PREVALENCE AND DIVERSITY OF *ARCOBACTER* SPP. IN POULTRY MEAT IN NEW ZEALAND

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Master of Science
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Errata sheet

- Both the terms "isolate" and "culture" are used to mean the same thing.
- Sections 3.4.2.1/3.4.2.2- Numbers of poultry sampled was 150, each were cultured by seven different methods. A total of 210 isolates were obtained as presumptive arcobacters out of which 189 were confirmed as *Arcobacter spp.* by PCR.
- Section 3.4.2- The number of isolates is the number out of 189 (i.e. PCR identified isolates).
- Page 59- The PFGE patterns are of the cultures isolated simultaneously from the same poultry sample by more than one method.
- Discussion- There are a number of *Arcobacter* genus specific PCR. When the study was designed there was no information on the species *A. cibarius,* so it was not thought necessary to include genus-specific PCR.
- Section 4.1.2.1- Poultry rearing shed surroundings like effluent or stagnant water, are a good source of arcobacters (Gude et al., 2006), from whence the crates and transportation vehicles may be contaminated. Once introduced in a processing plant, arcobacters may remain viable in processing equipments and water (Houf et al., 2002b; Houf et al., 2003). Thus, in a slaughterhouse with poor hygiene, these sources (processing equipments and water) may contribute to heavy contamination (also cross contamination). Similarly, improper packaging practices may result in cross-contamination contributing ultimately to high contamination rates.
- Section 4.1.3.1- A reviewer commented "The statement that the source of contamination for producers B and C appeared to be lower than A is not strictly true." This statement was made based on the diversity index as arcobacters from Producer B and Producer C were less diverse compared to those from Producer A. However, less diversity does not necessarily mean a common (or few) sources

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ABSTRACT

The microaerophilic bacterium *Arcobacter* has received increased attention in recent years as an emerging foodborne human pathogen. Although phenotypically related, arcobacters differ from campylobacters in their ability to grow aerobically and at lower temperatures. Poultry are considered a significant reservoir of this organism, with an isolation rate of up to 72% in faecal samples, and up to 100% in meat samples. To date, four species; *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. cibarius* have been isolated from poultry. The first three species have also been found to be associated with human and animal illnesses such as diarrhoea, bacteraemia, mastitis and abortions. The organisms are also found in raw meat products as well as in surface and ground water. Since most laboratories still do not use appropriate isolation techniques, the occurrence of this organism in food sources and their role in human illnesses is greatly underestimated.

This is the first investigation of the prevalence of arcobacters in poultry meat in New Zealand. The aim of this study was to compare the most commonly used *Arcobacter* isolation methods. In addition, this study aimed to estimate the prevalence of *Arcobacter* spp. in retail poultry in New Zealand. Other aims include comparison of genetic diversity of *Arcobacter* spp. isolated from three different poultry producers, and by different methods, and estimation of overall genetic diversity of arcobacters present in New Zealand.

During the period of May to October 2005, a total of 150 fresh, whole, retail poultry carcass produced by three different producers were purchased through two supermarket outlets in Palmerston North, New Zealand. Isolation of *Arcobacter* was done by seven different techniques. *Arcobacter*-like organisms were identified presumptively by phenotypic tests; temperature tolerance, aerotolerance, motility, and oxidase production. These presumptive arcobacters were confirmed by a species-specific multiplex PCR (m-PCR) either as *A. butzleri*, *A. cryaerophilus* or *A. skirrowii*. DNA sequencing was done for selected isolates from both species to further confirm the PCR results. The PCR positive isolates were subjected to Pulsed-Field Gel Electrophoresis (PFGE) following restriction digestion with *Eag*I.

It was found that 55.3 % of 150 retail poultry sold in New Zealand were harbouring *Arcobacter* species. Two species; *A. butzleri* and *A. cryaerophilus* were detected by m-PCR which was later confirmed by sequencing. A total of 189 isolates were detected by six methods from 83 retail poultry samples. *A. butzleri* was the predominant species and was detected in 51.3% of the samples, whereas *A. cryaerophilus* was detected only in 8% of the samples. *A. butzleri* and *A. cryaerophilus* accounted for 92.6% (n=175) and 7.4% (n=14) of the isolates, respectively. *A. butzleri* was the only *Arcobacter* species present in 46.6% samples, and *A. cryaerophilus* only in 3.3% of the samples. Both species were detected simultaneously in 4.6% of the samples. There was a wide variation among the prevalence rate of *Arcobacter* spp. in retail poultry from different producers varying from 30 to 98%. There was also a wide variation among the isolation rates of different methods varying from 3.3 to 39.3%. The best isolation method was found to be *Arcobacter*-broth enrichment followed by passive filtration through a sterile filter of 0.45μm, onto blood-agar plates. No single isolation method detected all arcobacters. PFGE of *Arcobacter* isolates demonstrated the occurrence of multiple genotypes of both *A. butzleri* and *A. cryaerophilus* in the retail poultry from the same producers, and even in a single poultry. The possible explanations for the large amount of heterogeneity include multiple sources of contamination, the occurrence of multiple parent genotypes for both species in a single poultry carcass, and a high degree of genomic recombination among the progeny of historical parent genotypes.

This study highlights the high prevalence of *Arcobacter spp.* in poultry meat in New Zealand. It also indicates prevalence of arcobacters in poultry carcass varies greatly with the choice of isolation method and none of the currently available methods are appropriate for the detection of all species of arcobacters in New Zealand. Therefore, two or more methods should be used in parallel. The level of contamination of poultry carcass may vary with the processing practices of a slaughterhouse. To eliminate or reduce arcobacters in retail poultry, maintenance of slaughter hygiene is of utmost importance. This may be achieved by regular microbiological monitoring of carcasses according to the HACCP principles. Further studies comparing the fingerprinting pattern of *Arcobacter* spp. isolates obtained from retails poultry with human isolates are necessary to test the hypothesis that poultry meat is an important source for *Arcobacter* infection in human.

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LIST OF ABBREVIATIONS

AA	*Arcobacter* agar
AB	*Arcobacter* broth
AFLP	Amplified fragment length polymorphism
ASB	*Arcobacter* selective broth
ASM	*Arcobacter* selective medium
ATCC	American type culture collection
BPW	Buffered peptone water
CAT	Cefoperazone-amphotericin-trimethoprim
CCDA	Charcoal-cefoperazone-deoxycholate agar
CHEF	Contour clamped homogenous electric field
CIN	Cefsulodin-irgasan-novobiocin
CLO	*Campylobacter* like organism
CVA	*Campylobacter*-cefoperazone-vancomycin-amphotericin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
ERIC	Enterobacterial repetitive intergenic consensus
ESR	Environmental Science and Research
FA	Fatty acid
FAME	Fatty acid methyl ester
FISH	Fluorescent *in situ* hybridization
HACCP	Hazard Analysis Critical Control Point
G+C	Guanine plus cytosine
ISH	*In situ* hybridization
Kb	Kilobase
MQ	Milli-Q
NCBI	National Centre for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TSI	Triple sugar iron
UPGMA	Unweighted pair group method using arithmetic averages

CHAPTER 1: LITERATURE REVIEW

1.1 General introduction

The family *Campylobacteraceae* includes the bacterial species belonging to the genera *Campylobacter* and *Arcobacter* (Vandamme and Ley, 1991). The genus *Arcobacter* (Latin for 'arc-shaped organism') includes bacteria that were formerly known as "aerotolerant campylobacters" because of their similarity with campylobacters, and ability to grow in atmospheric oxygen. The microbiological and clinical features of arcobacters are similar to campylobacters (Vandenberg *et al.*, 2004). However, members of the genus *Arcobacter* are differentiated from *Campylobacter* by their ability to grow at lower temperatures and in air (Neill *et al.*, 1985; Tee *et al.*, 1988; Kiehlbauch *et al.*, 1991a).

Arcobacters have received attention in recent years because of their association with food production, and animal and human illnesses. To date, seven species have been differentiated within the genus *Arcobacter*: *A. butzleri, A. skirrowii, A. cryaerophilus, A. cibarius, A. nitrofigilis, "Candidatus Arcobacter* sulfidicus*"* and *A. halophilus* sp. nov. Among these, the first three species have been isolated from various food-items as well as from animal and human illnesses. *A. cibarius* has recently been isolated from poultry meat (Houf *et al.*, 2005).

The remaining three species: *A. nitrofigilis, "Candidatus Arcobacter* sulfidicus*"* and *A. halophilus* are free-living and are not considered animal pathogens. *A. nitrofigilis* is a nitrogen-fixing bacterium occurring on the roots of *Spartina alterniflora,* a salt-marsh plant (McClung *et al.*, 1983). *"Candidatus Arcobacter* sulfidicus*"* is an autotrophic, sulphur oxidizing species found to be occurring in marine environments (Wirsen *et al.*, 2002). *A. halophilus* has recently been found to be occurring in hypersaline lagoon water (Donachie *et al.*, 2005).

In humans, arcobacters are mainly isolated from cases of gastroenteritis and septicaemia (Lehner *et al.,* 2005). These organisms have been associated with animal diseases including abortion (Ellis *et al.*, 1977; Ellis *et al.*, 1978; Neill *et al.*, 1985; Fernandez *et al.*, 1995; On *et al.*, 2002), mastitis (Logan *et al.*, 1982) and diarrhoea (Wesley *et al.*, 2000). Poultry is considered to be the most significant reservoir as up to 72% of the cloacal content samples (Atabay *et al.*, 2006) and up to 100% of poultry meat samples (Houf *et al.*, 2001a; Morita *et al.*, 2004) has been found to be harbouring arcobacters. Besides poultry meat, other food items like beef, pork, and lamb may also be contaminated with arcobacters (Golla *et al.*, 2002; Vytrasova *et al.*, 2003; Rivas *et al.*, 2004). Furthermore, surface and ground waters have also been found to be contaminated with different species of *Arcobacter* (Dhamabutra *et al.*, 1992; Jacob *et al.*, 1993; Musmanno *et al.*, 1997; Jacob *et al.*, 1998; Rice *et al.*, 1999; Frias-Lopez *et al.*, 2002; Amisu *et al.*, 2003; Moreno *et al.*, 2003; Diergaardt *et al.*, 2004; Fera *et al.*, 2004; Maugeri *et al.*, 2004; Morita *et al.*, 2004).

Among the arcobacters, *A. butzleri* is the most common species associated with human and animal illnesses, as well as food items (Ho *et al.*, 2006). In humans, *A. butzleri* has been associated with enteritis, abdominal cramps (Vandamme *et al.*, 1992a), appendicitis, septicaemia and bacteraemia (Taylor *et al.*, 1991; Lerner *et al.*, 1994; On *et al.*, 1995; Hsueh *et al.*, 1997; Vandamme, 2000; Yan *et al.*, 2000). *A. butzleri* has also been isolated from various animals including primates, pigs, horses, and cattle; and from various food products including poultry, pork, beef, and lamb (Lehner *et al.*, 2005).

A. cryaerophilus has been isolated from humans with abdominal illness, septicaemia, and pneumonia (Tee *et al.*, 1988; Hsueh *et al.*, 1997; Engberg *et al.*, 2000). This species has also been isolated from aborted foetuses of cattle, pigs and sheep (Fernandez *et al.*, 1995; Neill *et al.*, 1980); from pig faeces and from cattle with mastitis (Vandamme, 2000). Preputial fluid of boars has also been found to be harbouring this species of *Arcobacter* (De Oliveria *et al.*, 1999).

Recently, *A. skirrowii* has been isolated from a case of chronic diarrhoea in an elderly patient (Wybo *et al.*, 2004). Among animals, this species has been recovered from sheep and cattle with diarrhoea; from aborted porcine, ovine and bovine foetuses, and from preputial fluids of bulls (Vandamme, 2000).

The role of *Arcobacter* spp. has not been clearly defined in terms of human foodborne illness (Hsueh *et al.*, 1997; Yan *et al.*, 2000; Houf *et al.*, 2001a; Wybo *et al.*, 2004), and the infection rate in humans has not been clearly established (Vandenberg *et al.*, 2004). Although the pathogenicity of the organism is not clearly understood, the cytotoxic effects of the enterotoxin produced have been reported (Musmanno *et al.*, 1997).

Little is known about the risk factors associated with *Arcobacter* infection in humans. Transmission is believed to be by the oral route, through consumption of contaminated food or water (Marinescu *et al.*, 1996a; Jacob *et al.*, 1998; Rice *et al.*, 1999). Human-to-human transmission may also occur (Vandamme *et al.*, 1992a). It has been suggested that, because of the phylogenetic proximity, transmission mechanisms that have been described for *C. jejuni* may be applicable to *Arcobacter* spp. (Wesley, 1997).

Despite wide-occurrence and high isolation rate in different foods and water, data on the incidence and clinical importance of *Arcobacter* in humans are scare. This may be because most laboratories do not use appropriate culture conditions to detect all *Campylobacter* spp. and related organisms (Vandenberg *et al.*, 2004). Also, difficulty in assessing the infection rate may be due to the transient nature of the infection and similarity of symptoms with campylobacteriosis coupled with failure of *Campylobacter* isolation techniques to detect this organism. It has been suggested that, when the detection is based entirely on culturing on selective media, approximately 95% of *Campylobacter* infections are found to be caused by *Campylobacter jejuni* or *C. coli*. However, with modifications in isolation and identification techniques, other related species, including *Arcobacter* spp., may also be detected (Lastovica *et al.*, cited in Vandenberg *et al.*, 2004). Thus, it appears that lack of use of a suitable isolation technique is hindering the estimation of the true prevalence of different species arcobacters and their public health significance.

1.2 Taxonomy and historical review

The genus *Arcobacter* is one of the four genera of the family *Campylobacteraceae* (Vandamme and Ley, 1991). To date seven species have been differentiated within the genus *Arcobacter*: *A. butzleri, A. skirrowii, A. cryaerophilus, A. cibarius, A. nitrofigilis,* "*Candidatus Arcobacter* sulfidicus" and *A. halophilus* sp. nov. Among these species, the first three have been isolated from various food-items as well as from animal and human illnesses. These bacteria were called 'aerotolerant campylobacters' until the present name '*Arcobacter*' was given by Vandamme *et al.* in 1991.

Aerotolerant *Campylobacter*-like organisms were first isolated in the UK from aborted bovine and porcine foetuses in the 1970s (Ellis *et al.*, 1977; Ellis *et al.*, 1978). The workers were unable to further classify these organisms at that time.

In 1983, the species *Campylobacter nitrofigilis* was proposed for a group of *Campylobacter*-like organisms isolated from the rhizosphere of *Spartina alterniflora*, a salt marsh plant (McClung *et al.*, 1983).

In 1985, Neill *et al.* performed an extensive phenotypic characterization of aerotolerant *Campylobacter* strains isolated from various animal sources. These organisms were designated a single species, *Campylobacter cryaerophila*, on the basis of aerotolerance and ability to grow at 25 °C (Neill *et al.*, 1985). They found that the aerotolerant strains were only distantly related to strains of the other *Campylobacter* species examined and concluded that these strains formed a novel group.

In 1991, following extensive DNA homology studies, the species *Campylobacter butzleri* was proposed for aerotolerant *Campylobacter*-like organisms isolated from human enteritis (Kiehlbauch *et al.*, 1991a). The genus name '*Arcobacter*' was described as a second genus within the family *Campylobacteraceae* to encompass the bacteria *Campylobacter nitrofigilis*, *Campylobacter cryaerophila*, and an unnamed *Campylobacter* sp. strain, formerly known as aerotolerant campylobacters (Vandamme and Ley, 1991). Later, these two species were named as *Arcobacter nitrofigilis* comb. nov. (type species) and

Arcobacter cryaerophilus comb. nov., respectively (Vandamme *et al.*, 1991). In 1992, based on the results of DNA-DNA hybridization, *Campylobacter butzleri* was transferred to the genus *Arcobacter* as *A. butzleri* comb. nov., and a new species *A. skirrowii* was proposed (Vandamme *et al.*, 1992b).

Within the genus *A. cryaerophilus*, two subgroups referred to as subgroup 1 or group 1A and subgroup 2 or group 1B have been differentiated (Kiehlbauch *et al.*, 1991a; Vandamme *et al.*, 1992b). Strains of these subgroups vary in their whole-cell protein and fatty acid profiles, restriction fragment length polymorphism (RFLP) patterns, and DNA-DNA hybridizations (Vandamme, 2000). However, because these subgroups are phenotypically indistinguishable, they are regarded as a single species (Vandamme, 2000).

The publication of new species within the genus *Arcobacter* is ongoing. A novel group of bacteria occurring in sea water oxidizing sulphur derivatives were found to be phylogenetically related to *Arcobacter* and have been placed in the category *Candidatus* as "*Candidatus Arcobacter* sulfidicus" (Wirsen *et al.*, 2002). The occurrence of a "*Arcobacter skirrowii*-like" species in pig abortions and turkey faeces has been mentioned (On *et al.*, 2003). The existence of *Arcobacter cibarius* as a fourth species occurring in retail poultry carcasses has recently been published (Houf *et al.*, 2005). The nomenclature of a single bacterial isolate obtained from saline lagoon water has been published as *Arcobacter halophilus* (Donachie *et al.*, 2005).

1.3 Microbiology of arcobacters

1.3.1 Morphology

The members of the genus *Arcobacter* are Gram-negative, non-spore forming bacilli, curved, helicoid or S-shaped, 0.2 to 0.9 µm wide and 0.5 to 3 µm long (Vandamme, 2000). Cells in old cultures may form spherical or coccoid bodies and loose spiral filaments up to 20 µm long. The organisms display a corkscrew-like or darting motility by means of a single polar unsheathed flagellum at one or both ends of the cell (Ellis *et al.*, 1977; Vandamme, 2000).

A. butzleri has a diameter of 0.2 to 0.4μm and is 1 to 3 μm in length. After 3 days of incubation on blood agar, the colonies have a diameter of 2 to 4 mm, generally round shaped, and are whitish in colour (Euzeby, 2005).

A. cibarius is a slightly curved bacillus, having a diameter of 0.5μm and length of 1.5μm. The species is slightly motile although some cells have a very clear motility. After 3 days of incubation at 28°C in microaerobic atmosphere, the colonies obtained on blood-agar are whitish, slightly convex, round, smooth, nonhaemolytic, and about 2 mm in diameter (Euzeby, 2005).

A. cryaerophilus has an average size of 0.4×1.8 μm, with some forms longer than 20 μm. After 2-3 days of incubation, the colonies are smooth, convex and 1 mm in diameter, and have a regular contour (Euzeby, 2005).

A. skirrowii has a diameter of 0.2 to 0.4 μm and length of 1 to 3 μm. After 3 days of incubation, the colonies obtained on blood agar plates have a diameter of 2 to 3 mm and are often alpha-haemolytic. They are greyish and tend to spread out over the wet medium (Euzeby, 2005).

1.3.2 Growth and survival

In general, biochemical and physiological characteristics are similar in the members of the family *Campylobacteraceae* (Vandamme, 2000). Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, not from the carbohydrates since the latter are neither fermented nor oxidized (Ellis *et al.*, 1977). Cells have a respiratory and chemoorganotrophic type of metabolism. Microaerophilic conditions are needed for primary isolation, but upon subsequent subculture, the organisms become more tolerant to atmospheric oxygen (Tee *et al.*, 1988).

All the species of *Arcobacter* grow at 15, 25 or 30°C and growth is optimal in a microaerobic atmosphere (3 to 10% oxygen), but they can grow in atmospheric oxygen or in anaerobic conditions. Under aerobic conditions, these bacteria grow well at 15 and 30°C, and under anaerobic condition at 35 to 37°C (Euzeby, 2005). Optimum pH requirement ranges from 6.0 to 7.0 for *A. butzleri*, and 7.0 to 7.5 for *A. cryaerophilus* (D'Sa and Harrison, 2005).

Arcobacters can survive freezing for up to 6 months at -20°C and for up to 24 months at -70°C, but are rapidly inactivated by heating to 55°C and above (D'Sa and Harrison, 2005). They are susceptible to normal chlorination procedures used for water treatment plants (Rice *et al.*, 1999) and to γ irradiation (Collins *et al.*, 1996b).

1.3.3 Isolation

Because of their fastidious growth requirements, isolation of arcobacters from meat or environmental samples requires an enrichment step. Also, to suppress the accompanying contaminants in samples, a variety of antibiotic supplements are often needed to be incorporated in the media to make them 'selective'. As the cultural characteristics of campylobacters and arcobacters are similar, methods used for isolation of arcobacters have been derived from those developed for campylobacters. Most commonly used ingredients for *Arcobacter* media are shown in Table 1.

The first isolation of arcobacters was done by Ellis *et al.* (1977) from aborted bovine foetuses. They had used Ellinghausen–McCullough–Johnson–Harris (EMJH) isolation medium containing rabbit serum (2%), agar (0.15%), with and without 5-fluorouracil (100 mg/L). The incubation was done at 30°C. They were able to obtain arcobacters from the internal organs of 15 of the 34 aborted foetus samples, and nine of the 17 control foetuses.

An enrichment broth and selective plating medium for the isolation of arcobacters from food samples has been described (Lammerding *et al.* 1996). The enrichment broth contained peptone, Lab Lemco powder, yeast extract, NaCl, resazurin, and cefoperazone. The plating medium was a modification of CCDA (Charcoal-cefoperazone-deoxycholate agar), supplemented with cefoperazone (32 mg/L). The isolation protocol involved incubation in enrichment broth, filtration of the broth through 0.45µm pore size membrane, and plating onto modified CCDA plates. It was found that the enrichment broth and the modified CCDA plates (in combination with filtration) inhibited the growth of *P. aeruginosa*, *E. coli*, *S. aureus*, *Salmonella* sp., *C. jejuni*, and *L. monocytogenes*, but not that of arcobacters. Using this protocol, 97% of 125 poultry carcasses in Canada were found to be harbouring arcobacters.

Table 1. Commonly used ingredients and antibiotic supplements in *Arcobacter* media, and their specific properties

Ingredients		**Composition of *Arcobacter* media (mg/L)**					
Name	Properties	AB	CAT broth	AA	CAT agar	J&M broth	J&M agar
Bile salts	Makes media selective for Gram-negative enteric bacteria					250	
Charcoal	Quench toxic oxygen compounds					3%	
Lysed blood	Quench toxic oxygen compounds	5%	5%				5%
Sodium pyruvate	Source of carbon	500				500	500
Sodium thioglycolate	Maintains reducing conditions in media	500				500	500
5-fluorouracil	Inhibits campylobacters and promotes arcobacters	100		100			
Amphotericin B	Antifungal antibiotic	10	10	10	10		
Cefoperazone	Inhibit Gram-negative bacteria, mainly enteric flora	16	8	16	8	32	32
Novobiocin	Inhibit Gram-positive bacteria	32		32			
Teicoplanin	Inhibit Gram-positive bacteria		4		4		
Trimethoprim	Inhibit Gram-negative bacteria	64		64			

(AB=*Arcobacter* broth; AA=*Arcobacter* agar; J&M= Johnson and Murano medium)

The development of an isolation protocol for arcobacters based on their swarming ability on semisolid medium has been reported (De Boer *et al.* 1996). In this study, an *Arcobacter* selective enrichment broth (ASB) and an *Arcobacter* selective semisolid medium (ASM) was formulated for the recovery of *Arcobacter* from retail meat products. Basal media used for ASB and ASM were Brucella broth and Muller-Hinton agar, respectively. Both of these media contained cefoperazone (32 mg/L), piperacillin (75 mg/L), trimethoprim (20 mg/L), and cycloheximide (100 mg/L) as selective substances. The protocol involved enrichment of samples in ASB followed by transfer of 40µl of ASB onto ASM, and examination for the presence of motility zones. This method isolated arcobacters from 24% of 220 poultry meat samples.

A modified cefsulodin-irgasan-novobiocin (CIN) medium for the recovery of *Arcobacter* spp. from pork has been developed (Collins *et al.* 1996a). Enrichment was done using EMJH with 5-fluorouracil (200 mg/L), which was followed by plating on to three different media. Modified CIN was compared with CVA agar (brain heart infusion agar supplemented with 10% bovine blood and cephalothin (20 mg/L), vancomycin (10 mg/L), and amphotericin B (5mg/L)) as well as brain heart infusion agar supplemented with 10% bovine blood but without antibiotics. $MgCl_2$ was used at the rate of 2 g/L in the modified selective medium. Using this media, it was shown that 89% of the 149 pork samples were positive for *Arcobacter* spp.

A study comparing the growth performance of campylobacters and arcobacters on a variety of enrichment and direct isolation media has been published (Atabay and Corry, 1997). It was found that, enrichment, either in CAT broth or in ASB (Lammerding *et al.*, 1996) inhibited campylobacters, and allowed the growth of all of arcobacters from all 15 poultry carcasses tested, all of which were negative for arcobacters without enrichment. Plating onto CAT agar following enrichment was found to yield overgrowth of competitive organisms. Incubation at lower temperature (30 vs. 37°C) yielded wider variety of arcobacters. It was recommended that, when examining poultry for campylobacters and arcobacters, both direct plating and enrichment protocol should be included.

The CAT enrichment-filtration method developed by Atabay and Corry (Atabay and Corry, 1997) was modified by On *et al.* (2002) for use with biopsy samples taken from aborted porcine foetuses. The modifications included the use of two incubation temperatures (25 and 37°C), which improved the taxonomic diversity of isolates obtained compared with incubation at 37°C alone (On *et al.*, 2002). Here, tissue samples from liver and kidneys of aborted foetuses were enriched in CAT enrichment broth followed by spotting of broth onto blood agar plates upon which a cellulose acetate filter (pore size 0.65µm) had been placed. Arcobacters were detected in approximately 40% of the aborted foetuses.

The efficacy of Oxoid *Arcobacter* broth, supplemented with CAT was evaluated and its productivity was compared with two campylobacter enrichment media, Preston broth and LabM *Campylobacter* enrichment broth (Atabay and Corry, 1998). *Arcobacter* broth supported good growth of A. *butzleri*, *A. cryaerophilus*, and *A. skirrowii* although *A. nitrofigilis* grew poorly. It was revealed that Preston broth and to a lesser extent LabM *Campylobacter* enrichment broth, were not effective for detecting *Arcobacter* strains.

A range of solid (plating) media and enrichment broth were tested by Johnson and Murano (Johnson and Murano, 1999a; Johnson and Murano, 1999b). The solid medium containing cefoperazone (32 mg/L), thioglycolic acid (0.05%), sodium pyruvate (0.05%) and sheep's blood (5%; pH 6.9) added to a basal nutrient mix (J&M agar) was found to support the optimum growth of arcobacters at 30°C (Johnson and Murano, 1999b). The enrichment broth contained cefoperazone (32 mg/L), 5-fluorouracil (200 mg/L), activated charcoal (3%), thioglycolic acid (0.05%), sodium pyruvate (0.05%), and bile salts (0.25%). This broth, called 'JM enrichment broth', together with plating on this JM agar resulted in *Arcobacter* spp. being detected in 42 out of 50 poultry samples compared with 15 with method of De Boer *et al.* (1996), and 24 with method of Collins *et al.* (1996a). Johnson and Murano concluded that their method allowed the best recovery of *Arcobacter* and the greatest inhibition of other bacteria, and had the further advantage of using aerobic incubations, thereby eliminating the need for a modified atmosphere for incubations (Johnson and Murano, 1999a).

In 2001, Houf *et al.* (2001a) developed a selective supplement comprising amphotericin B (10 mg/L), cefoperazone (16 mg/L), 5-fluorouracil (100 mg/L), novobiocin (32 mg/L), and trimethoprim (64 mg/L). Using this supplement in enrichment and plating media, arcobacters were isolated from up to 100% of the poultry meat samples. The growth performance of *A. skirrowii* was however found to be poor with this supplement. Early studies by the same workers (Houf *et al.*, 2001b) had revealed that *A. skirrowii* is the species most susceptible to antimicrobial agents used in selective media. This may explain the low recovery rates reported to date for this organism.

Recently, Scullion *et al.*, (2004) compared protocol of Johnson and Murano (Johnson and Murano, 1999a) with other two protocols: Houf *et al.* (2001a) and On *et al.* (2002). It was found that Houf *et al.* method resulted in the highest recovery (68%) of arcobacters followed by Johnson and Murano (50%) and On *et al.* method (28%). Use of Houf *et al.* and Johnson and Murano method together increased the number of positive samples detected by approximately 25% compared with use of either method alone. Johnson and Murano method detected *A. cryaerophilus* in more samples than did the other two methods, and *A. skirrowii* was detected by only Johnson and Murano method.

While comparing the media used for isolation of *Arcobacter* spp. Houf *et al.* (2001a) technique appears to be the best in terms of high detection rates (up to 100%), and ease of preparation. In spite of detection of a range of species, the media used in the Johnson and Murano method is cumbersome and time consuming to prepare, and thus has not been used widely.

1.4 Identification and subtyping of arcobacters

Several phenotypic and molecular methods have been employed for the identification and/or subtyping of arcobacters. The most commonly employed methods are reviewed in the following sections.

1.4.1 Phenotypic identification methods

Observation of morphology, temperature tolerance, and biochemical tests are the most commonly used tests that have been used for the phenotypic characterization of arcobacters. The phenotypic tests that have been employed for the identification of arcobacters are shown in Table 2.

Identification of isolates of arcobacters to the species level, differentiating among species as well as between arcobacters and campylobacters, and subtyping by using classical phenotypic tests is difficult and may give erroneous results because of a lack of clear-cut differentiating tests (Vandamme *et al.*, 1991; Vandamme *et al.*, 1992b; Yan *et al.*, 2000). Thus, relying on conventional phenotypic methods may lead to considerable underestimation of the true incidence of arcobacters in food commodities, and in animal and human illness (Manke *et al.*, 1998).

Table 2. Differential phenotypic characteristics between *Arcobacter* and *Campylobacter* species*

Characteristics	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*	*A. nitrofigilis*	*A. halophilus*	*A. cibarius*	*C. jejuni*
Alpha-haemolysis	-	-	+	-	-	-	+
Catalase activity	V	V	+	+	-	V	+
Oxidase activity	+	+	+	+	+	+	+
Hippurate hydrolysis	-	-	-	-	-	-	+
Urease	-	-	-	+	-	-	-
Nitrate reduction	+	+	+	+	+	-	+
Selenite reduction	-	-	V	V	?	-	V
H_2S(TSI)	-	-	-	-	-	-	-
Indoxyl acetate hydrolysis	+	+	+	+	+	+	+
Growth at 15°C (air)	+	+	+	+	+	?	-
Growth at 25°C (air)	+	+	+	+	+	V	-
Growth at 37°C (microaerobic)	+	V	+	V	+	+	+
Growth at 42°C(microaerobic)	V	-	V	-	-	-	+
Growth on minimal medium	+	-	-	-	-	+	-
Growth on MacConkey agar	V	V	-	-	-	V	-
Growth in glycine (1%)	-	V	-	-	-	-	+
Growth in NaCl (4%)	-	-	+	+	+	-	-
Resistance to nalidixic acid	V	V	S	S	S	V	S
Resistance to cephalothin (32 mg/L)	R	R	R	S	S	R	R
Resistance to cefoperazone (64 mg/L)	R	R	R	S	S	R	R

+, Characteristic present in 90% of the strains examined; -, characteristic present in less than 11% of the strains examined; V, variable reaction; ?, not known; S, susceptible; R, resistant

(*Source: Vandamme *et al.*, 1991; Vandamme, 2000; Yan *et al.*, 2000; On *et al.*, 2003; Wybo *et al.*, 2004; Donachie *et al.*, 2005; Houf *et al.*, 2005).

1.4.1.1 Dark-field Microscopy

Rapid identification of arcobacters has been done by dark-field microscopy (Lammerding *et al.*, 1996; Schroeder-Tucker *et al.*, 1996; De Oliveria *et al.*, 1999; Atabay *et al.*, 2003; Fernandez *et al.*, 2004; Houf *et al.*, 2005). The technique involves direct examination of the presumptive colonies suspended in saline under a dark field microscope. The organisms are seen as small comma-shaped or spiral rods exhibiting characteristic darting or corkscrew motility. Dark-field microscopy is simple, rapid, and inexpensive, and is used for the presumptive diagnosis of *Campylobacter* enteritis in humans (Paisley *et al.*, 1982).

1.4.1.2 Temperature and aerotolerance test

The spiral or curved cellular morphology of arcobacters may not be a useful criterion to facilitate identification to genus level, as this characteristic is similar to campylobacters. Some of the phenotypic tests that differentiate arcobacters from campylobacters are aerotolerance, growth on MacConkey agar, growth at 15°C, 25°C, and 37°C, and no growth at 42°C (Vandamme *et al.*, 1992a; Marinescu *et al.*, 1996a; Schroeder-Tucker *et al.*, 1996; Hsueh *et al.*, 1997; Yan *et al.*, 2000; Atabay *et al.*, 2003).

1.4.1.3 Biochemical tests

Basic biochemical tests that are routinely used for the identification of campylobacters are also used for the identification of arcobacters to the species level. Commonly, *Arcobacter* isolates are tested for the presence of catalase and oxidase, tolerance to sodium chloride (3.5%), growth on MacConkey agar, and hydrolysis of indoxyl acetate (Schroeder-Tucker *et al.*, 1996).

Arcobacter spp. produce positive results for oxidase test, nitrate reduction test, and hydrolysis of indoxyl acetate (Marinescu *et al.*, 1996b; Euzeby, 2005). They give negative results for oxidation or fermentation of sugars, production of indole, production of lecithinase, Voges-Proskauer reaction, reduction of nitrites, production of hydrogen sulphide in TSI (Triple Sugar Iron) medium, hydrolysis of urea, hippurate, esculin, casein, tyrosine, and starch, and liquefaction of gelatine (Marinescu *et al.*, 1996b; Schroeder-Tucker *et al.*, 1996; Vandamme, 2000; Euzeby, 2005). A variable result is observed, according to the species, for the catalase test, reduction of nitrates, hydrolysis of DNA, growth in the presence of 1% glycine, 2% and 4% NaCl, and 1% bile, growth on MacConkey agar, and sensitivity to cadmium chloride (Marinescu *et al.*, 1996a; Euzeby, 2005).

Some biochemical tests are also useful for speciation of arcobacters. The most reliable biochemical tests to identify *A. butzleri* include growth in 1% glycine and in 1.5% NaCl, weak catalase activity, and resistance to cadmium chloride (Kiehlbauch *et al.*, 1991b; Vandamme *et al.*, 1992b; Schroeder-Tucker *et al.*, 1996). It has been suggested that *A. butzleri* (weak-to-negative catalase reaction) can be distinguished from other species of *Arcobacter* (strong catalase reaction) by the catalase test (De Oliveria *et al.*, 1997; 1999; Yan *et al.*, 2000).

The API CAMPY® system has been tested for the identification of arcobacters. Harrass *et al.* (1998) employed this system for the identification of *Arcobacter* isolates obtained from poultry carcasses. The authors argued that since the genus *Arcobacter* has not been included in the analytical profile index of the API CAMPY®, *Arcobacter* isolates cannot be identified suitably using this scheme. Yan *et al.* (2000) mentioned that this scheme had misidentified *A. butzleri* as *Campylobacter coli*.

The usefulness of biochemical tests is however hampered by the fastidious growth requirements of arcobacters and their relatively inert biochemical character (Vandamme, 2000).

1.4.1.4 Antibiotic sensitivity test

Antibiotic sensitivity tests may be used in combination with other phenotypic tests for the presumptive identification of arcobacters. As with campylobacters, the three most commonly used antibiotics for sensitivity testing are nalidixic acid, cephalothin and cefoperazone (Table 2). Disk diffusion test (On *et al.*, 1995; Hsueh *et al.*, 1997; Yan *et al.*, 2000) and agar dilution test (Houf *et al.*, 2001b; Houf *et al.*, 2004) have been used for testing antibiotic sensitivity of arcobacters. Although the agar dilution method is considered the reference method, the disk diffusion method could also be a reliable and convenient method (Gaudreau and Gilbert, 1997).

A variable sensitivity is observed with nalidixic acid (30 µg per disc) for *A. butzleri*, *A. cryaerophilus*, and *A. cibarius*; whereas *A. skirrowii*, and *A. nitrofigilis* are susceptible to it (Euzeby, 2005). With regards to cephalothin (30 µg per disc) and cefoperazone (30 µg per disc), *A. butzleri*, *A. cryaerophilus, A. skirrowii*, and *A. cibarius* are resistant, whereas *A.*

nitrofigilis and *A. halophilus* are susceptible (Euzeby, 2005). On *et al.* (1995) observed that *A. butzleri, A. cryaerophilus* and *A. skirrowii* were resistant to nalidixic acid (32 mg/L), metronidazole (4 mg/L), carbenicillin (32 mg/L) and cefoperazone (64 mg/L). Yan *et al.* (2000) observed that an isolate of *A. butzleri* from a human patient was susceptible to nalidixic acid and resistant to cefazolin in the disk test. Hsueh *et al.* (1997) found an isolate of *A. cryaerophilus* 1B obtained from a human patient was susceptible to nalidixic acid (30-μg disk) but resistant to cephalothin (30 μg disk).

Harrass *et al.* (1998) evaluated the usefulness of antimicrobial resistance tests to differentiate 87 isolates of *Arcobacter*. They observed that resistance to sulfamethoxazole/trimethoprim, cefazolin, and ampicillin were predominant, while resistance to nalidixic acid, chloramphenicol, and clindamycin were less frequent, and all 87 isolates were susceptible to aminoglycosides and minocycline. It was concluded that, antimicrobial resistance testing, in combination with growth and tolerance tests and plasmid analysis gave a highly specific and detailed characterization and differentiation of *A. butzleri* isolates obtained from poultry carcasses.

It should be noted in antimicrobial susceptibility testing that numerous factors may affect the result. Examples of such factors include the size of the inoculum and the composition of the basal medium (On and Holmes, 1991).

1.4.2 Biotyping

Phenotypic tests that evaluate the capability of a microorganism to generate or use biochemical substrates, for differentiating within a species, is referred to as biotyping.

A biotyping scheme has been developed for *A. butzleri* and *A. butzleri*-like isolates recognizing 16 biotypes numbered 1A, 1B to 8A, 8B, based on their ability to produce urease, rapid H_2S, DNase and the utilization of sodium acetate (Lior and Woodward, 1993). Using this scheme, Marinescu *et al.* (1996b) identified 3A, 4A, 6A, 7A and 8A biotypes among 162 *A. butzleri* and one *A. butzleri*-like isolate obtained from poultry samples. Out of these, biotype 8A was the most common followed by 7A and 4A. Lior's scheme differentiated the 44 strains of *A. butzleri* obtained from meat samples into the biotypes 2A, 3A, 3B, 4A, 4B, 5A, 6A, 7A, 7B, 8A, and 8B; 8A being the most common followed by 8B and 4A (De Boer *et al.* 1996). Similarly, this scheme was useful in subtyping 18 strains of *A. butzleri* obtained from river samples (Musmanno *et al.* 1997).

As with biochemical tests, the usefulness of biotyping is hampered by the fastidious growth requirements of arcobacters and their relatively inert biochemical character, so is not employed commonly.

1.4.3 Serotyping

Serotyping involves the use of specific antibodies to detect homologous antigens, and is most widely applied for typing of Gram-negative enteric bacterial pathogens. For most foodborne pathogens, agglutination techniques are employed. For campylobacters, a serotyping scheme, based on soluble heat-stable or heat-labile antigens, has been widely used (Penner and Hennessy, 1980; Lior *et al.*, 1982; On, 1996; Frost *et al.*, 1998).

A serotyping scheme for *A. butzleri* has been described by Lior and Woodward (Lior and Woodward, 1994). In Lior's approach, antisera produced from rabbits using heat-labile antigens were used for slide agglutination tests of live bacteria. This scheme recognized 65 serotypes (in 14 serogroups) of *A. butzleri* obtained from human and nonhuman sources. The same serotypes of *A. butzleri* were found to be common among human, poultry, pig, and water. No cross-reactivity was observed with the antisera against *C. jejuni*, *C. coli*, and *C. lari*.

Using Lior's scheme, 13 strains of *A. butzleri* obtained from 10 children from an outbreak of abdominal cramp has been serotyped (Vandamme *et al.* 1992a). Serotyping by using antiserum prepared against the outbreak strains revealed that all of the strains belong to serotype 1. This scheme has also been employed for typing of arcobacters obtained from poultry samples (Marinescu *et al.* 1996a, 1996b). Twenty-two different serogroups were recognized among 162 *A. butzleri* and one *A. butzleri*-like isolate; serotype 1 being the most predominant followed by 26 and 19 (Marinescu *et al.*, 1996b). The authors mentioned that *A. butzleri* isolated from poultry meat and from humans with diarrhoeal illness were belonging to the same serotype (serotype 1). Similar findings has been reported by Lammerding *et al.* (1996)

Serotyping is in limited use for subtyping of arcobacters. The main disadvantage of this method is lack of the availability of serotyping reagents. Production of antisera to the large number of strains would be too time consuming, costly and impractical.

1.4.4 Molecular/ Genotypic methods

These techniques involve detection and characterization of molecules (fatty acids, proteins, nucleic acids, and other chemicals) produced by bacteria. Genotyping, a commonly used molecular method, refers to the direct DNA-based analysis of chromosomal or extrachromosomal genetic material (Tyler and Farber, 2003). Molecular methods may be broadly classified into three categories on the basis of the type of macromolecules targeted for characterization (Swaminathan and Matar, 1993): fatty-acid based methods, protein based methods, and nucleic acid based methods.

1.4.4.1 Cellular fatty acid profiles

Since the fatty acid (FA) composition of bacterial cells may vary significantly between taxa, its profiling has been employed for classification and identification of several bacteria, including campylobacters (Vandamme, 2000). Briefly, the method involves saponification of the whole-cell FAs, esterification with an alcohol, extraction of FA methyl esters (FAMEs) with an organic solvent, separation by gas chromatography and identification by comparing their retention times with those of known standards (On, 1996).

Several authors used cellular fatty acid methyl ester analysis for the differentiation and identification of arcobacters. Lambert *et al.* (1987) described the use of cellular fatty acid analysis for the differentiation of *Campylobacter* and *Campylobacter*-like organisms, including *A. cryaerophilus*. Tee *et al.* (1988) used gas chromatography analysis of fatty acid for the identification of a human isolate of *A. cryaerophilus*. Kiehlbauch *et al.* (1991a) used this technique for the characterization of 78 strains of aerotolerant campylobacters and found them to be *A. butzleri*. Vandamme *et al.* (1992b) reported that fatty acid analysis was useful in distinguishing all species of arcobacters, with the exception of being unable to differentiate *A. butzleri* from *A. cryaerophilus* subgroup 2. Hsueh *et al.* (1997) employed this technique for the identification of a bacterial isolate recovered from a person with bacteraemia. In combination with biochemical tests, the isolate was identified as *A. cryaerophilus* 1B.

1.4.4.2 Protein profiling

Examination of the protein content of a living cell gives an indication of the genetic organization of an organism. Among the different types of protein profiling, profiles obtained from whole bacterial cell by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are most commonly used for identification of bacteria, including campylobacters (On, 1996).

The comparison of whole-cell protein patterns obtained by highly standardized SDS-PAGE has been used for screening and identifying a large number of strains of arcobacters. A good correlation has been observed between a high similarity in whole-cell protein content and level of DNA-DNA hybridization (Vandamme *et al.*, 1992b). Atabay *et al.* (2003) described the simultaneous use of a SDS-PAGE and a multiplex PCR for the detection of arcobacters from retail poultry carcass. Both the methods detected arcobacters from 42 samples out of 44, and the species were found to be *A. butzleri* by both methods. Wybo *et al.* (2004) mentioned SDS-PAGE profiling technique was useful for confirming the identification of *A. skirrowii* obtained from a patient with chronic diarrhoea. Houf *et al.* (2005) found this technique, in combination with DNA-DNA hybridization, rRNA gene sequencing, and DNA base composition analysis, to be useful in differentiating an *Arcobacter* isolate obtained from poultry carcass to be a novel strain.

In spite of being highly sensitive, protein profiling techniques are not suitable for routine identification studies since they are very laborious, time-consuming, and technically demanding to run patterns in an adequately standardized way (Vandamme, 2000).

1.4.4.3 DNA-base compositions

One of the distinctive features of DNA that has taxonomic significance is its mole percent guanine-plus-cytosine content (mol% G+C). Among the bacteria, the mol% G+C value is constant for a specific organism. All of the G+C values are determined by thermal denaturation method. Although closely related bacteria have similar mol% G+C values, two organisms that have similar mol% G+C values are not necessarily closely related.

The G+C content of the DNA of arcobacters ranges from 27 to 31 mol% (Vandamme, 2000). In one study, the G+C content the DNA of genus *Arcobacter* was found to be 28-31 mol% (Vandamme *et al.*, 1991). Kiehlbauch *et al.* (1991a) found this G+C content to be 29-32 mol% for five *A. cryaerophilus* reference strains. Tee *et al.* (1988) mentioned the G+C content of DNA of *A. cryaerophilus* from human faecal samples to be 31.1$\pm$1 mol%. Houf *et al.* (2005) found that G+C content of *A. cibarius* ranged between 26.8 and 27.3 mol%.

1.4.4.4 Hybridization techniques

Hybridization techniques depend on the detection of a signal generated after the binding of a labelled probe with the target nucleic acid. Hybridization takes place when the sequence of the probe is adequately similar to that of the target nucleic acid and that a duplex is formed and held together by hydrogen bonds from nucleotide pairing. The target nucleic acid as well as the probe may be single- or double-stranded RNA or DNA.

1.4.4.4.1 DNA-DNA hybridization

This technique involves hybridization of the entire DNA-contents of both organisms under examination. The degree of DNA-DNA binding is determined spectrophotometrically and is expressed as a percentage. DNA binding values of 70% or more indicate that there is significant DNA homology (Vandamme *et al.*, 1991), and indicates a direct relationship at species level.

The DNA-DNA hybridization technique has been found to be useful in speciation of *Arcobacter* spp. and differentiation of the two subgroups of *A. cryaerophilus*. Kiehlbauch *et al.*(1991a) found two distinct hybridization groups among the 78 aerotolerant campylobacters of human and animal origin by DNA-DNA hybridization. *A. cryaerophilus* belonged to a DNA hybridization group which was genetically and phenotypically heterogeneous, and was further differentiated as DNA hybridization group 1A and 1B; and *A. butzleri* belonged to DNA hybridization group 2. Employing this technique, Vandamme *et al.* (1992b) identified five groups of *Arcobacter* strains as *A. cryaerophilus* (two distinct subgroups), *A. butzleri*, *A. nitrofigilis*, and *A. skirrowii*.

This technique has been regarded as a reference method and has also been used to confirm the results of other techniques. In an outbreak of abdominal cramps in humans, the causative organisms identified as *A. butzleri* by SDS-PAGE of whole-cell proteins and cellular fatty acid analysis was confirmed by DNA-DNA hybridization (Vandamme *et al.*, 1992a). *A. cryaerophilus* obtained from faecal samples of a man which was presumptively identified by biochemical tests and liquid-gas chromatography was confirmed by this test (Tee *et al.* 1987; 1988). DNA-DNA hybridization test also confirmed the existence of a novel species of *Arcobacter* as the novel strain (*A. cibarius*) had binding percent of below 47 with *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Houf *et al.*, 2005).

Although it is generally regarded as the reference method, DNA-DNA hybridization technique has limited practical application in a routine laboratory or for examination of large numbers of strains in a reference laboratory.

1.4.4.4.2 In situ hybridization

In situ hybridization (ISH) involves hybridization of a labelled nucleic acid probe with a DNA or RNA sequence *in situ* (in the cells). The probe can be either radioactively labelled and detected by autoradiography or fluorescently labelled (abbreviated FISH) and detected by immunocytochemistry.

Fluorescent *in situ* hybridization (FISH) with rRNA oligonucleotide probes has been used for detection and identification of different microorganisms, including arcobacters. Using this technique, Snaidr *et al.* (1997) found that 4% of the microorganism cells present in an activated sludge plant were *Arcobacter* spp.

A rapid FISH protocol to detect arcobacters in naturally and artificially contaminated samples has been developed (Moreno *et al.* 2003). The probe was targeting partial 16S rRNA gene sequence. The detection range of FISH assay was found to vary between 10^2cells/ml (after culture enrichment) to 10^4cells/ml (without enrichment). It was found that 100% of the water samples (n=10) and sludge samples (n=10) were positive for *Arcobacter* spp.

The main advantage of FISH techniques is its rapidity as DNA is not necessary to be extracted from bacteria, so can be conducted without culture, and results may be directly observed in the samples.

1.4.4.4.3 Restriction fragment length polymorphisms (RFLP) and Ribotyping

These techniques involve southern blot hybridization of genomic DNA digested with a six-cutter restriction enzyme and hybridization with a universal rRNA probe (Swaminathan and Matar, 1993; Jay, 2000; Newell *et al.*, 2000). The occurrence of several copies of the rRNA genes (coding for 16S and 23S rRNA) at different locations on the chromosome and their high degree of conservation among bacteria make these genes ideal target for probing (Newell *et al.*, 2000).

RFLP and ribotyping has expedited the identification and/or subtyping of *Arcobacter* spp. from a variety of sources. Kiehlbauch *et al.* (1991b) have mentioned that RFLP patterns were useful in differentiating the species: *A. butzleri* and *A. cryaerophilus*, from other closely related bacteria (*Campylobacter* like organisms; CLOs). De Oliveria *et al.* (1999)

have described the use of ribotyping to identify *Arcobacter* spp. obtained from preputial fluids of pigs.

Besides speciation, the technique has also found useful to discriminate between the two hybridization groups of *A. cryaerophilus*. Out of 50 porcine abortion-related isolates, ribotyping identified 16% as *A. cryaerophilus* DNA group 1A, 60% as *A. cryaerophilus* DNA group 1B, and 8% as *A. butzleri* (Schroeder-Tucker *et al.* 1996). However, remaining 16% were not able to be classified by ribotyping patterns. In another study, out of 18 isolates of *Arcobacter* spp., two were identified as *A. butzleri*, six as *A. cryaerophilus* hybridization group A, and seven as *A. cryaerophilus* hybridization group B (De Oliveria *et al.* 1999).

PCR-RFLP is a modification of conventional RFLP technique which involves an additional step of PCR amplification of a target sequence. Hurtado and Owen (1997) reported a rapid two-step identification scheme based on PCR-RFLP analysis of the 23S rRNA gene. The scheme was found to useful in differentiating the isolates belonging to the *Campylobacter*, and *Arcobacter* genera. Marshall *et al.* (1999) described a PCR-RFLP analysis of the 16S rRNA gene for differentiating isolates belonging to the *Campylobacter*, *Arcobacter*, and *Helicobacter* genera. The technique also differentiated *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* by producing unique fingerprints for all three species.

1.4.4.5 Polymerase chain reaction (PCR)

PCR is an *in-vitro* method involving enzymatic amplification of specific DNA sequence using oligonucleotide primers that hybridize to the region of interest in the target DNA. Ribosomal RNA, an essential part of prokaryotic and eukaryotic ribosomes, is genetically stable and consists of conserved and variable regions. The latter may vary considerably among different bacterial species and are therefore targets for PCR amplification. PCR uses primers to get the copying process started. The extraordinary ability of PCR to exponentially and rapidly replicate a target DNA sequence has made it a very powerful tool for the detection of infectious agents. The difficulties in routine detection, isolation and identification make arcobacters ideal candidates for PCR identification.

Several investigators have targeted the 16S or 23S rRNA gene in order to identify the species level members of the *Arcobacter*. Based on a 23S rDNA area, Bastyns *et al.* (1995) developed a PCR assay for the identification of arcobacters,. They found the amplification of this 23S rDNA area was useful for genus-specific and species-specific detection of arcobacters. The species-specific assay was able to differentiate the three species *A. cryaerophilus*, *A. butzleri* and *A. skirrowii*.

A genus-specific PCR assay for the detection of *Arcobacter* spp has been described (Harmon and Wesley 1996). The assay was able to detect the four species of arcobacters, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. nitrofigilis*. The advantage of this protocol was it utilized either purified DNA or a crude bacterial cell lysate, and the amount of time required was reduced (8 h vs. several days). Later, a multiplex-PCR assay for the simultaneous detection of *Arcobacter* spp. and the differentiation of *A. butzleri* from other arcobacteria was developed by them (Harmon and Wesley, 1997). Two sets of primers were used in this protocol. The first set of primers targeted the 16S rRNA genes of *Arcobacter* spp., and the second set amplified the 23S rRNA genes unique to *A. butzleri*.

Surez *et al.* (1997) developed a nested PCR test for detection of arcobacters in gastric samples from swine. The primers were targeting the 16S rRNA gene of members of rRNA superfamily VI. The PCR products were differentiated and confirmed by hybridization with an internal oligonucleotide probe. The results of nested PCR were also compared with single step PCR and direct culture methods. *Arcobacter* spp. were recovered from 4 of 71 samples and the nested PCR test was found to be more sensitive than single step PCR.

Gonzalez *et al.* (2000) developed a genus-specific PCR-culture technique to detect *Arcobacter* spp. in fresh poultry meat. The primers were targeted to amplify the 16S rRNA gene from *Arcobacter* spp. PCR amplification was conducted following a short selective enrichment of poultry samples. Using this assay 53% of the 96 retail poultry samples were found to be positive for the presence of *Arcobacter* spp.

Using a variable 16S rRNA and 23S rRNA region, Houf *et al.* (2000) developed a species-specific multiplex-PCR assay for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*. Three primers sets were designed to amplify a 257 bp fragment of 23S rRNA gene from *A. cryaerophilus*, a 401 bp fragment of 16S rRNA gene from *A. butzleri*, and a 641 bp fragment of 16S rRNA gene from *A. skirrowii*. The assay was found to be specific as no PCR product was generated for closely related bacteria.

A genus-specific multiplex PCR assay for the simultaneous detection and identification of *Campylobacter* spp. and *Arcobacter* spp. has been described (Winters and Slavik 2000). The primer sets amplified a 159 bp fragment of 16S rRNA genes of *C. jejuni* and 1223 bp fragment of 16S rRNA genes of *A. butzleri*. The protocol was compatible with a variety of food products like poultry and pork, and fruits and vegetables.

Kabeya *et al.* (2003a) developed a species-specific PCR assay for the identification of the arcobacters. The one-step PCR assay was shown to be capable of providing a rapid species differentiation of *Arcobacter* strains. Moreover, by using this PCR assay, it was possible to differentiate between *A. cryaerophilus* 1A and 1B.

A PCR assay for identification of *Arcobacter* strains from environmental water and activated-sludge samples has been evaluated (Moreno *et al.* 2003). The assay was performed on naturally and artificially contaminated samples, with and without enrichment. The detection range of PCR assay varied between 1cell/ml (after enrichment) to 10^3cells/ml (without enrichment).

The use of a PCR technique combined with an enzyme-linked immunosorbent assay (PCR-ELISA) for the quantitative detection of *Arcobacter* spp. in poultry meat has been described (Antolin *et al.* 2001). The primers were targeted to amplify 181 bp DNA fragment of the 16S rRNA gene from *Arcobacter* spp. It was found that the detection threshold for the PCR-ELISA assay was 10 CFU/g.

Although highly discriminating PCR assays have been developed for species identification of *Arcobacter*, an inherent limitation often encountered with PCR assays in the inability to distinguish between bacterial strains. Among the PCR protocols mentioned here, the protocol of Houf *et al.* (2000) has been used extensively for speciation of *Arcobacter* spp. No PCR protocol has yet been published for the detection of a recently discovered species *A. cibarius*.

1.4.4.6 Repetitive element PCR (Rep-PCR)

This is a PCR-based fingerprinting method that targets the amplification of repetitive elements (rep elements) in the bacterial genome. The rep elements targeted for PCR amplification useful in subtyping of Gram-negative bacteria are enterobacterial repetitive intergenic consensus (ERIC) and the repetitive extragenic palindromic (REP) sequences (Versalovic *et al.*, 1991; Olive and Bean, 1999).

Rep-PCR has been used for assessing the genetic diversity and epidemiological relationships among *Arcobacter* spp. isolates. This technique revealed that 14 outbreak-related strains of *A. butzleri* obtained from the cases of abdominal cramps in children had an identical fingerprinting pattern (Vandamme *et al.* 1993). In another study, Rep-PCR employed for assessing the genetic diversity of 121 *A. butzleri* isolates from turkey meat revealed 86 different patterns, indicating multiple sources of contamination (Manke *et al.* 1998). Driessche *et al.* (2005) found this technique was useful in subtyping 164 isolates of *Arcobacter* spp. obtained from faecal samples of healthy cattle. A high degree of heterogeneity was observed among the isolates and it was suggested that animals could be colonized by multiple genotypes. It was further suggested that infection is transmitted by direct contact and no vertical transmission occurs in cattle.

Houf *et al.* (2002a) optimized Rep-PCR for subtyping of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* strains. Ninety-eight percent of the 228 *Arcobacter* isolates (182 *A. butzleri* and 46 *A. cryaerophilus*) from 24 poultry samples were typeable among which 131 types (91 *A. butzleri* and 40 *A. cryaerophilus*) were detected. The fingerprint profile was compared with random amplification of polymorphic DNA (RAPD) and both methods were found to be equally discriminatory.

1.4.4.7 Random amplification of polymorphic DNA (RAPD)

RAPD involves the use of arbitrary primers for amplification of target DNA sections by normal PCR. Whole genomic DNA is used and PCR is performed at low stringency allowing primer to bind at various positions of the target DNA resulting in several amplicons of various sizes (Swaminathan and Matar, 1993; Newell *et al.*, 2000).

RAPD has been successfully employed for identification and typing of *Arcobacter* spp (Houf *et al.* 2002a; 2003). Using this technique, 95% of the 228 *Arcobacter* isolates (182 *A. butzleri* and 46 *A. cryaerophilus*) from 24 poultry samples were typeable among which 128 types (89 *A. butzleri* and 39 *A. cryaerophilus*) were detected (Houf *et al.*, 2002a). Using RAPD together with ERIC-PCR, a total of 1,079 *Arcobacter* isolates obtained from various sources including slaughter equipment, processing water and the poultry carcass were differentiated into 159 *A. butzleri* types and 139 *A. cryaerophilus* types (Houf *et al.*, 2003). The extreme heterogeneity among the isolates suggested that arcobacters were acquired from different sources.

1.4.4.8 Amplified-fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) analysis involves digestion of chromosomal DNA with a combination of two restriction endonucleases followed by PCR amplification and detection of fragments between adjoining restriction sites in the whole genetic content of the given organism (Newell *et al.*, 2000).

The potential of AFLP has been examined for identification and subtyping of *Arcobacter* species. Numerical analysis of the AFLP pattern from the 72 strains produced five phenons at 29% similarity level, four of which represented the species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* (On *et al.* 2003). The remaining phenon suggested the existence of a new species for the isolates obtained from pig abortions and turkey faeces, and was called '*Arcobacter skirrowii*-like'. At 91% similarity level, AFLP differentiated five subtypes among the 73 strains obtained from six different sample types and six different countries (On *et al.* 2004). So, it was suggested that distinct subtypes of *A. butzleri* may be found in a given environment. In another similar study, at 90% similarity level, AFLP differentiated 12 genotypes among 20 *A. butzleri* strains obtained from poultry plant effluent (Amisu *et al.*, 2003)

AFLP is being increasingly employed routinely for subtyping of microorganisms with increased availability of automated DNA sequencers. The major advantage of this technique is that prior sequence knowledge of the amplification target is not necessary.

1.4.4.9 DNA sequencing

This is a common technique employed for identification of unknown organisms and involves sequence analysis of 16S rRNA and its comparison with rRNA sequences available in the international database (Vandamme, 2000). The similarity or diversity of two bacterial strains can also be determined by this technique.

A number of studies have employed sequencing of the 16S rRNA gene for the identification and differentiation of arcobacters. Using this technique, Yan *et al.* (2000) found that PCR product of two *Campylobacter*-like isolates obtained from human blood culture samples were having 100% sequence similarity with the 16S rRNA gene of *A. butzleri*. Lau *et al.* (2002) mentioned that 16S rRNA gene sequencing was useful in the identification of a strain of *A. butzleri* isolated from the blood culture of a patient with acute gangrenous appendicitis. On *et al.* (2003) employed this technique for identification of a groups of arcobacters that had distinct AFLP patterns than the known species. These strains were found to be novel species within the genus *Arcobacter* and were named "*Arcobacter skirrowii*-like". Similarly, Diergaardt *et al.* (2004) employed this technique for confirmative identification of *Campylobacter*-like isolates obtained from drinking and environmental water sources. Out of 22 *Campylobacter*-like isolates, 19 were identified as *A. butzleri*.

1.4.4.10 Pulsed-field gel electrophoresis (PFGE)

The technique involves embedding bacterial cells in agarose followed by *in-situ* lysis, digestion of the chromosomal DNA with restriction endonucleases that cleave infrequently, and electrophoresis of the DNA fragments in pulsed electric fields. The infrequent cutting enzymes generate 5-20 very large molecular weight DNA fragments (Tyler and Farber, 2003), and allows clear separation of DNA fragments ranging from 10 to 800 kb (Schwartz and Cantor, 1984).

To determine the relatedness (similarity or diversity) among strains, the DNA restriction patterns of the strains are compared with one another. Usually when strains have less than 3 band differences, they are considered to be closely related (Tenover *et al.*, 1995). However there are no standardized criteria for interpreting the fragment patterns. *Arcobacter* isolates that are >90% similar on the dendograms generated by specific software programs has been considered related for *Arcobacter* spp (On *et al.* 2004). Software packages such as BioNumerics are used in generating dendograms which employs dice similarity coefficient and the PFGE patterns are clustered by the unweighted pair group method using arithmetic averages (UPGMA). The total number of PFGE patterns in a given population, along with the values for total number of strains in the sample population, and number of strains belonging to a particular subtype may then be used for diversity index (DI) calculation. Simpson's index of diversity has been used commonly for this purpose (Hunter and Gaston, 1988). A DI with an absolute value of zero indicates that the population is clonal whereas a value closer to one indicates a high genetic diversity.

PFGE was first used to study the chromosomal DNA of *Arcobacter* spp. by Hume *et al.* (2001). Three endonucleases: *Ava*I, *Eag*I, and *Sac*II, were found to be useful for strain differentiation of arcobacters, *Eag*I and *Sac*II being more suitable for differentiation among the isolates. In this study multiple genotypes for the *A. butzleri* and *A. cryaerophilus* isolates were obtained from pigs of different ages at a farrow-to-finish pig farm, suggesting considerable genotypic variation and genetic rearrangement.

Rivas *et al.* (2004) employed PFGE for examination of the similarity of *A. butzleri* isolates recovered from ground poultry, pork, beef and lamb meats from different location and time-periods. Fingerprint patterns following restriction with the endonucleases *Sac*II, *Eag*I and *Sma*I were found to be useful for strain differentiation. Among the 31 *A. butzleri* isolates recovered from different sources, 15-18 different PFGE patterns were observed across all three enzymes. Among the three enzymes used, *Sma*I was found to be less discriminatory but when used in combination with other enzymes, the discriminatory power of the combination was increased. When compared with Rep-PCR and RAPD, PFGE was found to be the most discriminatory subtyping technique.

PFGE has also been employed for investigating the mode of transmission of *Arcobacter* spp. Ho *et al.* (2005) employed this technique for studying the transmission of *Arcobacter* species from carrying sows to their piglets. The genomic DNA of *Arcobacter* spp isolated from sows and newborns were digested with *Eag*I. High similarity of PFGE profile *Arcobacter* isolates from sows and their respective offspring revealed that *Arcobacter* spp. may be transmitted both vertically and horizontally.

Among the various molecular typing methods, PFGE and AFLP have been commonly used for subtyping of *Arcobacter* spp. PFGE is considered to be the most discriminatory molecular epidemiological tools available for subtyping of arcobacters (Son *et al.*, 2006) and is regarded as 'gold-standard' of all molecular typing methods (Olive and Bean, 1999).

1.5 Epidemiology of *Arcobacter*

1.5.1 Arcobacters and humans illness

Limited information is available on the worldwide contribution of *Arcobacter* spp. towards human illness. Regardless of the fact that specific techniques are rarely employed in routine laboratories to isolate and identity *Arcobacter*, evidence for its substantive role in human illness as an emerging pathogen is increasing. Table 3 list the cases of isolation of *Arcobacter* spp. in different countries of the world.

Table 3. Isolation of arcobacters from human illness in different countries of the world

Country	Description	Reference
Australia	*A. cryaerophilus* isolated from a 35-year-old man having intermittent diarrhoea for 4-6 months	Tee *et al.*, 1988
	A. butzleri isolated from two children and four adults having enteritis, diarrhoea, abdominal cramps, vomiting and fever	Lauwers *et al.*, 1996
Belgium	*A. skirrowii* found to be associated with chronic diarrhoea in a 73-old-man with chronic diarrhoea persisting for two months	Wybo *et al.*, 2004
	Out of 40,995 patients with abdominal illness, *A. butzleri* and *A. cryaerophilus* detected respectively in 67 and 10 patients	Vandenberg *et al.*, 2004
Chile	*A. butzleri* isolated from cases of chronic diarrhoea in two children having recurrent abdominal cramps, diarrhoea, and vomiting	Fernandez *et al.*, 2004
Denmark	*A. butzleri* and *A. cryaerophilus* isolated from faecal samples; no information on patient history and symptoms	Engberg *et al.*, 2000
France	*A. butzleri* and *A. butzleri*-like organisms isolated from two children (19 month and 3-year-old) having diarrhoea, but no abdominal cramps, fever or vomiting	Marinescu *et al.*, 1996a
Germany	*A. butzleri* detected in faecal samples of an adult man and a woman; both having severe abdominal cramps and profuse diarrhoea	Lerner *et al.*, 1994
Italy	*A. butzleri* isolated from 10 children with recurrent abdominal cramps, but no fever or diarrhoea	Vandamme *et al.*, 1992a
Taiwan	*A. cryaerophilus* 1B detected in blood sample of a 72-year-old women having uraemia and haematogenous pneumonia	Hsueh *et al.*, 1997
	A. butzleri isolated from a 60-year-old man with bacteraemia and liver cirrhosis; symptoms were fever and haematemesis	Yan *et al.*, 2000
Thailand	*A. butzleri* and *A. cryaerophilus* isolated from 15 (2.37%) of 631 children with mild diarrhoea	Taylor *et al.* 1991
UK	*A. butzleri* detected in blood samples of a neonate with bacteraemia; the neonate was experiencing hypotension and hypothermia	On *et al.*, 1995

There has been only one large scale study reporting the prevalence rate of arcobacters in patients with diarrhoeal illness (Vandenberg *et al.* 2004). In this study, out of 67,599 stool samples obtained from 40,995 patients, *A. butzleri* and *A. cryaerophilus* were found in 67 (97 isolates) and 10 patients (13 isolates), respectively, accounting for a prevalence rate of 0.18%. Arcobacters accounted for 4% of the 1,906 *Campylobacter* like organisms (CLOs) isolated. The most prominent clinical symptom observed was acute or persistent watery diarrhoea. Except bloody diarrhoea, other clinical features were similar to *C. jejuni* infection. The acute diarrhoea lasted for 3-15 days while the persistent one lasted for between 2 weeks to 2 months. Other important clinical features observed were: fever (temperature >38°C); nausea or vomiting or both; and abdominal pain.

Limited information is available on the risk factors for infection and transmission of *Arcobacter spp.* in humans. No particular age groups seemed to be susceptible to *Arcobacter* infection since the illness is found in neonates to 90-year-old patients (On *et al.*, 1995; Vandenberg *et al.*, 2004). Consumption of contaminated food or water is considered to be the most important source of infection (Marinescu *et al.*, 1996a; 1996b; Hsueh *et al.*, 1997). In a few occasions, person-to-person transmission (Vandamme *et al.*, 1992a), and intrauterine transmission (On *et al.*, 1995) has been suspected. There is no information about how arcobacters cause disease, their virulence factors or their pathogenicity. The difficulty encountered in the establishment of pathogenicity for each *Arcobacter* species, the sources and routes of infection are probably at least partly due to failure of their detection in routine laboratory tests.

1.5.2 Arcobacters in foods

Poultry meat is considered to be the most important source of arcobacters. *Arcobacter* spp. has been isolated from poultry carcasses with recovery rates of up to 100% (Table 4). In addition, there are small number of reports of *Arcobacter* detection in carcasses of other birds including 77% in turkey (Manke *et al.*, 1998), and 80% in ducks (Ridsdale *et al.*, 1998). Eggs are however considered to be free of arcobacters (Zanetti *et al.*, 1996; Phillips, 2001), and thus do not present a public health risk.

Table 4. Isolation rates of arcobacters from poultry carcasses in different parts of the world

Country	Species detected	Isolation rates (%)	No. of samples	Reference
Australia	All A. *butzleri*	73	22	Rivas *et al.*, 2004
Belgium	*A. butzleri* -64%, *A. cryaerophilus* -9%, both together -11% samples	90 100	71 (broiler) 34 (layer)	Houf *et al.*, 2001a
Brazil	*A. butzleri* -41%,	46	80	De Oliveria *et al.*, 2001
Canada	*A. butzleri* 67% isolates,	97	125	Lammerding *et al.*, 1996
Denmark	*A. butzleri* in 100% samples, *A. cryaerophilus* in 16%,	100	30	Atabay *et al.*, 2006
France	*A. butzleri* -99%	81	201	Marinescu *et al.*, 1996b
Japan	*A. butzleri*-55%, *A. cryaerophilus* –30%,	20	180	Maruyama *et al.*, 2001
	A. butzleri -15%, *A. cryaerophilus* -2%, and *A. skirrowii* in 1% of samples	23	100	Kabeya *et al.*, 2004
Mexico	*A. butzleri*- in 73%, *A. skirrowii*- in 23%	40	45	Villarruel *et al.*, 2003
Spain	No speciation	53	95	Gonzalez *et al.*, 2000
Thailand	No speciation	100	10	Morita *et al.*, 2004
Netherlands	All *A. butzleri* or *butzleri*-like	24.1	224	De Boer *et al.*, 1996
Turkey	All *A. butzleri*	95	44	Atabay *et al.*, 2003
UK	*A. butzleri* -in all 25, *A. cryaerophilus*- in 13, and *A. skirrowii*- in 2 samples	100	25	Atabay *et al.*, 1998
	A. butzleri - 33, *A. cryaerophilus* -3, and *A. skirrowii* -1 sample	68	50	Scullion *et al.*, 2004
USA	No speciation	84	50	Johnson and Murano, 1999a

Besides poultry meat, arcobacters are found to be occurring in a number of foods of animal origin; beef and pork being the most common (Table 5). In Chile, arcobacters have also been reported as occurring in oysters (Romero *et al.*, 2002).

Table 5. Prevalence rate of arcobacters in beef, pork and lamb meat in different parts of the world

Origin	Description	Reference
Australia	*A. butzleri* isolated from ground meat samples of pork 29% (n=21), beef 22% (n=32) and lamb 15% (n=13); no other species detected;	Rivas *et al.*, 2004
Canada	*Arcobacter* spp. isolated from 1.5% (n=68) of minced beef samples, 0.5% (n=194) pork samples, and 4.9% (n=61) of the mixed minced pork/beef samples	De Boer *et al.*, 1996
Czech Republic	*Arcobacter* spp. detected in 50% (n=9) of retail beef samples; two pork samples tested negative	Vytrasova *et al.*, 2003
Italy	*A. butzleri* detected in 3.7% (n = 27) of pork loin samples; no other species detected;	Zanetti *et al.*, 1996
Turkey	*A. butzleri* detected in 5% (n=97) of minced beef meat samples; no other species detected	Ongor *et al.*, 2004
USA	*Arcobacter* spp. detected in 90% (n=149) ground pork samples; no speciation done	Collins *et al.*, 1996a
USA	*Arcobacter* spp. detected in 32% (n=200) of ground pork samples; the detection rate ranged from 0-68% among different plants	Ohlendorf and Murano, 2002

As seen above, the abundant presence of *Arcobacter* spp. in foods of animal origin suggests an important role of contaminated food in the transmission of these bacteria.

1.5.3 Arcobacters in water and environment

Water is considered to have a significant role in transmission of arcobacters both to animals and humans. Table 6 lists the isolation of arcobacters from water and environmental samples in different parts of the world. Worldwide, arcobacters have been detected in various proportions in different types of water including surface water, ground water, sea water, sewage and sludge.

Some researchers have described *Arcobacter* spp. as ubiquitous organisms (On *et al.*, 1995). In one study, arcobacters were found to be more abundant than campylobacters in sludge samples (Moreno *et al.*, 2003). This may be because arcobacters are capable of growing in atmospheric oxygen and at lower temperatures than campylobacters (Wesley *et al.*, 2000). These organisms have been found occurring in poultry farm surroundings like stagnant water and sludge (Gude *et al.*, 2005), which may be a source of continuing infection in individual farms.

Table 6. Isolation of arcobacters from water and environment in different countries of the world.

Origin	Description	Reference
Belgium	*Arcobacter* spp. detected in 91% (n=24) water samples before being used in poultry processing plant	Houf *et al.*, 2003
Caribbean	*Arcobacter* spp detected in coral reefs	Frias-Lopez *et al.*, 2002
Germany	79% (n=147) of *Campylobacter*-like strains isolated from drinking water treatment plants identified as *Arcobacter* spp.; 100 strains were *A. butzleri;*	Jacob *et al.*, 1998
Germany	4% of all cells in activated sludge samples were *Arcobacter* spp.	Snaidr *et al.*, 1997
Japan	*A. butzleri* detected in 23% (n=17) of river water samples; no other species detected;	Morita *et al.*, 2004
Nigeria	26 (14%) of the poultry abattoir waste water samples positive for *A. butzleri*; no other species detected;	Amisu *et al.*, 2003
South Africa	*A. butzleri* isolated from 9% (n=11) of surface water samples and 54% (n=4) of sewage water samples; tap water samples (n=5) and ground water samples (n=4) free of arcobacters	Diergaardt *et al.*, 2004
Spain	100 % (n=10) of river water and 100% (n=10) activated sludge sample positive for *Arcobacter* spp; speciation not done;	Moreno *et al.*, 2003
Thailand	*A. cryaerophilus* and *A. cryaerophilus*-like organisms isolated from 47% and 26% (n=156) water samples, respectively; source was 36 canals of Bangkok metropolitan area; no seasonal variation on isolation rates	Dhamabutra *et al.*, 1992
Thailand	*A. butzleri* detected in 100% (n=7) canal water samples from Bangkok; no other species detected; the isolates were genetically diverse	Morita *et al.*, 2004
USA	*A. butzleri* isolated from contaminated well water; suspected to have been associated with abdominal illness in group of 117 girls	Rice *et al.*, 1999

Limited information is available on survival of *Arcobacter* spp. in environment. It has been reported that *A. butzleri* can remain viable for up to 16 days in groundwater (Rice *et al.* 1999). Houf *et al.* (2003) suggested that arcobacters survive the scalding water temperatures (52°C) in poultry processing plants, which have implications as to how cross-contamination between poultry carcass can be controlled during processing. However, these bacteria can easily attach to various water distribution pipe surfaces, like stainless steel, copper, and plastic (Assanta *et al.*, 2002) which makes them potentially difficult to control in processing plants. As these organisms are susceptible to chlorination (Rice *et al.*, 1999), chlorinated water may be considered free of arcobacters.

1.5.4 Arcobacters in animals

1.5.4.1 Poultry

Although live poultry are susceptible to infection in natural or experimental conditions (Wesley and Baetz, 1999), it has been argued that arcobacters may not be normal inhabitants of the poultry intestine (Atabay *et al.*, 1998; Eifert *et al.*, 2003; Gude *et al.*, 2005), or that their habitat in living birds in unknown (Houf *et al.*, 2000). A recent study indicated cloacal contents of poultry are naturally colonized by various species of *Arcobacter* (Atabay *et al.*, 2006). As with the retail carcasses, poultry faecal samples may have a high isolation rate of up to 72% (Table 7) indicating that contamination of carcasses occurs during processing (Gude *et al.*, 2005; Atabay *et al.*, 2006).

Table 7. Prevalence rate of arcobacters in poultry faecal/cloacal swab samples in different countries of the world.

Country	Species detected	Prevalence (%)	No of samples	Reference
Belgium	None	0%	30	Houf *et al.*, 2000
Denmark	*A. butzleri* (n=13) *A. cryaerophilus* (n=9)	72%	29	Atabay *et al.*, 2006
Japan	*A. butzleri*- 47.1% *A. cryaerophilus*- 55.9% isolates	14.5 %	234	Kabeya *et al.*, 2003b
UK	No speciation	1.6%	60	Atabay and Corry, 1997
USA	*A. butzleri* in 1% sample, others not speciated	15%	407	Wesley and Baetz, 1999

1.5.4.2 Pigs

Pigs are considered to be an important reservoir of arcobacters. Table 8 summarizes the isolation of arcobacters from pigs in different countries of the world. Since the first isolation of *Arcobacter*-like organisms from aborted porcine foetuses in the United Kingdom (Ellis *et al.*, 1978), a number of studies have been undertaken to estimate the occurrence of these organisms in pigs. Although the majority of the pigs are found to be healthy carrier of arcobacters (Driessche *et al.*, 2003; Kabeya *et al.*, 2003b; Driessche *et al.*, 2004), the organisms are also found to be associated with a variety of illnesses, such as infertility and reproductive problems (De Oliveria *et al.*, 1997), and gastric ulcers (Surez *et al.*, 1997). While their pathogenicity is not yet clearly established, arcobacters are found to be capable of colonizing neonatal piglets (Wesley *et al.*, 1996). Transmission may occur horizontally or vertically (Ho *et al.*, 2005).

Table 8. Prevalence rate of arcobacters in pigs in different countries of the world.

Origin	Description	Reference
Belgium	*Arcobacter* spp. detected in 16-85% (n=294) faecal samples from healthy pigs; excretion ranged from 0 to 10^4 CFU/g faeces; most predominant species- *A. butzleri,*	Driessche *et al.*, 2004
Brazil	17 *Arcobacter* isolates obtained from visceral organs of aborted foetus and sows with reproductive problems; 12 (71%) were *A. cryaerophilus* 1B, four (24%) were *A. cryaerophilus* 1A, and one (6%) was *A. butzleri.*	De Oliveria *et al.*, 1997
Brazil	24% (n=74) of the preputial swab samples positive for arcobacters; 8 were *A. cryaerophilus* 1B, 7 were *A. cryaerophilus* 1A and 2 were *A. butzleri.*	De Oliveria *et al.*, 1999
Denmark	*Arcobacter* spp. detected in >40% (n=55) aborted pig foetuses; 11 isolates were *A. cryaerophilus*, 10 were *A. skirrowii*,	On *et al.*, 2002
Japan	10% (n=250) of the faecal samples positive for arcobacters; *A. butzleri* the most prevalent species (60%) followed by *A. cryaerophilus* (36%); 13.3% (n=15) of the vaginal swab samples positive for *A. butzleri* only;	Kabeya *et al.*, 2003b
The Netherlands	>42% (n=144) sow's rectal swab samples positive for arcobacters; *A. skirrowii* - the predominant species, followed by *A. cryaerophilus* or *A. butzleri*; seasonality not found; infection of the newborn piglets ranged from 38.5 to 83.3% in each litter (litter size=4-17)	Ho *et al.*, 2006
UK	82% (n=17) of the aborted foetuses 18% (n=11) of the normal foetuses were harbouring *Arcobacter*-like organisms;	Ellis *et al.*, 1978
USA	*Arcobacter* spp. detected in 40.4% (n=952) of porcine faecal samples	Harmon and Wesley, 1996
USA	Arcobacters detected in 46% faecal samples (n=1,057) of market weight pigs; no species differentiation done	Wesley *et al.*, 1999
USA	*Arcobacter* spp. recovered from 43% (n=30) of porcine abortion cases; *A. cryaerophilus* 1B was the predominant species followed by *A. cryaerophilus* 1A and *A. butzleri.*	Schroeder-Tucker *et al.*, 1996
USA	2.85% (n=350) caecal samples from slaughtered pigs were positive for arcobacters; All were *A. butzleri*; pigs were colonized by multiple *Arcobacter* genotypes;	Hume *et al.*, 2001

1.5.4.3 Cattle

A summary of isolation of arcobacters from cattle in different parts of the world is shown in Table 9. In cattle, arcobacters has been isolated from a wide range of specimens including faeces (Driessche *et al.*, 2005), vaginal swabs (Kabeya *et al.*, 2003b), mastitic milk (Logan *et al.*, 1982), preputial sheath wash (Gill, 1983), and various visceral organs (Kiehlbauch *et al.*, 1991a). The prevalence rate in faecal samples in cattle has been found to range from

3.6 to 39%, with much higher rate in dairy cows (Wesley *et al.*, 2000; Golla *et al.*, 2002; Driessche *et al.*, 2003; Kabeya *et al.*, 2003b).

With the exception of a few abortions, diarrhoea and mastitis, a number of animals (11% of 276) have also been found to serving as healthy carrier of these organisms (Driessche *et al.*, 2005). Transmission may occur horizontally but vertical transmission has not been reported (Driessche *et al.*, 2005).

Table 9. Isolation of arcobacters from cattle in different countries of the world.

Origin	Description	Reference
Belgium	-Arcobacters detected in faecal samples of 11 % (n=276) of the animals; 5.9 to 11% in dairy cattle, 18.9 % in young cattle and 27.3% in calves; *A. cryaerophilus* predominant species followed by *A. butzleri* and *A. skirrowii*. - *Arcobacter* excretion ranged from 0 to 10^4 CFU/g faeces	Driessche *et al.*, 2005
Belgium	-Arcobacters detected in faecal samples of 39%(n=50) of the animals; *A. butzleri* isolated from 13, *A. cryaerophilus* from five and *A. skirrowii* from two samples	Driessche *et al.*, 2003
Canada	-4% of 198 isolates of campylobacters obtained from bovine faeces identified as arcobacters, four were *A. butzleri*, and *A. skirrowii*, and the remaining were *Campylobacter* spp.	Inglis and Kalischuk, 2003
Japan	-Arcobacters detected in 3.6% (n=332) faecal samples; *A. butzleri* (83.3% of isolates) the most prevalent species, followed by *A. cryaerophilus* 1B (16.7%); -Detected in 5 (8.1%) of 61 vaginal swab samples; four *A. butzleri*, one *A. cryaerophilus* 1B. -The seasonal positive rate varied from 1.4% in spring to 7.6% in summer	Kabeya *et al.*, 2003b
Turkey	-9.5% (n=200) of the rectal swab samples positive; *A. butzleri* most prevalent (7%) followed by *A. cryaerophilus* (2%) and *A. skirrowii* (0.5%)	Ongor *et al.*, 2004
UK	*Arcobacter* like organisms isolated from 44% (n=34) of aborted bovine foetuses	Ellis *et al.*, 1977
USA	*Arcobacter* spp. identified in 14.3% (n=1,682) of healthy cows; No information on different species	Wesley *et al.*, 2000
USA	9% (n=200) of the cattle sampled tested positive for *A. butzleri*; highest incidence for *A. butzleri* (i.e. 18%) observed for dairy cows; no other species detected	Golla *et al.*, 2002

1.5.4.4 Other animals

Besides poultry, pigs and cattle, arcobacters have also been recovered from other animal species. Driessche *et al.* (2003) isolated arcobacters from 16.1% (n=62) of ovine and 15.4% (n=13) of equine faecal samples. *A. butzleri* was the only species detected in equines whereas both *A. butzleri* and *A. cryaerophilus* were detected in ovines, the latter species being predominant. Wesley *et al.* (1995) have also mentioned the detection

Arcobacter spp. in aborted equine foetuses. A number of studies have recovered *A. butzleri* from primates with or without diarrhoeal illness (Kiehlbauch *et al.*, 1991a; Anderson *et al.*, 1993; Higgins *et al.*, 1999). Other animals from which arcobacters have been isolated include raccoon (Hamir *et al.*, 2004) tortoise and ostrich (Kiehlbauch *et al.*, 1991a).

There are several reasons behind the variation in isolation rates of *Arcobacter*, even from similar sample types. The most important reason may be the variation in isolation medium. Besides, hygienic practices during production and/or processing, sample size and sampling methods, and identification methods may influence the number of positive samples (Madden *et al.*, 2000; Atabay *et al.*, 2003; Ho *et al.*, 2006).

While the earlier laboratory methods favoured isolation of campylobacters instead of arcobacters, the precise role of the latter in human illness is still unknown. However, with improved isolation and identification methods, there is increasing evidence that *Arcobacter* is an emerging human pathogen (Phillips, 2001; Vandenberg *et al.*, 2004; Ho *et al.*, 2006). Nevertheless, currently available isolation techniques are not standardized and need further improvement as they are not optimal for all species of arcobacters (Atabay *et al.*, 1998; Houf *et al.*, 2001a; Houf *et al.*, 2001b). The widespread occurrence of arcobacters in food and water also requires the development of effective isolation methods for accurately assessing their prevalence and significance in human diseases.

While there are no epidemiological studies on the routes of transmission of arcobacters to humans, circumstantial evidence suggests that transmission results from consumption of contaminated food (mainly poultry) and water (Marinescu *et al.*, 1996b; Rice *et al.*, 1999). As the precise role of poultry meat in human *Arcobacter* infection is still unclear, molecular fingerprinting of these organisms may contribute to our knowledge of the epidemiological behaviour including contamination sources and transmission routes.

1.6 Aims and objectives

The isolation of *Arcobacter* species requires specific conditions, and the current methods may not be optimal for all species. The failure of isolation or very low isolation rate of these organisms may be due to the lack of information about the most appropriate isolation method. This study will thus compare the most commonly followed *Arcobacter* isolation protocols and recommend the most appropriate one for isolation of arcobacters from poultry meat in New Zealand.

Nothing is known about the prevalence of *Arcobacter* spp. in poultry meat in New Zealand, and its potential clinical significance as a foodborne pathogen. The overall objective of this study is to determine the potential role of poultry meat as a source of *Arcobacter* spp. in New Zealand.

The study aims:

- To establish the prevalence rate of *Arcobacter* species in poultry meat in New Zealand,
- To compare seven different techniques of *Arcobacter* isolation from poultry meat,
- To identify the species of *Arcobacter* prevalent in poultry meat in New Zealand,
- To compare the relatedness (similarity or diversity) among *Arcobacter* strains isolated from different poultry producers and by different methods.