lior scheme is based on heat-labile (HL) antigens by a bacterial agglutination method using live bacteria together with unabsorbed and absorbed antisera. The problems of both techniques are not only the high non-typeability strains of *Campylobacter* but also the production and quality control of antiserum are costly and not widely available. The Penner scheme was

developed by the Laboratory for Enteric Pathogens, Central Public Health Laboratory, UK. This serotyping assay based on the same principle of Penner scheme and still detected HS antigen but did not use a passive hemagglutination technique. The antigens were detected by direct bacterial agglutination of heated suspension and using specific antisera in microtiter plates. This developed scheme may be an improvement for routine use but still does not solve the problem of restricted reagent availability and the high level of non-typeability. With this serotyping scheme, 19% of human isolates were non-typeable, as same as the studies using the original Penner scheme typeability of human and veterinary strain is less than 20% (Newell *et al*., 2000; Wassenaar and Newell, 2000; Woodward and Rodgers, 2002).

Although there are some problems of serotyping, it has led to the identification of potentially unique strains of *C. jejuni* associated with GBS and Miller-Fisher syndrome (MFS) patients. Many studies have shown the lipopolysaccharides extracted of *C. jejuni* in particular O:14 and O:19 mimic human gangliosides in their structure. In the Penner scheme, O serotypes of GBS patients include O:1, O:2, O:4, O:4 complex, O:5, O:10, O:16, O:19, O:23, O:37, O:41, O:44 and O:64. In the Penner scheme and Lior scheme, the serotypes associated with GBS and MFS patients were O19:HL77, O37:HL28, O4,64:HL1, O23,36:HL5, O2:HL4, O4,50:HL7 and O13,65:HL7. The combination method of Penner and Lior schemes may help to identify the strains of *C. jejuni* which are associated with GBS or MFS (Woodward and Rodgers, 2002).

8.2 Phage typing

Phage typing is another phenotypic method developed for *Campylobacter* characterized isolates using bacteriophages. Phage typing was first used for *Campylobacter* in the U.S., England and Wales surveillance. The disadvantage of *Campylobacter* phage typing scheme is the high number of nontypeability and some strains reacted with the phage but do not conform to a designed type (Sopwith *et al*., 2003; Frost *et al*., 1999). Frost *et al*. (1999) identified 57 phage types from 2,407 *C. jejuni* isolated type. Approximately 60% of the strains were assigned to the 10

common phage types. There are 15% nontypeability rate and 7% of strain designated reacted with the phage but do not conform to a designed type. Some researches used the combination of serotyping and phage typing for increasing the level of discriminatory power. However, some isolates were untypable with both serotyping and phage typing. Sopwith *et al*. (2003) identified the strains of *Campylobacter* isolates from sporadic cases in the Northwest of England by using serotyping and phage typing methods. For serotyping, 56 different serotypes were identified but high proportion (25%) of each species being untypable. With combination of serotyping and phage typing, 60 phage types were identified giving 400 different serophage types. Forty-three isolates or 2% were untypable with both phenotyping.

8.3 PFGE

Digestion of bacterial chromosomes by restriction enzymes which cleave the DNA infrequently has proved to be useful for typing of many bacteria. PFGE is a modification from the traditional restriction enzyme analysis (REA) which has been used for bacterial subtyping for many years. PFGE and REA employ restriction site polymorphism in bacterial DNA but there are several differences between both methods. For PFGE, bacterial cells are embedded in agarose gel and lysed in situ to prevent DNA shearing. After washing to remove contaminating chemicals, thin slice of the DNA-containing blocks are cut and bacterial chromosomes are digested by restriction enzymes. Because the aim is to cut the DNA fragments comparatively large fragment and relatively few, therefore, rare cutting enzymes are used such as *Sma*I, *Sal*I and *Kpn*I which using for *Campylobacter*. Although the various fragments obtained are generally very large (20 to 200 kb), they can be separated upon the size of bacterial fragments by using special electrophoretic method. Variations in the presence of relevant restriction sites are referred to macrorestriction profile or genotypic profile. The DNA fragments are gently oriented and separated according to their sizes within the agarose gel matrix by the coordinated application of pulsed electric fields from different positions in the electrophoresis cell. DNA-restriction fragment patterns are visualized after staining in ethidium bromide. Any plasmids in *Campylobacter* spp. may be detected by PFGE accompanied by the chromosomal

DNA. The sensitivity of PFGE depend on whole genome restriction site polymorphism and the differentiation of strains are easier to determine when compared with the complex patterns of REA. PFGE was initially used to determine the genome size of *C. jejuni* and the technique was later adapted for *C. coli, C. hyointestinalis*, *C. fetus* and *C. upsaliensis* (Newell *et al*., 2000; Wassenaar and Newell, 2000).

Comparisons of the discriminatory power of PFGE with other typing methods in *C. jejuni* and *C. coli* consistently show extremely sensitive except AFLP which appears to be equal discriminatory capability with PFGE. PFGE typing appears to be at least twice as discriminatory as ribotyping with two to three times more sensitive than *fla* typing method (Newell *et al*., 2000). PFGE has been used to group *C. jejuni* isolates from poultry, cattle and human, and genotype dynamics in a broiler flock and slaughterhouse. Saito *et al*. (2005) found a possible link between sporadic human campylobacteriosis and *C. jejuni* from retail poultry and bovine bile and feces by using PFGE and serotyping method. Hook *et al*. (2005) investigated the genotype diversity and dynamics of *C. jejuni* in a commercial broiler flock during rearing and slaughter which were subtyped by PFGE. The results show multiple genotypes and genetic diversity of *C. jejuni* in both flock and slaughterhouse. There is no indication that any subtype excluded another during the rearing of the broiler flock.

Although the discriminatory power of PFGE profiling is good, some disadvantages are recognized. There have been inconsistent reports of *Campylobacter* which cannot be typed by PFGE. Because the DNase production of some *Campylobacter* strains can degrade DNA samples which may be overcome by formaldehyde treatment. The PFGE conditions vary in different studies. Differences in electrophoretic conditions are apparent dissimilarity in the profiles obtained even from the same DNA preparation. The restriction enzymes used for digestion of chromosomal DNA are also different in different studies. Interpretation of the results can be difficult from genetic instability during in vitro culture which can lead to minor or major changes in profiles (Newell *et al*., 2000; Wassenaar and Newell, 2000).

8.4 *fla* typing

The characteristic motility of *C. jejuni* is due to its having a single unsheathed polar flagellum at one end or both ends of the cell. The flagellar filaments are composed of repeats of a flagellin subunit which is encoded by a *fla* gene. The flagellin locus of *Campylobacter* contains two flagellin genes, designated *flaA* and *flaB*. These two flagellin genes are tamdemly arranged and separated by 170 nucleotides of an intervening segment. Normally, these genes are highly conserved (92% identity between *flaA* and *flaB* genes) in individual isolates. Nevertheless, the *fla* genes still vary between isolates which provides the basis of *fla* typing method. Because both variable regions and highly conserved are present, this locus is appropriate for restriction fragment length polymorphism (RFLP) analysis of a PCR product. In RFLP technique, primers can be synthesized based on the conserved sequences and a product incorporating both the conserved and variable regions of *fla* genes can be prepared by PCR. This product is digested with restriction endonucleases to reveal RFLP product after running by gel electrophoresis. The selection of restriction endonucleases enzymes has a significant effect on discrimination. *Dde*I is the best discrimination at least for veterinary isolates and the combination of *Dde*I and *Hin*fI can enhance the level of discrimination. The level of discrimination of *fla* typing appears to be much greater than that of serotyping but lower than that of PFGE (Newell *et al*., 2000; Wassenaar and Newell, 2000).

One of the most significant disasvantages of *fla* typing is genetic instability of *Campylobacter*. There are strong evidences of *C. jejuni* strains for intragenomic between *flaA* genes of different strains and intergenomic recombination between *flaA* and *flaB* genes of individual strains. Under natural condition, the frequency of such event is unknown but in long-term typing method *fla* typing cannot be particularly stable. Therefore, the interpretation of *fla* types should be undertaken with care and may need to compare with the alternative genotypic methods (Newell *et al*., 2000).

Table 5 Disadvantages and advantages of serotyping and genotyping methods for *Campylobacter* subtyping.

Method	Discriminatory power	Typeability (%)	Reproducibility	Sensitivity to genetic instability	Time	Cost	Availability
fla typing	Reasonable	100	Good	Yes	<1 day	Low	Good
PFGE	Good	100	Good	Yes	3-4 days	Average	Limited
Ribotyping	Poor	100	Good	Yes	3-4 days	Average	Complex method
Automated ribotyping	Good	NAV[a]	Good	NAV	8 h	High	Limited
RAPD	Average	80	Low	Yes	<1 day	Low	Good
AFLP	Good	100	Good	No	2-3 days	Average	Complex method
Serotyping	Average	80	Good	NA	<1 day	Low	Limited

[a] NAV, not available; [b]NA, not applicable
Source: Adapted from Wassennaar and Newell (2000)

The *fla* typing has proved to be valuable for the *C. jejuni* subsp. *jejuni* strains, majority of *C. coli* strains and some strains of *C. lari*, *C. helveticus* and *C. jejuni* subsp. *doylei*. Although *fla* typing is a reliable, useful and relatively simple subtyping technique, the variation in the procedure does not allow results obtained in different laboratory to be compared directly. The international standardization of, at least, primers and restriction enzymes used is essential for comparison of interlaboratory. *Fla* typing profiles can be stored in electronic databases so that they can be compared and global studies are possible (Newell *et al*., 2000; Wassenaar and Newell, 2000).

8.5 RAPD

The random amplification of polymorphic DNA (RAPD) method of typing bacteria is based on the use of arbitrary primers to amplify random DNA products under low-stringency PCR conditions. This method uses the entire genome of bacteria to generate amplified fragments. The size of fragments is partly controlled by regulating the stringency of PCR conditions which is manipulated by annealing temperature. Generally, randomly designed 10-mer primers are used under conditions which allow some mismatches to increase the number of primed sites. PCR products are produced with correct opposite orientation of primer which is situated within the amplification distance less than 5 kb. These lengths of PCR products, efficiency of annealing and amplification depend on sites primed. As a result, band patterns consist of weak and strong amplicons which complicate interpretation of the results. The band patterns obtained are appropriate to compare with bacterial isolates within species. Some bands are unique and can be separated into certain groups of microorganisms. The unique bands can be utilized for detection of specific organisms by developing species specific probes to identify *Campylobacter* spp. such as *C. jejuni, C. coli* and *C. lari*. Moreover, RAPD band patterns are used for subtyping and strain comparison. RAPD can provide a level of discrimination not lower than that of PFGE (Newell *et al*., 2000; Wassenaar and Newell, 2000).

Although RAPD is much cheaper than PFGE and less sensitive to genetic instability than *fla* typing, the major disadvantage of this method is poor reproducibility. This problem has largely limited to the widespread application of RAPD for *Campylobacter* subtyping. Minor differences in band patterns can be observed with both duplicated samples and can affect the interpretation of data. The causes of lack of reproducibility are multiple, *i.e.* inconsistencies in thermal cyclers, template purity and procedures have been implicated as possible causes. Nowadays, recent technology to control the thermal cyclers, standardized the procedure and the commercial of prepared reagents are now available. Nevertheless, the diversity of procedures and difficulties in interpretation of weak bands will not widespread acceptance of this technique (Newell *et al*., 2000; Wassenaar and Newell, 2000).

8.6 Ribotyping

The presence of multiple copies of the rRNA genes (coding for 5S, 16S and 23S rRNA) at different position on the chromosome, the strong conservation of regions in the rRNA genes and the presence of highly variable (noncoding) flanking regions make them suitable for subtyping purposes. The most commonly used technique in Southern blot hybridization of genomic DNA which is digested with restriction enzyme and hybridization with a probe specific for rRNA genes. This technique results in a high level of typeability in many bacteria but most *Campylobacter* spp. contain only three ribosomal gene copies. The discriminatory power of this method is limited. Nevertheless, ribotyping has been used successfully for subtyping in *C. jejuni*, *C. coli, C. helveticus*, *C. lari*, *C. upsaliensis* and aerotolerant *Campylobacter* spp. but it cannot distinguish between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* or differentiate strains within these subspecies. Generally, this method seems to be useful in *Campylobacter* spp. which is difficult to analyze phenotypically. The restriction enzymes used in ribotyping for chromosomal digestion are *Pst*I, *Hae*III, *Hin*dIII and *Pvu*II. They have been used alone, in pairs or in combination with three enzymes. Consequently, the differences in the restriction enzymes and probe used hamper ribotyping comparison between laboratories (Newell *et al*., 2000; Wassenaar and Newell, 2000).

The low discriminatory power of ribotyping and tedious nature of the technique make it unsuitable for routine *Campylobacter* subtyping. However, there are two improvements to add value to this method. First, rehybridization of the Southern blots with probes in the other hypervariable regions of the chromosome can be used to increase the level of discrimination. The second improvement of ribotyping involves automation, called riboprinting. It can overcome the labor-intensiveness, enhance the reproducibility of this method and provide a unique opportunity for the interlaboratory exchange of data from standard typing system. Nevertheless, the high cost of riboprinting and the low throughput will certainly restrict the use of this technology (Wassenaar and Newell, 2000).

8.7 AFLP

Amplified fragment length polymorphism (AFLP) analysis is a high resolution genotyping method. It was originally developed for genotyping of plants and has been adapted for genotyping bacteria. This method is based on complete digestion of the whole bacterial gene with two restriction enzymes, one with a 4-bp recognition site and the other with a 6-bp recognition site. PCR amplification of the digestion products are designed by adapter-specific primers that have an extension of one to three nucleotides at their 3' ends running into the unknown chromosomal restriction fragment. The adapters must allow ligation compatible with the restriction enzymes used for digestion, eliminate the restriction site after ligation and create a template sequence for subsequent PCR amplification. The PCR primer that spans the average frequency restriction site is labeled with fluorescent. The resultant labeled PCR products are analyzed on denaturing polyacrylamide gel (Figure 1). After polyacrylamide gel electrophoresis, a highly informative pattern of 50 to 500 bands is obtained. The number of bands generated typically 80 to 100 bands can be reduced by incorporating one or more specific nucleotides in the PCR primers adjacent to the restriction site. Therefore, those fragments containing the specific nucleotide adjacent to the restriction site are detected and analyzed. The method can be adapted with many bacterial species but the restriction enzyme and adjacent specific nucleotides

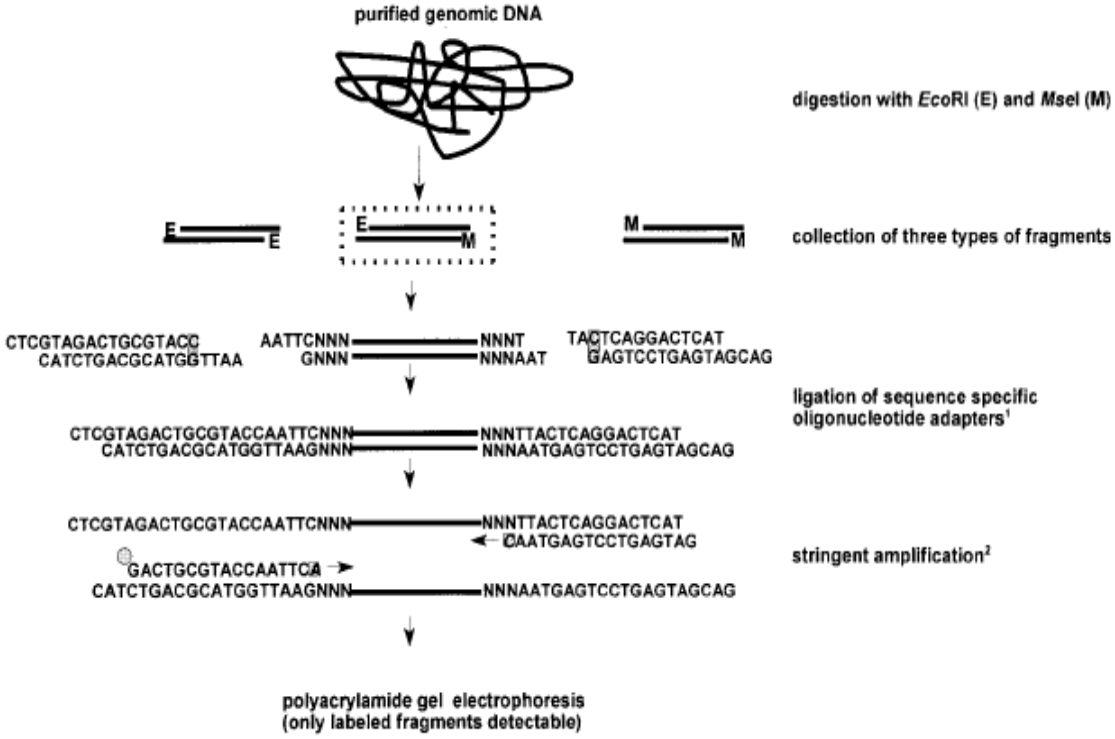

Figure 1 Schematic representation of the principle of AFLP analysis.

[1]point mutations incorporated in the adapter sequences to prevent digestion after ligation are shaded.

[2]one of the primers is labeled. In this presentation both primers contain one selective nucleotide (shaded) in the unknown fragment.

Source: Savelkoul *et al*.(1999)

used should be applied for each species. The value of AFLP analysis in the typing of *Campylobacter* has been reported (Savelkoul *et al*., 1999; Newell *et al*., 2000; Wassenaar and Newell, 2000).

Two AFLP methods which are different in both restriction enzymes have been described for the subtyping of *Campylobacter*. These methods use either *Hin*dIII and *Hha*I or *Bgl*II and *Csp6*I that differentiate selective nucleotides and generating either 50 fragments of 50 to 450 bp or 60 fragments of 35 to 500 bp respectively. The fragments generated in both cases were detected with fluorescently labeled primers and analyzed with an automated DNA sequencer. Both methods give

AFLP banding patterns with good discrimination and unrelated *C. jejuni* and *C. coli* strains show heterogeneous banding patterns. In the same research, AFLP was proved to be more advantage than other techniques such as *fla* typing or PFGE. AFLP analysis can also prevent overinterpretation and misinterpretation from point mutations or single-locus recombinations due to its banding patterns derived from the whole genome (Duim *et al*., 1999; Kokotovic and On, 1999).

The AFLP method was compared with PFGE and restriction fragment length polymorphism analysis on PCR products (PCR-RFLP). It was found that AFLP analysis of *C. jejuni* strains is a rapid method that offers better discriminatory power than PFGE and PCR-RFLP (Lindstedt *et al*., 2000). Duim *et al*. (1999) showed the result of AFLP analysis can distinguish genetically unrelated strains from genetically related strains of *Campylobacter* species. Clusters containing human and poultry *Campylobacter* strains, as well as some strains that were closely related genetically, were also obtained when AFLP analyis was used. Groups of outbreak strain, replicate subcultures and genetically identical strain of *C. coli* and *C. jejuni* from humans, poultry and cattle, proved to be indistinguishable by AFLP, but were differentiated from unrelated isolates (Kokotovic and On, 1999). Moreover, AFLP method was able to identify *Campylobacter* found in veterinary infection at the species, subspecies and strain levels such as *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli, C. helveticus*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. hyointestinalis* subsp. *lawsonii*, *C. mucosalis*, *C. sputorum*, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (Duim *et al*., 2001).

The advantage of AFLP technique is that a random portion of the whole genome is sampled. But the disadvantage is that this technique is complex and requires major capital investment for an automated DNA sequencer. However, the routine use of AFLP has become possible because of increasing automated DNA sequencer. The digitalization of AFLP results is accurate interpretation, simplicity for data storage and ease of data exchange for interlaboratories. Like other molecular subtyping methods, international standardization of procedures is essential to enable the exchange of AFLP data between laboratories. AFLP is the most promising

method for providing reproducible profiles and appears to be insensitive to the genetic instability which makes it less complicated than other molecular methods. Although major capital investment may restrict the use of this method in research laboratories, it seems like that this technique will be used more widely for global epidemiological studies (Newell *et al*., 2000; Wassennaar and Newell, 2000).

Therefore, AFLP is a tool which can be used for epidemiological investigation of *Campylobacter* strain from broiler farms and poultry slaughterhouses. Alter *et al*. (2005) showed the result of AFLP analysis can detect and determine the genotypes of *C. jejuni* strains at different stages of a turkey slaughter line. The genotype revealed a high diversity of *C. jejuni* strains which entered the slaughterhouse via the live turkeys and only some dominant subpopulation survived from environmental stresstors during slaughter and futher processing. Johnsen *et al*. (2007) also examined the occurance, diversity and tramsmission of *Campylobacter* in a poultry slaughterhouse by using AFLP. Genetic fingerprinting by AFLP of the 109 isolates obtained resulted in 28 different AFLP clones. The results strongly indicate that *Campylobacter* infected broilers contaminated the slaughterhouse environment, including the air which may cause a risk for the workers. The laying hen carcasses were infected with low contamination from slaughterhouse environment and together with the freezing of them. Therefore, broilers carcasses are a higher public health risk when compared with laying hens.

MATERIALS AND METHODS

1. Immunomagnetic separation and multiplex PCR

1.1 Bacterial strains and culture conditions

C. jejuni strain ATCC 33291 and *C. coli* strain ATCC 33559 were bought from Microbiologics (St. Cloud, MN, USA) and used as positive control strains. *Campylobacter* were grown under a microaerophilic atmosphere, which was generated by using a gas package (BBL; Becton Dickinson, Sparks, USA). Following multiplication in tryptone soya broth (TSB; Oxoid, Hampshire, England) for 24 h at 42°C, *Campylobacter* were plated onto CCDA and incubated for 48 h at 42°C. The agar was composed of *Campylobacter* blood-free selective agar base (Oxoid) and CCDA selective supplement (Oxoid), which contained 32 μg/ml cefoperazone and 10 μg/ml amphotericin B. One colony was transferred from the CCDA plate to a tryptone soya agar-containing tube (TSA; Oxoid) incubated for 24 h at 42°C to acquire pure *Campylobacter*. The identity of bacteria was confirmed using API Campy strips (BioMériuex, Lyon, France) developed according to the protocol of the manufacturer. Pure *Campylobacter* were stored at 4°C until used.

1.2 Sample preparation

Pure *Campylobacter* was grown in TSB as described above, and 1 ml of the suspension was decimally diluted with peptone physiological salt solution (Biotrading, Mijdrecht, The Netherlands) containing 5 g/l peptone and 8.5 g/l sodium chloride, to give cell concentrations ranging from 10^1 to10^6 cfu/ml. *Campylobacter* pure culture samples, containing 1 ml diluted *Campylobacter* suspension, were added to 225 ml Preston broth in a stomacher bag. For artificial inoculate chicken meat samples collected from the local market in Utrecht, The Netherlands, aliquots of 25 g minced chicken fillet were fortified with 1 ml diluted *Campylobacter* suspension followed by the addition of 225 ml Preston broth in the stomacher bags. A negative

control was prepared for each chicken meat sample without adding *Campylobacter* inoculation. The Preston broth was prepared according to the manufacturer's instructions and consisted of nutrient broth No.2 (Oxoid), which contained 5% (v/v) lysed horse blood (Oxoid), *Campylobacter* growth supplement (Oxoid) and modified Preston *Campylobacter* selective supplement (Oxoid). Both pure culture and chicken meat samples were homogenized for 90 s in a stomacher (Interscience, St.Nom, France), and the suspension was incubated under microaerophilic atmosphere at 42°C for incubation times indicated in the text.

1.3 Immunomagnetic separation

Following enrichment in Preston broth, each sample-containing stomacher bag was placed into an incubation pot of the IMS machine operating at 37°C (Pathatrix^{TM}; Microscience, Cambridgeshire, UK). *Campylobacter* were then captured according to the protocol of the manufacturer. In brief, 50 µl anti-*Campylobacter* magnetic beads (Pathatrix PC50; Microscience) were added to the sample and the bead-containing suspension was circulated for 30 min. The magnetically immobilized beads were released, washed with 100 ml of pre-warmed buffered peptone water, which was compose of 10 mg/ml peptone (Becton Dickinson), 5 mg/ml sodium chloride (Merck, Darmstadt, Germany), 4.5 mg/ml disodium hydrogen phosphate dehydrate (Merck) and 1.5 mg/ml potassium dihydrogen phosphate (Merck) adjusted to pH 7.2. After washing, the beads were drawn to the magnet again, and the wash solution was removed to leave 0.2 ml bead-containing suspension for analysis.

1.4 Microbial test

The number of *Campylobacter* cells in pure culture samples and chicken meat samples was determined by transferring 100 µl of a sample on a CCDA plate. The number of *Campylobacter* colonies with grayish and often with metallic sheen, smooth, moist, flat and effuse was counted following incubation at 42°C for 48 h, and

the result was expressed as colony forming units per volume or weight of the original sample. Microbial tests were conducted before and after IMS.

1.5 mPCR analysis

The identification of *Campylobacter* by its genus and species level was based on PCR amplification of *16S* rRNA (*Campylobacter* genus), *mapA* (*jejuni* species) and *ceuE* (*coli* species) genes (Denis *et al*., 1999). In short, *16S* rRNA gene-based MD16S1 (5′-ATC TAA TGG CTT AAC CAT TAA AC-3′) and MD16S2 (5′-GGA CGG TAA CTA GTT TAG TAT T-3′) oligonucleotides were used as genus-specific primers. The *mapA* gene-based oligonucleotide primers were MDmapA1 (5′-CTA TTT TAT TTT TGA GTG CTT GTG-3′) and MDmapA2 (5′-GCT TTA TTT GCC ATT TGT TTT ATT A-3′), whereas COL3 (5′-AAT TGA AAA TTG CTC CAA CTA TG-3′) and MDCOL2 (5′-TGA TTT TAT TAT TTG TAG CAG CG-3′) reflected the *ceuE* gene. All oligonucleotide primers were synthesized commercially by Isogen Bioscience (Maarsen, The Netherlands).

After enrichment in a chicken meat sample, bacterial DNA was extracted from 1 ml of Preston broth suspension using a QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The aimed genes were amplified using 30μl extracted DNA in an end volume of 50 μl containing 200 μM dNTPs (Amersham, Buckingshamshire, UK), 1.5 mM $MgCl_2$, 1X PCR reaction buffer, 2 U *Taq* DNA polymerase (Promega, WI, USA), 0.5 μM of each MD16S1 and MD16S2 primers, 0.42 μM of each MDmapA1, MDmapA2, COL3 and MDCOL2 primers. The amplification reactions were carried out in a Mycycler^{TM} thermocycler (Biorad, CA, USA). In brief, the sample was heated at 95°C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59°C for 1 min 30 s and extension at 72°C for 1 min. The PCR reaction was completed by the final extension step at 72°C for 10 min. This amplification step generated 857 bp, 589 bp and 462 bp DNA fragments for *Campylobacter* spp., *C. jejuni* and *C. coli*, respectively. These products were assessed by electrophoresis using 5 μl PCR product loaded onto a 1% (w/v)

agarose gel (Invitrogen, CA, USA) containing 0.05 μg/ml ethidium bromide (Sigma-Aldrich, MD, USA) run at 100 V for 30 min and photographed under UV light.

2. Molecular subtyping by AFLP

2.1 Bacteriological samples

Seven commercial broiler farms in one province of central region of Thailand were sampled by fecal sampling of 10 samples per farm. In each sample, one pooled sample from ten fecal samples from each farm was taken. All broiler farms were slaughtered in two slaughterhouses in the central region of Thailand. Ten carcasses from each step of six different slaughtering processes were collected (pre-evisceration, cecum, post-evisceration, post-chiller, chilling and freezing) (Figure 2). Cecal samples were collected aseptically during evisceration. In pre-evisceration, post-evisceration and post-chiller step, 25 g of neck skin from each carcass was taken. In chilling and freezing step, 25 g of chicken meat from each carcass was taken. Overall, 70 chicken samples were sampled at each farm of seven broiler farms. All samples were transferred to one sterile sample container and stored for one night at 0-4°C. Bacterial analyses were commenced immediately after the arrival at the laboratory.

2.2 Isolation of *Campylobacter*

Isolation and identification of *Campylobacter* spp. were based on the method described by the International Organization for Standardization (ISO 10272-1:2006(E), 2006). In brief, 25 g of fecal from each pooled sample was taken or 25 g of chicken meat was aseptically removed using sterile scissors and forceps. Samples were placed in 225 ml of Bolton broth. The samples in Bolton broth were homogenized in peristaltic bags for two minutes in a stomacher. The bags were incubated microaerophilically in anaerobic jars with gas-generating kits at 37°C for 4 h and then at 41.5°C for 44 h. The culture was streaked onto CCDA plate and

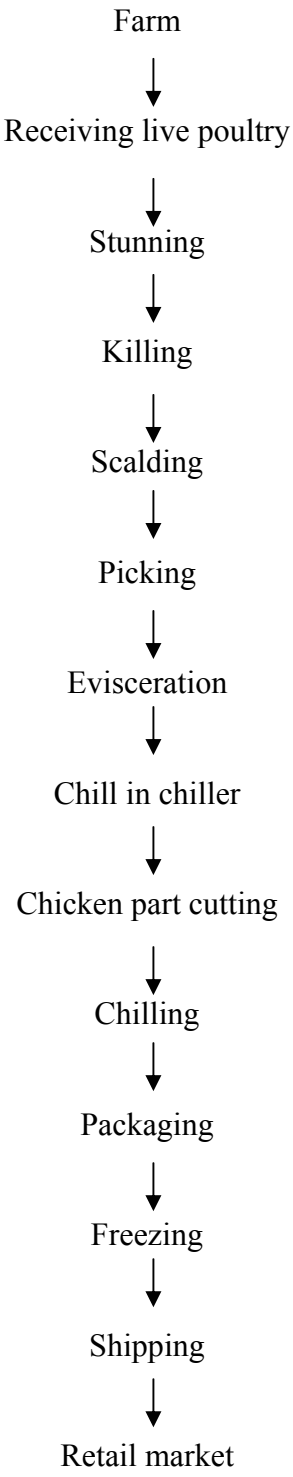

Figure 2 Flow diagram of slaughtering process in poultry slaughterhouse.

Karmali agar plate. The inoculated plates were incubated at 41.5°C for 48 h under microaerophilic environment.

The plates were taken from each selective medium at least 3 colonies considered to be typical or suspected as being *Campylobacter*. *Campylobacter* presumptive colonies of each sample were subcultured onto Columbia blood agar plate. The plates were incubated at 41.5°C for 48 h in microaerophilic atmosphere. The *Campylobacter* spp. were confirmed and identified by standard microbiological and biochemical procedures such as morphology, motility, Gram staining, oxidase and catalase activity, hippurate hydrolysis and indoxyl acetate hydrolysis and susceptibility to nalidixic acid and cephalothin (ISO 10272-1:2006(E), 2006).

2.3 Identification by mPCR

The identification of *Campylobacter* at its genus and species level was based on PCR amplification of *16S* rRNA (genus), *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) genes (Denis *et al*., 1999) as mentioned previously in section 1.5.

For isolation of chromosomal DNA, freshly grown cells were scraped from plates and washed with 100 µl TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). DNA was isolated using Phenol-Chloroform extraction method (Sambrook and Russell, 2001). The DNA was eluted with 50 µl TE pH 8.0 and stored at -20°C until processed. The PCR conditions as proposed by Denis *et al*. (1999) with minor modifications affected the dNTP concentration (200 instead 100 µM), *16S* rRNA gene primer concentration (0.5 instead 0.11 µM) and Taq DNA polymerase (1.2 instead 0.6 U) such that the PCR mixture (30 µl) contained 200 µM dNTPs (Fermentas, MD, USA), 1.5 mM $MgCl_2$, 1X PCR reaction buffer, 1.2 U Taq DNA polymerase (Invitrogen), 0.5 µM of each MD16S1 and MD16S2 primers, 0.42 µM of each MDmapA1, MDmapA2, COL3 and MDCOL2 primers. The amplification reactions were carried out in a Primus 96 plus thermocycler (MWG-Biotech, England) programmed as: heating at 95°C for 10 min, followed by 35 cycles consisting of

denaturation at 95 °C for 30 s, annealing at 59°C for 1 min 30 s and extension at 72°C for 1 min. The PCR reaction was completed with a final extension step at 72°C for 10 min. Amplification generated 857 bp, 589 bp and 462 bp DNA fragments corresponding to the genus *Campylobacter*, and the species *jejuni* and *coli*, respectively. These products were assessed by electrophoresis using 5 µl PCR product loaded onto a 1% (w/v) agarose gel (Seakem LE agarose; BMA, ME, USA) containing 0.05 µg/ml ethidium bromide (Amresco; OH, USA) run at 100 V for 30 min and photographed under UV light by using Biorad Gel Doc 1000 (Biorad).

2.4 Genotyping by AFLP

The AFLP analysis was performed by using a protocol adapted from as described previously (Duim *et al*., 1999). The concentration of DNA from Phenol-Chloroform extraction method was determined by measurement of optical density at 260 nm by using Gene Quant pro (Amersham Biosciences, Cambridge, England). AFLP was performed on 500 ng of genomic DNA digested in a total volume of 30 µl, which consisted of 5 U of *Hin*dIII endonuclease (New England Biolabs [NEB], Hertfordshire, England), 3 µl of 10X NEB buffer 2, and 1.5 µl of DNase-free RNase A (10 µg/µl) (Fermentas), for 1.5 h at 37°C. After being digested, added 5 U of *Hha*I (NEB) and 0.3 µl of 100X bovine serum albumin (BSA), and the reactions were incubated for a futher 1.5 h at 37°C. The endonucleases were inactivated (65°C for 10 min) prior ligation.

After double digested DNA, 25 µl of a solution containing 0.5 µl of 2 µM *Hin*dIII adapter (5'-CTC GTA GAC TGC GTA CC, 3'-CTG ACG CAT GGT CGA) (National Science and Technology Development Agency [NSTDA], Bangkok, Thailand), 0.5 µl of 20 µM *Hha*I adapter (5'-GAC GAT GAG TCC TGA TCG, 3'-G CTA CTC AGG ACT A [NSTDA]), 40 U of T4 DNA-ligase (NEB), and 5 µl of 10X T4 ligase buffer (NEB) was added. The reaction mixture was incubated at 16°C for 3 to 4 h, heated at 65°C for 10 min to inactivate the ligase, and store at -20°C. The forward primer (*Hin*dIII adapter specific), labeled with the D4 WellRED^{TM} dye,

contained an extra selective base at the 3' end (*Hin*dIII +A, 5'-GAC TGC GTA CCA GCT TA-3' [Proligo, CO, USA]). The reverse primer (*Hha*I adapter specific) also contained an extra selective base at the 3' end (*Hha*I +A, 5'-GAT GAG TCC TGA TCG CA-3' ([NSTDA]).

PCRs were performed in 25-µl volumes containing 2.5 µl of ligated DNA, 2.0 µl of 1.0 µM D4 dye-labeled *Hin*dIII +A primer, 1.0 µl of 5.0 µM *Hha*I +A primer, 0.5 µl of 10 mM concentrations of each of the four dNTPs (Fermentas), 2.5 µl of 10X PCR reaction buffer, 1.25 µl of 50 mM $MgCl_2$ and 1.25 U of Taq DNA polymerase (Invitrogen). Touchdown PCR cycling conditions were used for amplification as follows: denaturation for 2 min at 94°C (1 cycle), followed by 30 cycles of denaturation at 94°C for 20 s, a 30-s annealing step (see below), and a 2-min extension step at 72°C. The annealing temperature for the first cycle was 66°C; for the next nine cycles, the temperature was decreased by 1°C at each cycle. The annealing temperature for the remaining 20 cycles was 56°C. This was followed by a final extension at 60°C for 30 min. PCR was performed in a Biometra T-Gradient thermocycler (Whatman Biometra, Gottingen, Germany). The products from this amplification were stored at 20°C until used.

Amplified fragments were separated by capillary electrophoresis using the CEQ 8000 genetic analysis system (Beckman Coulter, CA, USA). For sample preparation, 25 µl of loading buffer solution (Beckman Coulter) was combined with 0.25 µl of CEQ DNA size standard-600 base pair ladder (Beckman Coulter), 1 µl of amplification product and was overlaid with one drop of mineral oil. Fragment separation and detection was performed at capillary temperature 50°C, denaturation at 90°C for 90 sec, injection at 2 kV for 30 sec, and separation 6 kV for 35 min. Fragment data were analyzed using the CEQ 8000 software with the analytical parameters calibrated to detect peaks with a slope of 10% and 10% of the height of

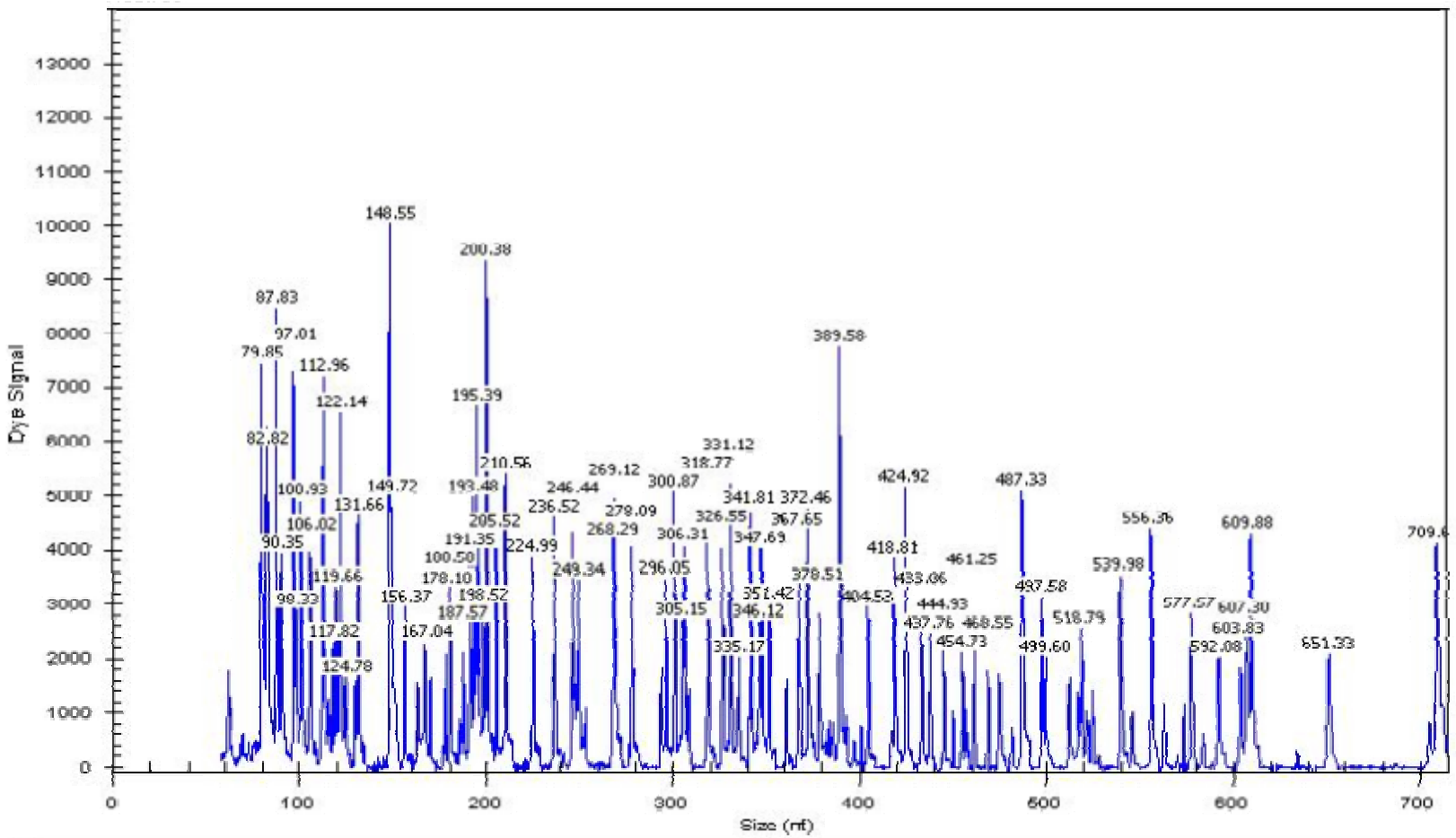

Figure 3 AFLP fragment pattern of a *Campylobacter* spp. strain. The horizontal fragment sizes in base pairs, and the vertical scale is relative fluorescence.

the second highest peak (Figure 3). The maximum bin width was adjusted to 2. Each sample was scored as "1" for each bin if a fragment of that size was present, and as "0" if not. Only AFLP profiles in the molecular size range of 60 to 640 bp were analyzed. A table containing this binary information was applied to calculate in lane similarity by Dice coefficient correlation because this method does not infer the direction or weight change of AFLP bands. Cluster analysis of AFLP banding pattern was done using the unweighted pair-group method with arithmetic averages (UPGMA) using NTSYS-pc software package version 2.2 (Exeter software, NY, USA). The bootstrap value was also generated using Winboot programme (IRRI, Los Banos, Philippines). The bootstrap presents the strength of formed groups in tree and the probability that two isolates belong to the same cluster are different (Yap and Nelson, 1996).

Correlation levels were expressed as percentage of similarity. Repeated analysis of same isolates revealed a similarity value of at least 90%, and therefore the cut-off for highly related strains was set at 90%, a value which is in accordance with finding of others (Duim *et al*., 1999; On and Harrington, 2000). Each of the 90% similarity group was called a phenon. All strains within a window of similarity between 95% and 100% homology were considered as identical and thus belonging to the same AFLP strain (Lindstedt *et al*, 2000). Individual phenons were designed numerically with Roman letters. A number letter was added when strains appeared clonal.

RESULTS AND DISCUSSION

1. Immunomagnetic separation and multiplex PCR

In order to assess the efficacy of IMS, Preston broth was spiked at various levels of *C. jejuni* and after IMS, beads were grafted on CCDA plates in order to count the number of captured viable cells after selective growth. It can be deduced from Table 6 that the recovery of added cells is only 0.5% to 9%, which may impair favorable sensitivity. Furthermore, despite the increasing spiking level, the number of colonies of retrieved cells did not show an upward trend revealing an unsatisfactory precision as well. From the ratio of the number of spiked and retrieved colonies, a detection limit could be calculated for each spiking level. This value ranged broadly and users applying IMS to detect *Campylobacter* might, therefore, reckon with a detection limit at least 2.1×10^2 cfu/ml instead of 11 cfu/ml.

In a similar way, 25 g minced chicken meat was inoculated at different levels of *C. jejuni*, mixed with 225 ml Preston broth, processed using IMS and the number of retrieved colonies determined using CCDA plates (Table 6). In this case, the recovery ranged between 0.02% and 0.1% of the original amount of added cells. The corresponding calculated detection limits were higher as well, and at least 5×10^3 cfu/g meat could be detected. Although IMS effectively remove debris and concentrate *C. jejuni* from chicken meat samples, chicken meat are complex media that may contain many substances as inhibitors for immunomagnetic capture such as lipids (Yu *et al*., 2001). Therefore, the detection limits of chicken meat samples were higher than pure culture samples.

This sensitivity was reflected in the number of *Campylobacter* colonies on CCDA plates following different incubation time intervals at different inoculation levels of medium and meat (Table 7). At any starting level, *C. coli* was detected faster giving rise to more colonies on the CCDA plate than *C. jejuni*. For example, at an inoculum level of 10^{-2} cfu/ml, *C. coli* was detected in Preston broth after an incubation time of 12 h whereas *C. jejuni* was firstly visualized after 19 h of

incubation. Likewise, *C. coli* was also detected earlier in meat than *C. jejuni*. Comparison to spiked broth, detection of *Campylobacter* in meat was strongly delayed, as expected from the results in Table 6.

To detect 1 to 9 (10^0) *Campylobacter* cells of both species in 1 g chicken meat, an incubation time of at least 12 h was necessary. However, at this inoculum level in pure culture, *C. jejuni* and *C. coli* were detected after 4 h incubation period (Table 7).

Table 6 Detection limit of *C. jejuni* pure culture and inoculated with chicken meat detected by the IMS followed by plating method.

Pure culture samples[a]			**Chicken meat samples**		
Inoculum level (cfu/ml)	After IMS (cfu)	Detection limit[b] (cfu/ml)	Inoculum level (cfu/g)	After IMS (cfu)	Detection limit (cfu/g)
2×10^2	1	2×10^2	2×10^3	1	2×10^3
3×10^2	13	23	4×10^3	4	1×10^3
7×10^2	38	18	1.5×10^4	3	5×10^3
2×10^3	1.8×10^2	11	4×10^4	14	2.9×10^3
5×10^3	1.1×10^2	45	8×10^4	28	2.9×10^3
6×10^3	29	2.1×10^2	1.5×10^5	91	1.7×10^3
7×10^3	77	91	3×10^5	81	3.7×10^3
8×10^3	3×10^2	27	1×10^6	4.2×10^2	2.4×10^3
9×10^3	75	1.2×10^2	2×10^6	9.2×10^2	2.2×10^3
1×10^4	4.1×10^2	24			

[a]Preston broth used without modified Preston *Campylobacter* selective supplement;
[b]ratio of the number of spiked colonies and the number of IMS-retrieved colonies

Using PCR, it is possible that not only reproductive cells but also the damaged and dead cells are detected. In addition, PCR offers a possibility to confirm the presence of *Campylobacter* spp. and the identification of the involved species in a so-called multiplex mode. To assess this sensitivity, mPCR analysis was performed before using IMS on the same chicken meat samples as well (Table 8). At the genus level, mPCR analysis was able to detect the bacterium following a 14 h incubation period in meat inoculated at 1 cfu/g, while at species level needed after 16 h of incubation. Without pre-enrichment, at least 10^5 of *C. jejuni* and *C. coli* in 1 g chicken meat can be detected with mPCR.

The efficacy of IMS followed by plating could detect at least 5×10^3 cfu/g of *C. jejuni* in chicken meat, which was not different from the other IMS methods, such as a combination of IMS with atomic force and fluorescence microscopy to enable the detection of 10^4 cfu/g in ground poultry meat (Yu *et al*., 2001) or IMS coupled with a tyrosinase modified enzyme electrode had a detection limit of 2.1×10^4 cfu/g in the chicken carcass samples (Che *et al*., 2001).

To compare the efficacy of IMS followed by plating between pure culture samples and chicken meat samples, we found that in pure culture samples the efficacy of IMS followed by plating gave better detection than in chicken samples. At the same inoculum level, such as 10^0 cfu/ml in Preston broth and 10^0 cfu/g in chicken meat, this method detected *C. jejuni* and *C. coli* in pure culture with less pre-enrichment time than when contaminated in chicken meat for
8 h.

In this study, mPCR conditions were used as mentioned in Denis *et al*. (2001), that reported the detection of artificial contamination of *C. jejuni* and *C. coli* of chicken samples and faecal samples after 24 h enrichment. The sensitivity was 1.5×10^3 cfu/ml and initially inoculated with 5 cfu/g of samples before the enrichment step. Our results showed that *C. jejuni* and *C. coli* contaminated in chicken meat samples were identified by mPCR at an inoculum level 10^0 cfu/g (1-9 cfu/g) after only

16 h enrichment. It meant that we could reduce the enrichment to 8 h in this mPCR assay condition.

Mateo *et al*. (2005) determined that detection limit of the mPCR method to detect 1 ml *C. jejuni* pure culture without pre-enrichment was 50 cfu/ml. However, the study showed that the mPCR method could detect chicken meat without pre-enrichment inoculated by 10^5 cfu/g of *C. jejuni* and *C. coli*. The difference in detection limits of both studies was caused by inhibitors of amplification reactions of PCR contained within chicken meat.

To date, real-time PCR assay was developed for the detection of *Campylobacter* in foods. This assay was demonstrated to be as sensitive as conventional culture and conventional PCR methods but reduced the time taken for detection. Sails *et al.* (2003) used a real-time PCR to detect *C. jejuni* in naturally and artificially contaminated food samples such as raw poultry meat, offal, raw shellfish and milk samples. The study showed that assay could detect enrichment broth samples which had total viable *Campylobacter* counts varied between 4×10^5 cfu/ml and greater than 10^7 cfu/ml and the total time required for real-time PCR assay approximately 2.5 h.

From Tables 2 and 3, the results showed the pre-enrichment time of chicken samples or pure culture samples detected by mPCR or IMS followed by plating were dependent on the inoculum levels of *C. jejuni* and *C. coli*. The high inoculum level could result in short pre-enrichment time.

Table 7 Number of counted colonies following CCDA plating of IMS beads used to capture *C. coli* and *C. jejuni* from either pure culture samples or chicken meat samples which were inoculated at indicated levels and incubated for indicated time intervals. The counted number of *C. jejuni* is given between brackets.

Incubation time (h)	Inoculum levels (cfu/ml or cfu/g)											
	10^{-2}	10^{-1}		10^{0}		10^{1}		10^{2}		10^{3}	
	Pure[a]	Pure	Meat[b]	Pure	Meat	Pure	Meat	Pure	Meat	Pure	Meat
1								12			2
2				nd[c]		3 (2)		184			
3									(nd)		
4		nd (1)		2 (1)		14 (14)	(nd)	379 (111)	1 (4)	(49)	23
6	(nd)	4 (2)		20		(45)	(nd)		5		
8	(nd)	17 (5)			(nd)	(184)	2 (nd)		118		
10		95									
12	19	438			9 (3)		13 (18)				
14	112	737			28						
15					(13)		(197)				
16	10^{2}		84		385						
17	(nd)		(nd)		(44)						
18	10^{3}		96								
19	(55)		(nd)								
20			830								
21			(332)								

[a]*Campylobacter* pure culture; [b]*Campylobacter* inoculated with chicken meat; [c]nd, not detected

Table 8 mPCR analysis to detect chicken meat inoculated with *C. coli* or *C. jejuni*, which were inoculated at indicated levels and incubated for indicated time intervals. The result of *C. jejuni* is given between brackets.

Incubation time (h)	Inoculum levels (cfu/g)																			
	10^{-1}		10^{0}		10^{1}		10^{2}		10^{3}		10^{4}		10^{5}		10^{6}		10^{7}	
0									nd[c]		nd	(nd)	G[a],S[b]	(G,S)	G,S	(G,S)	(G,S)	
2							nd		nd		nd	(nd)	G,S	(nd)		(G,S)		
4							nd		G,S	(nd)	G,S	(nd)		(G,S)				
6					nd				G,S	(G,S)								
8					nd		nd	(nd)	G,S	(nd)								
10			nd	(nd)	G,S	(nd)	G,S											
12			nd	(G)	G	(nd)												
14	G	(G)	G	(G)														
16	G,S	(G,S)	G,S	(G,S)		(G,S)												
17					G,S	(G,S)	G,S	(G,S)										
18	G,S		G,S		G,S			(G,S)		(G,S)								
20			G,S			(G,S)												

[a]G, PCR product detected in genus; [b]S, PCR product detected in species; [c]nd, not detected

2. Molecular subtyping by AFLP

2.1 Comparative identification of *Campylobacter* isolates by standard biochemical procedures and mPCR analysis

A total of 328 isolates of *Campylobacter* from 490 chicken fecal and carcass samples were used to compare between standard biochemical procedures and mPCR. Of 328 isolates, 263 samples were identified as *C. jejuni* and 65 samples were identified as *C. coli* by standard biochemical procedure. However, 118 samples were identified as *C. jejuni* and 210 samples were identified as *C. coli* by mPCR assay (Table 9).

Analysis data revealed that results were not in agreement for 155 (47.2%) isolates when tested by both methods. Using standard biochemical procedure, *C. jejuni* and *C. coli* were separated by hippurate hydrolysis. The difference of the results were notably due to a positive hippurate hydrolysis test for isolates identified as *C. coli*, instead of *C. jejuni*, by mPCR for 150 (45.7%). The positive identification by hippurate hydrolysis is the difficulties in interpreting the results. This is because *C. jejuni* can be weakly positive and other amino acids or peptides which are transported from culture media or produced during incubation can give false positive results (Denis *et al*., 1999). The negative hippurate hydrolysis test but identified as *C. jejuni* by mPCR for 5 (1.5%). The difference was due to the absence of hippurate hydrolysis test for some *C. jejuni*.

The results were not in agreement between standard biochemical procedures and mPCR which has been observed previously. Denis *et al.* (1999) found positive hippurate hydrolysis test result for isolates identified as *C. coli* by mPCR at 59% and negative hippurate hydrolysis test but isolates identified as *C. jejuni* at 7% out of 294 isolates of *Campylobacter* in fecal poultry samples. The results indicated the efficiency of the identification by the hippurate hydrolysis was 34% in comparison to 100% efficiency with the PCR.

Table 9 The Comparative results between multiplex PCR analysis and standard biochemical procedures to detect *C. coli* and *C. jejuni* in chickens.

Species identification		**mPCR**		**Total**
		C. jejuni	*C. coli*	
Standard biochemical method	*C. jejuni*	113 (95.8%)	150 (71.4%)	263 (80.2%)
	C. coli	5 (4.2%)	60 (28.6%)	65 (19.8%)
Total		118 (36.0%)	210 (64.0%)	328 (100.0%)

2.2 Isolation rates of *C. jejuni* and *C. coli* at farms and slaughterhouses

The isolation rates of *Campylobacter* by mPCR recovered from fecal samples and chicken carcasses during the slaughtering process were presented in Table 10. Of 328 positive samples (328/490, 66.9%) 118 samples (118/490, 24.1%) were identified as *C. jejuni* and 210 samples (210/490, 42.8%) were identified as *C. coli*. At farm level, only 6 (8.6%) of all fecal samples were positive for *Campylobacter* spp. but in cecal content samples were more positive at 44 (62.9%). The percentages of positive fecal samples were less than cecum samples because only some part of *Campylobacter* passing through feces and decreasing by environment pressure in intestinal tract and broiler houses.

At the beginning of slaughter, 65.8% of the chicken samples were positive for *Campylobacter* spp. at pre-evisceration step. Isolation rate at pre-evisceration step was high due to fecal contamination of feather and skin from transportation through defeathering step. Then, at post-evisceration step, *Campylobacter* spp. positive increased to 70% from possible fecal recontamination by intestinal content leakage. Isolation rates of samples at post-chiller and chilling step were 81.4% and 92.9%, respectively which increasing from cross contamination between carcasses. However, at the final freezing step *Campylobacter* isolation rate was decreased to 87.2% due to temperature decreasing at -18°C.

High prevalences of *Campylobacter* were detected on chicken carcasses during processing at the slaughterhouse as observed previously. Berndtson *et al*. (1996) found 100% (*n* = 40) *C. jejuni* positive samples at pre-chiller step and 95% (*n* = 38) *C. jejuni* positive samples at post-chiller step. The proportions of *Campylobacter*-positive carcasses at chilling step from U.S. poultry producers ranging from 21 to 40.9% (Stern *et al*., 2001).

Even though the *Campylobacter* load on chicken carcasses is commonly decreased during slaughter and processing from 10 to 1000 fold by slaughter specific stressors such as scalding bath temperature, presence of oxygen, drying of chicken skin or chilling at 0-4 °C (Alter *et al*., 2005). The reduction of bacterial load by these stressors cannot completely eliminate contamination in each processing step and also arising due to cross contamination between carcasses. Our results showed the continued increase of *Campylobacter* positive on chicken carcasses from pre-evisceration step to chilling step but a small decrease after the final freezing step from 92.9% to 87.2%. Consequently, the high isolation rate of chicken meat in the last step still remains a high risk factor for human infection.

Table 10 Isolation rates of *Campylobacter* by mPCR recovered from fecal samples and chicken carcasses during the slaughtering process.

Species	**Sampling stages**							
	Feces (*n* = 70)	**Cecum (*n* = 70)**	**Pre-evisceration (*n* = 70)**	**Post-evisceration (*n* = 70)**	**Post-chiller (*n* = 70)**	**Chilling (*n* = 70)**	**Freezing (*n* = 70)**	**Total (*n* = 490)**
Campylobacter spp.	8.6[a]	62.9	65.8	70.0	81.4	92.9	87.2	66.9
C. jejuni	4.3	8.6	22.9	28.6	27.1	42.9	34.3	24.1
C. coli	4.3	54.3	42.9	41.4	54.3	50.0	52.9	42.8

[a]Number of positive samples/number of samples examined (%)

2.3 Analysis with polymorphism of AFLP fragments

Using AFLP, 109 isolates of *C. jejuni* and 205 isolates of *C. coli* presented 48 and 95 distinct patterns, respectively (Figure 4 and 5). The fingerprints generated by the *Hin*dIII and *Hha*I restriction enzymes gave sharp and distinguishable peaks at 50 to 80 DNA fragments for *C. jejuni* and 80 to 120 DNA fragments for *C. coli* ranging from 60 to 640 bp. Similarly, Duim *et al*. (1999) obtained highly reproducible 40 to 50 DNA fragments, sized between 50 to 450 bp, using *Hin*dIII and *Hha*I together with selective primers *Hin*dA and *Hha*A. Our results had higher number of fragments than this research because the increased counting numbers of 450 bp to 640 bp. Duim *et al*. (1999) were tested with several combination of restriction endonuclease to find the optimal length distribution of DNA fragments. *Hin*dIII together with *Mse*I resulted in more than 80 DNA fragments ranging from 50 to 200 bp long with many and small DNA fragments. The combination of *Hin*dIII and *Taq*I resulted in 40 to 65 DNA fragments ranging from 50 to 500 bp long. Nevertheless, after being used repeatedly with this combination of enzymes, unacceptable variation occurred in high molecular weight portions of DNA fragments due to irreproducible digestion of *Taq*I.

AFLP of *C. jejuni* strains had 18 common amplified fragments while AFLP of *C. coli* strains had 30 common amplified fragments (Table 11). The intralinkage homologies of AFLP pattern among all *C. coli* strains was between 73% and 87% (Appendix Figure 1 to 7) and the AFLP banding patterns of *C. coli* strains showed more common fragments and more homologous than the patterns for *C. jejuni* strains. In addition, we found that *C. coli* strains from broilers and slaughtering process were more clonal than *C. jejuni* strains. Fragments of 199, 329 and 486 bp were presented in all *C. jejuni* and *C. coli* strains. These specific fragments could be used for identification of species-specific genetic marker of *C. jejuni* and *C. coli* which are common causes of gastrointestinal disease in human.

2.4 AFLP analysis of genetically related *C. jejuni* and *C. coli* strains

AFLP analysis of *C. jejuni* and *C. coli* strains from farm number 1 to 7 were performed and used to construct the resulting dendogram. The intralinkage homologies of the AFLP patterns among *C. jejuni* isolates in each farm were ranging from 68.5% to 96% and among all *C. jejuni* isolates were 65% (Appendix Figure 1 to 7 and Figure 4). The intralinkage homologies of the AFLP patterns among *C. coli* strains in each farm were ranging from 73% to 87% and among all *C. coli* isolates were 73% (Appendix Figure 1 to 7and Figure 5).

The AFLP banding patterns of *C. coli* strains contained many closely distributed bands which were more homologous than the patterns for *C. jejuni*, similarly shown by other authors previously (Duim *et al*., 1999, 2001). The *C. coli* strains from broilers and slaughtering process seem to be more closely related to each other than *C. jejuni* strains, and this finding suggests that *C. coli* strains are more clonal than *C. jejuni* strains. Similarly, Duim *et al*. (1999) found *C. coli* strains from poultry were more closely related to each other and more clonal than *C. jejuni* strains.

Distinction of these two species based in biochemical tests is often uncertain. *C. jejuni* and *C. coli* were identified as separate species that exhibit 25 to 49% homology on the basis of hybridization testing and multilocus enzyme electrophoresis typing (Duim *et al*., 1999). Our results showed that *C. jejuni* strains and *C. coli* strains produced very distinct AFLP banding patterns with interlinkage homologies of the AFLP patterns ranging from 35% to 42% (Appendix Figure 1 to 7). The use of the AFLP analysis resulted in a high degree of discrimination and seems to be useful and practical for identification of *C. jejuni* and *C. coli* strains.

Table 11 Common amplified DNA fragments of *C. jejuni* and *C. coli* isolates from fecal samples and chicken carcasses during the slaughtering process.

Presence of common amplified DNA fragments (bp)	***C. jejuni***	***C. coli***
95	+[a]	–[b]
104	–	+
118	–	+
130	–	+
139	+	–
148	–	+
155	–	+
167	+	–
168	+	–
186	–	+
190	–	+
192	–	+
194	–	+
198	–	+
199	+	+
209	–	+
223	–	+
233	+	–
235	–	+
236	+	–
245	–	+
248	–	+
277	+	–
290	+	–

Table 11 (Continued)

Presence of common amplified DNA fragments (bp)	***C. jejuni***	***C. coli***
299	–	+
304	+	–
309	+	–
317	–	+
322	+	–
325	–	+
329	+	+
346	–	+
350	–	+
366	–	+
382	+	–
388	–	+
417	–	+
418	+	–
420	+	–
423	–	+
459	–	+
486	+	+
507	+	–
538	–	+
555	–	+

[a] +, a fragment characteristically present in *C. jejuni* or *C. coli* isolates;

[b] –, characteristic absent of fragment from *C. jejuni* or *C. coli* isolates

2.5 Strain diversity and AFLP analysis of *C. jejuni* and *C. coli*

Numerical analysis differentiated the AFLP patterns of *C. jejuni* into 5 distinct clusters, representing genogroups I to V and AFLP patterns of *C. coli* into 4 distinct clusters, representing genogroups VI to IX and grouped 5 strains (473c1, 487c1, 521k1, 247k1, 495c2) separately (Figure 4 and 5). For *C. jejuni*, all AFLP banding pattern of cluster II strains collected from farm 1 and all of cluster IV strains collected from farm 2 were homogeneous and exhibited more than 87% and 78% similarity. The pattern of cluster I, III and V collected from at least two farms were highly diverse and exhibiting less than 77% similarity (cluster I; mostly collected from farm 3 and 7, cluster III; mostly collected from farm 6 and 7, cluster V; mostly collected from farm 2 and 6). For *C. coli*, all AFLP banding pattern of cluster VIII strains collected from farm 6 were homogeneous and exhibited more than 83%. The pattern of cluster VI, VII and IX collected from at least two farms were highly diverse and exhibited less than 83% similarity (cluster VI; mostly collected from farm 4 and 6, cluster VII; mostly collected from farm 3 and 5, cluster IX; mostly collected from farm 3 and 4).

From seven farms, the 109 isolates of *C. jejuni* from fecal samples and chicken carcasses during the slaughtering process were distributed into 27 different AFLP phenons and 48 different AFLP strains (Table 12). In most farms, AFLP type distribution changed during processing and broad diversity of *C. jejuni* strains were found except for farm 1, 2, 3. In farm 1, AFLP type was found to have only 3 phenons and 6 strains. The dominating genotypes were found from cecum, pre-evisceration, post-evisceration, post-chiller, chilling and freezing step (i1, i2, i3 and k1). Genotype i2 was found in every step from pre-evisceration to freezing and genotype k1 was found from cecum and pre-evisceration to chilling step. The closely related strains of phenon i (i1, i2 and i3) and phenon k (k1 and k2) were isolated along the processing line of farm 1.

Table 12 Number of *C. jejuni* isolated studied per farm, and number of different AFLP clones (95-100% similarity) and phenons (>90% similarity).

	Farm 1	**Farm 2**	**Farm 3**	**Farm 4**	**Farm 5**	**Farm 6**	**Farm 7**	**Total**
No. of isolates studied	42	24	5	2	4	13	19	109
No. of phenons	3	3	1	2	4	8	8	27
No. of AFLP strains	6	7	4	2	4	10	15	48

Table 13 Number of *C. coli* isolated studied per farm, and number of different AFLP clones (95-100% similarity) and phenons (>90% similarity).

	Farm 1	**Farm 2**	**Farm 3**	**Farm 4**	**Farm 5**	**Farm 6**	**Farm 7**	**Total**
No. of isolates studied	0	16	51	56	28	42	12	205
No. of phenons	0	2	6	5	8	10	7	33
No. of AFLP strains	0	4	23	15	22	33	8	95

In farm 2, genotype t1 was found only at pre-evisceration and post-evisceration steps and genotype u1 was found at post-evisceration, chilling and freezing. The only closely related strain of phenon a (a1, a2, a3 and a5) was found along the processing line of farm 3. Genotype e1, i1 and k1 were found in cecum and visible in the other steps of processing line. These genotypes could be proved for the source of *C. jejuni* contamination in chicken intestine and transmission to chicken meat by processing method in slaughterhouse (Table 14).

From farm 2 to 7, the 205 isolates of *C. coli* from fecal samples and chicken carcasses during the slaughtering process were distributed into 33 different AFLP phenons and 95 different AFLP strains (Table 13). *C. coli* cannot be detected from samples of farm 1. In most farms, AFLP type distribution changed during processing and broad diversity of *C. coli* strains were found except for farm 2. Dominating genotypes (A1, A3, A4 and Y1) were found along the line from at least two farms. It was different from *C. jejuni* strains diversity which was found dominating genotype only in one farm. Genotype A1 was found from cecum of broilers, pre-evisceration step and post-evisceration step of farm 3. This dominating genotype was also found at post chiller of farm 4 and chilling step of farm 2. Genotype A3 that closely related with subtype A1 was found from cecum of broilers of farm 3 and also found at pre-evisceration, post-evisceration, chilling and freezing step of farm 4. Genotype A4 was found from cecum of broilers of farm 3 and chilling step of farm 2. Genotype Y1 was observed in many samples of cecum, pre-evisceration, post-evisceration and freezing step of farm 3 and also found from cecum, pre-evisceration, post-evisceration, post-chiller and chilling step of farm 4.

The closely related strains of phenon A (A1 to A13) were isolated along the line in farm 3, 4 and some processing steps in farm 2, 6. In most phenon Y (Y1 to Y11) also found along the line in farm 2, 3, and 4. Many genotypes (genotypes A1, A2, A3, Y1, Y7, Y8 and AE1) of *C. coli* were found in cecum and transmission to chicken carcasses in many steps of processing line like *C. jejuni* (Table 15).

Table 14 AFLP type of *C. jejuni* strains recovered from fecal samples and chicken carcasses during the slaughtering process.

Sources	Sampling stages						
	Feces	**Cecum**	**Pre-evisceration**	**Post-evisceration**	**Post-chiller**	**Chilling**	**Freezing**
Farm 1 (A) [a]		i1, j1, k1	2i1, 2i2, 3k1	i2, 4k1, k2	4i2, 5k1	4i2, i3, 3k1, 2k2	4i2, 3i3
Farm 2 (B) [b]			2t1	5t1, 5u1		5u1, u2, v1, v2	2u1, v3, v4
Farm 3 (B)			a1	a2	a2, a5	a3	
Farm 4 (A)	b1	c1					
Farm 5 (A)					a4, w1	l1	aa1
Farm 6 (A)			4p1, q1	r1, z1		x1, x2, y1	d1, s1, s2
Farm 7 (A)		a6, e1			m1, n1, n2, n6, o1	3f1, h1, 2n3	e1, f2, g1, g2, n4, n5

[a]A = Slaughterhouse A; [b]B = Slaughterhouse B

Table 15 AFLP type of *C. coli* strains recovered from fecal samples and chicken carcasses during the slaughtering process.

Sources	Sampling stages						
	Feces	**Cecum**	**Pre-evisceration**	**Post-evisceration**	**Post-chiller**	**Chilling**	**Freezing**
Farm 1 (A) [a]							
Farm 2 (B) [b]		5Y7, Y8	2Y7, Y8		5Y7	A1, A4	
Farm 3 (B)		2A1, 2A3, A4, A5, A6, 2Y1, AG1	A1, 5Y1, 2Y9	A1, 2Y1, 3Y9, Y10	L1, 6AA1, AA5	A10, L2, L3, L4, 2L5, L7, W1	L6, 2Y1, Y2, 4AA1, AA2, AA4
Farm 4 (A)		8Y1	A3, 7Y1	A3, A11, 9Y1	A1, 3A11, A12, A13, Y1, Y3, Y4	6A3, 2Y1, AC1, Y11	3A3, 2A11, F1, Y5, Y6, Z1, Z2
Farm 5 (A)			2J2, J4, M1, M7, M10	M1, M3, M8, N1	J2, M3, M4	I1, J4, M5, M9, M11, O1, AA3, AB1	J1, J2, J3, K1, M2, M6, M11
Farm 6 (A)	A9, C1	A2, 2A8, B1, 3D1	A2, A7, D3, Q1	D2, D8, 2Q2, R1	D5, D7, E1, E2, G1, 2Q2, Q3, R2, S1	D4, D6, Q2, Q4, Q6, R3	D1, D4, D6, H1, 3Q5, T1
Farm 7 (A)	U1	U2, AD1, AE1	X1, AE1		AE1, AF1	V1	P1, 2AE1

[a]A = Slaughterhouse A; [b]B = Slaughterhouse B

A limited diversity of *Campylobacter* genotypes occured within the poultry population which was found formerly by *fla*-typing, PFGE and AFLP. Although the broad diversity of clones of *Campylobacter*, the persistent of farm specific clones were detected in broiler houses, colonization in broiler flocks and around the environment of broiler houses (Alter *et al*., 2005). Some AFLP strains of *Campylobacter* were not detected in the early stage of the slaughter line but appeared later on the carcasses, possibly caused by cross contamination between carcasses or more than one strain of bacteria contaminated on individual carcasses. In our study, we collected one colony of bacteria from each sample so it is possible to find only one bacterial strain isolate from one sample or carcass.

In most farms, many genotypes (A1, A3, Y1, M1, i2, k1 and t1) were isolated in pre-evisceration and post-evisceration steps which can be concluded that both species of *Campylobacter* contamination not only by the leakage of intestinal content in evisceration step but also by feces or environments contamination from transportation step until the pre-evisceration step. Only some of *C. jejuni* and *C. coli* subpopulations may survive and remain in the food chain which can resist environmental stressors such as scalding, chilling and freezing period in processing line. Enrichment is the best method to recover injured *Campylobacter*. Therefore, the long pre-enrichment period provides an opportunity for fast growing strains to outgrow slow growing strains. Conversely, sub-lethally injured cells will be detected by the pre-enrichment procedure which is impossible to detect by the direct method (Alter *et al*., 2005). Different genotypes not only exhibit a different potential for colonization but also different potential to survive environment stressors. The local predominant genotypes in the same clonal groupings were share specific characteristics which were advantageous for colonization in poultry, pathogenicity in humans and the survival in environment (Alter *et al*., 2005).

Form Appendix Figure 8 and 9, bootstrap analysis of *C. jejuni* strains revealed that most of the branches in the dendogram have bootstrap values of 11.7%-100% and most of the branches in *C. coli* strains have bootstrap values of 5.4%-99.7%. Most of the bootstrap value of both species for each group branch was low.

However some clusters and phenons showed a high bootstrap value that validated the robustness reliability of the branching pattern of them such as; cluster II (96.9), IV (88.6) and VIII (67.1); phenon a (98.9), e (99.7), f (78.3), g (84.9), p (96.3), s (86.0), t (100), u (100), v (88.3), x (92.9), U (97.7) and AE (99.7)

An automated sequencer is an important advantage of AFLP fingerprinting method because a database can be compiled automatically and fingerprint data can be compared and exchanged. For this reason, using AFLP analysis of *Campylobacter* spp. for creation of a national database will be possible. It is essential to provide an accurate reflection of bacterial population from different source in Thailand. This database represents a useful tool for the development of control strategies for *Campylobacter* spp. and to traceback infection source. However, the prevention of campylobacteriosis will be accomplished by hazard analysis and critical control point principle throughout the different stages of food process, good hygiene and proper cooking methods. These methods are also important to decrease the other foodborne pathogens and limit the increasing of antibiotic resistant bacteria to human.

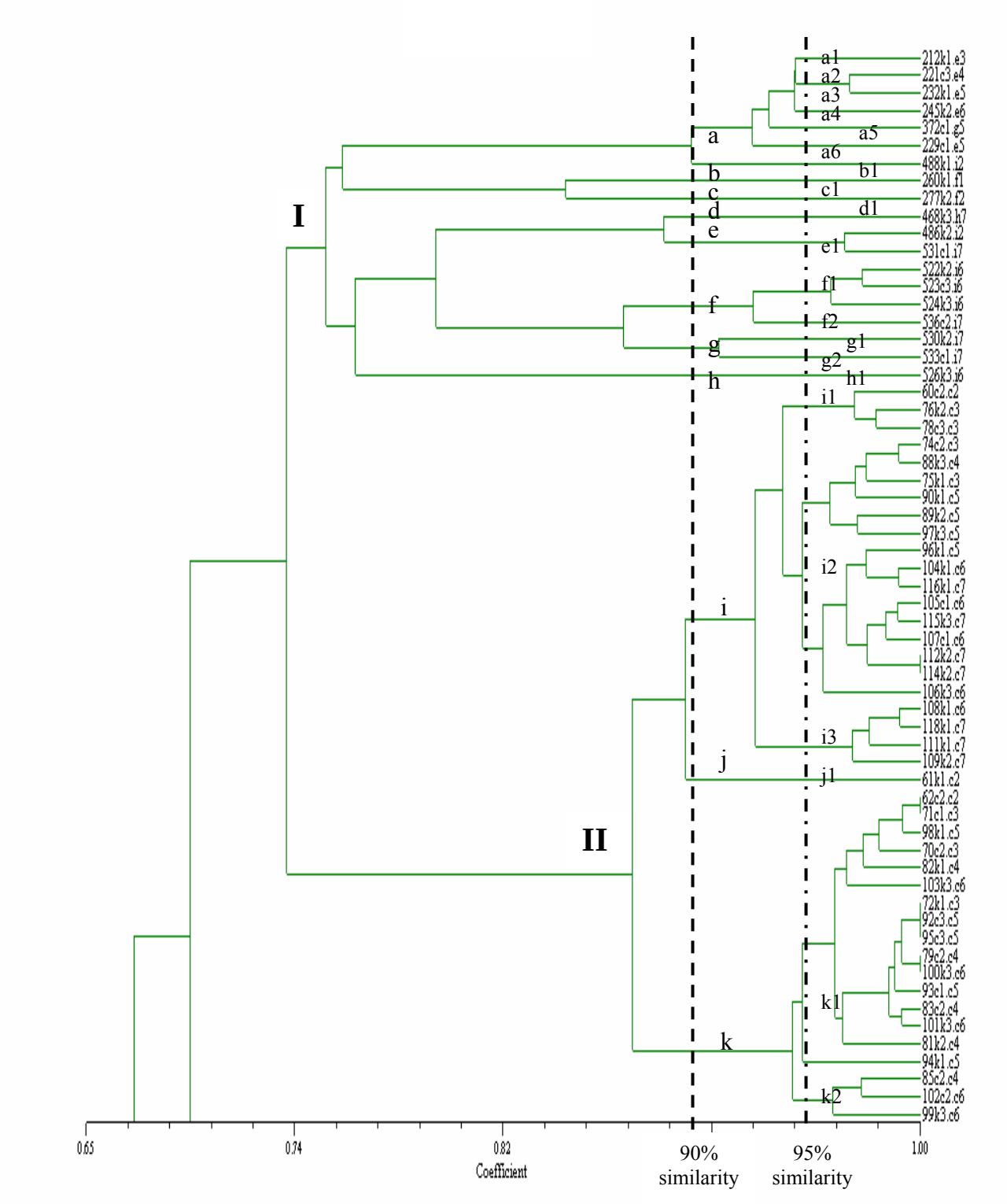

Figure 4 Dendogram of AFLP fingerprints of *C. jejuni* strains. The dotted vertical line indicates 90% similarity and 95% similarity. Roman letters a-aa represents groups of isolates sharing more 90% genetic similarity (phenon). The distinct AFLP strains (>95% genetic similarity) are indicated by Roman letters and numbers.

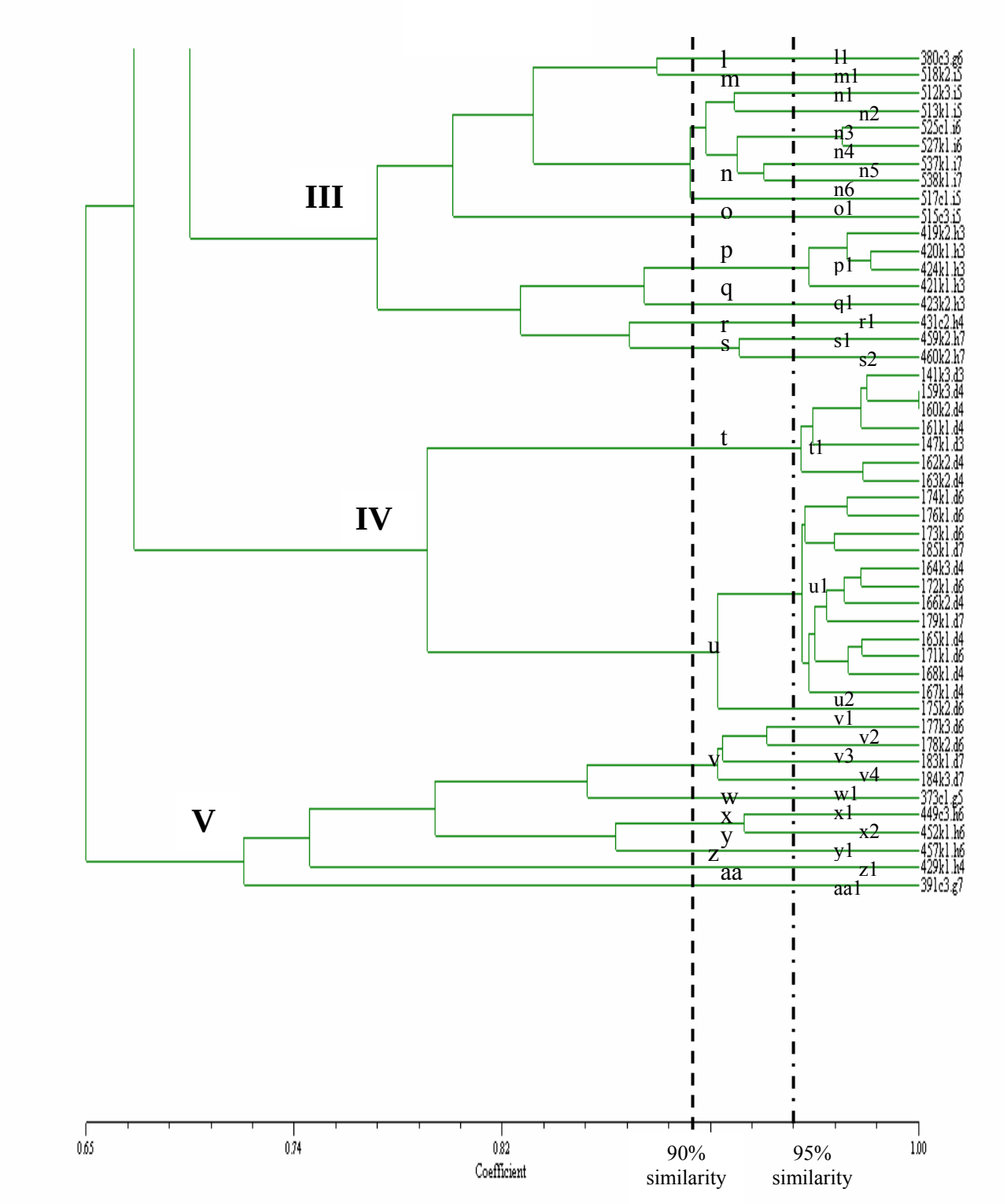

Figure 4 (Continued)

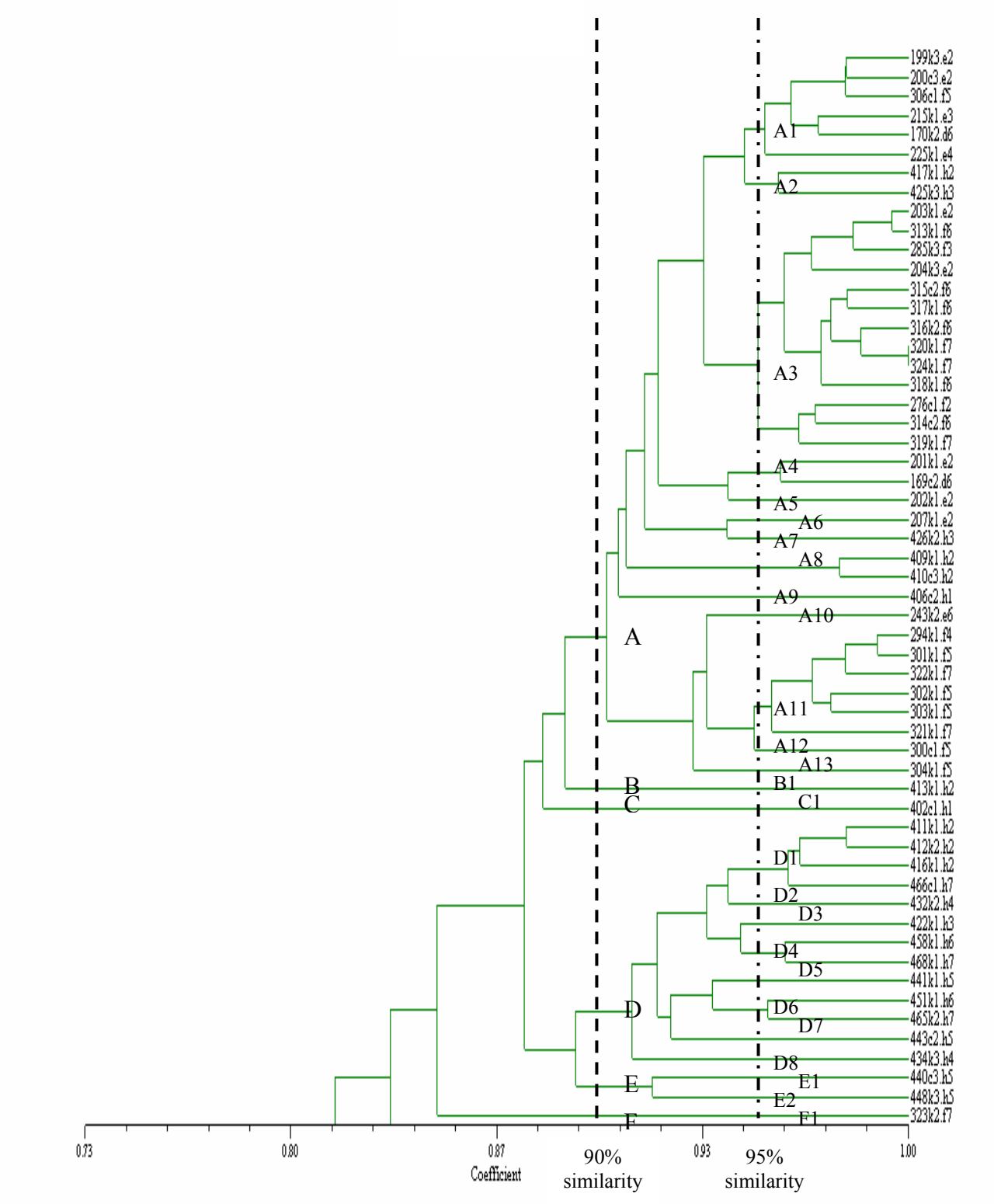

Figure 5 Dendogram of AFLP fingerprints of *C. coli* strains. The dotted vertical line indicates 90% similarity and 95% similarity. Roman letters a-aa represents groups of isolates sharing more 90% genetic similarity (phenon). The distinct AFLP strains (>95% genetic similarity) are indicated by Roman letters and numbers.

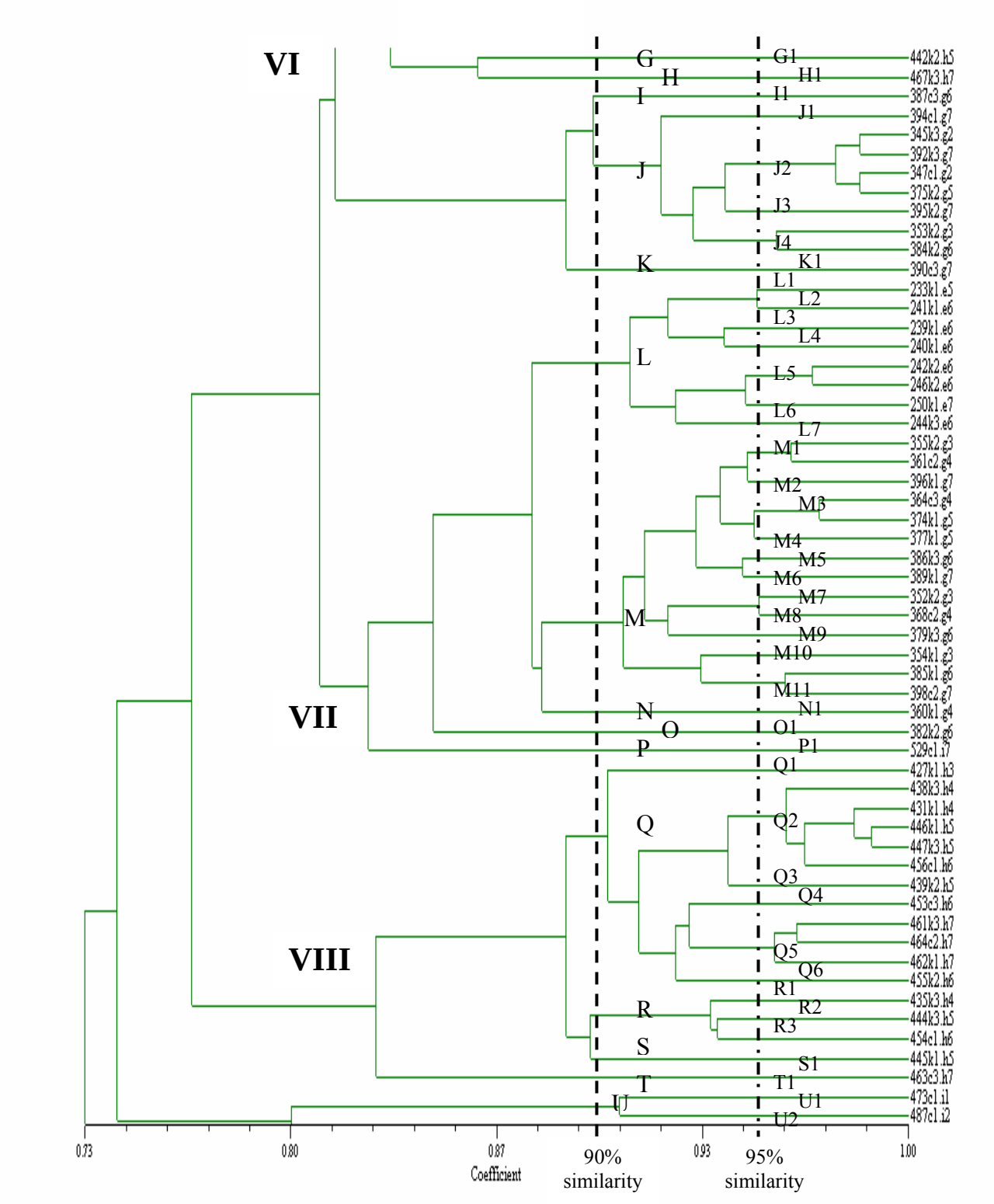

Figure 5 (Continued)

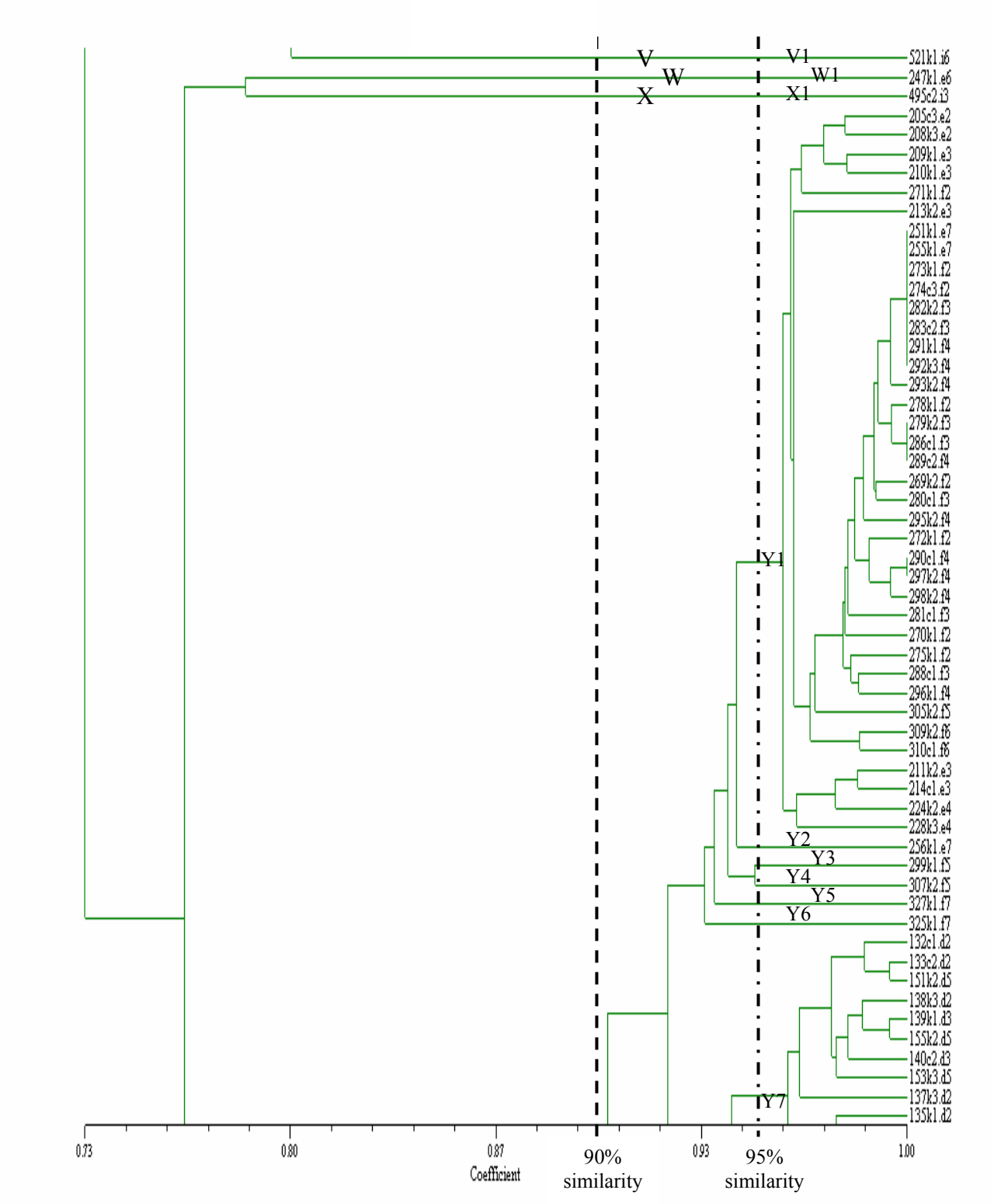

Figure 5 (Continued)

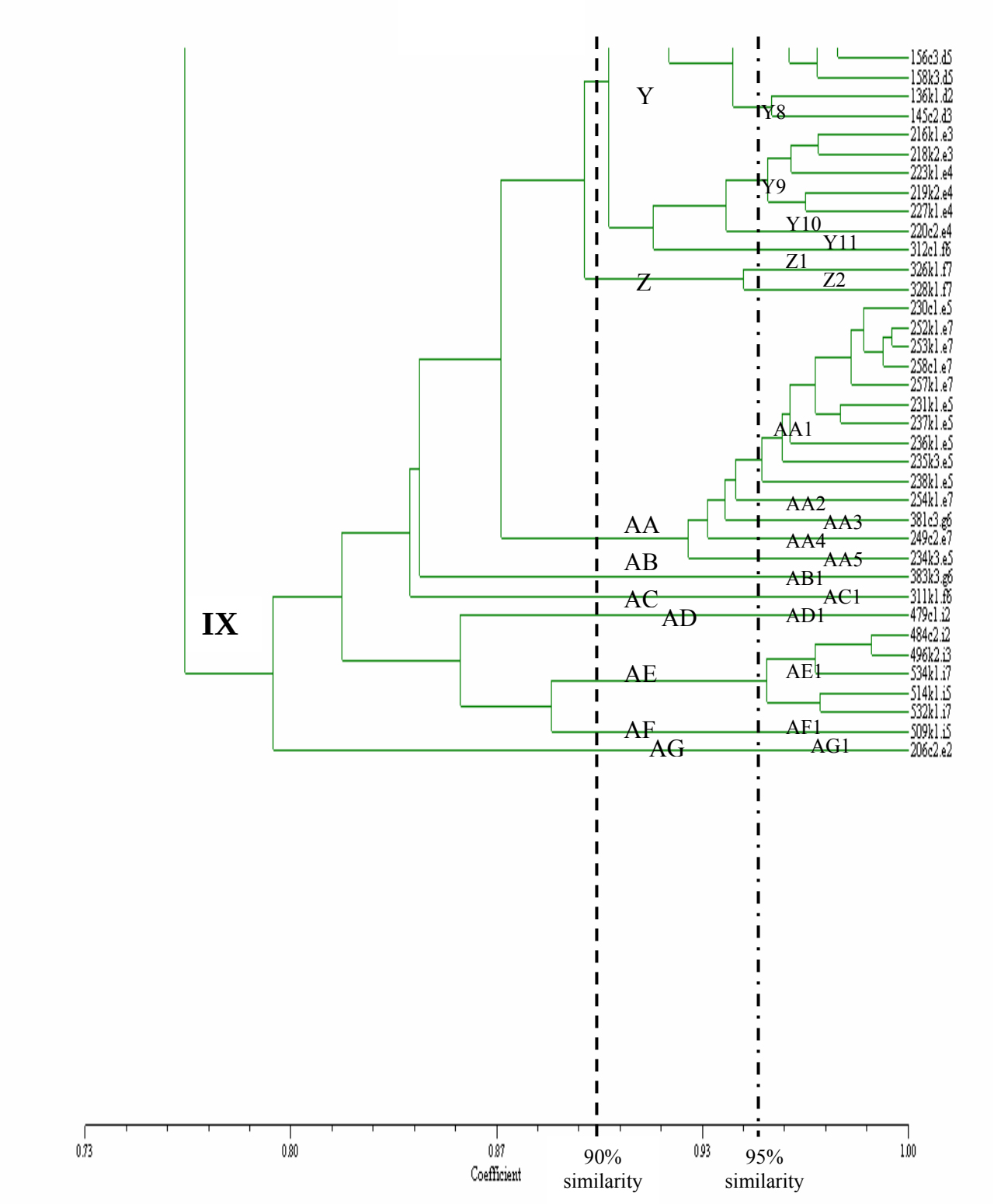

Figure 5 (Continued)

CONCLUSION

Two alternative methods, multiplex PCR (mPCR) and immunomagnetic separation (IMS) followed by plating to charcoal cefoperazone-deoxycholate agar (CCDA), were compared for their suitability to detect *Campylobacter jejuni* and *C. coli* in chicken meat. IMS followed by plating could detect *C. jejuni* and *C. coli* inoculated at 10^0 cfu/g in meat after 12 h of incubation whereas the mPCR method could detect both species at the same inoculation level after 16 h of incubation. However, the total analytical time to identify *C. jejuni* and *C. coli* in chicken meat using IMS followed by plating was 72-96 h while the time used by mPCR was only 22 h. Thus, the mPCR method for the detection of *C. jejuni* and *C. coli* in chicken meat could be performed with less total analytical time than IMS followed by plating.

Additional study was conducted to further investigate *C. jejuni* and *C. coli* isolated from seven commercial poultry farms and two slaughterhouses by molecular epidemiological analysis using high-resolution genotyping method of amplified fragment length polymorphism (AFLP). A total of 328 isolates of *Campylobacter* from 490 chicken fecal and carcass samples from all farms and slaughterhouses were used to comparae between standard biochemical procedures and mPCR. The standard biochemical procedure identified 263 isolates as *C. jejuni* and 65 isolates as *C. coli*, while the mPCR identified 118 isolates as *C. jejuni* and 210 isolates as *C. coli*. Analysis data revealed that results were not in agreement for 155 (47.2%) isolates by both methods which may be due to lacking of the efficiency of hippurate hydrolysis test in biochemical procedures.

The isolation rates of *Campylobacter* at different stages of slaughter line were high such as 62.9% cecal content, 65.8% at pre-evisceration step, 70% at post-evisceration step, 81.4% at post-chiller step, 92.9% at chilling step and 87.2% at freezing step. Consequently, chicken meat at retail market remains a high risk factor for human infection. AFLP analysis of 314 *Campylobacter* isolates revealed 48 AFLP strains of *C. jejuni* and 95 AFLP strains of *C. coli*. The intralinkage homologies of the AFLP patterns among all *C. jejuni* isolates were 65%. The

intralinkage homologies of the AFLP patterns among all *C. coli* isolates were 73%. The AFLP banding patterns of *C. coli* strains contained many closely distributed bands, which were more homologous than the patterns for *C. jejuni*. The *C. coli* strains from broilers and slaughtering process seem to be more closely related to each other than *C. jejuni* strains, and this finding suggests that *C. coli* strains are more clonal than *C. jejuni* strains.

In most farms, broad diversity of *C. jejuni* and *C. coli* strains were found and AFLP type distribution changed during the slaughter line. Some genotypes of both species were found in chicken intestine and may be the source of contamination of chicken meat during slaughtering and cutting process in slaughterhouse. Contamination of *C. jejuni* and *C. coli* in chicken meat occur directly from intestinal content and feces or indirectly from bird to bird and environment in slaughterhouse. AFLP fingerprint is the effective method to discriminate between *C. jejuni* and *C. coli* strains in which the interlinkage homology of the AFLP pattern is only 35 to 42%. In addition, it can distinguish genetically unrelated- from related-strains. Therefore, AFLP analysis is a suitable epidemiological tool for investigations of *Campylobacter*.

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APPENDICES

Appendix A

Chemical reagents, substances and the standard methods

1. Chemical reagents and substances

1.1 Boton broth

Basic medium 1000 ml

(Enzymatic digest of animal tissues, lactalbumin hydrolysate, yeast extract, sodium chloride, sodium pyruvate, sodium metabisulphite, sodium carbonate, α-ketoglutaric acid, haemin and water)

Antibiotic solution 5 ml

- Cefoperazone 0.02 g
- Vancomycin 0.02 g
- Trimethoprim lactate 0.02 g
- Amphotericin B 0.01 g
- Ethanol/sterile distilled water 50/50 (volume fraction) 5 ml

Sterile lysed defibrinated horse blood 50 ml

1.2 Preston broth

Basic medium 1000 ml

(Lab-Lemco meat extract, peptone, sodium chloride, sodium pyruvate, sodium metabisulphite, ferrous sulphate and water)

Antibiotic solution 4 ml

- Polymixin B 5000 i.u.
- Rifampicin 10.0 mg
- Trimethoprim 10.0 mg
- Amphotericin B 10.0 mg

Sterile lysed defibrinated horse blood 50 ml

1.3 CCDA

Basic medium	1000 ml

(Meat extract, enzymatic digest of animal tissues, sodium chloride, charcoal, enzymatic digest of casein, sodium deoxycholate, iron (II) sulfate, sodium pyruvate, water and agar)

Antibiotic solution	5 ml
Amphotericin B	0.01 g
Cefoperazone	0.032 g

1.4 Columbia blood agar

Basic medium	1000 ml

(Enzymatic digest of animal tissues, starch, sodium chloride, water and agar)

Sterile defibrinated sheep blood	50 ml

1.5 Karmali agar

Columbia agar base	39 g/l
Activated charcoal	4 g/l
Haemin	32 mg/l
Sodium pyruvate	100 mg/l
Cefoperazone	32 mg/l
Vancomycin	20 mg/l
Cycloheximide	100 mg/l

1.6 Brucella broth

Enzymatic digest of casein	10 g
Enzymatic digest of animal tissues	10 g
Glucose	1 g
Yeast extract	2 g

Sodium chloride	5 g
Sodium hydrogen sulfite	0.1 g
Water	1000 ml

1.7 Mueller Hinton blood agar

Basic medium	1000 ml

(Enzymatic digest of animal tissues, Enzymatic digest of casein, starch, sodium, water and agar)

Sterile defibrinated sheep blood	50 ml

1.8 Reagent for the detection of oxidase

N,*N*,*N*′,*N*′-Tetramethyl-1,4-phenylenediamine dihydrochloride	1 g
Water	100 ml

1.9 Reagent for the detection of hydrolysis of hippurate

Sodium hippurate	10 g
Phosphate-buffered saline (PBS) consisting of:	
sodium chloride	8.5 g
disodium hydrogen phosphate dihydrate	8.98 g
sodium dihydrogen phosphate monohydrate	2.71 g
Water	1000 ml

1.10 Ninhydrin solution 3.5% (mass/volume)

Ninhydrin	1.75 g
Acetone	25 ml
Butanol	25 ml

1.11 Indoxyl acetate discs

Indoxyle acetate	0.1 g
Acetone	1 ml

1.12 20X TAE buffer pH 8.3 (1 litre)

0.8 M Tris	96.9 g
0.4 M Sodium acetate	32.8 g ($NaOAc\text{-}3H_2O$)
0.04 M Na_2EDTA	14.9 g

Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre with distilled water.

1.13 10X loading buffer/dye

20% glycerol
0.01% bromphenol blue
Add TE to final volume.

2. The Standard methods

2.1 International standard ISO 10272-1:2006(E) of the confirmation and identification of *Campylobacter* spp.

2.1.1 Examination of morphology and motility

1. Suspend one colony from the Columbia agar plate in 1 ml of Brucella broth and examine for morphology and motility using a microscope.

2. Retain for further examination all cultures in which curved bacilli with a spiraling “corkscrew” motility are found.

2.1.2 Detection of oxidase

1. Using platinum/iridium loop or glass rod, take a portion of a well-isolated colony from each individual plate and streak it onto a filter paper moistened with the oxidase reagent; the appearance of a mauve, violet or deep blue colour within 10 s indicates a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer's instructions.

2. Confirm the result using positive and negative controls. Examples of suitable control strains are *Pseudomonas aeruginosa* NCTC 10662 (positive control), *Escherichia coli* NCTC 9001 (negative control).

2.1.3 Detection of catalase

1. For each colony, deposit a loop of culture into a drop of hydrogen peroxide solution on a clean microscopic slide.

2. The test is positive if bubbles appear within 30 s.

3. Confirm the results using positive and negative controls. Examples of suitable control strains are *Staphylococcus aureus* NCTC 8532 (positive control), *Enterococcus faecalis* NCTC 755 (negative control).

2.1.4 Detection of sensitivity to nalidixic acid and to cephalothin

1. For each colony, use a loop to prepare a suspension in Brucella broth of density 0.5 on the McFarland scale.

2. Dilute this suspension 1/10 with the same broth.

3. Flood the surface of MuellerHinton 5% blood agar plate with the suspension.

4. Leave in contact for 5 min and then drain off excess suspension.

5. Dry the plates in a drying cabinet and set at 37°C for 10 min.

6. On the surface of the agar, place a disc of nalidixic acid and a disc of cephalothin.

7. Incubate the plates, with lids uppermost, at 37°C for 22h ± 2 h in a microaerobic atmosphere

8. Interpret the bacterial growth in following manner: growth that is in contact with the disc is classified as resistant; the presence of a zone of any size due to inhibition of growth is classified as susceptible.

2.1.5 Detection of hippurate hydrolysis

1. For each colony, use a loop with a heavy inoculum to prepare a suspension in a haemolysis tube containing 0.4 ml of a sodium hippurate solution, taking care not to incorporate any agar.

2. Shake in order to mix thoroughly and incubate for 2 h in a water bath at 37°C or in an incubator at 37°C.

3. Carefully add 0.2 ml of a ninhydrin solution on the top of the sodium hippurate solution. Do not shake.

4. Interpret after an additional incubation of 10 min a water bath at 37°C or in an incubator at 37°C.

5. A dark violet colour indicates a positive reaction and a pale violet colour or no colour change indicates a negative reaction.

6. Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* NCTC 11351 (positive control), *Campylobacter coli* NCTC 11366 (negative control).

2.1.6 Detection of indoxyl acetate hydrolysis

1. Place a colony on an indoxyl acetate disc and add a drop of sterile distilled water. A loopful of colony material is required for a clear reaction.

2. If the indoxyl acetate is hydrolysed, a colour change to dark blue occurs within 5 min to 10 min. No colour change indicates hydrolysis has not taken place.

3. Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* NCTC 11351 (positive control), *Campylobacter coli* NCTC 11352 (negative control).

2.1.7 Interpretation

Campylobacter jejuni and *Campylobacter coli* are identified according to Appendix Table A1

Appendix Table A1 Characteristic of *Campylobacter jejuni* and *Campylobacter coli*

Chracteristic	*C. jejuni*	*C. coli*
Catalase	+	+
Oxidase	+	+
Hippurate hydrolysis	+	–
Indroxyl acetate hydrolysis	+	+
Nalidixic acid	S[a]	S[a]
Cephalothin	R	R

+ = positive; – = negative; S = Sensitive; R = Resistant.
[a] An increase in the resistance to nalidixic acid of *C. jejuni* and *C. coli* strains has been shown.

2.2 Phenol-Chloroform extraction of DNA and ethanol precipitation (Sambrook and Russell, 2001)

1. DNA was extracted from one loop of bacterial colony which washed with 100 µl TE, add 500 µl of denature solution and shaking for 5 to 10 minutes.

2. Add DNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.

3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.

4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

5. Take 2.2 to 2.4 again.

6. Remove 400 µl of the upper, aqueous layer to clean tube, add absolute ethanol 1,000 µl and invert gently up side down and keep in -80°C for 30 minutes.

7. Centrifuge at 13,000 rpm for 10 minutes and carefully decant the supernatant.

8. Wash the DNA pellet with 75% ethanol. Centrifuge at 13,000 rpm for 5 minutes. Decant the supernatant and dry the pellet by air.

9. Resuspend the pellet with 30-50 µl TE pH 8.0 and store at -20°C until use.

2.3 Agarose gel electrophoresis

1. Prepare an agarose gel by combining the agarose powder and water in a Ehrlenmeyer flask and heating in a microwave for 2 to 4 minutes until the agarose powder is dissolved.

2. Pour the gel onto a taped plate with casting combs in place. Allow 20 to 30 minutes for solidification.

3. Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1X TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

4. Add at least one-tenth volume of 10X agarose gel loading dye to each DNA sample, mix and load into the wells. Electrophorese the gel at 50 to 100 V/cm until the required separation has been achieved. Visualize the DNA fragments on a long wave UV box.

2.4 AFLP analysis parameters of CEQ 8000 software (default fragment analysis parameters)

2.4.1 General

Peak criteria
Slope threshold – 10
Relative peak height threshold – 10%
Size estimation and allele ID confidence
Confidence level – 95%

2.4.2 Analysis methods

Size standard – 600
Model – cubic
Migration variable – migration time

2.4.3 Quantitation

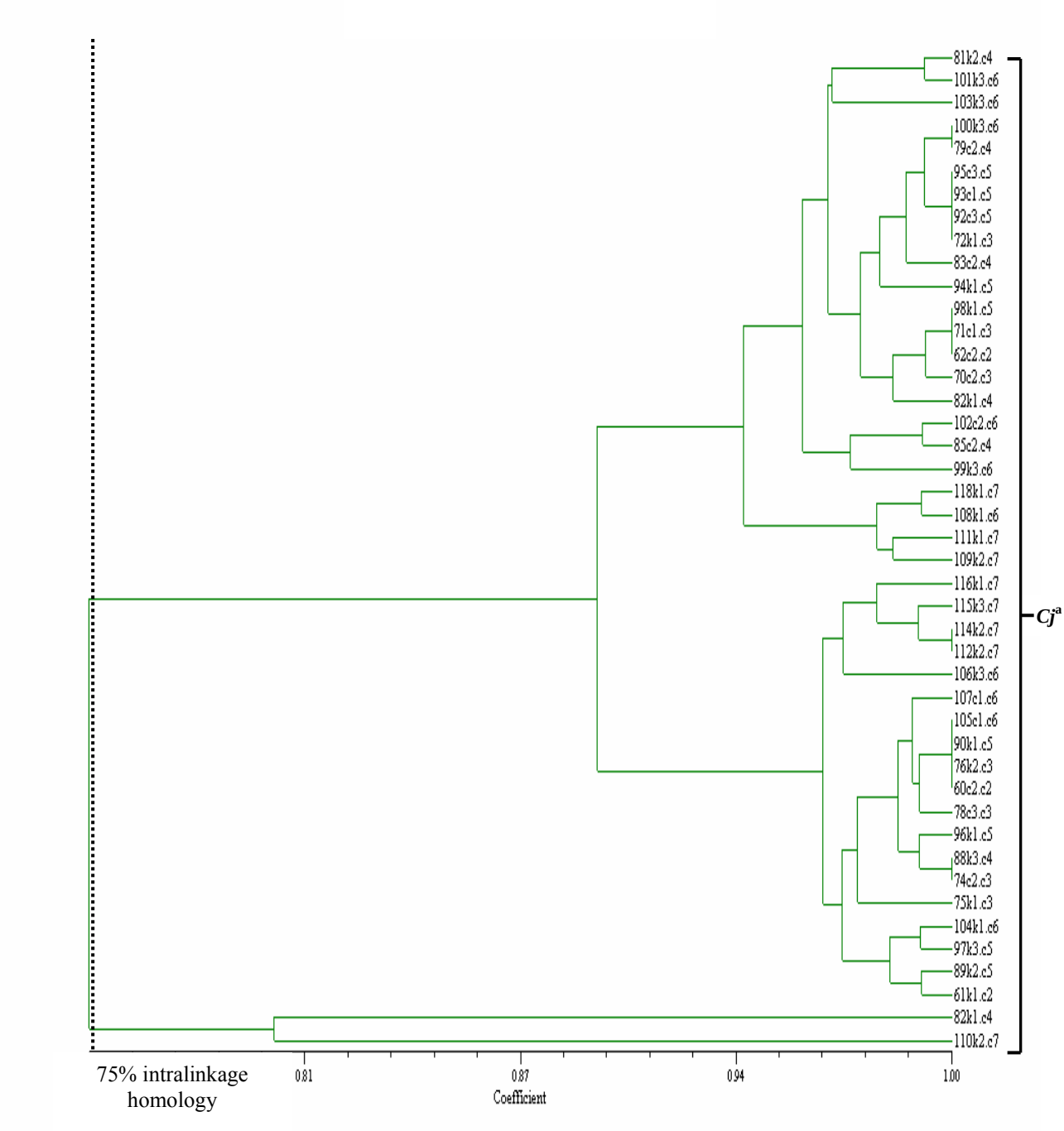
Identity standard – time
Calculate using – area

2.4.4 Advanced

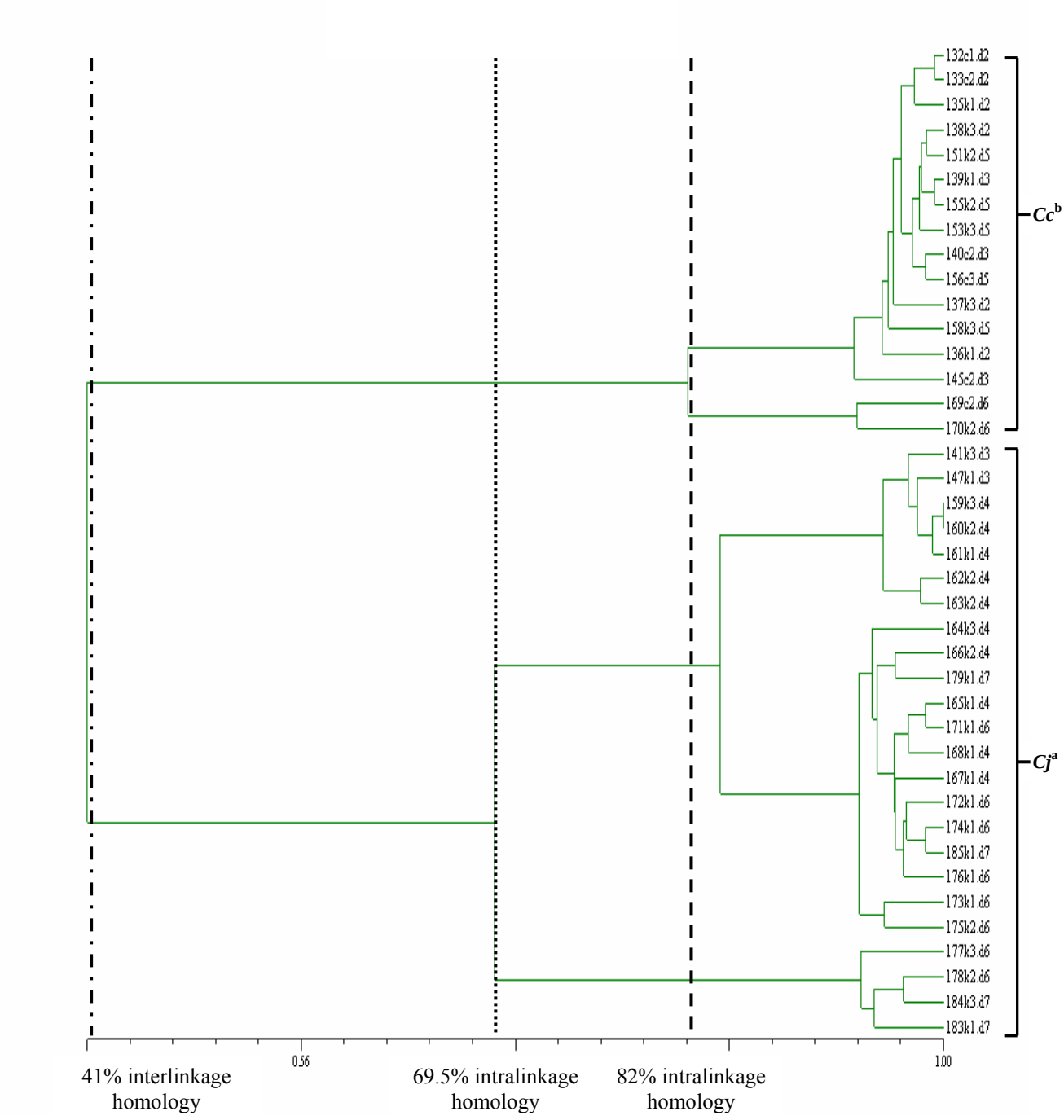
Mobility
Dye mobility calibration – PA ver. 1
Standard mobility reference – none
Dye spectra – use system dye spectra

Appendix B

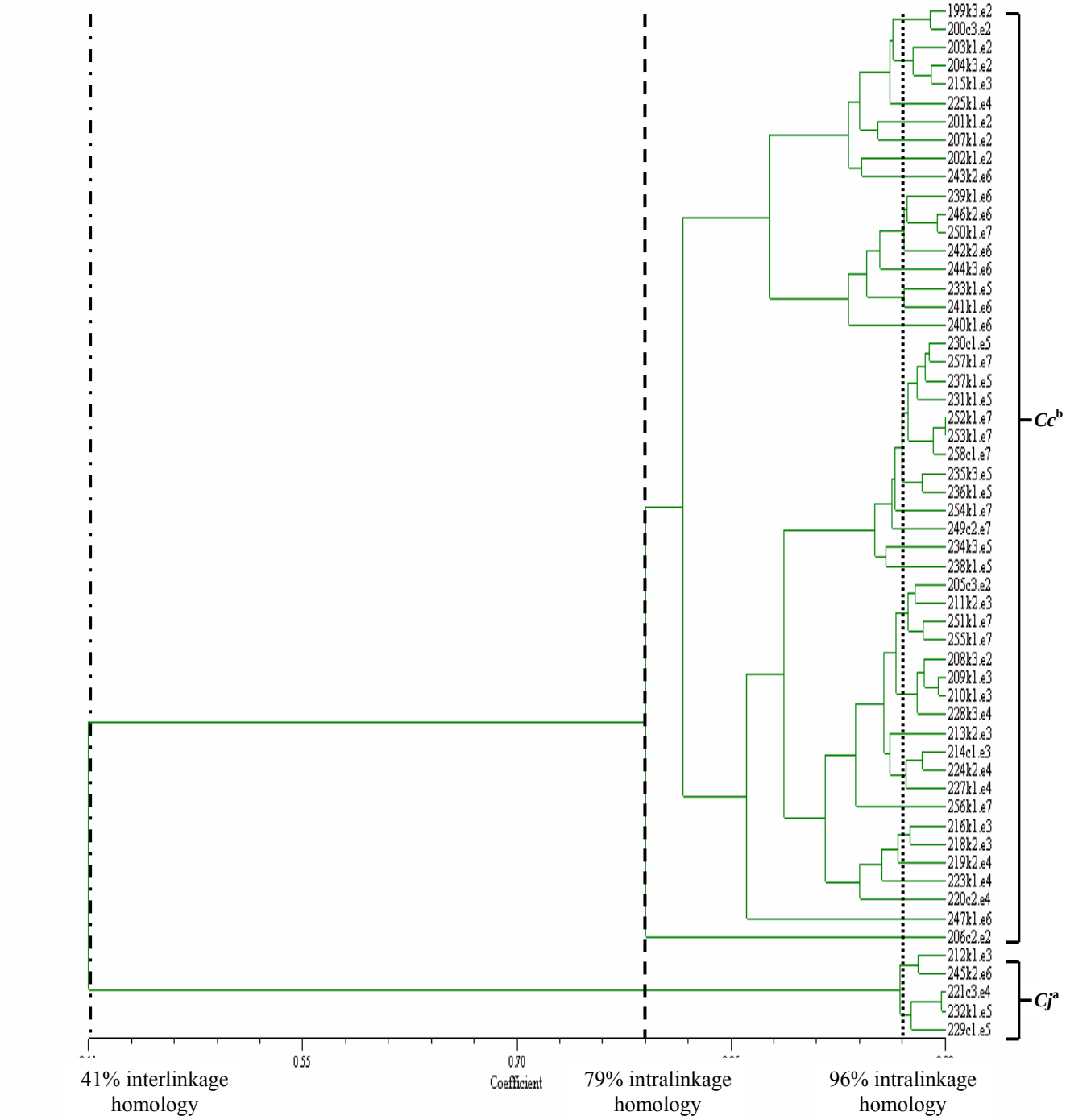
AFLP analysis results



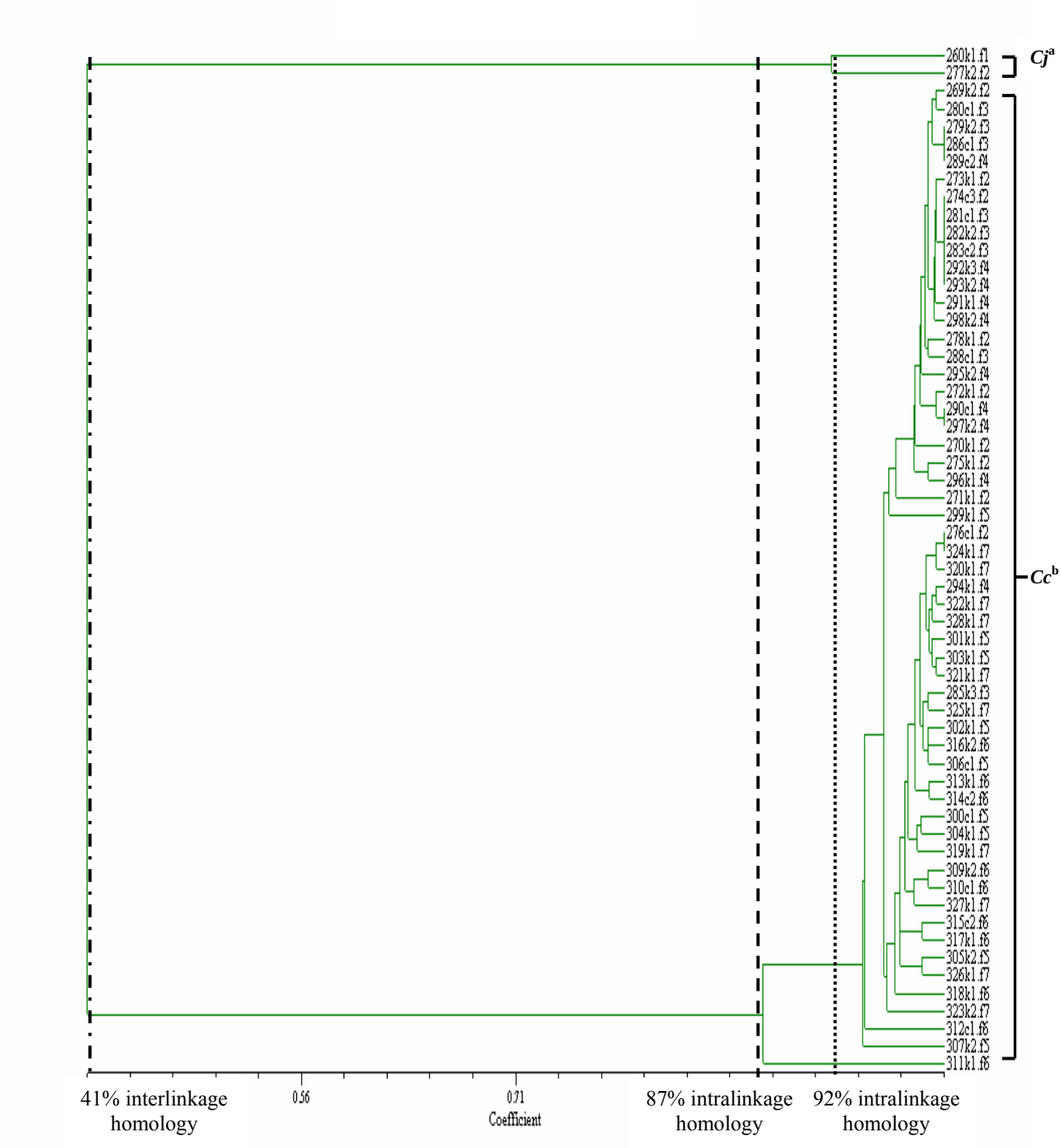
Appendix Figure 1 Dendogram of AFLP fingerprints of *Campylobacter* from farm 1. The dotted vertical line indicates the percentage intralinkage homology of *C. jejuni*.
[a]*Cj = C. jejuni*



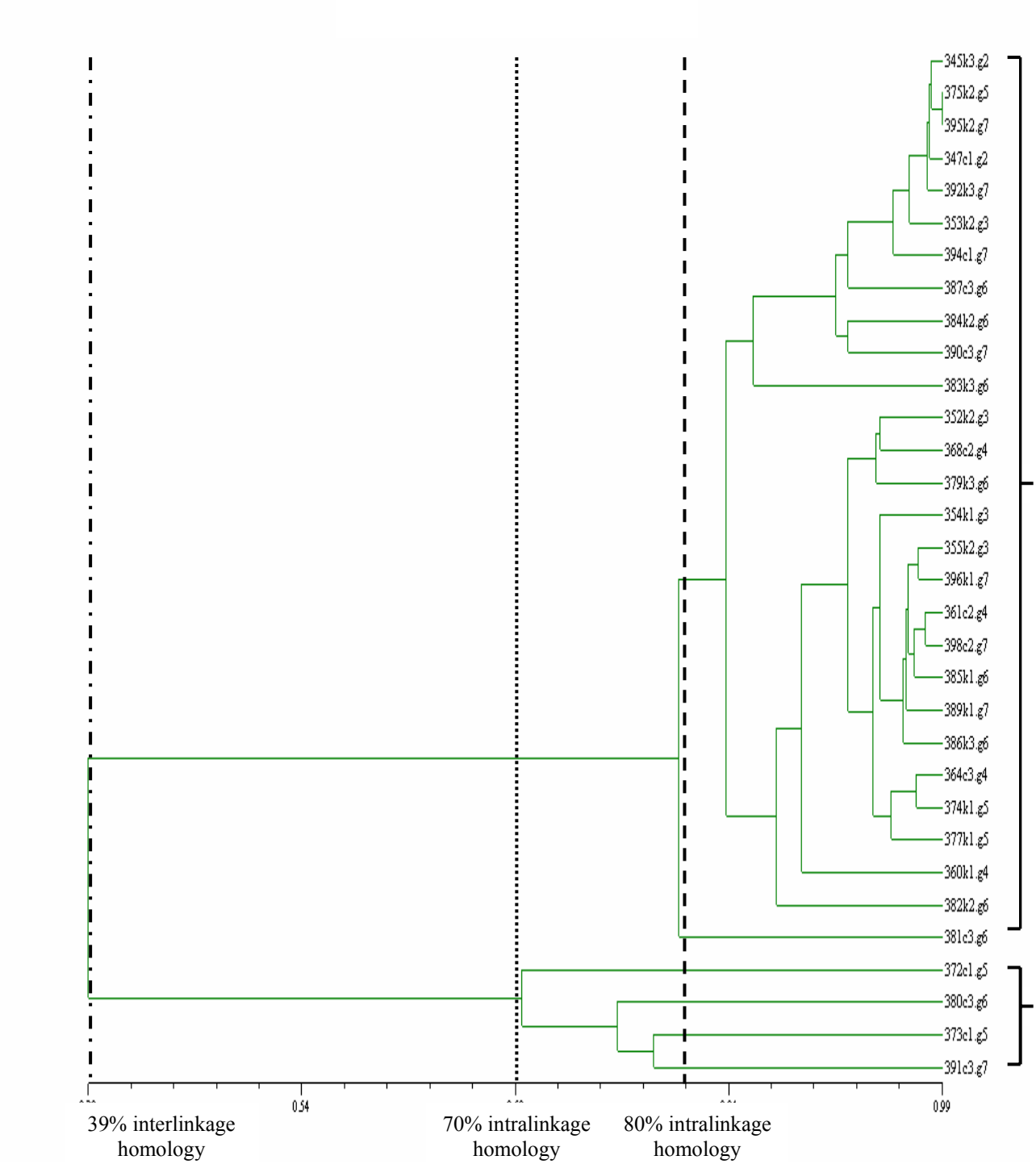
Appendix Figure 2 Dendogram of AFLP fingerprints of *Campylobacter* from farm 2. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



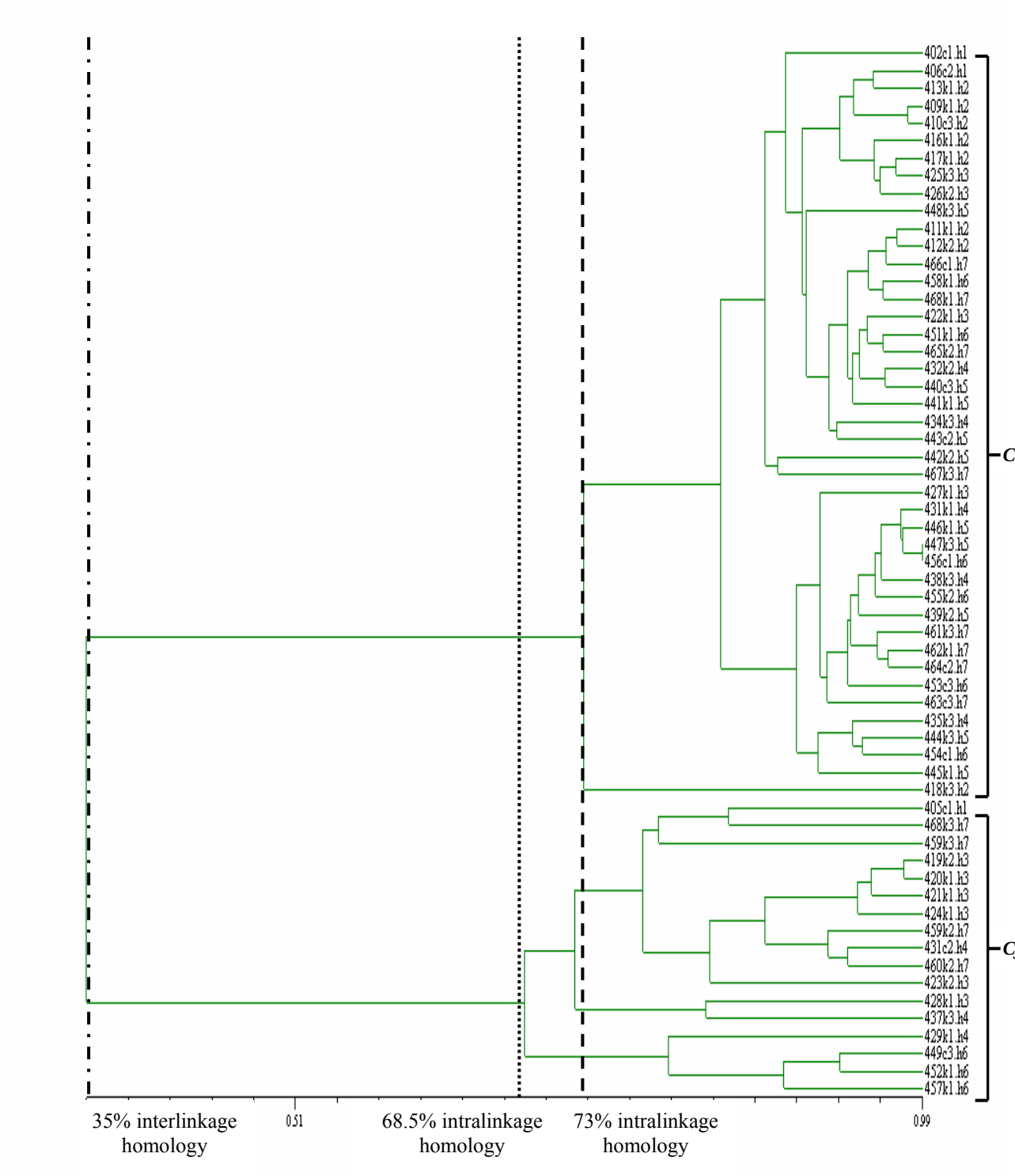
Appendix Figure 3 Dendogram of AFLP fingerprints of *Campylobacter* from farm 3. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



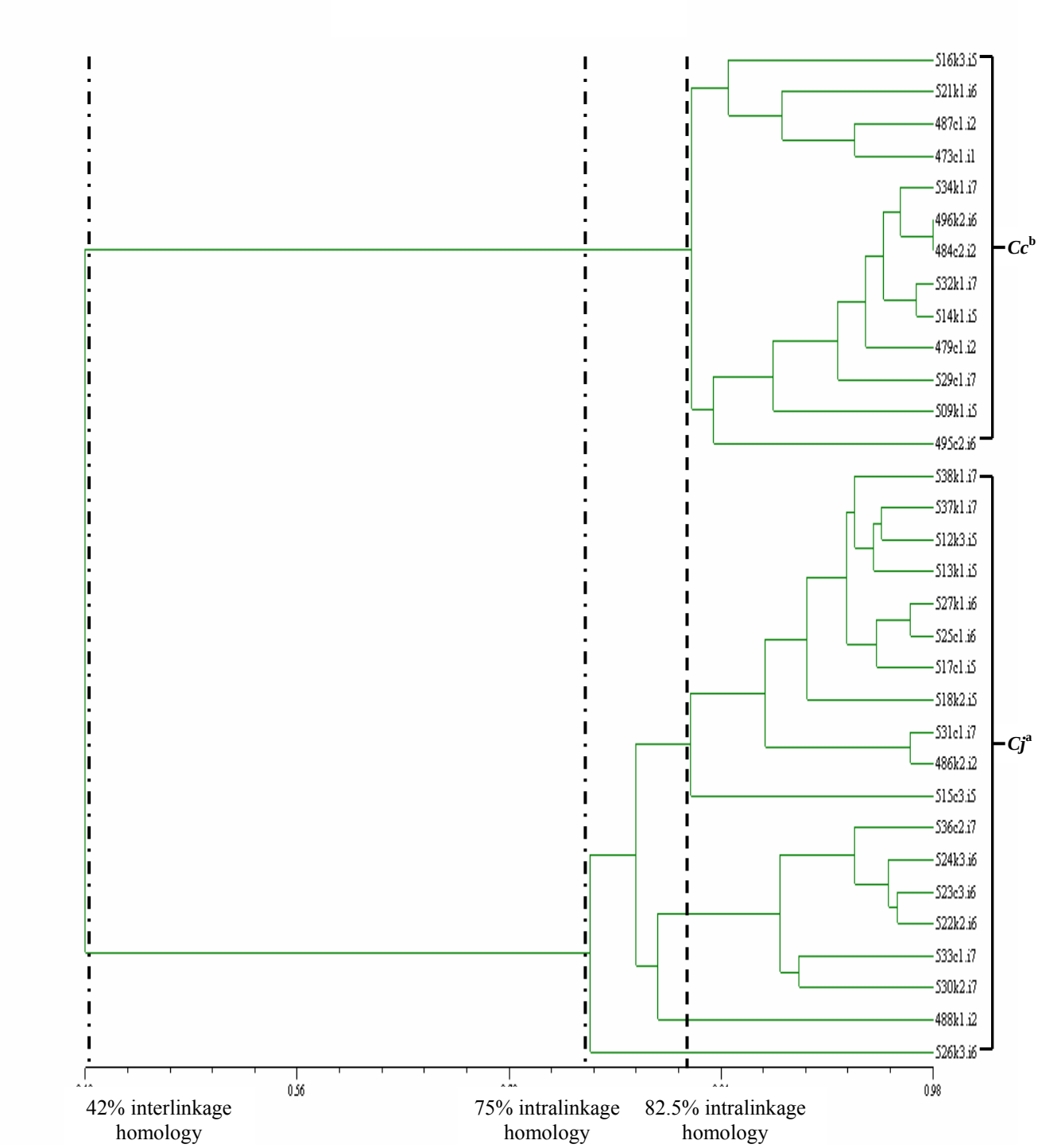
Appendix Figure 4 Dendogram of AFLP fingerprints of *Campylobacter* from farm 4. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



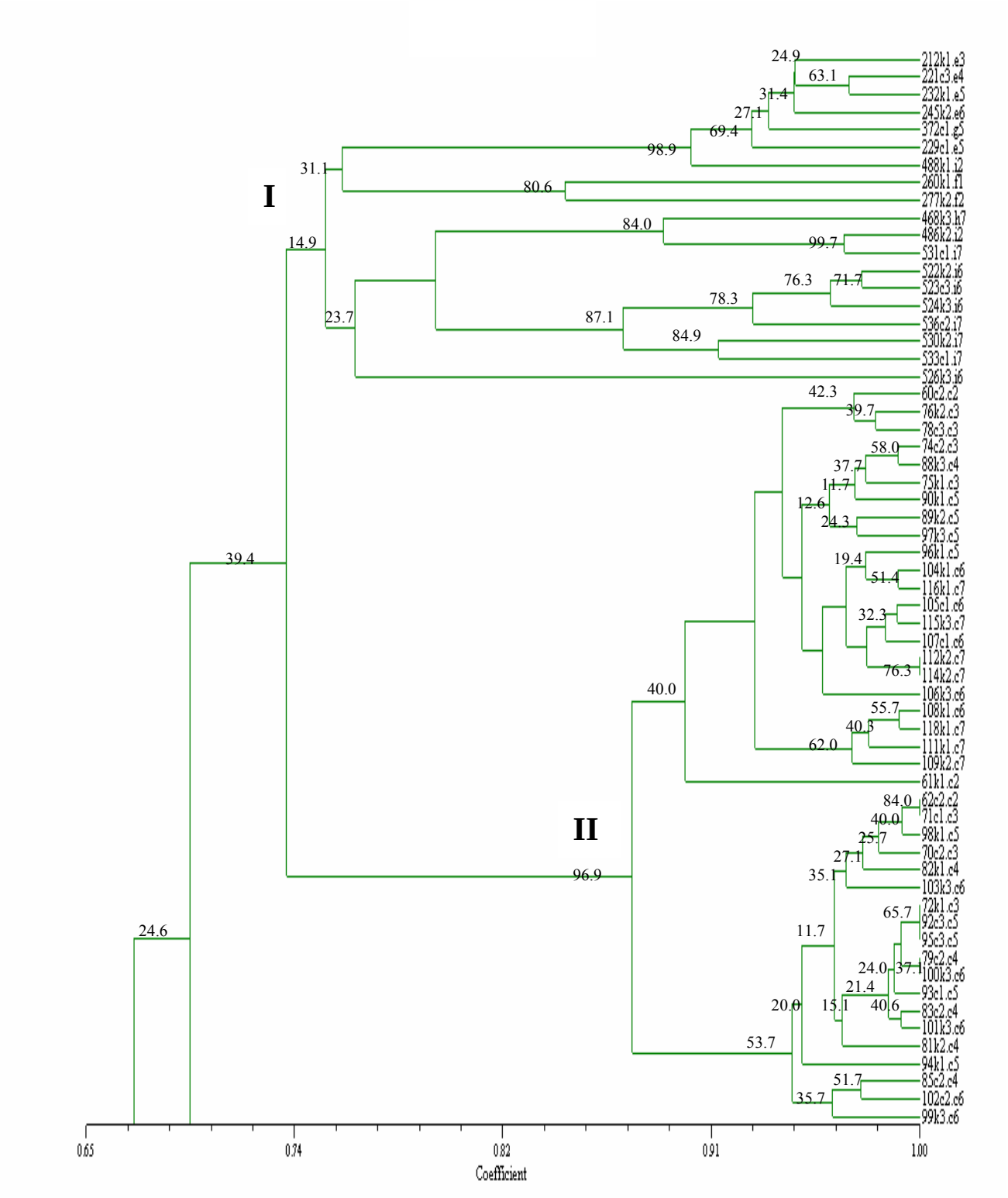
Appendix Figure 5 Dendogram of AFLP fingerprints of *Campylobacter* from farm 5. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



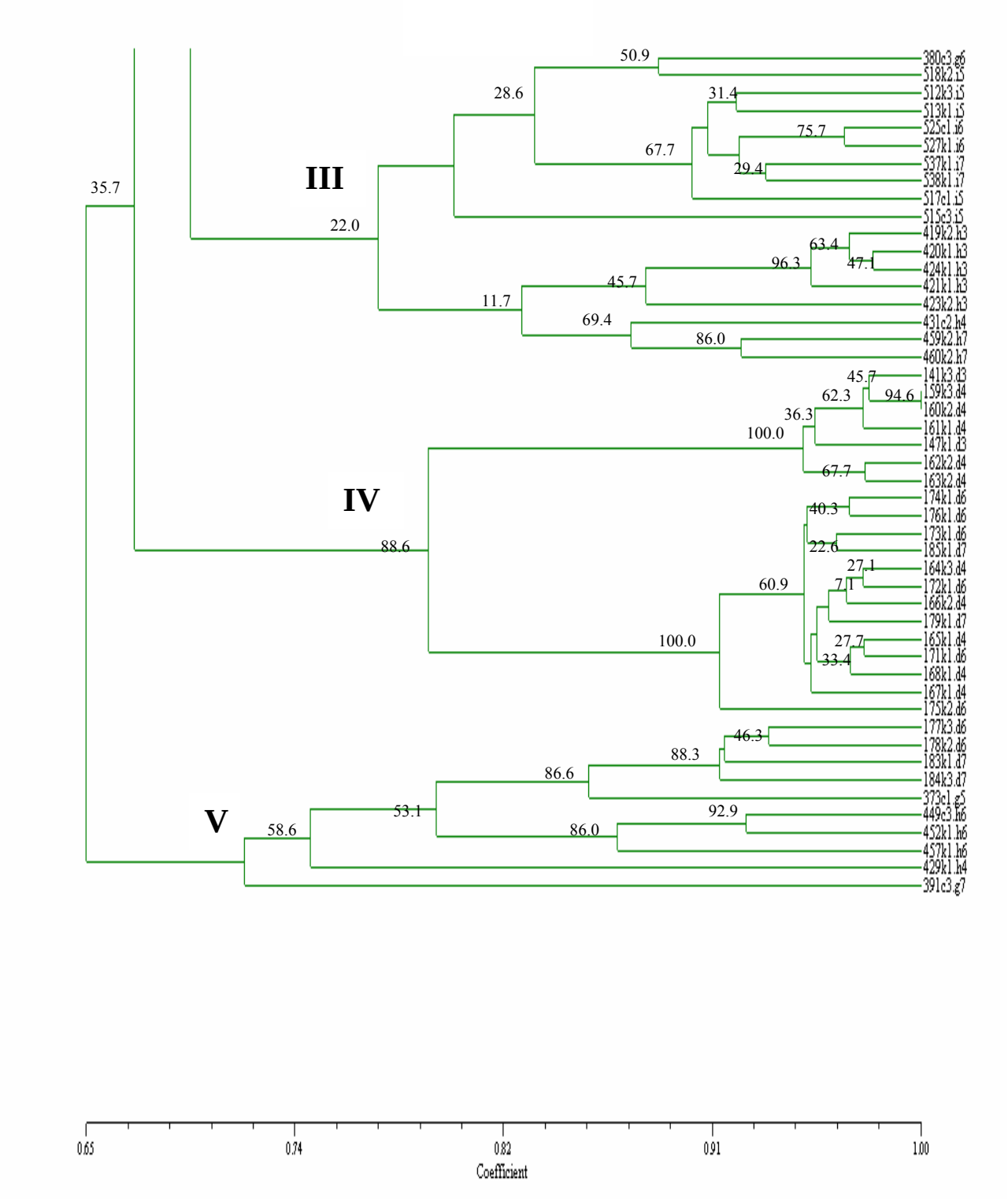
Appendix Figure 6 Dendogram of AFLP fingerprints of *Campylobacter* from farm 6. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



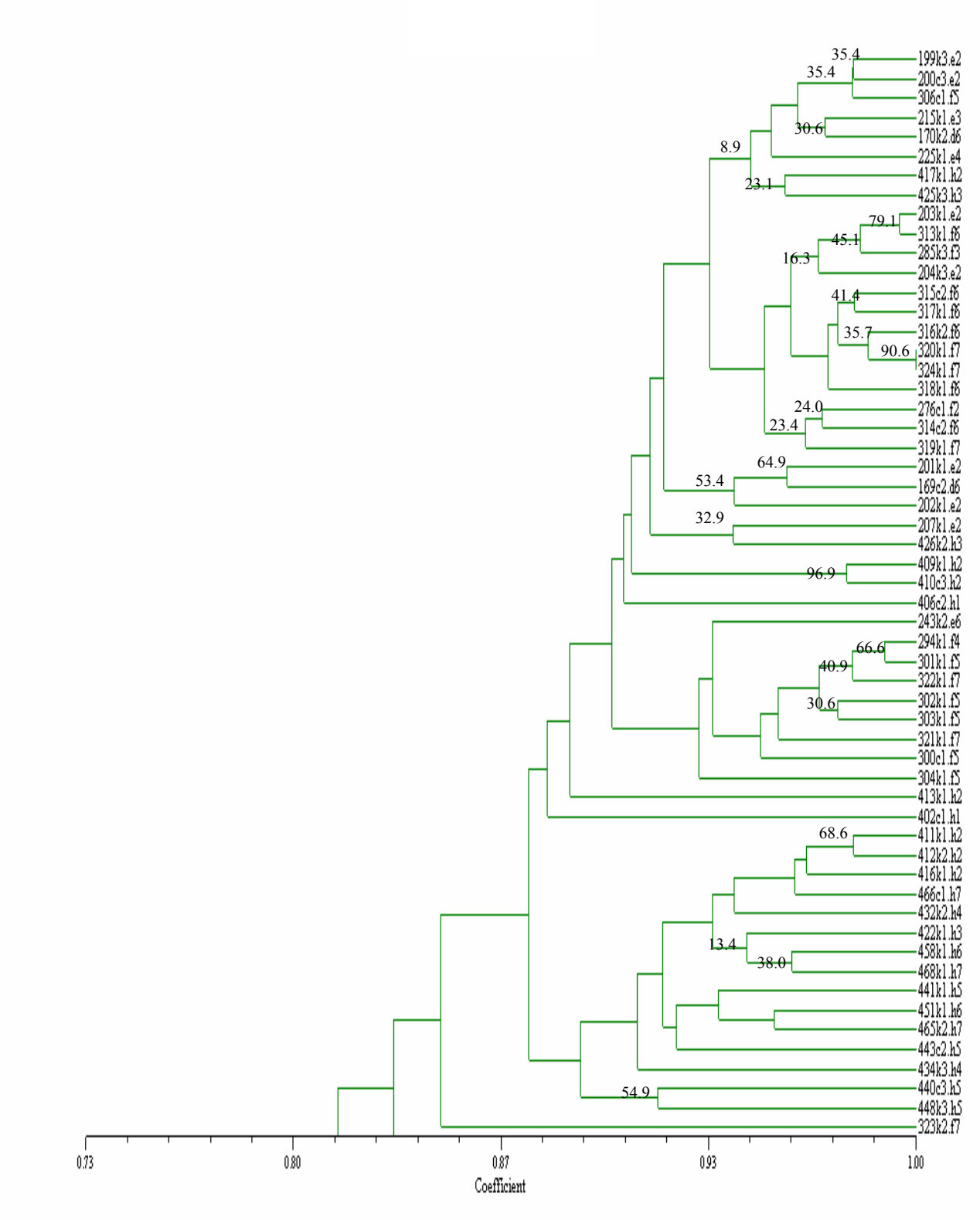
Appendix Figure 7 Dendogram of AFLP fingerprints of *Campylobacter* from farm 7. The dotted vertical line indicates the percentage of interlinkage homology of both *Campylobacter* spp. and intralinkage homology of *C. jejuni* and *C. coli*.
[a]*Cj = C. jejuni*; [b]*Cc = C. coli*



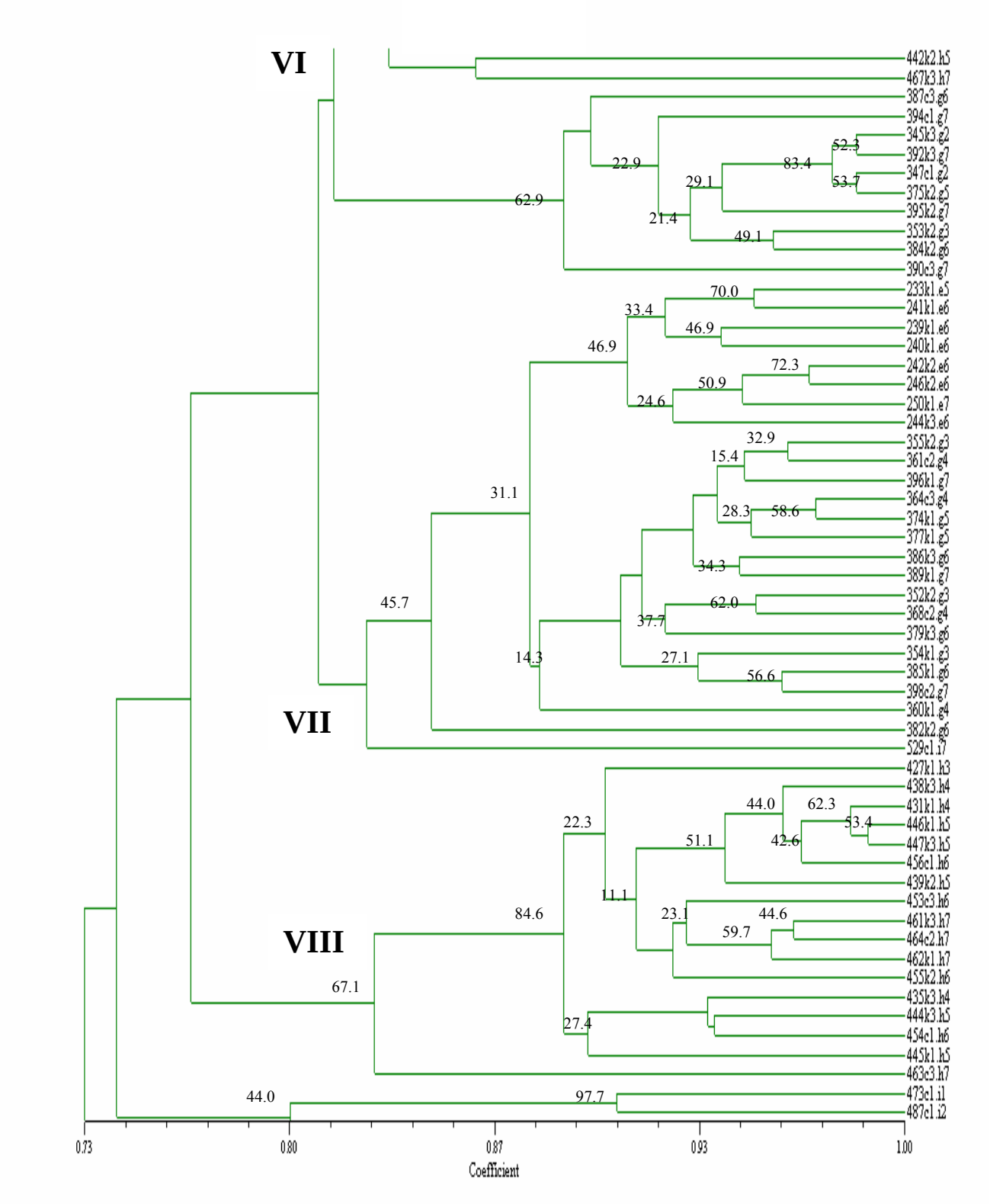
Appendix Figure 8 Dendogram of AFLP fingerprints of *C. jejuni* strains. Values appearing above the branches are percentages of 350 bootstrap analyses which replicate the reliability of the branching pattern.



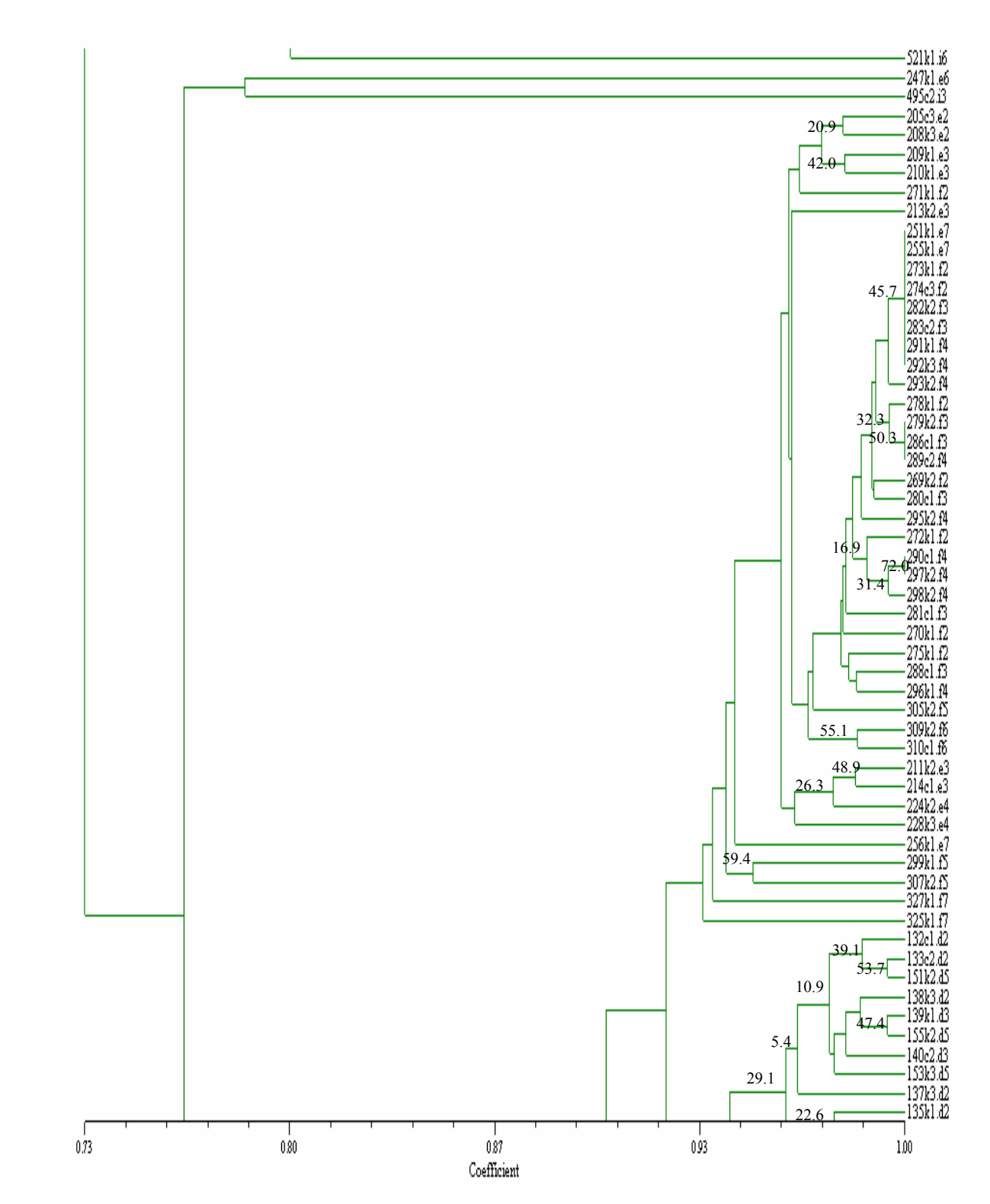
Appendix Figure 8 (Continued)



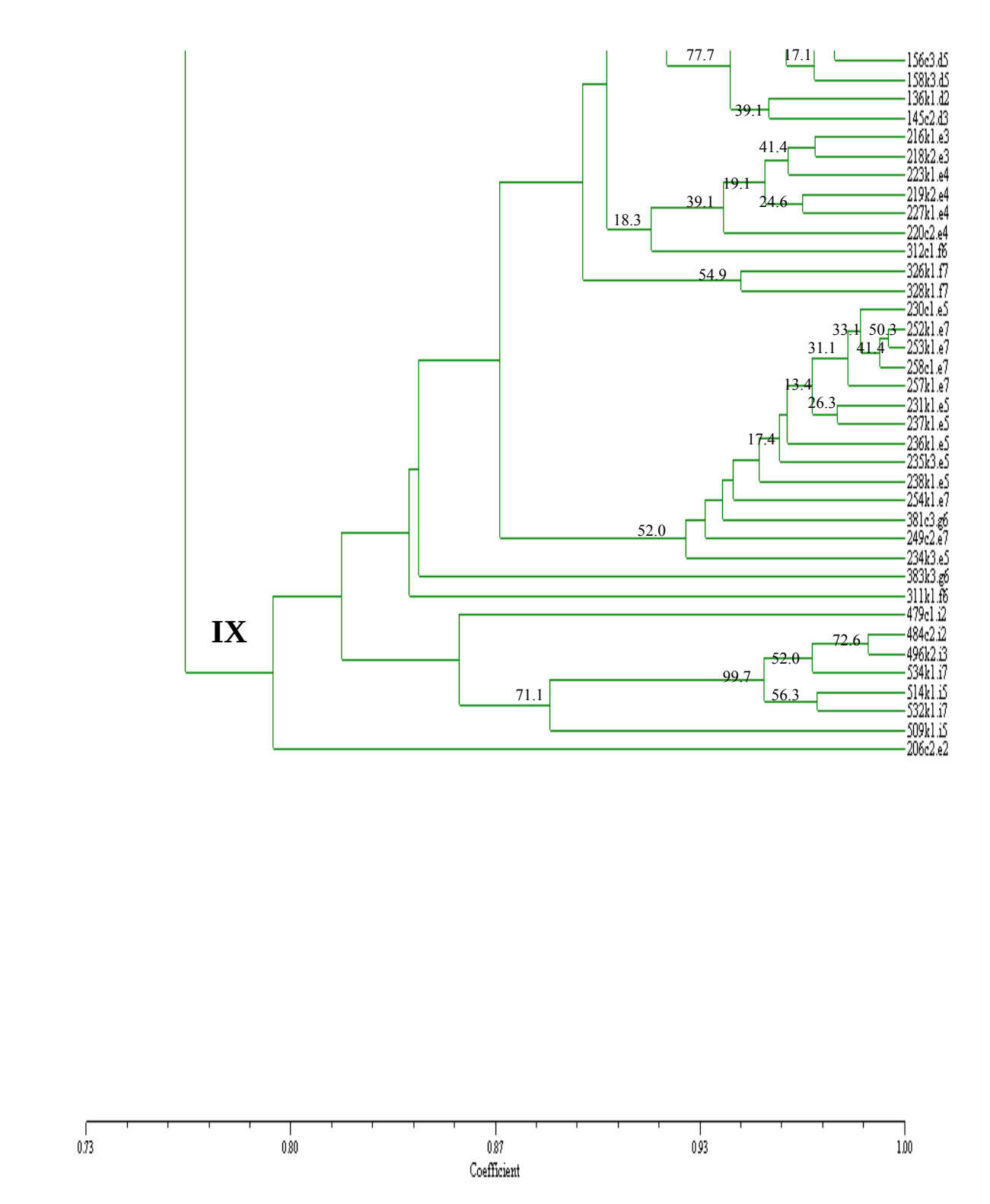
Appendix Figure 9 Dendogram of AFLP fingerprints of *C. coli* strains. Values appearing above the branches are percentages of 350 bootstrap analyses which replicate the reliability of the branching pattern.



Appendix Figure 9 (Continued)



Appendix Figure 9 (Continued)



Appendix Figure 9 (Continued)

CIRRICULUM VITAE

NAME : Mr. Chalermkiat Saengthongpinit

BIRTH DATE : May 11, 1973

BIRTH PLACE : Bangkok, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE/DIPLOMA**
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WORK PLACE : Department of Veterinary Public Health and Diagnostic services, Faculty of Veterinary Medicine, Kasetsart University

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