อุบัติการณ์ การดื้อยา และรูปแบบทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์ที่แยกได้จากเนื้อไก่ในเขตกรุงเทพมหานคร

นางสาวพรรณวิภา ภาษีผล

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OCCURRENCE, ANTIBIOTIC RESISTANCE AND GENETIC PROFILES OF *ARCOBACTER* ISOLATED FROM CHICKEN CARCASSES IN BANGKOK

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พรรณวิภา ภาษีผล: อุบัติการณ์ การดื้อยา และรูปแบบทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์ที่แยกได้จากเนื้อไก่ในเขตกรุงเทพมหานคร. (OCCURRENCE, ANTIBIOTIC RESISTANCE AND GENETIC PROFILES OF *ARCOBACTER* ISOLATED FROM CHICKEN CARCASSES IN BANGKOK) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: น.สพ.ดร. ธราดล เหลืองทองคำ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.นิภา โชคสัจจะวาที, 103 หน้า.

การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาอุบัติการณ์ การดื้อยา และลักษณะทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์ในเนื้อไก่ในเขตกรุงเทพมหานคร โดยทำการเก็บตัวอย่างไก่สดจำนวน 180 ตัวอย่างจากตลาดสด 15 แห่ง และห้างสรรพสินค้า 19 แห่ง ในระหว่างปี พ.ศ. 2553 - 2554 เชื้อที่เพาะแยกได้ถูกนำมาทดสอบการดื้อต่อยาปฏิชีวนะ 6 ชนิด ได้แก่ ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid และ tetracycline ด้วยวิธี Agar dilution method และศึกษาลักษณะทางพันธุกรรมโดยเทคนิค Repetitive element sequence-based PCR (rep-PCR) และ Pulsed-field Gel Electrophoresis (PFGE) ผลการศึกษาพบว่าไก่สดที่จำหน่ายในเขตกรุงเทพมหานครมีการปนเปื้อนของเชื้ออาร์โคแบคเตอร์เป็นจำนวนมาก โดย 100% และ 73.3% ของตัวอย่างไก่สดที่มาจากตลาดสดและห้างสรรพสินค้ามีการปนเปื้อนของเชื้ออาร์โคแบคเตอร์ตามลำดับ ผลการทดสอบการดื้อต่อยาปฏิชีวนะแสดงให้เห็นว่าเชื้ออาร์โคแบคเตอร์จำนวน 114 ตัวอย่าง (76.0%) ดื้อต่อยา nalidixic acid และเชื้ออาร์โคแบคเตอร์จำนวน 69 ตัวอย่าง (46.0%) ดื้อต่อยา ciprofloxacin ในขณะที่อัตราการดื้อยาต่อยาปฏิชีวนะชนิดอื่นอยู่ในระดับที่ต่ำกว่า 3% ผลการศึกษาลักษณะทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์ด้วยเทคนิค rep-PCR และ PFGE แสดงให้เห็นว่าเชื้ออาร์โคแบคเตอร์ที่ปนเปื้อนในเนื้อไก่ในเขตกรุงเทพมหานครมีความหลากหลายทางพันธุกรรมเป็นอย่างมาก จากผลการศึกษาดังกล่าว มีความเป็นไปได้ที่เชื้ออาร์โคแบคเตอร์ที่ปนเปื้อนในเนื้อไก่จะมีแหล่งที่มาแตกต่างกัน ดังนั้นการศึกษาถึงแหล่งที่มาของการปนเปื้อนจึงเป็นสิ่งจำเป็นเพื่อที่จะได้ทำการควบคุมและลดอัตราการปนเปื้อนของเชื้ออาร์โคแบคเตอร์ในเนื้อไก่ได้อย่างมีประสิทธิภาพ

ภาควิชา..... สัตวแพทยสาธารณสุข.....ลายมือชื่อนิสิต.....

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PANVIPA PHASIPOL : OCCURRENCE, ANTIBIOTIC RESISTANCE AND GENETIC PROFILES OF *ARCOBACTER* ISOLATED FROM CHICKEN CARCASSES IN BANGKOK.
ADVISOR : TARADON LUANGTONGKUM, D.V.M., Ph.D., CO-ADVISOR : NIPA CHOKESAJJAWATEE, Ph.D., 103 pp.

The objective of the present study was to determine the occurrence, antibiotic resistance and genetic profiles of *Arcobacter*. One hundred and eighty whole chicken carcasses were collected from 15 fresh markets and 19 supermarkets located in Bangkok during 2010 - 2011. *Arcobacter* isolated strains were tested for their antimicrobial resistance to 6 antimicrobial agents including ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid and tetracycline using the agar dilution method. In addition, repetitive element sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) were also performed to study the genetic characteristics of *Arcobacter*. The results showed that chicken carcasses sold in Bangkok were highly contaminated with *Arcobacter*. All of chicken samples from fresh markets (100%) and 73.7% of chicken carcasses from supermarkets were positive for *Arcobacter*. The most common antimicrobial resistance observed in the present study was nalidixic acid resistance which was found in 114 *Arcobacter* isolates (76.0%), followed by ciprofloxacin resistance which was found in 69 *Arcobacter* isolates (46.0%), while the resistance rate to other antimicrobials tested was less than 3%. The results of rep-PCR and PFGE were in agreement which revealed a high degree of genetic diversity of *Arcobacter*. The presence of a variety of *Arcobacter* strains may reflect that there were multiple sources of contamination. Further investigations are needed to identify the source of contamination in order to effectively control and reduce the occurrence of this emerging foodborne pathogen.

Department : ……Veterinary Public Health……. Student's Signature ……...

Field of Study : ….Veterinary Public Health…….... Advisor's Signature…….

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LIST OF ABBREVIATION

A.	*Arcobacter*
CAT	cefoperazone-amphotericin B-teicoplanin
CLSI	The Clinical and Laboratory Standards Institute
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
NARMS	The National Antimicrobial Resistance Monitoring System
PEG	polyethylene glycol
PFGE	Pulsed-Field Gel Electrophoresis
Rep-PCR	Repetitive sequence-based polymerase chain reaction
spp.	species
TE	Tris-EDTA
TBE	Tris-Borate EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Chapter I

INTRODUCTION

Arcobacter is a gram-negative spiral-shaped bacterium that has been receiving more attention in recent years for its potential role as an emerging foodborne pathogen. This organism has been frequently isolated from food of animal origin and from cases of human enteritis. Although three species of the genus which are *Arcobacter butzleri* (*A. butzleri*), *Arcobacter cryaerophilus* (*A. cryaerophilus*) and *Arcobacter skirrowii* (*A. skirrowii*) have been implicated in human illnesses (On et al., 1995; Wybo et al., 2004; Lehner et al., 2005), *A. butzleri* is the most prevalent species and is more often isolated from clinical samples than the other species (Houf et al., 2003; Samie et al., 2007). *A. butzleri* was originally isolated from humans and animals with diarrhea (Kiehlbauch et al., 1991) and occasionally from bacteremic patients (On et al., 1995). The symptoms of patients with *A. butzleri* infections include abdominal pain, nausea, vomiting and fever which are similar to those of *Campylobacter jejuni* infections. However, *A. butzleri* infection is likely to cause more watery and persistent diarrhea (Vandenberg et al., 2004). In addition to *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*

have also been isolated from stool of diarrheic patients (Vandenberg et al., 2004; Wybo et al., 2004). *Arcobacter* species particularly *A. butzleri* has been categorized as an emerging foodborne pathogen by the International Commission on Microbiological Specifications for Foods (ICMSF) since 2002 (ICMSF, 2002).

Like other enteric bacteria, *Arcobacter* infections in humans likely occur by the oral route via contaminated food or water. Food of animal origin has been considered the most important source of *Arcobacter* transmission to humans (Ho et al., 2006a; Snelling et al., 2006). It has been shown that *Arcobacter* is frequently isolated from a wide variety of meats including chicken, pork, beef, lamb and even seafood (Kabeya et al., 2004; Rivas et al., 2004; Patyal et al., 2011). Similar to *Campylobacter* spp., this bacterium is more prevalent in poultry meat than pork and beef (Kabeya et al., 2004; Rivas et al., 2004). Since poultry is considered a major reservoir of *Arcobacter*, handling raw poultry or consumption of undercooked poultry products seems to play a key role in *Arcobacter* infection in humans (Ho et al., 2006a).

Even though the majority of enteritis cases caused by *Arcobacter* spp. do not require antimicrobial treatment and are usually self-limited, antimicrobial therapy might be warranted in some cases especially in patients with severe and prolonged symptoms (Collado and Figueras, 2011). Among antimicrobial agents commonly used for treatment of foodborne bacterial diseases, fluoroquinolones (e.g. ciprofloxacin) have been suggested as the most effective agent for the treatment of *Arcobacter* infections

(Vandenberg et al., 2006). Unfortunately, ciprofloxacin-resistant *Arcobacter* strains have been reported over the last few years (Abdelbaqi et al., 2007b). Moreover, it has been shown that some of *Arcobacter* strains especially the isolates from broiler chickens were also resistant to other antibiotics commonly used for treatment of bacterial infections in humans and animals, i.e. ampicillin, amoxycillin, amoxycillin/clavulanic acid, azithromycin, clindamycin and erythromycin (Atabay and Aydin, 2001; Houf et al., 2004; Son et al., 2007a). Because antimicrobial-resistant *Arcobacter* strains may transfer resistance genes to susceptible strains or to other bacteria, especially their closely related genus *Campylobacter*, the presence of antimicrobial resistance in *Arcobacter* is a concern for public health (Snelling et al., 2006).

In Thailand, the information on *Arcobacter* is very limited. Besides having been isolated from diarrheic patients (Taylor et al., 1991), the bacterium has also been recovered from cooked meals served in restaurants in Bangkok (Teague et al., 2010). Teague and colleagues (2010) revealed that the contamination of *Arcobacter* in meals was more frequently observed than the contamination of *Salmonella* or *Campylobacter*, the two most common causes of foodborne disease worldwide. Although it has previously shown that poultry is an important source of *Arcobacter* infections in humans, studies focusing on *Arcobacter* in chicken meat in Thailand were barely established. Neither antimicrobial resistance patterns nor genetic profiles of *Arcobacter* strains isolated from chicken meat in Thailand have been investigated.

Therefore, the aims of the present study are to determine the occurrence of *Arcobacter* in chicken carcasses in Bangkok and to determine their antimicrobial resistance patterns and genetic profiles. Such information will increase the awareness of this new emerging foodborne pathogen and help elucidate the current situation of antibiotic resistance and genetic characteristics of *Arcobacter* isolated from chicken meat in Bangkok metropolitan area.

Chapter II

LITERATURE REVIEW

2.1 General characteristic of *Arcobacter* spp.

Arcobacter is a Gram-negative S shaped bacterium that belongs to the family *Campylobacteraceae*. Due to the similar colony morphology between *Arcobacter* and *Campylobacter*, a leading cause of foodborne bacterial pathogen worldwide (Humphrey et al., 2007), *Arcobacter* was formerly known as “aerotorelant *Campylobacter*”. The most important characteristic that differentiates these two bacteria is the ability of *Arcobacter* to grow in the presence of air and at lower temperature ranging between 15 – 37 °C (Vandamme et al., 1991). *Arcobacter* has unsheathed flagella at one or both ends of the cell which facilitate its corkscrew motility. The size of *Arcobacter* is 0.2 to 0.9 μm wide and 1 to 3 μm long. *Arcobacter* yields positive results to oxidase and catalase tests and has nitrate reduction activity. The G+C contents of *Arcobacter* DNA range between 28 – 31 mol% (Vandamme et al., 1991).

2.2 Habitats of *Arcobacter* spp.

Arcobacter was originally isolated from aborted fetus of farm animals (Ellis et al., 1977). However, later studies show that the organism can also be found in a wide variety of habitats and hosts. *Arcobacter* was isolated from feces of both healthy animals and animals with diarrhea (Vandamme et al., 1992b; Kabeya et al., 2003b; van Driessche et al., 2003), along with other veterinary specimens such as reproductive tracts of cattle and swine (Vandamme et al., 1992b; De Oliveria et al., 1999; Kabeya et al., 2003b), milk of cows with mastitis (Logan et al., 1982) and oral cavities of felines and canines (Houf et al., 2008; Fera et al., 2009). Apart from the presence of *Arcobacter* in live animals, *Arcobacter* has been frequently isolated from food of animal origins especially meat product which is regarded as the major transmission vehicle of *Arcobacter* to humans (Ho et al., 2006a). In humans, the organism has been recovered from diarrheic stool samples and occasionally from bacteremic patients (On et al., 1995; Hsueh et al., 1997; Woo et al., 2001). In addition, *Arcobacter* has also been isolated from diverse environmental samples such as roots of *Spartina alterniflora*, a salt marsh plant (McClung et al., 1983), sewage treatment plant (Stampi et al., 1993), hypersaline lagoon (Donachie et al., 2005), sea water, seaweeds and starfish (Kim et al., 2010). The recent proposed *Arcobacter* species were recovered from shellfishes, pork meat and sewage in Spain (Figueras et al., 2011b; Levican et al., 2012a; Levican et al., 2012b). Presently,

genus *Arcobacter* contains 17 recognized *Arcobacter* species. Table 1 is the list of *Arcobacter* species and their source of isolation.

2.3 *Arcobacter* in humans

An association between *Arcobacter* and human diarrhea was first reported in 1991 when Kiehlbauch and colleagues cultured aerotolerant *Campylobacter* from stool samples of diarrheic patients. The discovered isolates were proposed as *Campylobacter butzleri* sp. nov. and *Campylobacter cryaerophilus* sp. nov. (Kiehlbauch et al., 1991), which subsequently designated as *A. butzleri* and *A. cryaerophilus*, respectively (Vandamme et al., 1992b). *Arcobacter* has been isolated from diarrheic stools of patients in several countries such as Belgium, France, South Africa and Thailand (Taylor et al., 1991; Vandenberg et al., 2004; Prouzet-Mauleon et al., 2006; Samie et al., 2007). The largest scale study on *Arcobacter* in humans was conducted in Belgium. Almost 67,600 stool specimens were collected and cultured for enteropathogens. *A. butzleri* was the fourth common organism isolated from stool samples of diarrheic patients (Vandenberg et al., 2004). Although the symptoms of patients with *A. butzleri* infections are similar to those of *C. jejuni* infections, the diarrhea caused by *Arcobacter* tends to be more watery and persistent (Vandenberg et al., 2004). One recent study has shown that *Arcobacter* was also involved with traveler's diarrhea. The study showed that *Arcobacter* was detected in diarrheic stool samples of

European and U.S. travelers who had traveled to Mexico, Guatemala and India (Jiang et al., 2010). Three species of *Arcobacter* that have been implicated in human illnesses are *A. butzleri, A. skirrowii* and *A. cryaerophilus*. *A. butzleri* was the most frequently observed species (Vandenberg et al., 2004; Samie et al., 2007; Collado and Figueras, 2011) and has been classified as a foodborne pathogen by the International Commission on Microbiological Specifications for Foods (ICMSF) since 2002 (ICMSF, 2002), whereas *A. skirrowii* and *A. cryaerophilus* were reported at lower rates (Ho et al., 2006a; Collado and Figueras, 2011). The isolation of *Arcobacter* from feces of healthy humans has also been reported in a few studies (Vandenberg et al., 2004; Houf and Stephan, 2007; Samie et al., 2007). Indeed, the true role of *Arcobacter* in human illnesses may be underestimated due to false identification of *Arcobacter* with other pathogens such as *C. fetus* as both organisms can grow at 25 °C (Prouzet-Mauleon et al., 2006; Collado and Figueras, 2011).

Apart from the association between *Arcobacter* and enteritis cases, invasive *Arcobacter* infections have also been described (On et al., 1995). Bacteremic cases involving *Arcobacter* infections were mostly found in patients with underlying diseases such as liver cirrhosis (Yan et al., 2000), chronic renal failure (Hsueh et al., 1997), diabetes (Fera et al., 2010) and heart disease (Wybo et al., 2004). In addition, a fatal *Arcobacter* case was reported in the patient infected with *A. cryaerophilus* (Woo et al., 2001). Association between *Arcobacter* and human illnesses is summarized in Table 2.

2.4 Transmission of *Arcobacter* to humans

Similar to other foodborne pathogens, the major transmission route of *Arcobacter* is oral route via consumption of contaminated food and water (Collado and Figueras, 2011). Foods of animal origins particularly chicken meat have been frequently contaminated with *Arcobacter* and speculated to be the most important source of infection in humans (Ho et al., 2006a). Apart from *Arcobacter* contamination in foods of animal origin, the organism was also found in fresh vegetables such as lettuce (Gonzalez and Ferrus, 2011). Since lettuce is usually consumed without cooking, it may serve as a source of *Arcobacter* infection in humans. Recently, *Arcobacter* was found in oral cavities of dogs and cats. Thus, it is possible that this organism may be transmitted to their owners via direct contact (Houf et al., 2008; Fera et al., 2009). Other possible routes of *Arcobacter* transmission included infection via placenta and respiratory tract (On et al., 1995; Woo et al., 2001).

Arcobacter infections in humans are usually sporadic. However, an outbreak of *A. butzleri* infection was reported in Italy where 10 school students were affected with recurrent abdominal cramps without diarrhea. The same strain of *A. butzleri* was isolated from stool samples of all cases (Vandamme et al., 1992a).

Table 1. List of *Arcobacter* species and their source of isolation

Species	Type strain	Other designation	Source of isolation	Reference
A. bivalviorum	LMG 26155	CECT 7835	Mussels	Levican et al., 2012a
A. butzleri	ATCC 49616	CCUG 30485; CIP 103493; CIP 103537; DSM 8739; LMG 10828; NCTC 12481	Feces of human with diarrhea	Kiehlbauch et al., 1991 Vandamme et al., 1992a
A. cibarius	LMG 21996	CCUG 48482	Broilers	Houf et al., 2005
A. cloacae	LMG 26153	CECT 7834	Sewage	Levican et al., 2012b
A. cryaerophilus	ATCC 43158	CCUG 17801; CCUG 17801; CIP 104014; DSM 7289; LMG 7536; LMG 9904; NCTC 11885	Aborted bovine fetus	Neill et al., 1985 Vandamme et al., 1991
A. defluvii	LMG 25694	CECT 7697	Sewage	Collado et al., 2010
A. ellisii	LMG 26155	CECT7837	Mussels	Figueras et al., 2011b
A. halophilus	ATCC BAA-1022	CIP 108450	Hypersaline lagoon	Donachie et al., 2005
A. marinus	JCM 15502	KCCM 90072	Seawater, Seaweeds, Starfish	Kim et al., 2010
A. molluscorum	LMG 25693	CECT 7696	Mussels, Oysters	Figueras et al., 2011a
A. mytili	LMG 24559	CECT 7386;F2075	Mussels	Collado et al., 2009
A. nitrofigilis	ATCC 33309	LMG 7604; CCUG 15893; CECT 7204	Roots of *Spartina alterniflora*	McClung et al., 1983 Vandamme et al., 1991
A. skirrowii	ATCC 51132	CCUG 10374; CIP 103538	Feces of lamb with diarrhea	Vandamme et al., 1992
A. suis	LMG 26152	CECT 7833	Pork	Levican et al., 2012b
A. thereius	LMG 24486	CCUG 56902	Pigs, Ducks	Houf et al., 2009
A. trophiarum	LMG 25534	CCUG 59229	Fattening pigs	De Smet et al., 2011
A. venerupis	LMG 26156	CECT 7836	Clams	Levican et al., 2012a

ATCC, American Type Culture Collection, Rockville, Md; CCUG, Culture Collection of the University Goteborg, Goteborg,Sweden; CECT, Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CIP, CollectionBacterienne de l'Institut Pasteur, Paris, France; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Gent, Belgium; JCM, Japan Collection of Microorganisms; KCCM, Korean Culture Center of Microorganisms, Seoul, Korea; NCTC, National Collection of Type Cultures, Central Public Laboratory Service, London, UK; DSM, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

(Modified from L. Collado, unpublished Ph.D. dissertation)

Table 2. Association between *Arcobacter* and human illness

Countries	*Arcobacter* species	Symptoms	Reference
USA	*A. butzleri, A. cryaerophilus*	Diarrhea	Kielhbauch et al., 1991
Germany	*A. butzleri*	Diahhrea, Abominal cramp	Lerner et al., 1994
Taiwan	*A. butzleri*	Bacteremia	On et al., 1995
Taiwan	*A. cryaerophilus*	Bacteremia	Hsueh et al., 1997
Thailand	*A. cryaerophilus*	Diarrhea	Taylor et al., 1991
Taiwan	*A. butzleri*	Bacteremia	Yan et al., 2000
	A. butzleri	Bacteremia,	Lau et al., 2002
Belgium	*A. butzleri*	Diarrhea	Vandenberge et al., 2004
Belgium	*A. skirrowii*	Chronic diarrhea	Wybo et al., 2004
France	*A. butzleri*	Diarrhea	Prouzet-Maleon et al., 2006
Hong Kong	*A. cryaerophilus*	Bacteremia	Woo et al., 2001
South Africa	*A. butzleri, A. skirrowii, A.cryaerophilus*	Diarrhea	Samie et al., 2007
Italy	*A. butzleri, A. cryaerophilus*	Diabetes	Fera et al., 2010
India	*A.* species	Diarrhea	Patyal et al., 2011

(Modified from Collado and Figueras, 2011)

2.5 Pathogenicity of *Arcobacter*

Despite the fact that *Arcobacter* has been discovered for many years, the pathogenicity of the organism still needs much further clarification (Collado and Figueras, 2011). A study of *Arcobacter* infection in experimental animals showed that orally infected rats with *A. butzleri* strains were suffered from diarrheal illness. Histopathology lesions of the infected rats included hepatic necrosis, villous erosion, desquamation and necrosis of small intestine (Adesiji et al., 2009). Another experimental infection of *Arcobacter* in animals was conducted in rainbow trout. *A. cryaerophilus* strains were administered into the muscle of the fish, which subsequently resulted in

degenerated opercula and gills, liver damage, hemorrhagic kidney and swollen intestine (Yildiz and Aydin, 2006).

The adhesion capacity, invasiveness and cytotoxicity of *Arcobacter* were investigated in several types of cell lines (Musmanno et al., 1997; Johnson and Maruno, 2002; Villarruel-Lopez et al., 2003; Ho et al., 2007). Among these virulence mechanisms, cytotoxicity was the most apparent characteristic of *Arcobacter* followed by adhesion and invasion (Collado and Figueras, 2011). In order to determine the pathogenicity of *Arcobacter* causing diarrhea in humans, a study using human colonic epithelial cells (HT-29/B6) was performed. Interestingly, an expression of claudins, tight-junction proteins, of epithelial cells was decreased which led to dysfunction and apoptosis of those cells and increase in paracellular transport (Bucker et al., 2009). The ability of *Arcobacter* to induce production of proinflammatory cytokine interleukin-8 (IL-8), an important virulence factor in *Campylobacter* and *Helicobacter*, was also observed (Ho et al., 2007). Moreover, some putative virulence genes homologous to those of *C. jejuni* were discovered in the genome of *A. butzleri* RM4018. However, it is unknown whether these putative virulence determinants are function or not (Miller et al., 2007).

2.6 *Arcobacter* in animals and foods of animal origin

Arcobacter has been found in animals since 1977 when it was recovered from aborted bovine fetuses (Ellis et al., 1977). After the first detection of *Arcobacter* in

veterinary specimens, several reports continue to identify the association between *Arcobacter* and animal illnesses. *Arcobacter* was isolated from aborted swine fetuses (Schoeder-Tucker et al., 1996; DeOliveira et al., 1997; On et al., 2002) as well as from placenta and oviductal tissues of sows with reproductive problems (De Oliveira et al., 1997). Clinical signs of *Arcobacter* infections in sow included infertility, a chronic discharge during estrous, chronic stillborn problems and late-term abortion (Schroeder-Tucker et al., 1996). In cattle, *Arcobacter* was associated with reproductive problems and diarrhea (Ellis et al., 1977; Vandamme et al., 1992b). In addition, an intramammary inoculation of *A. cryaerophilus* was proven to be able to induce acute mastitis in dairy cows. However, the clinical symptoms spontaneously resolved after 5 days without antimicrobial treatment (Logan et al., 1982). Other animals that were infected with *Arcobacter* and showed clinical symptoms included sheep, horses, ostriches, tortoises and non-human primates (Vandamme et al., 1992b; Anderson et al., 1993; Ho et al., 2006a).

Although *Arcobacter* were frequently isolated from animals with clinical symptoms, the organism was found in healthy farm animals as well (Gill, 1983; De Oliveira et al., 1999; Wesley et al., 2000; Kabeya et al., 2003b). A study in the US showed that 71% of dairy cow managements were positive for *Arcobacter* with 14.3% of healthy individual dairy cattle were *Arcobacter* positive (Wesley et al., 2000). The organism was also isolated from preputial sheath washing of healthy bulls and vaginal

swabs of cows with no reproductive problems (Gill, 1983; Kabeya et al., 2003b). In swine, *Arcobacter* was recovered from preputial fluid of boars and fattening pigs (De Oliveria et al., 1999) and approximately 42% of sows were found to be carriers of *Arcobacter* in their intestine which could be transmitted to piglets (Ho et al., 2006b). Although poultry is presumed to be the major reservoir of *Arcobacter*, the presence of this organism in poultry intestine is quite controversial. Some studies indicated that *Arcobacter* was rarely or could not be isolated from intestinal tracts of chickens (Atabay and Corry, 1997; Wesley and Baetz, 1999; Houf et al., 2002b; Adesiji et al., 2011), while other reports showed the evidence of fecal shedding of *Arcobacter* in poultry (Kabeya et al., 2003b; Atabay et al., 2008; Ho et al., 2008). An experimental oral infection of *Arcobacter* in 3 days-old chickens demonstrated that the organism cannot be detected in cloacal swabs or in cecal samples of those inoculated chickens up to 10 days post-infection. Compared to the control chickens which were inoculated with *Campylobacter*, the presence of *Campylobacter* was detected at only 3 days post-inoculation (Wesley and Baetz, 1999). According to these paradoxical findings, *Arcobacter* is currently not recognized as normal flora of chickens and more likely to be a transient colonizer of chicken intestines (Adesiji et al., 2011).

The presence of *Arcobacter* in foods of animal origins is mainly observed in fresh meat products. Poultry meat is more often contaminated with *Arcobacter* than red meat or meat from other animals (Kabeya et al., 2004; Rivas et al., 2004). The

contamination rates of *Arcobacter* in chicken meat varied among studies ranging from 12% in India (Patyal et al., 2011) to 100% in the UK (Atabay et al., 1998). One study in Japan showed that the prevalence of *Arcobacter* in chicken meat was 23% which was significantly higher than that in beef and pork in which the observed prevalence was 2.2% and 7%, respectively (Kabeya et al., 2004). Likewise, a similar study conducted in Australia also found the highest contamination rate of *Arcobacter* in chicken meat samples which was 73%, while the recovery rates of *Arcobacter* in pork, beef and lamb was 29%, 22% and 15%, respectively (Rivas et al., 2004). A high contamination rate of *Arcobacter* in chicken meat was also evident in the Netherlands, Belgium, France, Spain, UK, Turkey and the US (De Boer et al., 1996; Atabay et al., 1998; Gonzalez et al., 2000; Atabay et al., 2003; Son et al., 2007b). The cross-contamination between meat species could be one of the most important factors contributing to a spreading of *Arcobacter* in retail meats. Genotypic patterns of some *Arcobacter* isolates recovered from chicken meat were indistinguishable from those of the strains isolated from pork and beef that were sold in the same establishment by pulsed-field gel electrophoresis (PFGE) method (Rivas et al., 2004).

In addition to the fresh meat products, contamination of *Arcobacter* was also found in raw milk and seafood (Scullion et al., 2006; Collado et al., 2009b; Ertas et al., 2010). Up to 46% of raw milk samples in Northern Ireland were positive for *Arcobacter*

(Scullion et al., 2006). Likewise, 41.1% of mussel in Spain were contaminated with *Arcobacter* (Collado et al., 2009b).

2.7 The introduction of *Arcobacter* into chicken meat

Although the contamination of *Arcobacter* in chicken meat has been frequently recognized, the introduction of *Arcobacter* into chicken meat is still unclear (Houf et al., 2002b). Slaughterhouse environment as well as slaughtering equipments was found to be highly contaminated with *Arcobacter* and may serve as an important source of *Arcobacter* contamination in chicken carcasses (Houf et al., 2002b). One study in Belgium revealed that *Arcobacter* was rarely or could not be isolated from cloacal swabs of chickens at farm level before entering a slaughterhouse, but when neck skins of chicken carcasses during processing were examined, all of the samples were positive for *Arcobacter* (Van Driessche and Houf, 2007). The number of *Arcobacter* cells presented on the neck skins of chickens was observed as high as $>10^3$ cfu/g of skin (Van Driessche and Houf, 2007). On the contrary, Ho and colleagues (2008) proposed that *Arcobacter* in gut system of chickens could be the potential source of carcass contamination. The researchers revealed that *Arcobacter* can be recovered from intestinal tract of chickens at farm level ranging from 20% to 85% in hens and 3.3% to 51% in broilers. In addition, genetic characterization by ERIC-PCR demonstrated that *Arcobacter* isolated strains from intestinal tracts of chickens were indistinguishable from

the strains isolated from carcasses (Ho et al., 2008). Although some *Arcobacter* isolates showed identical DNA fingerprint pattern, the majority of the isolates produced distinct genetic profiles which may indicate multiple sources of contamination. More in depth epidemiological studies of *Arcobacter* on chickens are needed to better define the source of carcass contamination.

2.8 Isolation of *Arcobacter* spp.

Presently, there is no standardized protocol for isolation of *Arcobacter*. The isolation techniques for *Arcobacter* are mainly adopted and adjusted from those of *Campylobacter*. The most commonly used technique is a combination between using an enrichment technique and a filtration technique. To isolate *Arcobacter*, samples are usually enriched in *Arcobacter* enrichment broth containing cefoperazone, amphotericin B and teicoplanin (CAT) as selective supplements and then filtered through a membrane to reduce other contaminants (Collado and Figueras, 2011).

Before this technique is widely used, the first culture medium applied for isolation of *Arcobacter* was the Ellinghausen-McCullough-Johnson-Harris (EMJH) *Leptospira* medium which was used to isolate *Arcobacter* from aborted bovine fetus (Ellis et al., 1977). Later in 1999, Johnson and Maruno developed a new technique for isolation of *Arcobacter* which was proven to be more effective than the EMJH medium for isolation of *Arcobacter* from chicken meat (Johnson and Maruno, 1999). Another

technique that was developed for isolation of *Arcobacter* from poultry products is the method of Houf and colleagues (2001a). This method uses a new formula of antimicrobial mixture, which consists of amphotericin B, cefoperazone, 5-fluorouracil, novobiocin and trimethoprim, together with the use of *Arcobacter* selective agar plates. The technique effectively inhibited the growth of other contaminants in chicken carcasses and was useful for isolation of *Arcobacter* from poultry. This method was subsequently adopted and validated for isolation of *Arcobacter* from human stools (Houf and Stephan, 2007) and animal fecal samples (van Driessche et al., 2003).

Although selective enrichment medium is commonly used for *Arcobacter* isolation (Collado and Figueras, 2011), it can reduce the genetic diversity of *Arcobacter* isolates recovered (Houf et al., 2002a). It has been shown in the past that *A. butzleri* was more resistant to antimicrobials used in selective media than *A. cryaerophilus* and *A. skirrowii* and was able to grow faster than other *Arcobacter* species in several enrichment media (Houf et al., 2001b).

2.9 Identification of *Arcobacter* spp.

Species of *Arcobacter* can be differentiated by using a combination of biochemical tests. Biochemical based identification technique of genus *Arcobacter* was published in Bergey's Manual of Systematic Bacteriology in 2005 (Vandamme et al., 2005). However, this technique is time-consuming and provides quite unreliable results

(Phillips, 2001). Due to biochemical inertness of *Arcobacter*, results of biochemical tests are often ambiguous and hard to interpret (Yan et al., 2000; Gonzalez et al., 2007). API-*Campy* system, a commercial biochemical-based *Campylobacter* and related organisms identification kit, was found to be ineffective for identification of *Arcobacter* species. Several *A. butzleri* isolates were misidentified as *A. cryaerophilus* using the API system (Gonzalez et al., 2007).

Due to the limitations of biochemical tests, molecular-based techniques have been developed and widely used for *Arcobacter* identification (Phillips, 2001). Several PCR-based techniques were designed for detection and identification of *Arcobacter* species. Multiplex-PCR using a combination of primer sets specific for several species of *Arcobacter* has been the most commonly used technique for identification of *Arcobacter* species (Collado and Figueras, 2011). Other techniques such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE), real-time PCR, 16S rDNA-RFLP, DNA microarray and matrix-associated laser absorption ionization-time-of-flight (MALDI-TOF) mass spectrometry have also been developed for identification of *Arcobacter* (Abdelbaqi et al., 2007a; Petersen et al., 2007; Quinones et al., 2007; Figueras et al., 2008; Aliphasic et al., 2010). Molecular methods used for detection and identification of *Arcobacter* are summarized in Table 3.

Table 3. Molecular-based techniques for *Arcobacter* identification

Methods	Gene(s) targeted	Species discriminated	Reference
PCR	*gyrA*, 16S rRNA	*A. butzleri*, *A. cryaerophilus* *A. skirrowii*, *A. cibarius*	Pentimalli et al., 2009
PCR	*hsp60*	*A. trophiarum*	De Smet et al., 2011
Multiplex PCR	16S rRNA, 23S rRNA	*Arcobacter* sp., *A. butzleri*	Harmon and Wesley, 1997
Multiplex PCR	16S rRNA, 23S rRNA	*A. butzleri, A. cryaerophilus* *A. skirrowii*	Houf et al., 2000
Multiplex PCR	23S rRNA	*A. butzleri, A. cryaerophilus* 1A *A. cryaerophilus* 1B, *A. skirrowii*	Kabeya et al., 2003a
Multiplex PCR	*rpoBC*, 23S rRNA	*A. butzleri, A. cryaerophilus*	Brightwell et al., 2007
Multiplex PCR	23S rRNA, *gyrA*	*A. butzleri, A. cryaerophilus* *A. skirrowii, A. cibarius , A. thereius*	Douidah et al., 2010
RFLP, Southern blotting	16S rRNA, 23S rRNA	*A. butzleri*	Kiehlbauch et al., 1991
PCR-RFLP	16S rRNA	*A. butzleri*	Cardarelli-Leite et al., 1996

Table 3. Molecular-based techniques for *Arcobacter* identification (continued)

Methods	Gene(s) targeted	Species discriminated	Reference
PCR-RFLP	23S rRNA	*A. butzleri, A. nitrofigilis*	Hurtado and Owen, 1977
PCR-RFLP	16S rRNA	*A. butzleri, A. cryaerophilus, A. skirrowii*	Marshall et al., 1999
PCR-RFLP	*groEL*	*A. butzleri*	Karenlampi et al., 2004
PCR-RFLP	16S rRNA, 23S rRNA	*A. butzleri*	Gonzalez et al., 2006
PCR-RFLP	16S rRNA	*A. butzleri, A. cryaerophilus* 1A *A. cryaerophilus* 1B, *A. skirrowii* *A. cibarius, A. nitrofigilis , A. halophilus,* *A. cibarius, A. mytili*	Figueras et al., 2008
PCR-hybridization	*glyA*	*A. butzleri*	Al Rashid et al., 2000
PCR-DGGE	16S rRNA	*A. cryaerophilus* 1B, *A. nitrofigilis*	Petersen et al., 2007
MALDI-TOF MS	Proteins	*A. butzleri, A. cryaerophilus, A. skirrowii*	Alispahic et al., 2010
Real time PCR	*rpoBC*, 23S rRNA	*A. butzleri, A. cryaerophilus*	Brightwell et al., 2007
Real time PCR	*gyrA*	*A. butzleri, A. cryaerophilus, A. cibarius* *A. nitrofigilis*	Abdelbaqi et al., 2007a

2.10 Molecular genotyping techniques and genetic characteristics of *Arcobacter* spp.

Several genotyping techniques including repetitive element sequence-based polymerase chain reaction (rep-PCR), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA-PCR (RAPD-PCR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Houf et al., 2003; Gonzalez et al., 2007; Miller et al., 2009; Gonzalez and Ferrus, 2011) have been used for determining genetic characteristics of *Arcobacter* spp. Results from different genotyping methods were in agreement which reveal a great genetic variation among *Arcobacter* isolates (Houf et al., 2003; Gonzalez et al., 2007; Miller et al., 2009; Gonzalez and Ferrus, 2011). Although many typing methods have been used, the gold standard for *Arcobacter* has not been specified.

Among several typing techniques, PFGE has been regarded as the gold standard typing method for several organisms including those in the family *Campylobacteraceae* since the technique has good discriminatory ability and reproducibility (Majella et al., 2006). The results obtained from different laboratories can be compared if the standardized PFGE protocol is followed. The principle of PFGE is based on the digestion of whole genomic DNA by using restriction enzyme and electrophoresis of the DNA fragments in a special electrophoresis chamber. Nevertheless, PFGE is expensive, laborious and time-consuming. In addition, the technique requires special equipments and chemical reagents that are not available in

most laboratories. Thus, PFGE may not be suitable for studying genetic profiles of *Arcobacter* in every occasion.

On the other hand, rep-PCR is a simple typing method which has been the most frequently used technique for typing *Arcobacter* strains (Collado and Figueras, 2011). The principle of rep-PCR technique is based on the amplification of DNA fragments that lay between interspersed repetitive sequences in prokaryotic genome (Versalovic et al., 1994). Rep-PCR is rapid and cheap and has high throughput ability. The technique requires only basic equipments that available in most laboratories such as thermal cyclers, gel electrophoresis chambers and gel documentation systems. Rep-PCR has been proven to be highly reproducible (Houf et al., 2002a) and sufficient for studying genetic relatedness of *Arcobacter* on several occasions such as outbreak investigation, strains characterization and description of new *Arcobacter* species (i.e. *A. mytili* and *A. molluscorum*) (Vandamme et al., 1992a; Ho et al., 2008; Collado and Figueras, 2011).

2.11 Antimicrobial susceptibility testing and antibiotic resistance of *Arcobacter*

Currently, there is no standard recommendation for antimicrobial susceptibility testing of *Arcobacter*. Several techniques including agar dilution, broth microdilution, disc diffusion and E-test have been applied to *Arcobacter* (Atabay and Aydin, 2001; Fera et al., 2003; Vandenberg et al., 2006; Son et al., 2007a). Due to the differences in susceptibility testing techniques, breakpoints, incubation period and conditions used in

each laboratory, comparison of results among studies is difficult. Since only limited numbers of studies in antimicrobial resistance of *Arcobacter* are available, it is hard to determine a prospective trend of antibiotic resistance in *Arcobacter*.

Although *Arcobacter* infection is generally self-limiting and does not require antimicrobial treatment, some patients may develop invasive or prolonged infection that antibiotics are necessary. Flouroquinolones and tetracyclines have shown good activities against *Arcobacter* and have been recommended for the treatment of *Arcobacter* infections in both human and veterinary medicine (Vandenberg et al., 2006; Son et al., 2007a). Other antibiotics such as carbapenems, cefepime and aminoglycosides can also be used for treatment of severe *Arcobacter* infections (Fera et al., 2003). Unfortunately, *Arcobacter* has increasingly become resistant to antimicrobial agents commonly used for treatment of bacterial infections in humans and animals (Houf et al., 2004). Antimicrobial-resistant *Arcobacter* strains have been reported in several countries such as Belgium, Italy, Turkey and USA (Atabay and Aydin, 2001; Fera et al., 2003; Vandenberg et al., 2006; Son et al., 2007a). Approximately, 21% of *A. butzleri* isolated from diarrheic patients in Belgium were resistant to ampicillin and erythromycin (Vandenberg et al., 2006). *Arcobacter* strains isolated from animals such as broilers were also shown resistance to different antimicrobial agents including amoxycillin, amoxycillin/clavulanic acid, ampicillin, azithromycin, clindamycin, nalidixic acid, penicillin G and trimethoprim (Atabay and Aydin, 2001; Son et al., 2007a). In addition,

Arcobacter isolated from brackish environments was also found to be highly resistant to several antimicrobials such as chloramphenicol, macrolides, penicillins, trimethoprim and vancomycin (Fera et al., 2003).

2.12 Studies of *Arcobacter* in Thailand

In Thailand, the information on *Arcobacter* spp. is very limited. Only few studies were previously conducted. Nevertheless, it has been demonstrated that *Arcobacter* could be isolated from food and environment (Morita et al., 2004; Teague et al., 2010) as well as from stool samples of diarrheic patients (Taylor et al., 1991). In 2004, a comparison study of the prevalence of *Arcobacter* in environment between Thailand and Japan revealed that the prevalence of *Arcobacter* in canal water in Thailand was 100%, while 23% of canal water samples in Japan were *Arcobacter* positive. In addition, this study also revealed a high prevalence of *Arcobacter* in ground chicken meat samples collected from retail markets in Thailand (Morita et al., 2004). Apart from the contamination of *Arcobacter* in environment and fresh food products, the contamination of *Arcobacter* in cooked meals has also been reported. One recent study has shown that the contamination of *Arcobacter* in meals served in restaurants in Bangkok was observed at higher frequency than the contamination of other common entero-pathogens, such as *Salmonella* or *Campylobacter*. The exposure risk of consumer to *Arcobacter* was found at 13% per meal eaten and the risk was increased to 75% per ten

meals eaten. Furthermore, the majority of *A. butzleri* isolates in the study were resistant to azithromycin, a commonly prescribed antibiotic for the treatment of traveler's diarrhea in Thailand (Teague et al., 2010). In terms of *Arcobacter* in humans, 2.4% of stool samples from Thai children who went to the Children's hospital in Bangkok with the symptom of acute diarrhea were *Arcobacter* positive (Taylor et al., 1991).

Although chicken meat appears to be a key role for transmission of *Arcobacter* to humans, the information on *Arcobacter* in chicken meat in Thailand is still limited. Thus, studies focusing on *Arcobacter* in chicken meat should be established. The information on contamination rates of *Arcobacter* in chicken meat as well as antibiotic resistance and genetic characteristics of the isolated strains will provide better understanding of the organism and promote the awareness of this new emerging foodborne pathogen in Thailand.

Chapter III

MATERIALS AND METHODS

3.1 Sample collection

From October 2010 to January 2011, 90 whole chicken carcasses were purchased from fresh markets and 90 whole chicken carcasses were purchased from supermarkets. Fifteen fresh markets and 19 supermarkets located in Bangkok metropolitan area were included in the present study. Fresh markets sampled in this study are ones of the well known markets located in Bangkok. For supermarkets, 5 out of 8 major supermarket chains in Thailand were sampled. One fresh market and 1 - 3 supermarkets were visited for sample collection each week.

In fresh market where there were more than 6 chicken meat stalls, one whole chicken carcass was randomly purchased from each stall throughout the market (no more than 6 stalls per fresh market). However, if there were fewer than 6 chicken meat stalls in a market, more than one carcass per stall were sampled. For supermarkets, 2 – 9 chicken carcasses were sampled as available. Chicken samples were put in plastic bags and covered with ice in a shipping box and transported to the laboratory within 2 hours of purchase.

3.2 Isolation of *Arcobacter*

The presence of *Arcobacter* spp. in chicken carcasses was determined by selective enrichment method and membrane filtration method as described previously (Atabay et al., 2003) with some modifications.

Twenty-five grams of chicken skin were aseptically excised and placed in a sterile plastic bag. Then, the rest of the carcass was rinsed with 225 ml of buffered peptone water (BPW) and manually agitated for 2 min. After agitation, BPW was transferred to the previously excised skin sample and homogenized in a stomacher for 1 min. Ten ml of the homogenate were added to 90 ml of *Arcobacter* enrichment broth containing cefoperazone (8 mg/l), amphotericin (10 mg/l) and teicoplanin (4 mg/l) as selective supplements (Appendix A) and incubated at 25°C for 48 h under aerobic conditions. Thereafter, 200 µl of each enrichment sample were dispensed onto 47 mm diameter 0.45 µm pore size cellulose acetate membrane filter laid on the surface of the modified charcoal cefoperazone deoxycholate agar (mCCDA) plate. The membrane was removed after 30 min. The inoculated plates were incubated at 25 °C aerobically for 24 to 48 h. Colonies of typical morphology of *Arcobacter* (grayish, pin-point colonies) per chicken sample were selected and purified by subculturing onto blood agar. Each purified *Arcobacter* isolate was kept in a cryovial tube containing skim milk and 30% glycerol at -80 °C for further study.

3.3 Identification of *Arcobacter*

Arcobacter genus confirmation was performed by PCR according to the protocol previously published (Neubauer and Hess, 2006) with some modifications. Briefly, each 25-µl PCR reaction mixture contained 1× PCR buffer (Kapa Biosystems, Boston, USA), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates, 400 pM of each primer specific for *Arcobacter* spp. and 0.75 U *Taq* DNA polymerase (Kapa Biosystems, Boston, USA). DNA fragments were amplified at an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C (2 min), annealing at 65°C (1 min) and extension at 72 °C (1 min). The final extension is at 72 °C for 10 min. Primers used for genus confirmation are shown in Table 4.

Species of each *Arcobacter* isolate was further determined by species-specific multiplex PCR as described previously (Douidah et al., 2010) with some modifications. PCR reactions were performed in a 25-µl reaction mixture composed of 1× PCR buffer (Kapa Biosystems, Boston, USA), 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphates, 25 pmol of each primer and 0.75U *Taq* DNA polymerase (Kapa Biosystems, Boston, USA). Primers for species-specific multiplex PCR are presented in Table 4. The PCR amplification was started with an initial denaturation at 94 °C for 3 min and then 30 cycles of denaturation at 94 °C (45 sec), annealing at 58 °C (45 sec) and extension at 72 °C (2 min), followed by a final extension at 72 °C for 5 min. *A. butzleri*

NCTC 12481, *A. skirrowii* NCTC 12731, and *A. cryaerophilus* NCTC 11885 were used as positive control strains for PCR reactions.

Table 4. Primers for genus confirmation and species identification

Primers	Sequence (5' – 3')	Fragment size (bp)
Primers for genus confirmation (Neubauer and Hess, 2006)		
REVERS	GTG GAG TAC AAG ACC CGG GAA	822
ARCOB1	TGT AGG CGG ATT GAT AAG TTT GAA	
Primers for species identification (Douidah et al., 2010)		
A. butzleri		
ArcoF	GCY AGA GGA AGA GAA ATC AA	2061
ButR	TCC TGA TAC AAG ATA ATT GTA CG	
A. skirrowii		
ArcoF	GCY AGA GGA AGA GAA ATC AA	198
SkiR	TCA GGA TAC CAT TAA AGT TAT TGA TG	
A. cryaerophilus		
GyrasF	AGA ACA TCA CTA AAT GAG TTC TCT	395
GyrasR	CCA ACA ATA TTT CCA GTY TTT GGT	

3.4 Antimicrobial susceptibility testing

Eighty six *Arcobacter* isolates from fresh markets and 64 *Arcobacter* isolates from supermarkets were tested for their antimicrobial susceptibility to 6 antimicrobial agents including ciprofloxacin (CIP), clindamycin (CLN), erythromycin (ERY), gentamicin (GEN), nalidixic acid (NAL) and tetracycline (TET) using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI,

2008). Briefly, the isolates were subcultured onto blood agar and incubated at 37 °C for 48 h in anaerobe jars containing a gas mixture of 10% CO_2, 5% O_2 and 85% N_2. Then, the isolates were suspended in sterile physiological saline and adjusted to the turbidity of 0.5 McFarland standard. These suspensions were inoculated onto Mueller-Hinton agar containing a 2-fold dilution series of each antimicrobial (Appendix B) and supplemented with 5% (V/V) defribrinated sheep blood using a multipoint inoculator. The inoculated plates were incubated at 37 °C under microaerobic atmosphere as described previously for 48 h. *C. jejuni* ATCC 33560 was used as a quality control strain for each susceptibility testing. The minimum inhibitory concentration (MIC), defined as the lowest concentration of antimicrobial agent that completely inhibits the visible growth of bacteria on the plate, was determined after 48 h of incubation. Since the breakpoints for *Arcobacter* spp. have not been established, the resistance breakpoints used in this study were adopted from those of *Campylobacter*. The resistance breakpoint for ciprofloxacin, erythromycin and tetracycline was from CLSI established guideline (CLSI, 2008), while the resistance breakpoints of the National Antimicrobial Resistance Monitoring System (NARMS) were used for the other antmicrobial agents (NARMS, 2009). Antimicrobial concentration test range and MIC breakpoints of each antimicrobial agent used in this study are shown in Table 5.

Table 5. Antimicrobial concentration test range and MIC breakpoints of antimicrobial agents

Antimicrobial agents	Range (µg/ml)	MIC breakpoints (µg/ml)
Ciprofloxacin	0.008 - 128	$\geq$ 4
Clindamycin	0.008 - 128	$\geq$ 8
Erythromycin	0.06 - 512	$\geq$ 32
Gentamicin	0.015 - 128	$\geq$ 8
Nalidixic acid	0.25 - 512	$\geq$ 64
Tetracycline	0.015 - 128	$\geq$ 16

3.5 Genetic profiles of *Arcobacter*

In the present study, *Arcobacter* isolates were examined for their genetic profiles using two molecular genotyping techniques which are repetitive sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE). All *Arcobacter* isolates were characterized by rep-PCR before a subset of the strains were selected for further characterization by PFGE.

3.5.1 Repetitive sequence-based PCR (rep-PCR)

3.5.1.1 DNA preparation

Whole cell lysate of *Arcobacter* strains was used as DNA template for rep-PCR amplification in the present study. The cell lysis was performed using alkaline PEG reagent which was prepared as described previously

(Chomczynski and Rymaszewski, 2006). In brief, *Arcobacter* strains were grown on Mueller-Hinton agar supplemented with 5% sheep blood at 30 °C for 24 - 48 h under aerobic conditions. Approximately a quarter loopful of *Arcobacter* colonies were added into 500 µl of the alkaline PEG reagent. Then, the mixture was heated at 90 °C for 10 min and centrifuged at 12,000 rpm for 5 min. Two microliters of the supernatant were used as DNA template in PCR mixture.

3.5.1.2 Rep-PCR reaction

Rep-PCR reaction was carried out in a 25-µl reaction volume using Takara *Ex Taq* DNA polymerase (Takara Bio Inc, Shiga, Japan). The final PCR mixture consisted of 1× PCR buffer, 200 µM of each deoxynucletide triphosphates, 20 µM $(GTG)_5$ primers, 0.625 U *Ex Taq* DNA polymerase and 2 µl of DNA sample. PCR amplification was performed with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C (45 sec), annealing at 40 °C (1 min) and extension at 65 °C (10 min) and a final extension step at 65 °C for 20 min. The banding patterns were examined using 1% agarose gels in 0.5× Tris-borate-EDTA buffer. Gel electrophoresis was performed at 135 V for 2.2 h. The gels were stained with 5 µg/ml ethidium bromide for 5 min and destained with tap water for 10 min and then visualized by gel scanner (Typhoon 9410, Amersham Pharmacia Biotech Inc., New Jersey,

USA). For the analysis of DNA fingerprints, the Gelcompar®II 5.1 software package (Applied Maths, Belgium) was used. Similarity values among *Arcobacter* isolates were calculated using Pearson's correlation and the dendrogram was constructed by unweighted pair group of arithmetic mean (UPGMA) method.

3.5.2 Pulsed-field gel electrophoresis (PFGE)

The previously described pulsed-field gel electrophoresis protocol modified from the PulseNet protocol for *Campylobacter jejuni* (Son et al., 2006) was employed for studying genetic characteristic of *Arcobacter* isolates in this study (Appendix C). The selection of a subset of *Arcobacter* isolates for further characterization by PFGE was based on the results of rep-PCR. One to three *Arcobacter* isolates from each cluster of rep-PCR dendrogram at a cut off value of 75% were selected to be representatives of each cluster. In addition, some *Arcobacter* isolates that had similar rep-PCR patterns were also selected. *KpnI* was used as a restriction enzyme and *Salmonella* Braenderup H9812 digested with *XbaI* was used as a universal PFGE molecular marker as recommended by the PulseNet. The restriction fragments of *Arcobacter* isolates were separated on 1% PFGE-grade agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer for 18 h at 14 °C using CHEFF Mapper apparatus (Bio-Rad Laboratory, USA). PFGE gels

were visualized and photographed with a UV gel documentation system (Gene Genious, Syngene, MD, USA).

The program GelCompar® II 5.1 was used for normalization of the gels and for analyzing genetic relatedness of the banding patterns obtained. The dendrogram of PFGE patterns was constructed using UPGMA method. The degree of similarity between PFGE patterns was quantified by Dice coefficient.

3.6 Determination of discriminatory ability and concordance of typing techniques

3.6.1 Discriminatory ability

The discriminatory ability of antibiotic resistance pattern, rep-PCR and PFGE was determined using the Simpson's index of diversity (SID). The SID demonstrates the probability of two unrelated strains sampled from the test population will be of a different type which can be used for describing the discriminatory power of typing techniques (Hunter and Gaston, 1988).

3.6.2 Concordance between antibiotic resistance pattern, rep-PCR and PFGE

The Adjusted Rand and Wallace coefficients were used for calculation of the concordance of the typing methods used in the present study. Adjusted Rand coefficient shows the overall congruence between typing methods (Carrico et al., 2006). Wallace coefficient also measures the agreement of

clustering techniques, but the direction of concordance was also taken into account. Wallace coefficient can be used for predicting the results of one technique by the results of another technique (Pinto et al., 2008).

In the present study, SIDs, Adjusted Rand and Wallace coefficients were calculated for the subset of 58 *Arcobacter* isolates for which the results of antimicrobial resistance pattern, rep-PCR and PFGE were available. Calculation was performed using the online tool for quantitative assessment of classification agreement available at

http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool.

Chapter IV

RESULTS

4.1 Occurrence of *Arcobacter*

Occurrence of *Arcobacter* in chicken carcasses collected from fresh markets and supermarkets located in Bangkok is shown in Table 6 and 7, respectively. The overall occurrence was 86.67%. Occurrence of *Arcobacter* in fresh markets was higher than that in supermarkets. *A. butzleri* was the most prevalent species contaminating in both fresh markets and supermarkets which accounts for almost 97% of the isolates. Only three *A. skirrowii* and two *A. cryaerophilus* were recovered in this study.

Table 6. Occurrence of *Arcobacter* in fresh markets

Fresh market	Numberof positive samples/tested samples (%)	Number (%) of positive samples		
		A. butzleri	*A. cryaerophilus*	*A. skirrowii*
M1	6/6 (100.0)	6 (100.0)	0	0
M2	6/6 (100.0)	6 (100.0)	0	0
M3	6/6 (100.0)	6 (100.0)	0	0
M4	6/6 (100.0)	5 (83.3)	0	1 (16.6)
M5	6/6 (100.0)	6 (100.0)	0	0
M6	6/6 (100.0)	4 (66.6)	0	2 (33.3)
M7	6/6 (100.0)	6 (100.0)	0	0
M8	6/6 (100.0)	6 (100.0)	0	0
M9	6/6 (100.0)	6 (100.0)	0	0
M10	6/6 (100.0)	6 (100.0)	0	0
M11	6/6 (100.0)	6 (100.0)	0	0
M12	6/6 (100.0)	6 (100.0)	0	0
M13	6/6 (100.0)	6 (100.0)	0	0
M14	6/6 (100.0)	6 (100.0)	0	0
M15	6/6 (100.0)	6 (100.0)	0	0
Total	90/90 (100.0)	87 (96.6)	0	3 (3.3)

Table 7. Occurrence of *Arcobacter* in supermarkets

Supermarket	Number of positive samples/tested samples (%)	Number (%) of positive samples		
		A. butzleri	*A. cryaerophilus*	*A. skirrowii*
S1	4/6 (66.6)	4 (100.0)	0	0
S2	8/8 (100.0)	8 (100.0)	0	0
S3	3/3 (100.0)	3 (100.0)	0	0
S4	3/4 (75.0)	3 (100.0)	0	0
S5	6/6 (100.0)	6 (100.0)	0	0
S6	1/3 (33.3)	0	1 (100.0)	0
S7	4/9 (44.4)	4 (100.0)	0	0
S8	3/3 (100.0)	3 (100.0)	0	0
S9	2/3 (66.6)	2 (100.0)	0	0
S10	4/6 (66.6)	3 (75.0)	1 (25.0)	0
S11	1/6 (16.6)	1 (100.0)	0	0
S12	3/4 (75.0)	3 (100.0)	0	0
S13	2/2(100.0)	2 (100.0)	0	0
S14	4/8 (50.0)	4 (100.0)	0	0
S15	6/6 (100.0)	6 (100.0)	0	0
S16	3/3 (100.0)	3 (100.0)	0	0
S17	2/4 (50.0)	2 (100.0)	0	0
S18	4/4 (100.0)	4 (100.0)	0	0
S19	3/3 (100.0)	3 (100.0)	0	0
Total	66/90 (73.3)	64 (96.9)	2 (3.0)	0

Nineteen supermarkets included in the current study belonged to five supermarket chains. The occurrence of *Arcobacter* based on supermarket chain is shown in Table 8.

Table 8. Occurrence of *Arcobacter* based on supermarket chain

Supermarket chain	Supermarkets	Number (%) of positive samples/ examined samples
A	S1, S4, S5, S10, S11, S15, S17	26/37 (70.2)
B	S2, S3, S9, S12, S13, S14	22/28 (78.5)
C	S7, S18	8/13 (61.5)
D	S8, S16, S19	9/9 (100.0)
E	S6	1/3 (33.3)
Total		66/90 (73.3)

According to the results of *Arcobacter* isolation, chicken carcasses sold in Bangkok were highly contaminated with *Arcobacter*. Every fresh market had *Arcobacter* contamination rate of 100%, while chicken carcasses in supermarkets had lower contamination rates ranging from 16.6% to 100%. When the contamination rate of each supermarket chain was compared, supermarket chain D had the highest occurrence of *Arcobacter* (100.0%), while supermarket chain E had the lowest occurrence (33.3%).

4.2 Antibiotic resistance of *Arcobacter*

MICs distribution, MIC_{50} and MIC_{90} and antimicrobial resistance rates of *Arcobacter* strains isolated from fresh markets and supermarkets are summarized in Table 9 and 10, respectively. Most *Arcobacter* isolates were resistant to nalidixic acid and ciprofloxacin (Figure 1). Almost 84% of *Arcobacter* isolates from fresh markets and 65.6% of *Arcobacter* isolates from supermarkets were resistant to nalidixic acid. For

ciprofloxacin, the resistance rate was found in 50.0% and 40.6% of *Arcobacter* isolates from fresh markets and supermarkets, respectively. None of *Arcobacter* isolates in this study were resistant to erythromycin. In addition, clindamycin, gentamicin and tetracycline also effectively inhibited the growth of *Arcobacter* in the present study. Less than 3% of *Arcobacter* isolates from fresh markets and supermarkets in Bangkok were resistant to clindamycin, gentamicin and tetracycline. The most common resistance pattern observed in this study was ciprofloxacin-nalidixic acid resistance (Table 11). Among 86 *Arcobacter* isolates from fresh markets and 64 *Arcobacter* isolates from supermarkets tested, ciprofloxacin-nalidixic acid resistance was accounted for 46.5% and 40.6% of *Arcobacter* isolates from fresh markets and supermarkets, respectively. On the contrary, 13 *Arcobacter* isolates out of 86 isolates (15.1%) from fresh markets and 21 out of 64 isolates (32.8%) from supermarkets were susceptible to all antimicrobial agent tested. No multidrug-resistant *Arcobacter* (isolate that is resistant to $\geq$ 3 classes of antimicrobial agents) was identified in the present study.

Table 9. MICs distribution and antimicrobial resistance rates of *Arcobacter* isolates from chicken carcasses in fresh markets[a]

Antimicrobial agent	No. of isolates inhibited at the following MIC (µg/ml)[b]																	
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512	MIC50/90[c]	%R[d]
Ciprofloxacin																		
A. butzleri	11	9	16	4					15	15	8	5					8/32	51.8
A. skirrowii			3														0.12/0.12	0
Total	**11**	**9**	**19**	**4**					**15**	**15**	**8**	**5**					**4/32**	**50.0**
Clindamycin																		
A. butzleri			2	3	7	23	29	19									2/4	0
A. skirrowii					1	1		1									1/4	0
Total			**2**	**3**	**8**	**24**	**29**	**20**									**2/4**	**0**
Erythromycin																		
A. butzleri					5	24	17	8	17	12							2/16	0
A. skirrowii						2	1										1/2	0
Total					**5**	**26**	**18**	**8**	**17**	**12**							**2/16**	**0**
Gentamicin																		
A. butzleri			1	9	44	27						1	1				0.5/1	2.4
A. skirrowii				1	1	1											0.5/1	0
Total			**1**	**10**	**45**	**28**						**1**	**1**				**0.5/1**	**2.3**
Nalidixic acid																		
A. butzleri					1				4	1	7	21	7	7	18	17	256/>512	84.3
A. skirrowii											1	1				1	64/>512	66.6
Total					**1**				**4**	**1**	**8**	**22**	**7**	**7**	**18**	**18**	**256/>512**	**83.7**
Tetracycline																		
A. butzleri				2	24	22	25	8		1	1						1/4	2.4
A. skirrowii					1	1	1										1/2	0
Total				**1**	**25**	**23**	**26**	**8**		**1**	**1**						**1/4**	**2.3**

[a] *A. butzleri* (n = 83) and *A. skirrowii* (n = 3), [b] The grey shading indicates resistant isolates

[c] MICs required to inhibit the growth of 50% and 90% of isolates, [d] Resistance rates

Table 10. MICs distribution and antimicrobial resistance rates of *Arcobacter* isolates from chicken carcasses in supermarkets[a]

Antimicrobial agent	No. of isolates inhibited at the following MIC (μg/ml)[b]																	
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512	MIC50/90[c]	%R[d]
Ciprofloxacin																		
A. butzleri	6	14	14	4				1	2	11	7	1	2				0.12/32	38.7
A. cryaerophilus									2								8/8	100.0
Total	**6**	**14**	**14**	**4**				**1**	**4**	**11**	**7**	**1**	**2**				**0.12/32**	**40.6**
Clindamycin																		
A. butzleri				12	12	19	6	12	1								1/4	1.6
A. cryaerophilus					1		1										0.5/2	0
Total				**12**	**13**	**19**	**7**	**12**	**1**								**1/4**	**1.5**
Erythromycin																		
A. butzleri				1	14	21	10	6	9	1							1/8	0
A. cryaerophilus					1	1											0.5/1	0
Total				**1**	**15**	**22**	**10**	**6**	**9**	**1**							**1/8**	**0**
Gentamicin																		
A. butzleri		2	2	8	37	12					1						0.5/1	1.6
A. cryaerophilus			1			1											0.12/1	0
Total		**2**	**3**	**8**	**37**	**13**					**1**						**0.5/1**	**1.5**
Nalidixic acid																		
A. butzleri								2	1	3	16	14	5	6	12	3	64/>512	64.5
A. cryaerophilus													1	1			128/256	100.0
Total								**2**	**1**	**3**	**16**	**14**	**6**	**7**	**12**	**3**	**64/>512**	**65.6**
Tetracycline																		
A. butzleri				5	28	10	10	8	1								0.5/4	0
A. cryaerophilus			1	1													0.12/0.25	0
Total			**1**	**6**	**28**	**10**	**10**	**8**	**1**								**0.5/4**	**0**

[a] *A. butzleri* (n = 62) and *A. cryaerophilus* (n = 2), [b] The grey shading indicates resistant isolates

[c] MICs required to inhibit the growth of 50% and 90% of isolates, [d] Resistance rates

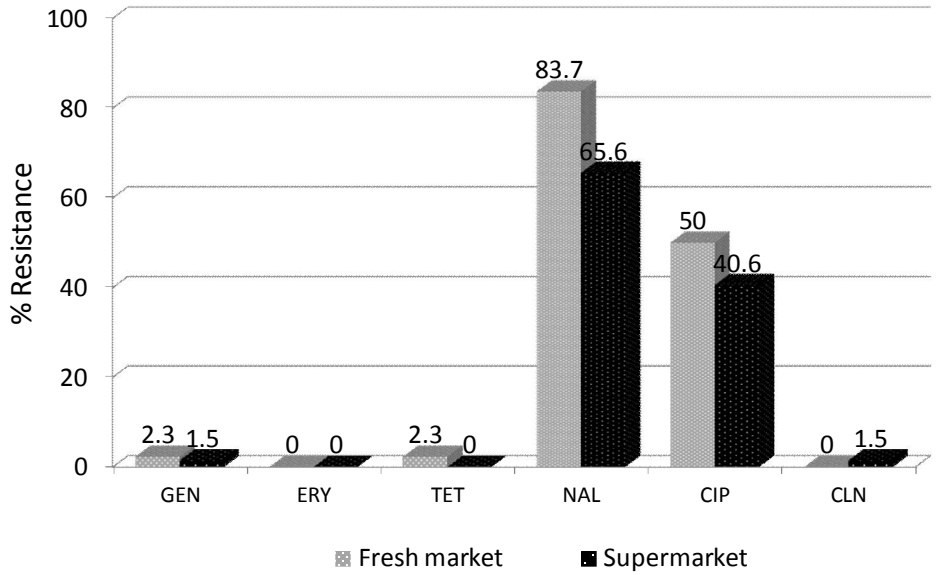

Figure 1. Comparison of antimicrobial resistance rates of *Arcobacter* strains isolated from fresh markets and supermarkets

Table 11. Antimicrobial resistance pattern of *Arcobacter* isolates from fresh markets and supermarkets

Resistance pattern	Number of isolates (%)	
	Fresh market	Supermarket
NAL	28 (32.5)	15 (23.4)
CIP	1 (1.1)	-
CLN	-	1 (1.5)
CIP-NAL	40 (46.5)	26 (40.6)
GEN-NAL	-	1 (1.5)
NAL-TET	1 (1.1)	-
CIP-NAL-GEN	2 (2.2)	-
CIP-NAL-TET	1 (1.1)	-
No resistance	13 (15.1)	21 (32.8)
Total	86 (100.0)	64 (100.0)

4.3 Genetic profiles of *Arcobacter*

4.3.1. Rep-PCR

High degree of genetic diversity of *Arcobacter* was observed. No dominant rep-PCR pattern was present in each fresh market or supermarket. The banding patterns obtained were composed of 8 – 15 fragments with the sizes ranging from approximately 300 to 9,000 bp.

4.3.1.1 *Arcobacter* isolated from chickens in fresh markets

Among 87 *Arcobacter* isolates from fresh markets that were typed by rep-PCR, 73 rep-PCR patterns were obtained. At 75% cutoff value on the rep-PCR dendrogram, *Arcobacter* isolates from fresh markets comprised of 19 clusters (Figure 2).

An extreme genetic heterogeneity was noticed among *Arcobacter* strains recovered from fresh markets. The majority of *Arcobacter* originated from the same fresh market produced distinct rep-PCR profiles. None or only a pair of isolates with the similar rep-PCR pattern was observed in each market (Figure 3 - 7). Additionally, most *Arcobacter* isolates recovered from the same chicken meat stall also had different rep-PCR patterns. In fresh market M15 where chicken samples were obtained from one meat stall, all of six *Arcobacter* isolates examined yielded divergent rep-PCR profiles (Figure 7C). Although the high genetic diversity was noticed among *Arcobacter* strains isolated from fresh markets, similar rep-PCR patterns were shared by some *Arcobacter*

isolates originated from different chicken meat stalls (Figure 4A, 5A, 5B and 6B). The dendrogram of *Arcobacter* strains isolated from different fresh markets is shown in Figure 3 to 7.

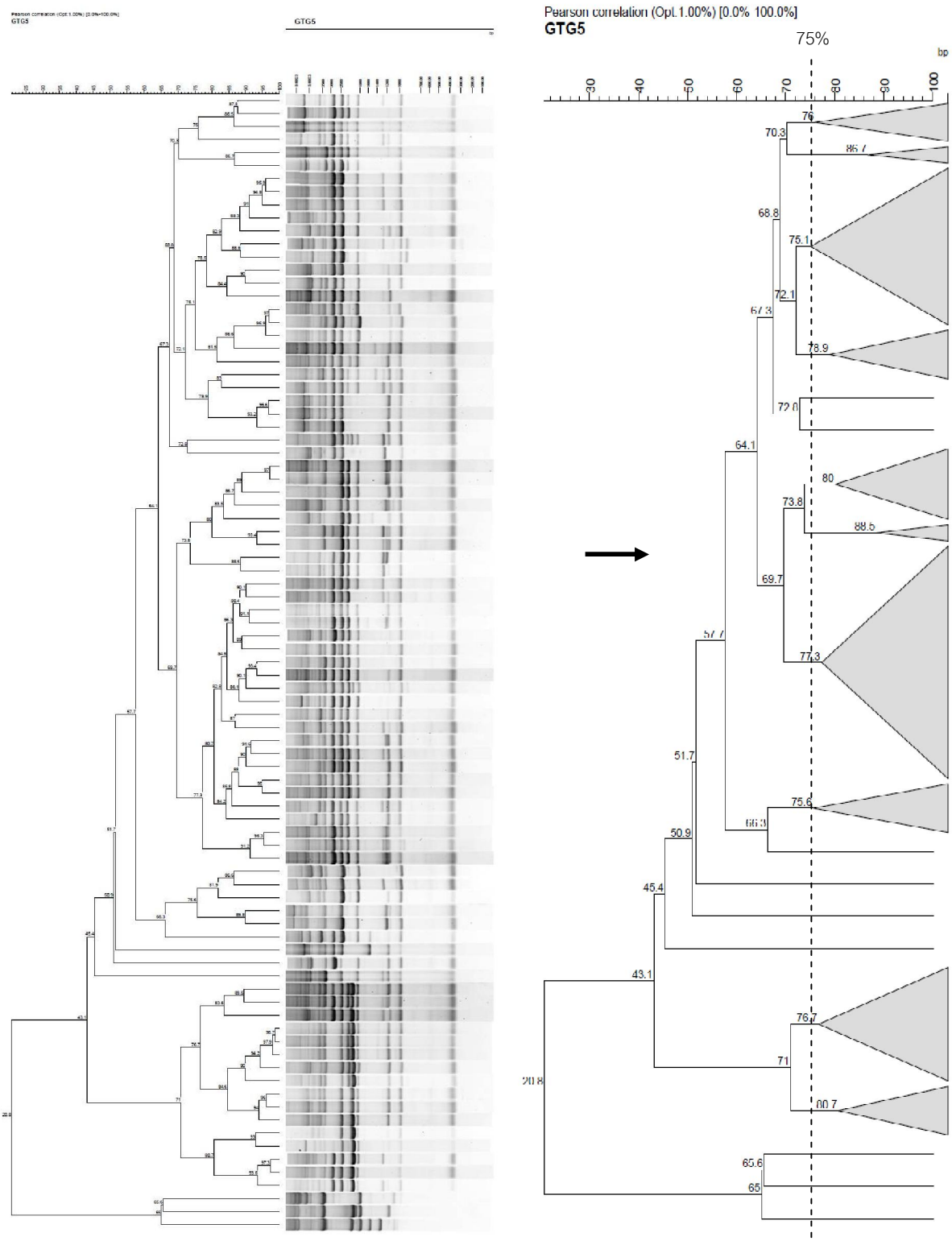

Figure 2. Dendrogram of *Arcobacter* strains isolated from chicken carcasses in fresh markets based on the results of rep-PCR

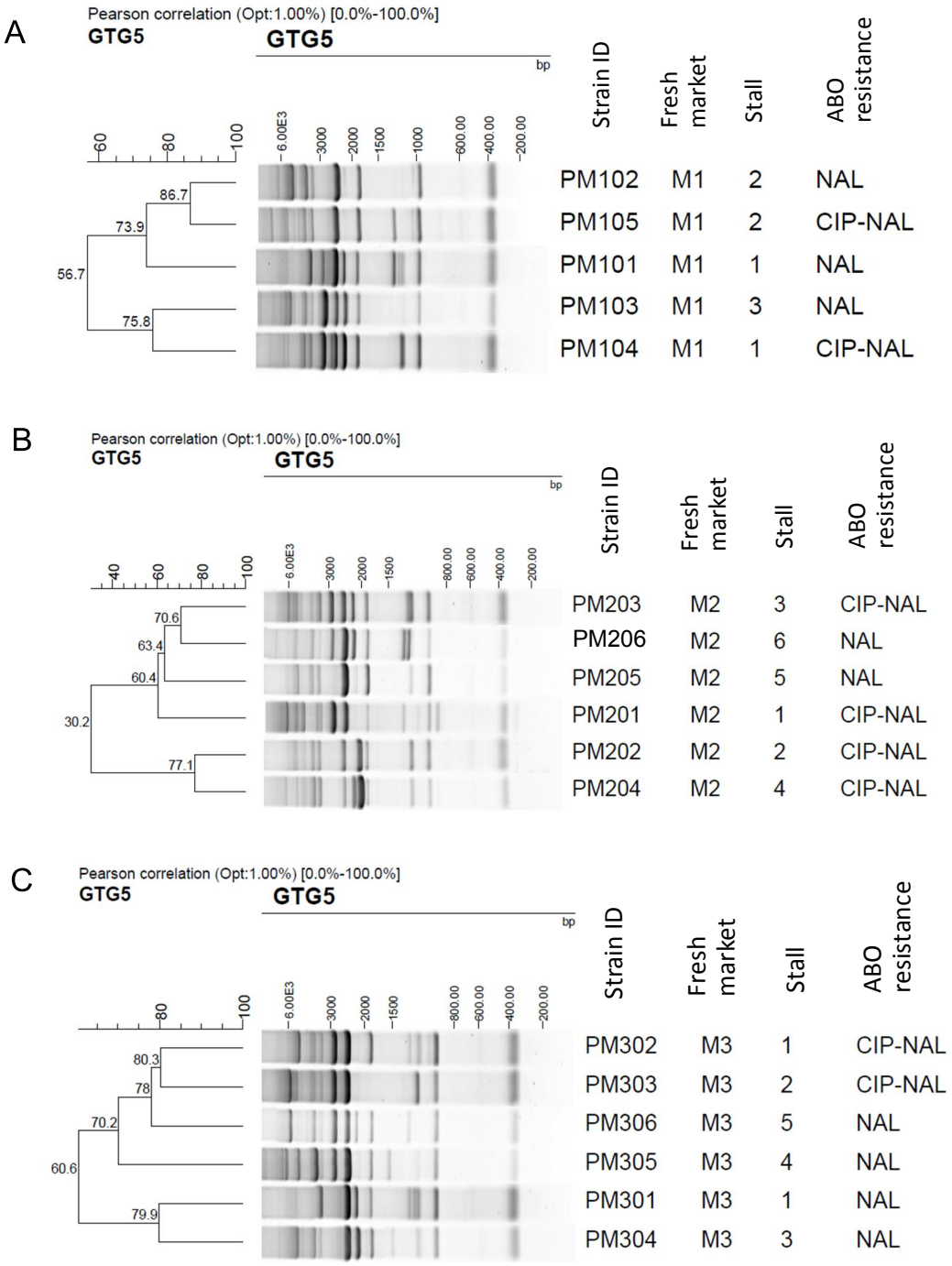

Figure 3. Dendrogram of *Arcobacter* isolates from fresh market M1 (A), M2 (B) and M3 (C)

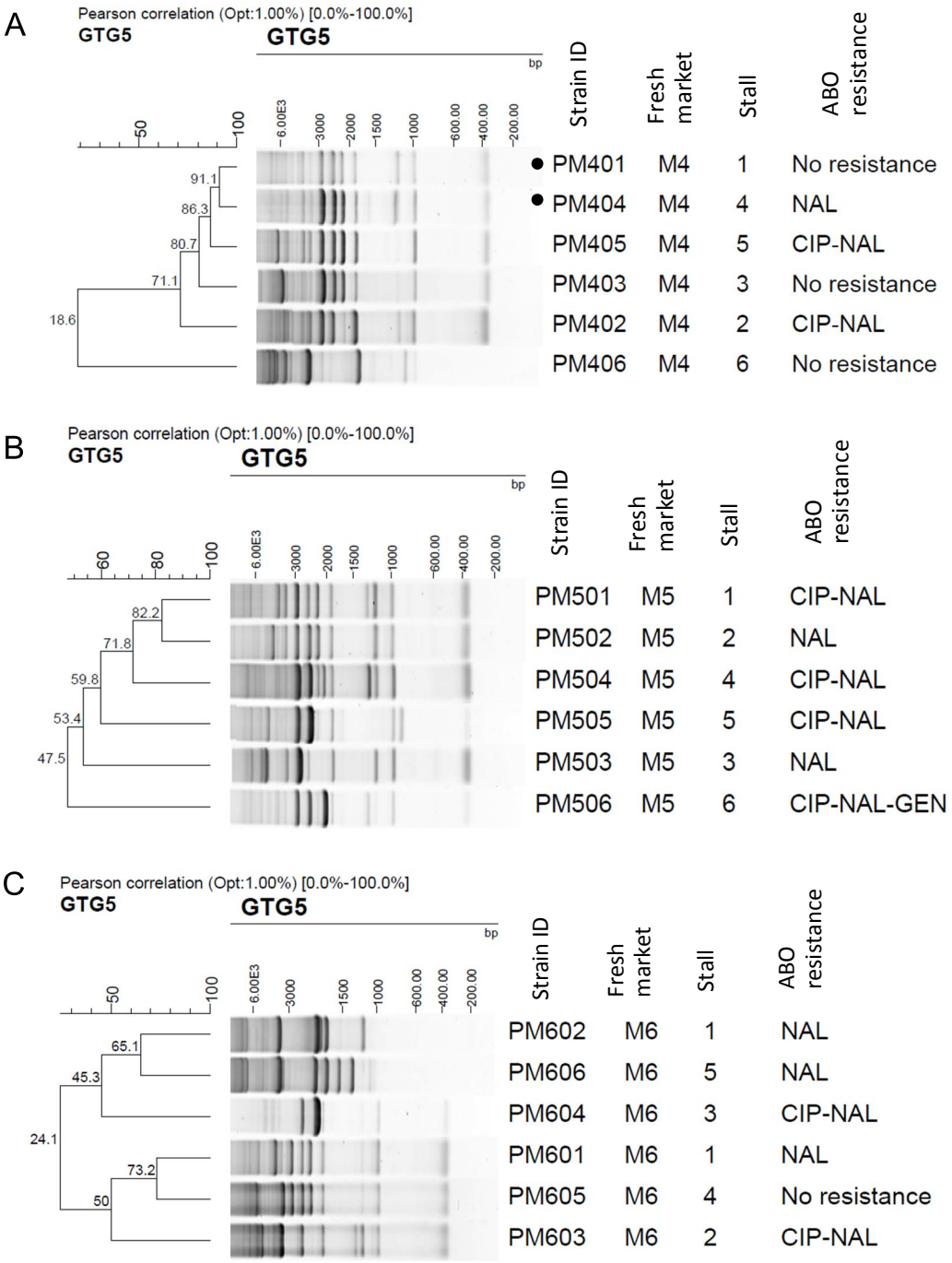

Figure 4. Dendrogram of *Arcobacter* isolates from fresh market M4 (A), M5 (B) and M6 (C). (●) The isolates that had similar rep-PCR patterns but were recovered from different chicken meat stalls.

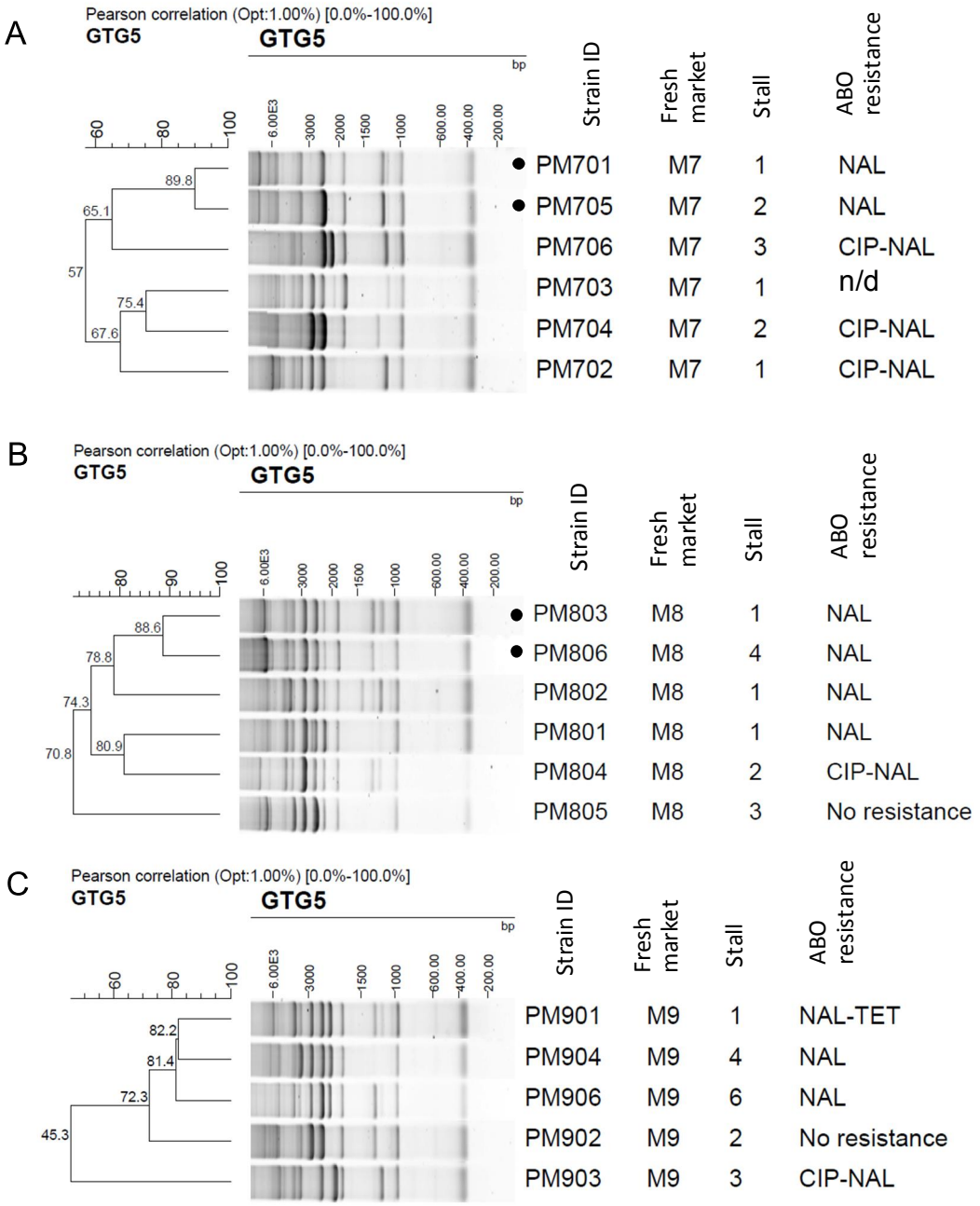

Figure 5. Dendrogram of *Arcobacter* isolates from fresh market M7 (A), M8 (B) and M9 (C). (●) The isolates that had similar rep-PCR patterns but were recovered from different chicken meat stalls. n/d means that the antimicrobial susceptibility was not determined.

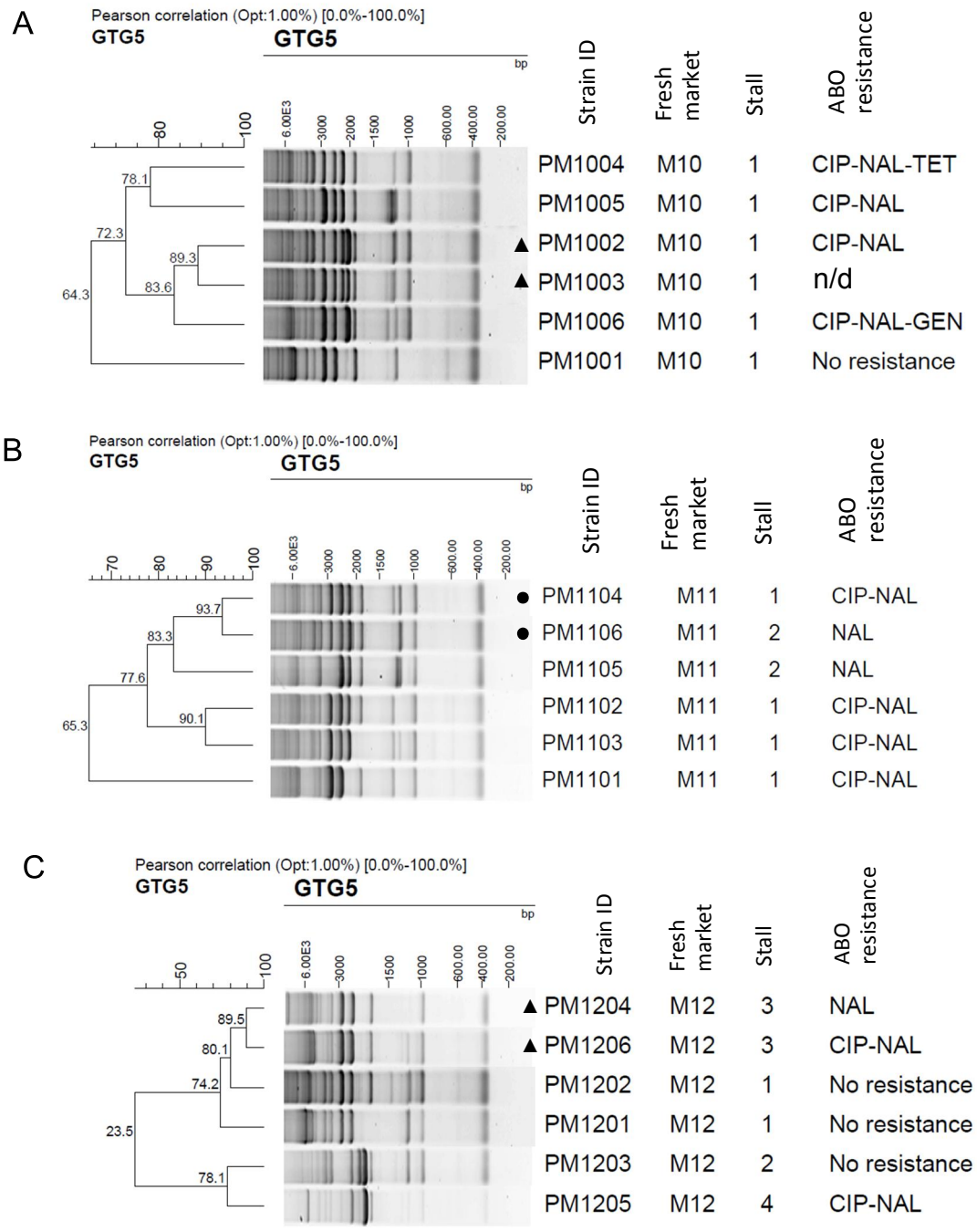

Figure 6. Dendrogram of *Arcobacter* isolates from fresh market M10 (A), M11 (B) and M12 (C). (▲) The isolates that had similar rep-PCR patterns and were recovered from the same chicken meat stall; (●) The isolates that had similar rep-PCR patterns but were recovered from different chicken meat stalls. n/d means that the antimicrobial susceptibility was not determined.

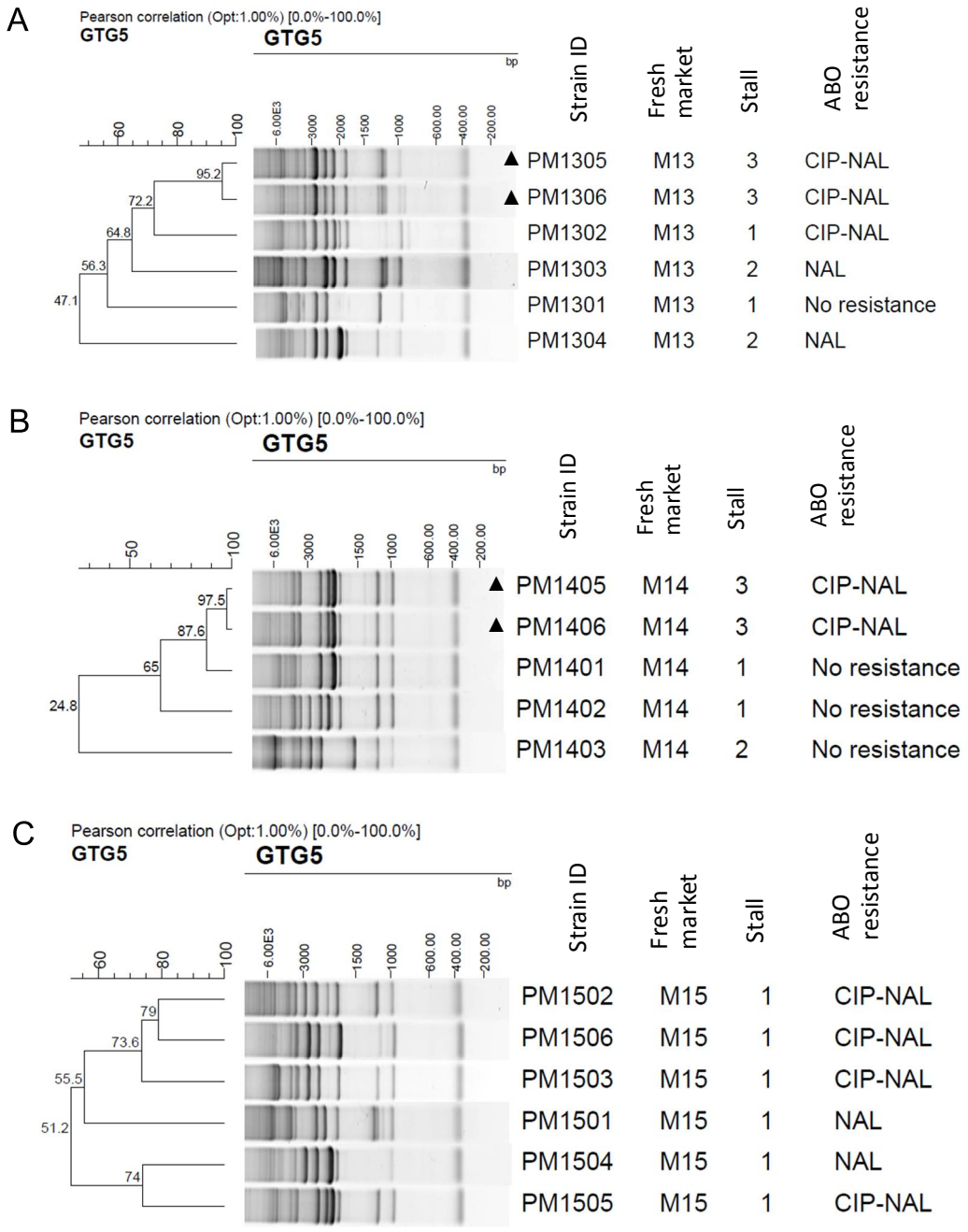

Figure 7. Dendrogram of *Arcobacter* isolates from fresh market M13 (A), M14 (B) and M15 (C). (▲) The isolates that had similar rep-PCR patterns and were recovered from the same chicken meat stall.

4.3.1.2. *Arcobacter* isolated from chickens in supermarkets

Sixty-four *Arcobacter* isolates from supermarkets were typed by rep-PCR. Fifty-three rep-PCR patterns were obtained which were grouped into 17 clusters at the similarity value of 75%. The phylogenetic analysis of *Arcobacter* isolates from supermarkets is shown in Figure 8. Similar to *Arcobacter* isolates from fresh markets, the genetic profiles of *Arcobacter* isolates from supermarkets were also highly diverse. No dominant pattern was identified in each supermarket chain. Generally, *Arcobacter* isolates that had indistinguishable rep-PCR patterns were isolated from chicken carcasses sampled from the same establishment on the same date. Dendrogram based on rep-PCR results of *Arcobacter* isolates from supermarket chain A, B, C and D is shown in Figure 9 to 12.

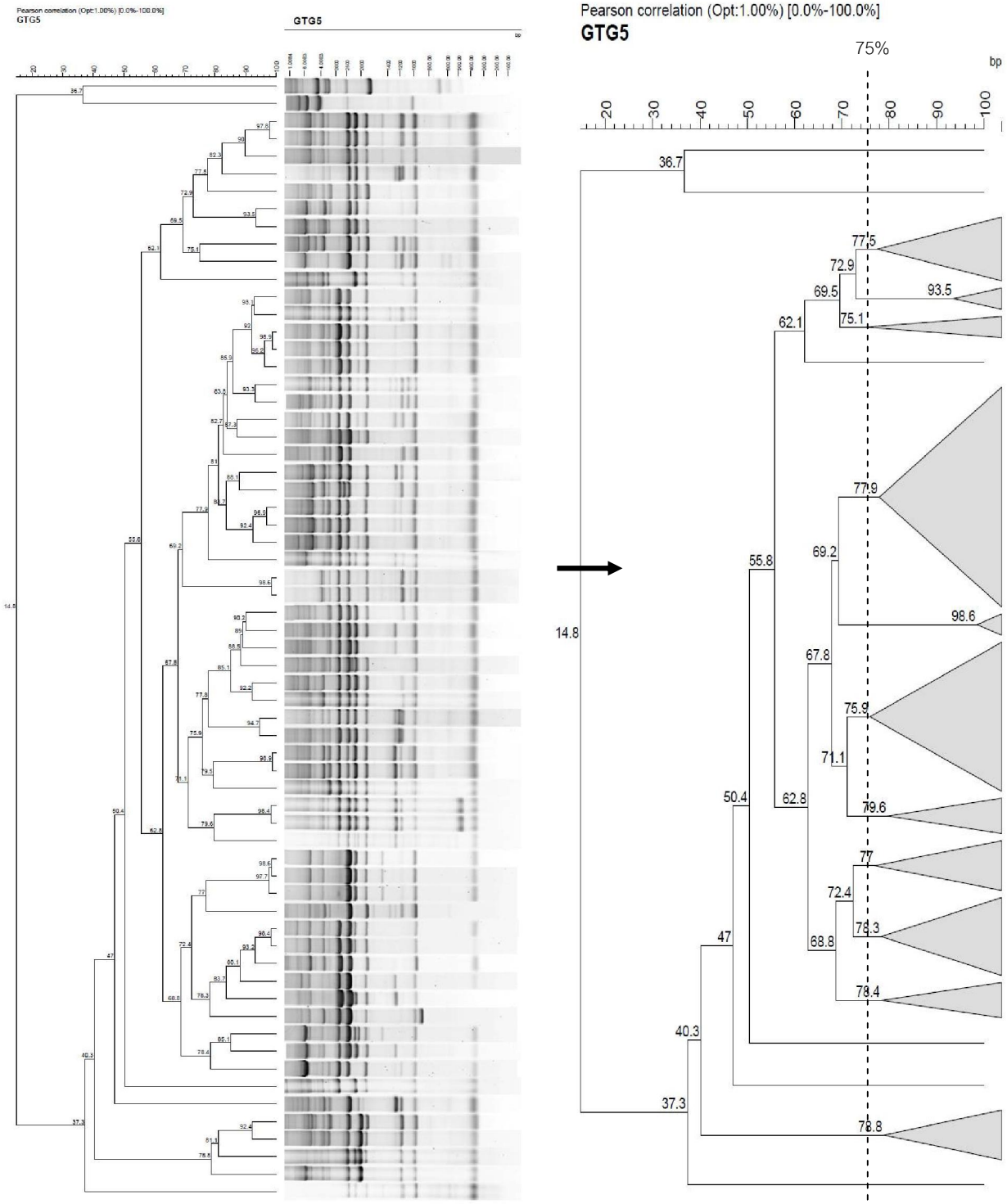

Figure 8. Dendrogram of *Arcobacter* strains isolated from chicken carcasses in supermarkets based on the results of rep-PCR

Although the majority of *Arcobacter* isolates in each supermarket chain produced divergent rep-PCR profiles, some *Arcobacter* isolates within each supermarket chain had indistinguishable rep-PCR pattern. All of the indistinguishable *Arcobacter* isolates in each supermarket chain were recovered from chicken carcasses sampled from the same location (Figure 9 to 12) except two isolates from supermarket chain A (PS10205 and PS15401) that had indistinguishable rep-PCR pattern, although these two isolates were recovered from different locations (Figure 9). Interestingly, when all *Arcobacter* isolates from every supermarket were clustered, some *Arcobacter* strains originated from different supermarket chains showed indistinguishable rep-PCR patterns (Figure 13). For example, PS154 was isolated from supermarket S1 (supermarket chain A), while PS393 was isolated from supermarket S3 (supermarket chain B). But, these two isolates showed indistinguishable rep-PCR patterns. Antimicrobial resistance patterns of *Arcobacter* isolates that shared the same rep-PCR profiles were not always identical.

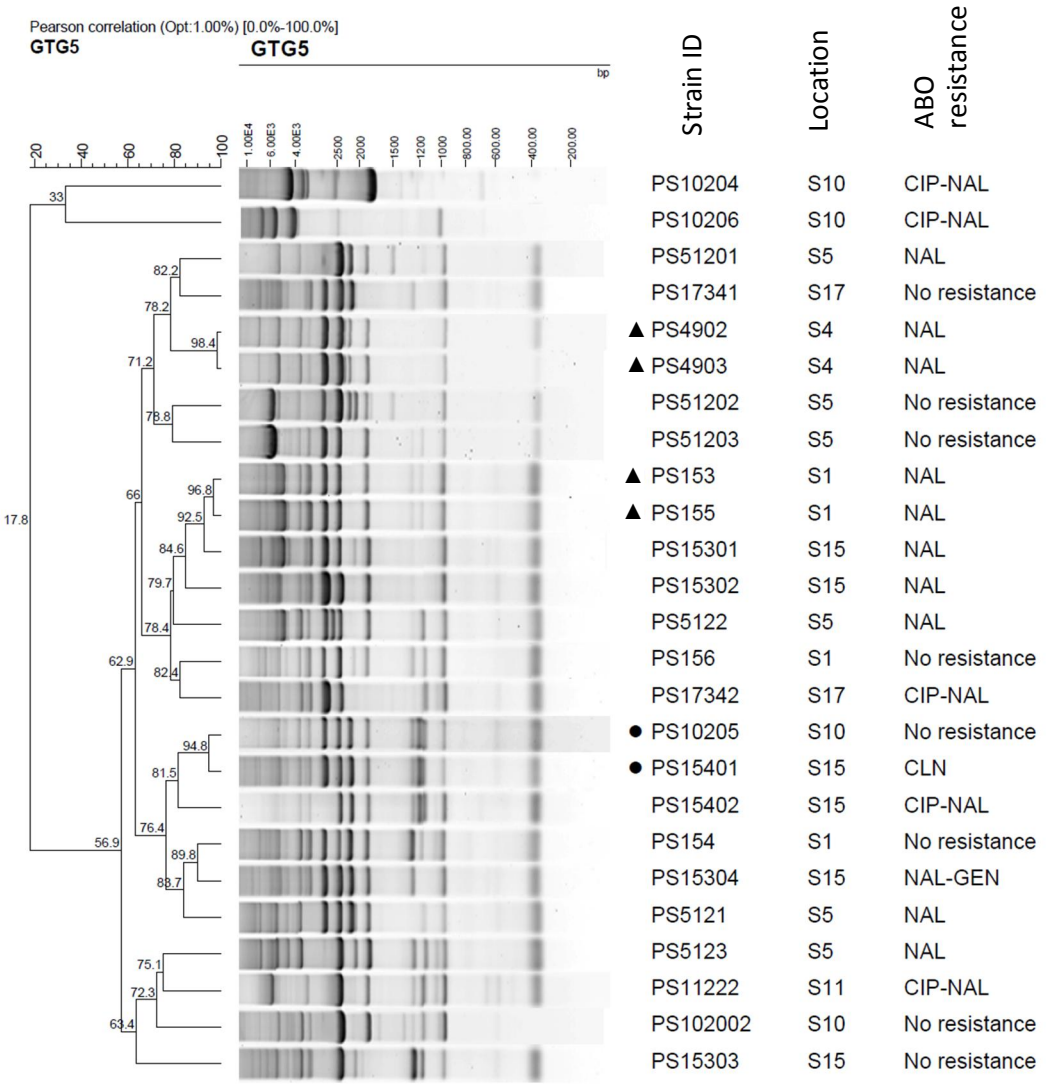

Figure 9. Dendrogram of *Arcobacter* strains isolated from supermarket chain A (S1, S4, S5, S10, S11, S15 and S17). (▲) The isolates that had indistinguishable rep-PCR pattern and were recovered from the same location; (●) The isolates that had indistinguishable rep-PCR pattern but were recovered from different locations.

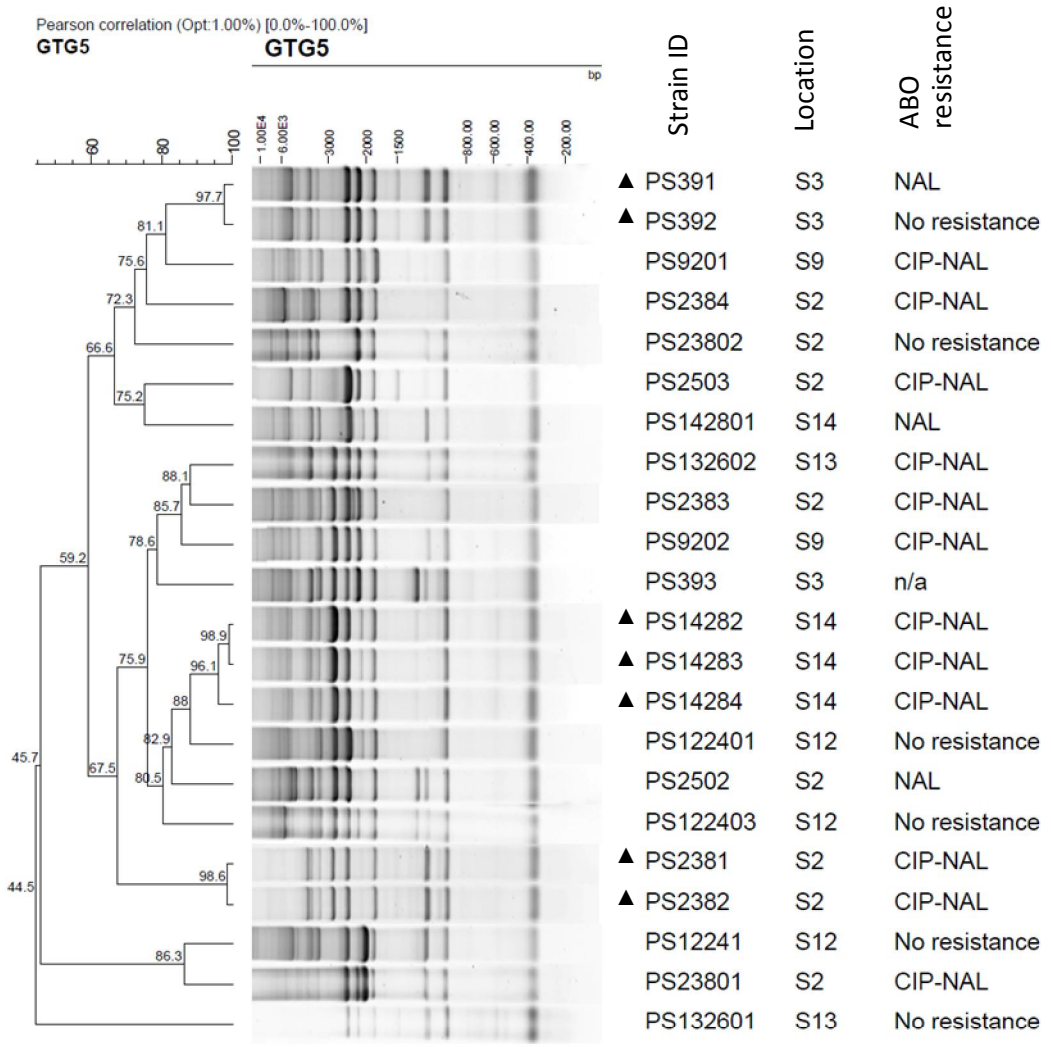

Figure 10. Dendrogram of *Arcobacter* strains isolated from supermarket chain B (S2, S3, S9, S12, S13 and S14). (▲) The isolates that had indistinguishable rep-PCR pattern and were recovered from the same location.

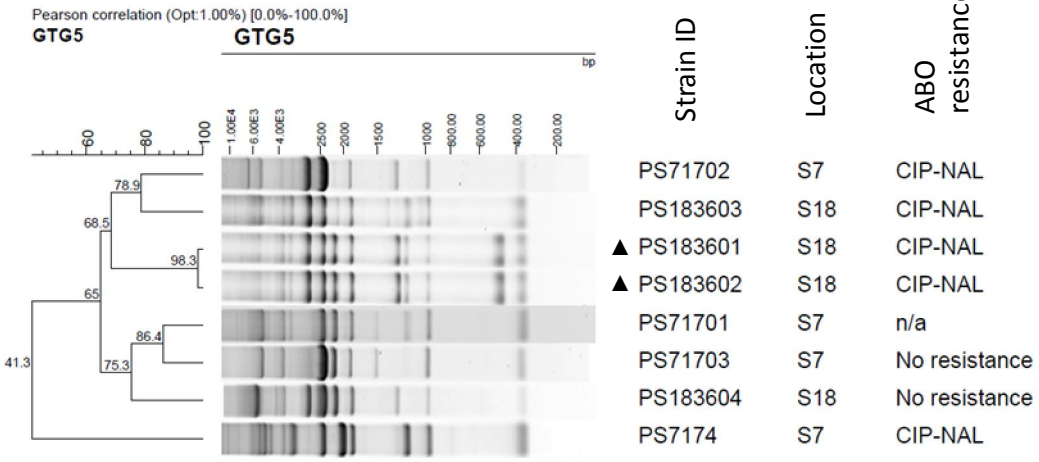

Figure 11. Dendrogram of *Arcobacter* strains isolated from supermarket chain C (S7 and S18). (▲) The isolates that had indistinguishable rep-PCR patterns and were recovered from the same location.

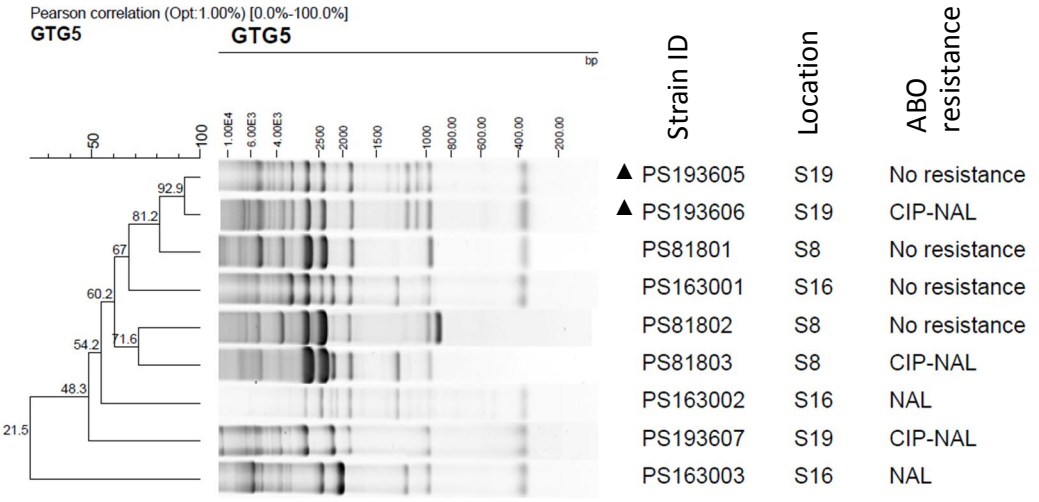

Figure 12. Dendrogram of *Arcobacter* strains isolated from supermarket chain D (S8, S16 and S19). (▲) The isolates that had indistinguishable rep-PCR patterns and were recovered from the same location.

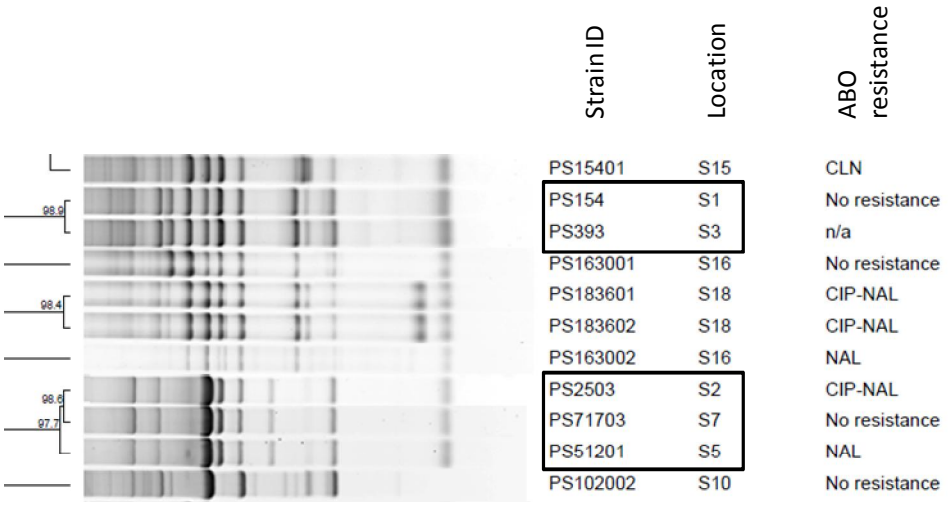

Figure 13. *Arcobacter* isolates originated from different supermarkets but had indistinguishable rep-PCR pattern.

4.3.2 PFGE

Arcobacter isolates were selected for PFGE typing according to the results of rep-PCR. At 75% cutoff value on the rep-PCR dendrogram, a few *Arcobacter* isolates were selected as representatives of each cluster. In addition, some *Arcobacter* isolates that had indistinguishable rep-PCR pattern were also included to determine whether the results obtained from PFGE were similar to those obtained from rep-PCR.

In total, 58 *Arcobacter* isolates were examined of which 32 isolates were from fresh markets and 26 isolates were from supermarkets. The dendrogram of *Arcobacter* isolates from fresh markets and supermarkets is shown in Figure 14 and 15, respectively.

PFGE results of *Arcobacter* originated from fresh markets revealed a great genetic diversity of *Arcobacter*. *Arcobacter* isolates showing different rep-PCR patterns also produced different PFGE patterns. Interestingly, all *Arcobacter* isolates recovered from chicken carcasses sampled from the same fresh market had different PFGE patterns. One pair of *Arcobacter* isolates that had indistinguishable rep-PCR pattern (PM1105 and PM1303) was further discriminated into different PFGE types (Figure 16).

Similar to the results of fresh markets, the PFGE results of *Arcobacter* isolates from supermarkets concurred with the results of rep-PCR. *Arcobacter* isolates that had different rep-PCR patterns were also had different PFGE patterns and *Arcobacter* isolates that had similar rep-PCR patterns also had indistinguishable PFGE patterns. Although type assignments of PFGE and rep-PCR were mostly concordant, some *Arcobacter* isolates that had indistinguishable rep-PCR patterns were further discriminated into different types by PFGE (Figure 17). For example, PS15401 and PS10205 were indistinguishable by rep-PCR but the two isolates were further discriminated into different PFGE types (Figure 18A). All isolates that had indistinguishable PFGE patterns were mostly recovered from chicken carcasses sampled from the same location except two isolates (PS154 and PS393) that were originated from different supermarkets (Figure 18B and 15). PS154 was isolated from supermarket S1, while PS393 was isolated from supermarket S3. However, these two isolates produced indistinguishable PFGE pattern (Figure 18B).

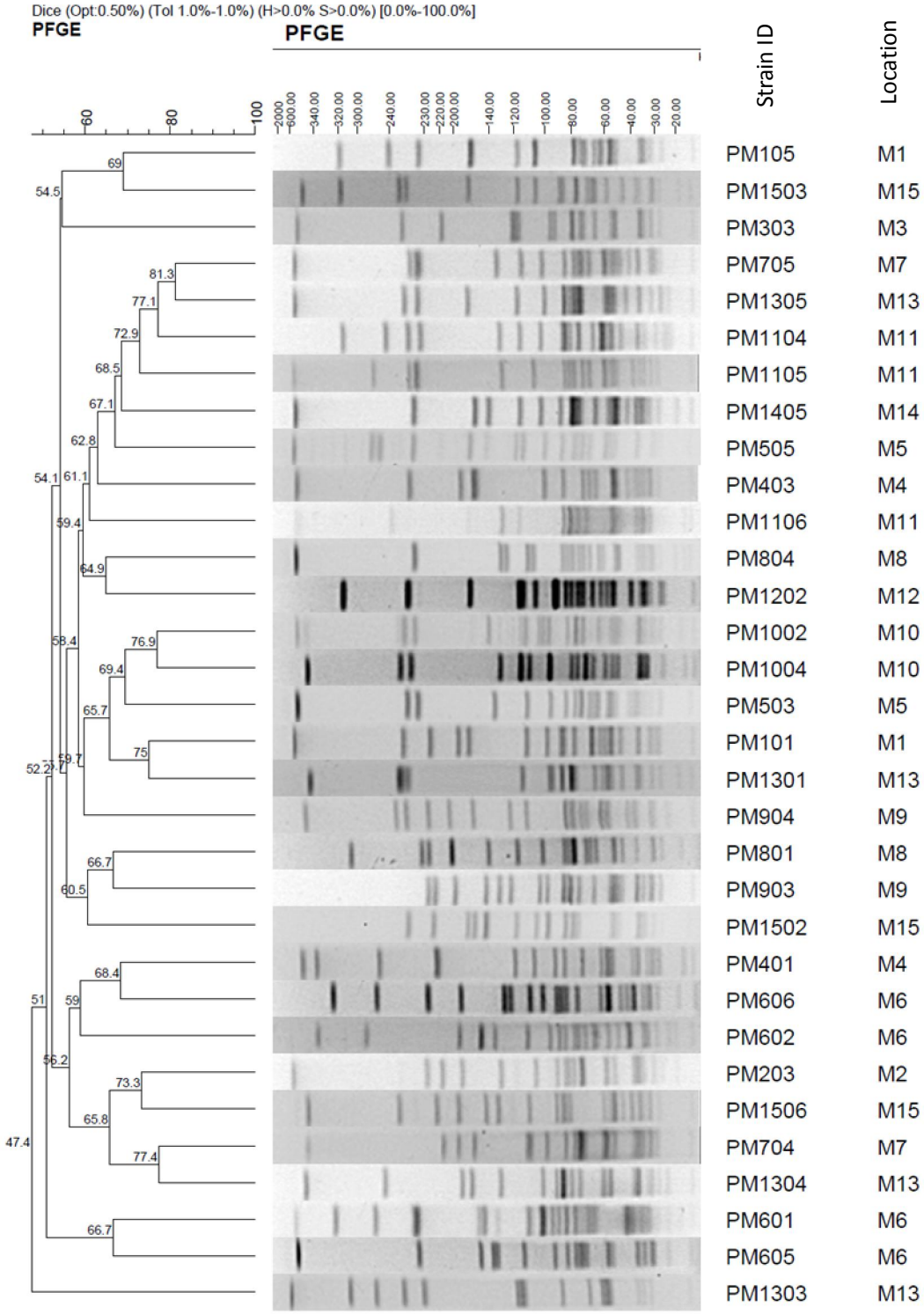

Figure 14. Dendrogram of PFGE patterns of 32 *Arcobacter* strains isolated from chicken carcasses sampled from fresh markets

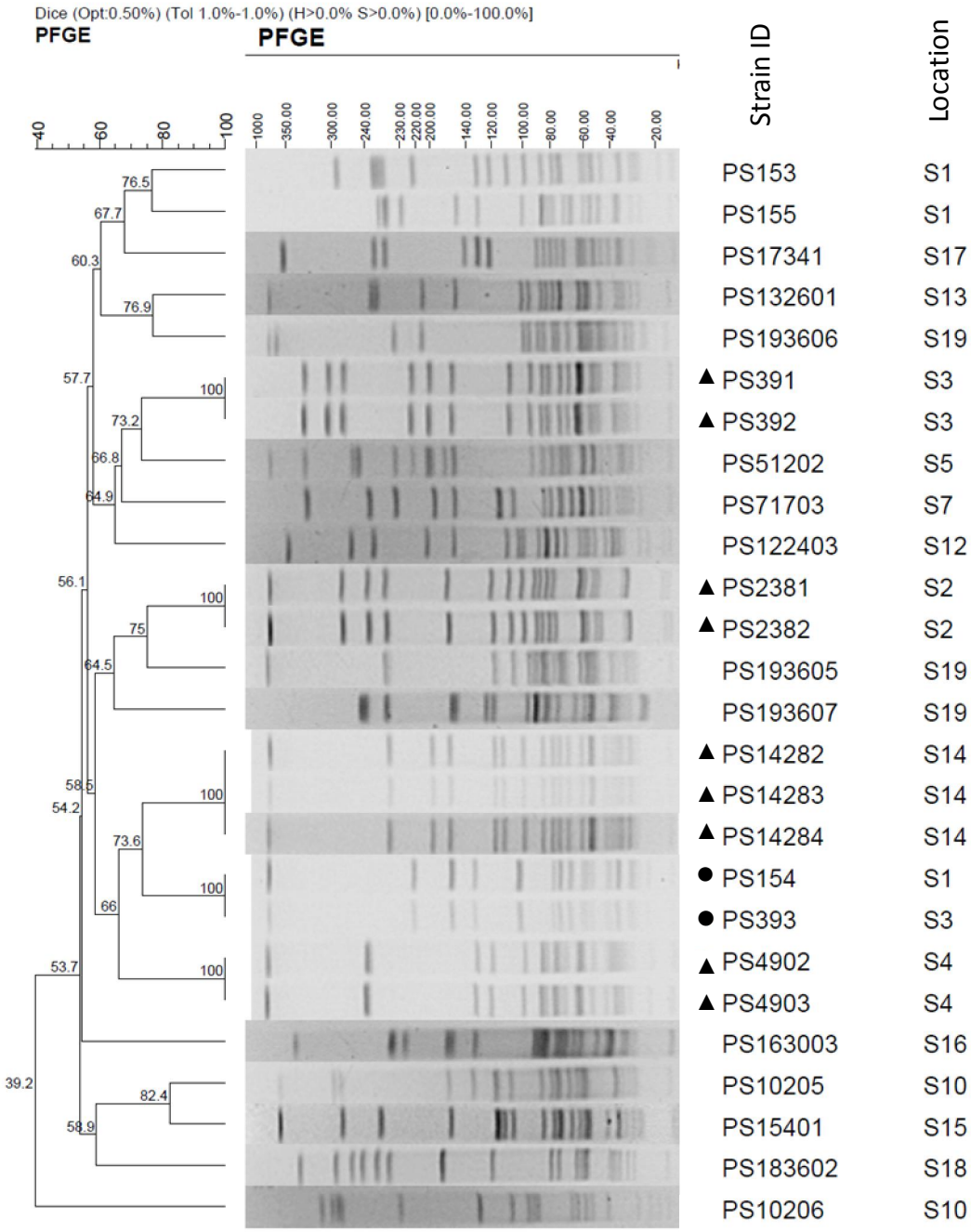

Figure 15. Dendrogram of PFGE patterns of 26 *Arcobacter* strains isolated from chicken carcasses sampled from supermarkets. (▲) The isolates that had indistinguishable PFGE patterns and were recovered from the same location; (●) The isolates that had indistinguishable PFGE pattern but were recovered from different locations.

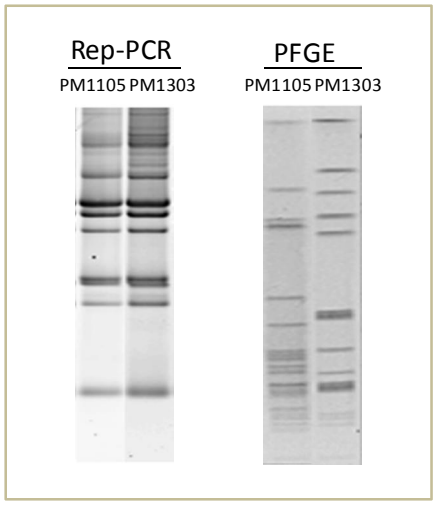

Figure 16. *Arcobacter* isolates from fresh markets that had indistinguishable rep-PCR pattern but different PFGE patterns. PM1105 was isolated from fresh market M11, while PM1303 was isolated from fresh market M3.

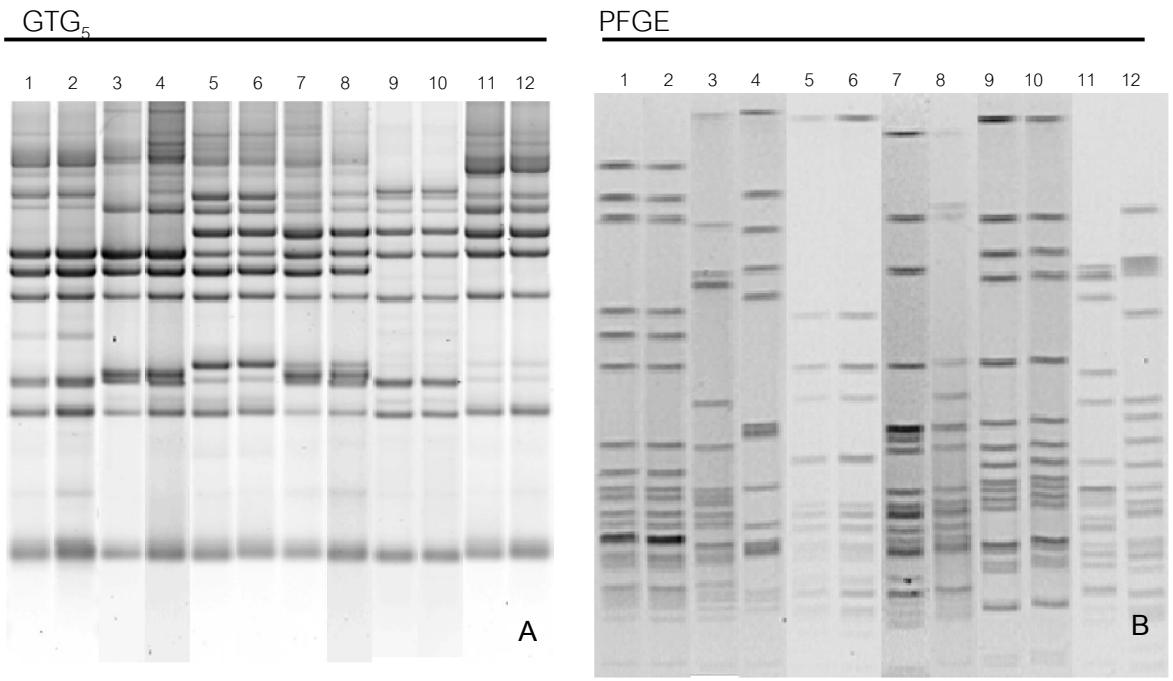

Figure 17. The banding patterns of rep-PCR (A) and PFGE (B). Some *Arcobacter* isolates that produced the same rep-PCR pattern were further differentiated by PFGE (lane 3 and 4; 7 and 8; and 11 and 12).

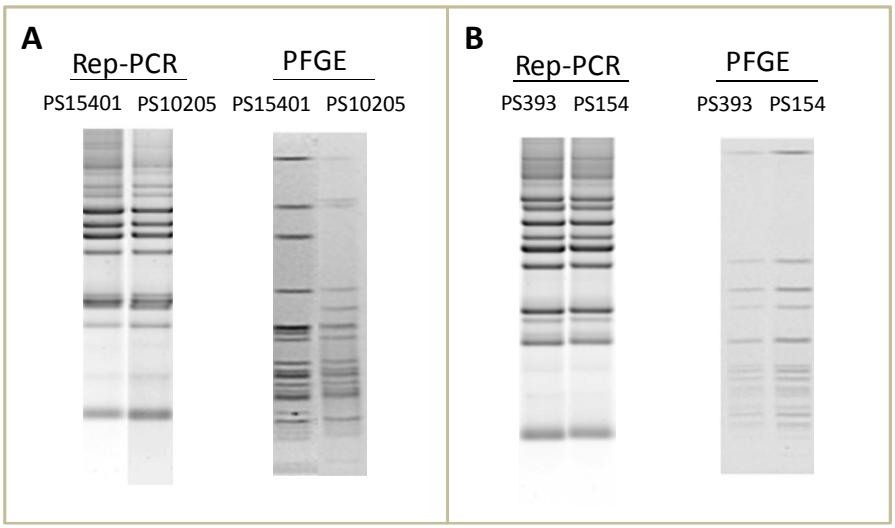

Figure 18. Rep-PCR and PFGE patterns of *Arcobacter* isolates from supermarkets. (A) *Arcobacter* isolates that had similar rep-PCR patterns but different PFGE types. PS15401 was originated from supermarket S15, while PS10205 was originated from supermarket S10; (B) *Arcobacter* isolates that had similar rep-PCR and PFGE patterns. PS393 was originated from supermarket S3, while PS154 was originated from supermarket S1.

4.4 Discriminatory power of antimicrobial resistance pattern, rep-PCR and PFGE

The discriminatory power of antimicrobial resistance pattern, rep-PCR and PFGE for typing of *Arcobacter* strains was measured using Simpson's index of diversity (SID). The SID of antimicrobial resistance pattern, rep-PCR and PFGE was 0.691, 0.993, 0.996, respectively (Table 12). The SID of rep-PCR was as high as that of PFGE suggesting that both techniques have excellent discriminatory ability and can be used effectively for studying genetic profiles of *Arcobacter*. On the other hand, since the SID of

antimicrobial resistance pattern was quite low, this technique is less suitable for typing of *Arcobacter* strains.

Table 12. Simpson's Index of Diversity (SID) with 95% Confidence Interval (CI)

Method	SID	95%CI
PFGE	0.996[a]	0.991-1.000
Rep-PCR with $(GTG)_5$ primer	0.993[a]	0.987-0.998
Antibiotic resistance pattern	0.691[b]	0.649-0.733

[a,b] The different superscript letter indicates significant difference ($P < 0.001$).

4.5 Concordance of antimicrobial resistance pattern, rep-PCR and PFGE

To determine the concordance between type assignments of the different typing methods, the Adjusted Rand coefficient (AR) and Wallace coefficient were calculated (Table 13 and 14).

The Adjusted Rand coefficient showed that rep-PCR was more congruent with PFGE (95% CI 0.390 < AR < 1.000) than antibiotic resistance pattern (95% CI 0.000 < AR < 0.037). The Adjusted Rand coefficient of comparison between antimicrobial resistance pattern and rep-PCR or PFGE was very low (0.017 and 0.015), indicating a poor agreement between these typing techniques.

Table 13. Adjusted Rand coefficient with 95% CI

	PFGE	rep-PCR	ABO resistance pattern
PFGE	1.000 (1.000 – 1.000)		
rep-PCR	0.735 (0.390 - 1.000)	1.000 (1.000 – 1.000)	
ABO resistance pattern	0.015 (0.000 - 0.037)	0.017 (0.000 – 0.041)	1.000 (1.000 – 1.000)

The Wallace coefficient of PFGE to rep-PCR was 1.000 which showed that *Arcobacter* strains that were assigned in the same PFGE type had 100% chances of being assigned in the same rep-PCR type. On the contrary, the Wallace coefficient of rep-PCR to PFGE was 0.583 which indicated that *Arcobacter* isolates that were fallen into the same rep-PCR type had 58.3% chances of being fallen into the same PFGE type. This finding reflects that PFGE was more discriminatory than rep-PCR. For antimicrobial resistance pattern, the Wallace coefficient of antimicrobial resistance pattern to rep-PCR or PFGE was very low (0.016 and 0.012) indicating that antibiotic resistance pattern is a poor predictor of rep-PCR type or PFGE type (Table 17).

Table 14. Wallace coefficient with 95% CI

Wallace coefficient	PFGE	rep-PCR	ABO resistance pattern
PFGE		1.000 (1.000 – 1.000)	0.857 (0.424 –1.000)
rep-PCR	0.583 (0.118 – 1.000)		0.667 (0.233 – 1.000)
ABO resistance pattern	0.012 (0.000 – 0.037)	0.016 (0.000 – 0.042)	

Chapter V

DISCUSSION

The present study provides information on occurrence, antimicrobial resistance and genetic profiles of *Arcobacter* isolated from chicken carcasses in fresh markets and supermarkets located in Bangkok metropolitan area. Chicken carcasses in Bangkok particularly the ones obtained from fresh markets were highly contaminated with *Arcobacter*. All of the samples from fresh markets (100.0%) were *Arcobacter* positive, while 73.3% of chicken carcasses from supermarkets were contaminated with this organism. The high contamination rate of *Arcobacter* in retail chicken meat was previously reported in other countries such as Turkey (95%), Japan (48%) and Australia (73%) (Atabay et al., 2003; Kabeya et al., 2004; Rivas et al., 2004). Among *Arcobacter* species commonly found in chicken carcasses, *A. butzleri* was the most common species identified (Atabay et al., 2003; Kabeya et al., 2004). Approximately 79% and 100% of *Arcobacter* isolates recovered from retail meat in Japan and Turkey were *A. butzleri*, respectively (Atabay et al., 2003; Kabeya et al., 2004). In the present study, *A. butzleri* was also the most dominant species observed which accounted for 96.8% of

Arcobacter isolates examined. The high prevalence of *A. butzleri* observed in this study is consistent with other studies (Atabay et al., 2003; Kabeya et al., 2004) and likely due to the fact that *A. butzleri* seems to be more resistant to antimicrobials used in culture medium and can grow faster than *A. cryaerophilus* and *A. skirrowii* (Houf et a., 2001b).

The difference in the occurrence of *Arcobacter* between fresh markets and supermarkets may be contributed to several factors such as the difference in source of chicken carcasses and hygienic practices in fresh markets and supermarkets. Chicken carcasses in fresh markets may come from small-scale slaughterhouses providing meat for local consumption, while chicken carcasses sold in supermarkets more likely come from medium- and large-scale poultry companies which have modern equipments for slaughtering process. In term of hygienic practices, chicken carcasses sold in fresh markets in Thailand are generally left at room temperature or placed on ice without any coverage which would lead to higher chances of cross-contamination. In addition, chicken carcasses sold in fresh markets may be contaminated with *Arcobacter* from intestinal contents during evisceration, which is usually performed at fresh markets upon customers' request. On the other hand, chicken carcasses sold in supermarkets are eviscerated at slaughterhouse and packed in individual package and refrigerated. These different managing systems may influence the introduction and survival of *Arcobacter* on chicken carcasses as well as the amount of other contaminants which could affect the recovery rates of *Arcobacter*.

In the present study, the occurrence of *Arcobacter* is not relevant to the size of markets. Every chicken carcass from fresh markets was *Arcobacter* positive whether the carcass was collected from small- or large-scaled fresh market. For supermarkets, the highest *Arcobacter* contamination rate was found in supermarket chain D and the lowest contamination rate was found in supermarket chain E. However, it should be noted that the number of samples collected from these supermarket chains was much lower than the number of samples collected from supermarket chain A, B and C. It is possible that the occurrence of *Arcobacter* would have changed if more samples were collected and examined. Therefore, interpretation of the results has to be done carefully.

The most common antimicrobial resistance of *Arcobacter* isolates observed in the present study was nalidixic acid resistance followed by ciprofloxacin resistance. Although nalidixic acid resistance and ciprofloxacin resistance in *Arcobacter* have been previously reported in other countries (Atabay and Aydin, 2001; Son et al., 2007a), the frequencies of resistance were lower than the resistance rates observed in the present study. About 24% of *Arcobacter* isolates from the US were resistant to nalidixic acid (Son et al., 2007a), while 74.6% of *Arcobacter* isolates examined in this study were resistant to this antimicrobial agent. The high rate of nalidixic acid resistance found in the present study concurs with the results of a previous study conducted in Thailand which showed that 80% of *A. butzleri* isolated from foods served in restaurants in Bangkok were resistant to nalidixic acid (Teague et al., 2010). Similar to nalidixic acid

resistance, ciprofloxacin resistance was also more frequently observed among *Arcobacter* isolates in Thailand than *Arcobacter* isolates examined in other countries (Vandenberg et al., 2006; Son et al., 2007a; Mandisodza et al., 2012). Ciprofloxacin resistance was found in 40 – 50% of *Arcobacter* isolates in the present study, while much lower resistance or no resistance rates were observed in the US (0.6%), Belgium (5.9%) and New Zealand (0%) (Vandenberg et al., 2006; Son et al., 2007a; Mandisodza et al., 2012). Unlike ciprofloxacin and nalidixic acid, none of *Arcobacter* isolates in this study and less than 4% of *Arcobacter* isolates in other countries were resistant to erythromycin (Atabay and Aydin, 2001; Son et al., 2007a). Likewise, only 2 out of 150 isolates from fresh markets and supermarkets in the present study were resistant to tetracycline. This finding is in agreement with the results of other studies which reported that tetracycline resistance was rarely or not identified (Vandenberg et al., 2006; Shah et al., 2012). Similar to erythromycin and tetracycline, only small numbers of *Arcobacter* isolates were resistant to aminoglycosides (Atabay and Aydin, 2001; Kabeya et al., 2004; Son et al., 2007a). Out of 150 *Arcobacter* isolates examined in this study, only two isolates from fresh markets and one isolate from supermarket in Bangkok were resistant to gentamicin. For clindamycin, although a high proportion of *Arcobacter* strains isolated from broiler carcasses in the US were resistant to this antimicrobial agent (88.5%)(Son et al., 2007a), only one *Arcobacter* isolate in the present study was resistant to clindamycin. Despite the difference in antimicrobial susceptibility results among studies,

it should be noted that the standard recommendation of antimicrobial susceptibility testing for *Arcobacter* has not been established yet. Different testing techniques, incubation period and conditions as well as breakpoints used make the comparison of antimicrobial resistance between studies difficult. It is necessary to standardize the antimicrobial susceptibility testing protocol for *Arcobacter*, so the differences in antimicrobial resistance of *Arcobacter* between countries can be accurately compared.

In Thailand, antibiotic use in livestock production is controlled by the Department of Livestock Development. The use of fluoroquinolones such as enrofloxacin has been regulated and reserved for the treatment purpose only in chicken production in Thailand since 1999. Interestingly, nalidixic acid and ciprofloxacin resistance of *Arcobacter* observed in the present study was quite high. It is possible that some chicken producers may not follow the standard of antimicrobial use regulation strictly or the quinolone resistance in *Arcobacter* may persist although the use of quinolone had been stopped for several years. It has been previously demonstrated that the level of quinolone resistance in *Campylobacter* was not decreased despite the withdrawal of quinolone use (Zhang et al., 2003). The persistence of quinolone resistance may also be the same for *Arcobacter* but further investigations are needed.

The divergent rep-PCR and PFGE patterns in the present study clearly showed that *Arcobacter* had a high degree of genetic heterogeneity. Rep-PCR has been the most widely use typing technique for *Arcobacter* (Collado and Figueras, 2011). Rep-

PCR with ERIC primer (ERIC-PCR) has been applied to strain characterization of *Arcobacter* in several studies (Houf et al., 2002a; Van Driessche et al., 2004; Van Driessche et al., 2005; Aydin et al., 2007). However, the most commonly performed ERIC-PCR protocol (Houf et al., 2002a) used very low annealing temperature (25 °C) and a high concentration of $MgCl_2$, which can lead to questionable results and doubtful reproducibility. From the results of our preliminary study, the rep-PCR patterns obtained by using conditions mentioned in the previous protocol (Houf et al., 2002a) were not reproducible. In this study, $(GTG)_5$ primer and higher annealing temperature were used for rep-PCR amplifications. The conditions used in the present study generated highly reproducible results. In addition, the technique also had a high discriminatory index which was comparable to that of PFGE. Therefore, rep-PCR with $(GTG)_5$ primer is another promising technique for studying genetic relatedness of *Arcobacter*. Although PFGE is considered as a gold standard for typing several bacterial pathogens (Majella et al., 2006), the technique is expensive, complicated and time and labor intensive. In addition, it also requires special equipments as well as chemical agents which are not available in most laboratories. Rep-PCR, on the other hand, is a simple, fast and inexpensive. This technique only requires standard equipments such as thermal cyclers, gel electrophoresis chambers and gel documentation systems, which are available in most laboratories. From the advantages of rep-PCR technique and the extreme genetic heterogeneity of *Arcobacter*, rep-PCR can be a useful typing technique for screening

genetic profiles of *Arcobacter* especially when large numbers of samples are needed to be examined.

In the present study, every *Arcobacter* isolate was screened by rep-PCR before some *Arcobacter* isolates were selected for further characterization with PFGE. At a cut off value at 75% on the rep-PCR dendrogram, *Arcobacter* from fresh markets and supermarkets were categorized into 19 and 17 clusters, respectively. One to three *Arcobacter* isolates from each cluster were chosen for further typing. Generally, the results of rep-PCR were in agreement with the results of PFGE. *Arcobacter* isolates that yielded different rep-PCR patterns were also yielded different PFGE patterns, except some isolates that were indistinguishable by rep-PCR were further differentiated by PFGE. In the present study, *Arcobacter* isolates from fresh markets had a wide genetic variation. There was no association between the origin of chicken carcasses and the patterns observed. Although *Arcobacter* strains were isolated from chicken carcasses purchased from the same chicken meat stall, rep-PCR patterns of these *Arcobacter* isolates were different. However, we noticed that some *Arcobacter* strains that were recovered from different fresh markets produced indistinguishable rep-PCR patterns. Similar to *Arcobacter* recovered from chicken carcasses in fresh markets, *Arcobacter* strains isolated from supermarkets also had an extreme genetic diversity. There was no dominant pattern observed in each supermarket chain. Indistinguishable PFGE patterns were found only among *Arcobacter* isolates from the same supermarket at the same

collection time except one pair of *Arcobacter* isolates that had identical rep-PCR and PFGE patterns, although they were isolated from different supermarket chains.

Conclusion and suggestion

Retail chicken carcasses in Bangkok metropolitan area were highly contaminated with *Arcobacter*. The occurrence of *Arcobacter* was significantly higher for chicken samples collected from fresh markets than supermarkets. The difference in condition and environment as well as sources of chicken between fresh markets and supermarkets could influence the recovery rate of *Arcobacter*. *Arcobacter* isolates in the present study showed variable susceptibility to different antimicrobials. The majority of *Arcobacter* isolates were resistant to nalidixic acid and ciprofloxacin. The resistance rates were generally higher among *Arcobacter* isolates recovered from chicken carcasses in fresh markets than those isolates originated from supermarkets. Although the present study revealed that most *Arcobacter* isolates were susceptible to the commonly used antimicrobials, the antimicrobial resistance in *Arcobacter* should be monitored periodically. In terms of genetic profiles of *Arcobacter* isolates, rep-PCR and PFGE are valuable techniques for studying genetic characteristic of *Arcobacter*. Rep-PCR is a simple and robust technique which is suitable for screening a large amount of samples, while PFGE is more appropriate for studying closely-related *Arcobacter*

strains. Based on the results of rep-PCR and PFGE, the present study revealed that *Arcobacter* had a great genetic diversity. *Arcobacter* strains isolated from chicken carcasses sold in the same place were generally produced different fingerprint patterns. The presence of different *Arcobacter* strains on chicken carcasses may indicate that there were multiple sources of carcass contamination. The high contamination rate of *Arcobacter* in the present study suggests that consumers should cook chicken meat thoroughly and avoid cross-contamination during food preparation. According to the results in this study, further studies should focus on the sources of *Arcobacter* contamination in chicken carcasses and the antimicrobial resistance mechanisms of *Arcobacter*. In order to effectively control and reduce the occurrence of *Arcobacter* in chicken carcasses, sources of contamination are needed to be identified. The understanding of antimicrobial resistance mechanisms would help elucidate the spread of antimicrobial resistance trait among *Arcobacter* isolates which will be essential for controlling the antimicrobial resistance.

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APPENDICES

APPENDIX A

Culture media used for *Arcobacter* isolation

1. *Arcobacter* enrichment broth (CM0965; Oxoid)

   Typical formula (gm/litre)

Peptone	18.0
Yeast extract	1.0
Sodium chloride	5.0

   pH 7.2 ± 0.2 @ 25°C

2. CAT selective supplement

   (mg /litre of *Arcobacter* enrichment broth)

Cefoperazone	8.0
Amphotericin B	4.0
Teicoplanin	10.0

3. Blood agar base no. 2 (CM0271; Oxoid)

   Typical Formula (gm/litre)

Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

   pH 7.4 ± 0.2 @ 25°C

4. Campylobacter blood-free selective agar base (mCCDA) (CM0739; Oxoid)

   Typical Formula (gm/litre)

Nutrient Broth No.2	25.0
Bacteriological charcoal	4.0
Casein hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0

   pH 7.4 ± 0.2 @ 25°C

5. CCDA selective supplement

   (mg/litre of mCCDA)

Cefoperazone	32 mg
Amphotericin B	10 mg

Illustration of *Arcobacter* isolation procedures

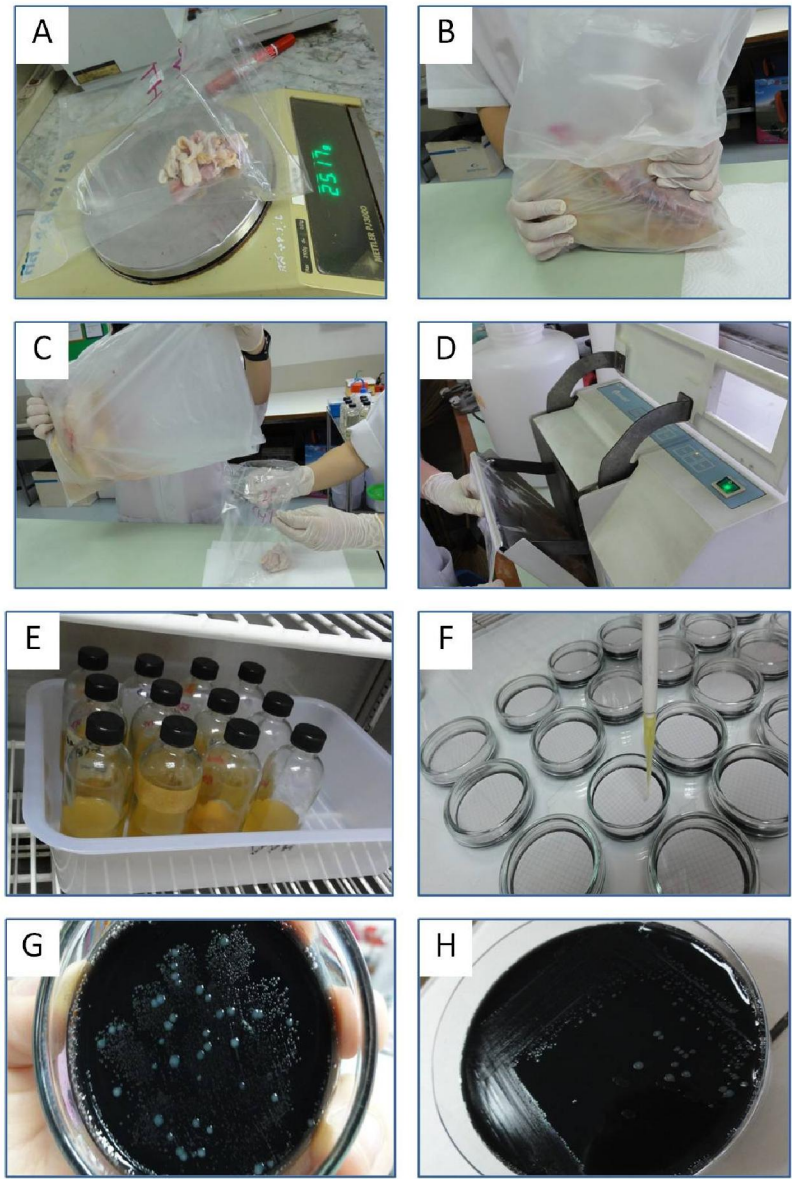

Illustration of *Arcobacter* isolation from chicken carcasses. A, aseptically excise 25 grams of chicken skins from each carcass; B, rinse the rest of the carcass with BPW; C, mix carcass rinsed BPW with chicken skins; D, homogenize the mixture in stomacher; E, inoculate the mixture into *Arcobacter* enrichment broth and incubate at 25 °C for 48 hours; F, dispense *Arcobacter* enrichment broth on filter membrane which laid on mCCDA plates; G, colonies of *Arcobacter* after incubation; H, resubculture onto mCCDA to obtain pure *Arcobacter* isolates.

APPENDIX B

Diluents of antimicrobial agents used in the present study

Antimicrobial agent	Diluent
Cefoperazone	Distilled water
Amphotericin B	Diethyl ether
Teicoplanin	Distilled water
Ciprofloxacin	0.1N HCl and distilled water
Clindamycin	Distilled water
Erythromycin	95% ethanol and distilled water
Gentamicin	Distilled water
Nalidixic acid	1M NaOH indistilled water
Tetracycline	Distilled water

APPENDIX C

Chemicals for PFGE

1. 1 M Tris-HCl, pH 8.0

Tris base 121.1 g

Dissolve in 650 - 700 ml ultrapure water

Add approximately 80 ml of 6N HCl

Let solution come to room temperature

Make final adjustments to pH 8.0

Dilute to 1000 ml with ultrapure water

Sterilize by autoclaving

2. 0.5M EDTA, pH 8.0

$Na_2EDTA.2H_2O$ 186.1 g

Add 800 ml ultrapure water

Mix and adjust pH to 8.0 with approximately 50 ml of 10 N NaOH

Sterilize by autoclaving

3. Phosphate buffer saline (PBS), 0.01M, pH 7.2 - 7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g

Dissolve in 800 ml ultrapure water

Mix and adjust pH to 7.2 or 7.4 with HCl

Adjust final volume to 1000 ml with ultrapure water

Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

4. Cell suspension buffer (CSB)

100 mM Tris-HCl:100 mM EDTA, pH 8.0

1 M Tris-HCl, pH 8.0	10 ml
0.5 M EDTA, pH 8.0	20 ml

Adjust to 100 ml with sterile ultrapure water

5. Cell lysis buffer

50 mM Tris-HCl:50 mM EDTA, pH 8.0 + 1% N-Lauroyl-Sarcosine, sodium salt (Sarcosyl) 0.1 mg/ml Proteinase K (add just before use).

1 M Tris-HCl, pH 8.0	25 ml
0.5 EDTA, pH 8.0	50 ml
10% Sarcosyl	50 ml

Dilute to 500 ml with sterile ultrapure water

Add 25 µl proteinase K stock solutions (20 mg/ml) per 5 ml of cell lysis buffer just before use.

6. Ethidium bromide 5 µg/ml

   Add 75 µl ethidium bromide stock solutions (10mg/ml) into 1.5 litres of 0.5× TBE

7. Molecular grade water	(Hyclone, USA)
8. ProteinaseK	(Thermoscientific, Lithuania)
9. *KpnI* restriction enzyme	(New England Biolabs, Canada)
10. *XbaI* restriction enzyme	(New England Biolabs, Canada)
11. SeaKem® gold agarose gel	(Lonza, Switzerland)
12. Pulsed-field certified agarose	(Biorad, Canada)

Pulsed-Field Gel Electrophoresis (PFGE) procedures

The PulseNet 24-h standardized PFGE protocols for *C. jejuni* was followed for characterization of *Arcobacter* isolates in this study, except for electrophoresis running conditions that was performed according to the protocol previously published (Son et al., 2007) The procedures are described as follow:

1) Culture *Arcobacter* isolates on blood agar plates under aerobic conditions at 25°C for 48 hours.
2) Use a moisten cotton swab to harvest and suspend colonies of *Arcobacter* in 5 ml of phosphate buffered saline (pH = 7.4). Gently spinning the cotton swab for an evenly dispersion. Adjust the optical density of the cell suspension to 0.65 at 610 nm wavelength.
3) Transfer 200 µl of cell suspensions into microcentrifuge tubes and place the tubes on ice. Add 10 µl of 20 mg/ml stock proteinase K (Thermoscienctific, USA) into cell suspensions and mix gently with pipette to avoid DNA shearing.
4) Add 200 µl of 1% melted Seakem® Gold agarose (Lonza, Switzerland) into cell suspensions and quickly pipette the mixture for a few times before dispense the mixture into plug molds immediately. Allow the plugs to solidify for 10 – 15 minutes at room temperature.
5) Prepare a master mix of cell lysis buffer by adding 25 µl of proteinase K (20 mg/ml stock) into 5 ml of cell lysis buffer to get a final concentration of proteinase K at 0.1 mg/ml. Pipette 500 µl of the cell lysis buffer mixture into each microcentrifuge tube.

6) Push plug slides out of the plug molds and submerge plug slides in the previously prepared mixture of cell lysis buffer/proteinase K in microcentrifuge tubes.
7) Incubate the plug slides at 55°C and constantly shake (300 rpm) for 30 minutes.
8) Pre-heat sterile ultrapure water and Tris-EDTA (TE) buffer at 55°C in water bath. The amount of sterile ultrapure water and TE buffer have to be sufficiently prepared for washing the plugs two times with sterile ultrapure water and four times with TE buffer (15 - 20 ml/tube/time).
9) Pour off the mixture of cell lysis buffer/proteinase K from plug slides. Plug slides can be held with screened cap (Biorad, Canada). Pour sterile ultrapure water on plug slides for a few seconds and then submerge the plug slides in 15 to 20 ml of TE buffer in centrifuge tubes. Shake the centrifuge tubes in 55°C water bath for 10 – 15 minutes.
10) Pour off the water and repeat washing step with sterile ultrapure water for one more time.
11) Pour off water and use TE buffer for washing instead. Wash plug slides with TE buffer for 4 times.
12) Plug slides can be kept in 5 – 10 ml of fresh TE buffer at 4°C if the restriction step will not be performed right after the washing step.
13) Prepare pre-restriction mixture by diluting 10X restriction buffer 1:10 with molecular grade water (Hyclone, USA) (20 µl of restriction buffer and 180 µl of molecular grade water per plug slide). Dispense 200 µl of pre-restriction buffer to each microcentrifuge tube.

14) Remove plug slides from TE buffer and place them on Petri dish. Cut plug slides into 2 mm-wide pieces with a sharp razor blade and put them into the pre-restriction mixture. Leave at room temperature for 10 minutes and then discard the pre-restriction mixture using pipette.

15) Prepare restriction enzyme mixture as follow :

	µl per sample
Molecular grade water	88
Buffer 4 (NEB, Canada)	10
KpnI (20U/µl)	2
Total	100

16) After removal of pre-restriction buffer, add 100 µl of restriction enzyme mixture into each microcentrifuge tube. Plug slides must be submerged in the restriction cocktail. Incubate plug slides at 37 °C for 5 hours.

17) Approximately 1 hour before the restriction is completed, prepare 1% pulsed-field certified agarose. Incubate the agarose at 55 °C in water bath for at least 30 minutes before use.

18) Rinse the gel casting apparatus and comb with tap water and distilled water and use tissue paper to wipe off water.

19) After 5 hours of incubation, remove restriction enzyme mixture from the plug slides and then add with 200 µl of 0.5× TBE buffer in each sample. Allow the samples to sit at room temperature for 10 minutes.

20) Remove plug slides from 0.5× TBE buffer. Use dust free tissue to absorb excess water from plug slides. Load plug slides on the bottom of the comb teeth and allow plug slides to air dry on the comb for 5 – 10 minutes. Load standard plug slides[†] on the 1st, 8th and 15th lane.

21) Put the comb up right and pour the 1% pulsed-field certified agarose into the gel casting apparatus. Allow the gel to solidify for 30 – 45 minutes.

22) Place gel frame inside the electrophoresis chamber. Pour 2 – 2.2 liters of 0.5× TBE buffer into the chamber and turn on the Chef Mapper (Biorad, Canada) apparatus, pump and the cooling module approximately 30 minutes before use. Set the temperature of the cooling module at 14 °C and set the pump to 70 for a circulation of the buffer at 1 liter/minute. Select auto algorithm mode and set the running conditions as follow :

Initial switch time 6.76s

Final switch time 13.68s

Gradient 6 V/cm

Included angle 120 °

Range 30 – 400 kbp

Duration 18 hours

[†] Standard plugs are *Salmonella* Braenderup H9812 restricted with enzyme *XbaI*

23) After the run is completed, stain the gel with ethidium bromide for 20 minutes in a closed container. Destain the gel with 500 of distilled water for three times (each time approximately 20 minutes).

24) Visualize and take the image under UV light gel documentation system (Synoptic, Ltd., UK).

Illustration of PFGE procedures

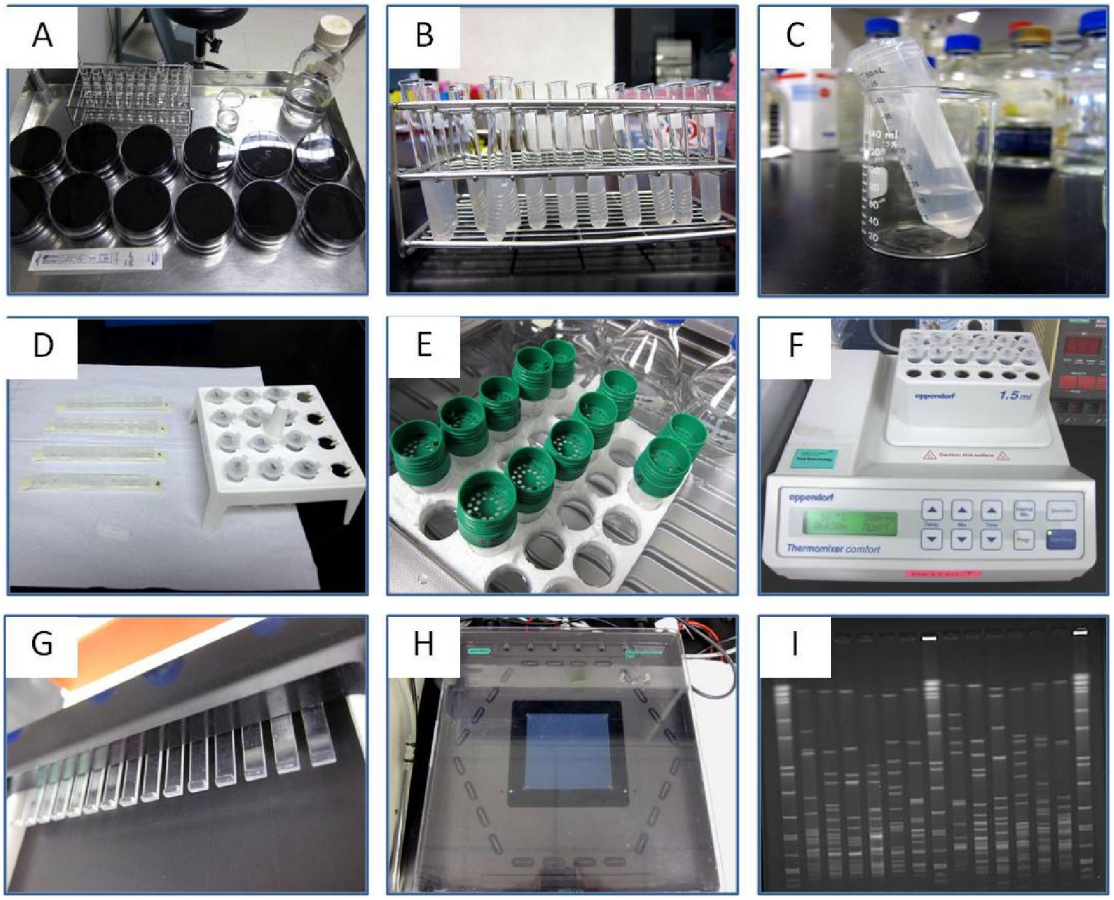

Illustration of PFGE procedures. A, culture *Arcobacter* isolates on mCCDA plates; B, adjust the optical density to 0.65 at 610 nm; C, prepare 1% Seakem (Lonza, Switzerland) agarose gel; D, mix cell suspensions with Seakem agarose gel and put into plug molds; E, wash the agarose plugs with sterile water and TE buffer; F, incubate the agarose plugs with *KpnI* restriction enzyme; G, after digestion, load plug slides onto comb teeth; H, perform electrophoresis for 18 hours; I, visualize PFGE patterns under UV light .

Biography

Miss Panvipa Phasipol was born on June 13, 1985 in Bangkok, Thailand. She got the degree in Doctor of Veterinary Medicine (D.V.M.) with 1st class honour from the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2009. After that, she enrolled in the Master of Science Program in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University in the academic year 2010. Miss Panvipa is a recipient of the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej.