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Arcobacter spp in food: isolation, identification and control

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The genus *Arcobacter* was proposed to describe organisms previously classified as 'aerotolerant campylobacters'. There have been reports of the organism being implicated in human illness although a definitive association has not, as yet, been established. The organism is routinely isolated from a range of foods of animal origin, such as pork and poultry, as well as water sources. This article reviews methods of isolation and identification of *Arcobacter* spp. from food, its control as a contaminant and discusses a possible role for this organism in human disease.

Introduction

Members of the family *Campylobacteraceae* are motile, gram-negative, non-sporing, curved, occasionally straight, rods, which may appear as spiral. They include the genera *Campylobacter* and *Arcobacter* (Vandamme & De Lay, 1991). The classification of *Arcobacter* was first proposed by Vandamme *et al.* (1991a) to describe those organisms previously classified as 'aerotolerant campylobacters'. There are four species: *Arcobacter butzleri*, *Arcobacter cryaerophilus* (Groups 1A and 1B), *Arcobacter skirrowii* and *Arcobacter nitrofigilis* (Vandamme *et al.*, 1992). *A. nitrofigilis* is a free-living nitrogen fixing organism, first described as *Campylobacter nitrofigilis* (McClung & Patriquin, 1980; McClung, Patriquin, & Davis, 1983) and subsequently transferred to the genus *Arcobacter* (Vandamme *et al.*, 1991a). There have been reports of isolation of *A. nitrofigilis*-like organisms from sea sediments (Thamdrup, Rossello-Mora, & Amann, 2000), petroleum-contaminated groundwater (Watanabe, Kodama, Syutsubo, & Harayama, 2000) and oil field brine (Gevertz, Telang, Voordouw, & Jenneman, 2000). *A. butzleri* and *A. cryaerophilus* have been associated with human illness (Burnens, Schaad, & Nicolet, 1992; Lauwers, Breynaert, Etterijck, Revets, & Mets, 1996; Lerner, Brumberger, & Preac-Mursic, 1994; Marinescu, Collignon, Squinazi, Derimay, Woodward, & Lior, 1996; On, Stacey, & Smyth, 1995; Taylor, & Kiehlbauch, 1991; Tee, Baird, Dyall-Smith, & Dwyer, 1988; Vandamme *et al.*, 1992b; Yan, Wang, Huang, Chen, Jin, & Wu, 2000). *Arcobacter* spp. have similarities with both *Campylobacter* and *Helicobacter* and are included in the RNA Superfamily VI of the Proteobacteria. However, unlike thermophilic pathogenic campylobacters, they are generally unable to grow at 42°C but grow between 15 and 37°C. Originally the group-2 'aerotolerant campylobacters' were differentiated from the 'true' campylobacters by their ability to grow at 30°C after primary isolation in an micro-aerobic environment (Neill, Ellis, & O'Brien, 1978). The key distinguishing features of the genus *Arcobacter* used to differentiate them from *Campylobacter* are: the ability to grow at 15°C but not at 42°C; the ability to grow optimally aerobically at 30°C; G+C contents of 27 to 30 mol% and methyl-substituted menaquinone-6 not present as a major isoprenoid quinone (Ursing, Lior, & Owen, 1994; Vandamme & De Lay, 1991; Vandamme *et al.*, 1991a).

Isolation

Arcobacter spp. are generally 1–3 by 0.2–0.9 μm in size and display a corkscrew or darting motility by means of a single unsheathed polar flagellum. After incubation at 30°C aerobically, the organism produces 2–4 mm grey or whitish colonies on blood agar. Swarming may occur on fresh agar and the colony size varies (Mansfield & Forsythe, 2000).

Although CIN (cefsulodin-irgasan-novobiocin) agar, selective for *Yersinia* spp., has been used to recover *Arcobacter* from pork meat samples (Collins, Wesley, & Murano, 1996) and from a human case of enteritis (Burnens *et al.*, 1992), the original methods used for isolation of *Arcobacter* spp. were mainly based on those developed for *Campylobacter* spp. Many of the proto-

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cols were time-consuming, lacked specificity and, although aerotolerance is a characteristic of *Arcobacter* spp. differentiating them from *Campylobacter* spp., this was not always observed on initial isolation by these methods (DeBoer, Tilberg, Woodward, Lior, & Johnson, 1996). Traditionally identification required the isolation of a pure culture of the organism and many techniques have been used to isolate *Arcobacter* spp. from a variety of sources (Table 1). The main features of an isolation method must include specificity and it must not be too resource and time-laden for routine practical use. Species and strain identification may not always be required in clinical or food monitoring situations but in epidemiological studies it will be necessary to further identify strains and clones in order to determine the source(s) of infection. If *Arcobacter* spp. are shown to be human pathogens of significance and screening of specimens is to become routine, specific and rapid isolation and identification methods must be developed to monitor their occurrence in food, environmental and clinical samples.

Protocols have been developed for isolating Arcobacter *per se* (Table 1), usually incorporating aerobic growth at lower temperatures, for example, 30°C. One such procedure, based on the swarming of arcobacters on a semi-solid medium, similar to that suggested as a sensitive and rapid method for isolation of campylobacters from stool specimens (Goossens, Vlaes, Galand, Borre, & Butzler, 1989) and chicken products (DeBoer, Heerwaarden, & Tilburg, 1989), has been assessed for its effectiveness for isolating arcobacters from poultry and other meats. Enrichment in an Arcobacter selective broth (ASB), followed by plating onto semisolid Arcobacter selective medium using cefoperazone, trimethoprim, piperacillin and cycloheximide and incubation at 24°C, resulted in *Arcobacter* spp. being isolated from 24.1% poultry meat, 1.5% minced beef, 0.5% pork and 4.9% minced mixed pork/beef samples (DeBoer *et al.*, 1996).

Enrichment micro-aerobically at 30°C in an Arcobacter enrichment broth (AEB) containing cefoperazone as the selective agent together with a filter method onto mCCDA (modified cefoperazone, charcoal, deoxycholate) agar, incubated aerobically at 30°C, is effective for recovery of the organism from raw poultry, with no growth of competing microflora (Lammerding, Harris,

Table 1. Efficiency of Isolation methods used to recover *Arcobacter* spp. from meats

Meat	Isolation method	Efficiency (%) (*n*)	References
Pork	Modified CIN/ CAT agar.	0–89.9 (299)	Collins *et al.* (1996)
	Pre-enrichment in ASB, plating onto semi-solid medium, incubating at 24 °C.	0.5 (194)	DeBoer *et al.* (1996)
	Enrichment in Preston medium followed by culturing on mCCDA, both at 37 °C micro-aerobically.	3.7 (27)	Zanetti *et al.* (1996)
	Enrichment in Arcobacter broth (+ CAT), incubated at 24 °C microaerobically.	76 (54)	Houf *et al.* (2000)
Chicken	Pre-enrichment in ASB, plating onto semi-solid medium, incubating at 24 °C.	22.1 (220)	DeBoer *et al.* (1996)
	Enrichment micro-aerobically at 30 °C in AEB containing cefoperazone, filter onto mCCDA.	97 (125)	Lammerding *et al.* (1996)
	Enrichment in CAT broth, plating onto CAT/blood agar with/without filter.	110 (25)	Atabay and Corry (1997)
	Enrichment in JM broth followed by plating onto JM solid medium 30 °C.	84 (50)	Johnson and Murano (1999a, 1999b)
	Bacto Leptaspira medium/enrichment EMJH, subculture onto Karmali agar supplemented with cefoperazone, colistin, amphotericin and 5-fluorouracil at 25 °C.	80.5 (201)	Marinescu *et al.* (1996b)
	Enrichment in AB supplemented with CAT micro-aerobically, subculture onto mCIN/CAT agar incubating at 30 °C aerobically.	53 (96)	Gonzalez *et al.* (2000)
	Enrichment in Preston medium followed by culturing on mCCDA, both at 37 °C micro-aerobically.	0 (32)	Zanetti *et al.* (1996)
		52.3 (170)	Harrab, Schwarz, and Wenzel (1998)
Turkey	EMJH enrichment at 30 °C, filter onto BHI agar incubating at 30 °C.	77 (395)	Manke *et al.* (1998)
	Enrichment in Preston medium followed by culturing on mCCDA, both at 37 °C micro-aerobically.	0 (30)	Zanetti *et al.* (1996)
	Campylobacter enrichment broth (+CAT) microaerobic, 30 °C for 2 days followed by filter onto blood agar, incubation aerobically for up to 7 days.	24 (17)	Atabay *et al.* (2001)
Duck	Enrichment micro-aerobically at 30 °C in AEB containing cefoperazone, filter onto mCCDA.	80 (10)	Ridsdale *et al.* (1998)
	Campylobacter enrichment broth (+CAT) microaerobic, 30 °C for 2 days followed by filter onto blood agar, incubation aerobically for up to 7 days.	70 (10)	Atabay *et al.* (2001)
Beef	Pre-enrichment in ASB, plating onto semi-solid medium, incubating at 24 °C.	1.5 (68)	DeBoer *et al.* (1996)

Lior, Woodward, Cole, & Muckle, 1996). However, although both mCCDA and CAT (cefoperazone, amphotericin, teicoplanin) agars support growth of *Arcobacter* spp., the latter tends to support a wider range of *Arcobacter*s and *Campylobacters* than the former. Although *Arcobacter* spp. are resistant to the level of cefoperazone present in mCCDA (32 μg ml^{-1} compared with 8 μg ml^{-1} in CAT) they generally grow better on CAT, suggesting that there might be a synergistic inhibitory effect of deoxycholate and cefoperazone at 32 μg ml^{-1} present in mCCDA (Corry & Atabay, 1997).

A modification of the Lammerding method (Lammerding *et al*., 1996) using enrichment in CAT broth micro-aerobically followed by plating on CAT agar and incubating at 30°C aerobically has been used to isolate a wider range of arcobacters than the original method which was devised to detect *A. butzleri*. However, the results of a small-scale study suggested a lower isolation rate. Ten out of 10 supermarket chicken carcasses were positive by the original method compared with 8 out of 10 for the modified method. After enrichment in CAT broth, similar recovery rates are obtained substituting blood agar for CAT agar and also using a 0.65 μm diameter pore membrane filter (Steele & McDermott, 1984) instead of a 45 μm pore size filter (Lammerding *et al*., 1996) onto blood agar at 37°C. An enrichment step is important for maximal isolation since conventional direct plating onto CAT agar is not successful in recovering *Arcobacter* from poultry samples (Atabay & Corry, 1997).

A commercial enrichment broth (Arcobacter broth (AB)) has been developed which, when supplemented with either cefoperazone, amphotericin, teicoplanin (CAT) or modified cefoperazone, charcoal, deoxycholate (mCCD), may be used for the isolation of *Arcobacter* spp. or *A. butzleri*, respectively. When supplemented with CAT this medium supports good growth of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* although *A. nitrofigilis* grows poorly. More importantly, when eight strains of *Campylobacter* spp. were tested, none of these grew in the AB, probably because this medium contains no oxygen-quenching system, such as blood, which neutralizes the effect of atmospheric oxygen (Atabay & Corry, 1998; Corry, Post, Colin, & Laisey, 1995). Another advantage of AB is that *Arcobacter* can reach higher population densities than using biphasic growth methods (i.e. a solid phase of 10% bovine blood agar and a liquid phase overlay of brain heart infusion broth in tissue culture vessels), previously suggested as the method for increasing bacterial population densities over the rather low densities achieved in brain heart infusion broth (Dickson, Manke, Wesley, & Baetz, 1996).

Recently, a combination of new enrichment and solid media has been developed by Johnson and Murano (1999a,b) (JM formulation) allowing, it is suggested, optimal aerobic growth at 30°C. In the solid medium, the addition of 0.05% thioglycolic acid, 0.05% sodium pyruvate, 5% sheep's blood and 32 mg l^{-1} cefoperazone (pH 6.9) to a formula of special peptone no. 2 results in an growth medium that supports *A. butzleri*, *A. cryaerophilus* and *A. nitrofigilis*, although *A. skirrowii* was not tested. There is an added characteristic in that a deep red colour develops around the *Arcobacter* colonies making presumptive positives easier to recognize.

In a comparative study of the De Boer *et al*. (1996) and the JM methods for isolating *Arcobacter* spp. from chicken samples, 14 out of 50 broiler chicken samples were positive with the former while 42 were positive with the latter. In the JM method, both the total method time (including PCR confirmation) is reduced substantially from 6–9 days to 4 days and the specificity is increased. 14% of samples produced plates containing other microflora using the JM formulation compared with 53% in the de Boer method and 2% were negative for *Arcobacter* spp. with the plates completely overgrown compared with 16% (Johnson & Murano, 1999a,b). The JM method therefore shows promise as a routine method of isolation for *Arcobacter* spp. since it appears both rapid and specific fulfilling the most important criteria for an isolation method.

Identification

The original 'biotyping' identification scheme for *Campylobacter* spp. (including, at that time, *C. cryaerophilia*) was developed as a method of differentiation of strains of thermophilic campylobacters by Lior (1984), based on reaction to a small number of biochemical tests. This was later extended to be used for speciation of campylobacters (Bolton, Wareing, Skirrow, & Hutchinson, 1992). Biochemical tests, protein profiles and fatty acid profiles have been used to differentiate arcobacters (Vandamme *et al*., 1992a) and serotyping, based on heat-labile antigens has been developed (Lior & Woodward, 1991). A combination of serotyping and biotyping was used successfully to identify biotypes and serotypes of *A. butzleri* isolates from poultry in France (Marinescu, Collignon, Squinazi, Woodward, & Lior, 1996).

Arcobacter species are now described as gram negative curved rods which are oxidase positive and grow aerobically at 25°C. The main phenotypic tests used for species identification are: catalase activity; nitrate reduction; cadmium chloride susceptibility; micro-aerobic growth at 20°C; growth on McConkey agar and in the presence of 3.5% NaCl and 1% glycine (Kiehlbauch, Plikaytis, Swaminathan, Cameron, & Wachsmuth, 1991; Vandamme *et al*., 1992a).

Identification of species within the family *Campylobacteraceae* using standard biochemical tests is prob-

lematical because of the low metabolic activity generally observed within the Proteobacteria together with a variability and atypical reaction of some strains. Therefore, combining a systematic range of biochemical tests with a probabilistic matrix comparing the characteristics of the unknown isolate with those of defined taxa, provides a method of differentiating between species. However, this procedure is beneficial only if there is a match between the taxa in the scheme and the unknown isolate. The original described scheme (Barrett, Patton, & Morris, 1988) and the commercially available scheme (API Campy, Biomérieux SA, France) only include 16 and 18 named taxa, respectively. The latter, although effective for differentiation of *Campylobacter* spp., does not allow similar identification of *Arcobacter* spp. *A. cryaerophilus* is the only *Arcobacter* species in the scheme and because of strain variability it is not a reliable means of identification (Jacob, Lior, & Feuerpfeil, 1993; Ridsdale, Atabay, & Corry, 1998).

Numerical analysis of phenotypic characters (On & Holmes, 1995) together with the use of an extensive probabilistic identification matrix provides a systematic approach to identification and confirms pathogenic arcobacters as a distinct group from campylobacters. Although *A. nitrofigilis* clusters with *C. curvis*, *C. rectus* and *C. showae* in one group, the former can be distinguished by growth aerobically at 25°C emphasising aerobic growth as an useful species-specific marker (On, Holmes, & Sackin, 1996).

Campylobacter, *Helicobacter* and *Arcobacter* may be distinguished by whole cell protein profiling (Vandamme, Pot, & Kersters, 1991). After differentiating *Campylobacteraceae* from other genera, the use of SDS-PAGE of whole cell proteins has been shown to be effective in speciating *Arcobacter* isolated from poultry at the abattoir, compared with API Campy strips or biotyping by the Bolton method (Bolton *et al.*, 1992). In the former 100% of *Arcobacter* isolates were mis-identified while the latter does not speciate *A. butzleri* and *A. skirrowii* (Ridsdale *et al.*, 1998).

Molecular techniques are the method of choice if definitive identification of species or particularly strain is required since, in the classical typing methods of biotyping and serotyping, different strains frequently belong to the same type. Although *A. butzleri* may be distinguished from *A. cryaerophilus* by the strong reaction of the latter to the catalase test compared with the weak or negative reaction of the former, the two groups of *A. cryaerophilus* cannot be distinguished by any phenotypic test. All the protocols developed to date have advantages and disadvantages and some of these are shown in Table 2. Ideally if a molecular typing technique is to be used in a routine laboratory it must be robust, reliable, relatively inexpensive, discrimatory and, preferably, rapid.

Wesley and co-workers have designed several molecular probes for identification purposes. The oligonucleotide primers designed for the rRNA superfamily (R01–R05). R01 and R05 are broadly reactive with *Campylobacter*, *Helicobacter* and *Arcobacter*. R03 under low stringency screens for *Arcobacter* spp. and *Helicobacter* spp. while under high stringency it was specific for *A. butzleri* (Suarez, Wesley, & Larson, 1997).

Two genus specific 16S rRNA-based oligonucleotide DNA probes and an *A. butzleri* species specific DNA probe have proved effective at identifying field isolates from animal and human sources. The 27-mer probe (for *Arcobacter* spp.) generates reliable RFLP patterns with key restriction fragments (kb) to identify *A. butzleri* (~3.0 kb), *A. cryaerophilus* 1A (~3.2 kb) and *A. cryaerophilus* 1B (~3.2 and ~2.6 kb). Although these differentiate *A. cryaerophilus* Group 1A from *A. cryaerophilus* Group B they do not allow the identification of *A. skirrowii*. However, they do differentiate between the two species associated with human illness and therefore may be useful in clinical studies (de Oliveria, Wesley, Baetz, Harmon, Kader, & de Uzeda, 1999; Wesley, SchroederTucker, Baetz, Dewhirst, & Paster, 1995).

Ribotyping has been shown to be reliable for identification of *Arcobacter* isolates (Kiehlbauch *et al.*, 1991) although its use in routine laboratories was originally hampered by two factors. Firstly, the procedure had to be carefully optimised for the organism under investigation and secondly, the original protocols involved the use of hazardous chemicals or radioisotopes. A modified procedure has been developed using a guanidium DNA extraction method and hybridization with non-radioactive digoxigenin-labelled probes. Ribotyping involving the hybridization of *Pvu*II-digested chromosomal DNA or *Cla*I-digested DNA with probes for the highly conserved 16S rRNA gene, discriminates between strains of *Campylobacter*, *Helicobacter* and *Arcobacter* or maximally between *Arcobacter* strains, respectively (Table 2). The modified method does not use hazardous chemical or radioisotopes which makes it more acceptable for use in a routine clinical or research laboratory (Kiehlbauch, Cameron, & Wachsmuth, 1994).

A two-step typing scheme based on PCR-RFLP analysis of the 16S rRNA gene involving digestion of the amplicon with *Dde*I and *Taq*I produced unique fingerprints for *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. However, as the scheme does not differentiate between *C. jejuni* and *C. coli*, it probably will not prove useful in a wider, routine laboratory setting (Marshall, Melito, Woodward, Johnson, Rodgers, & Mulvey, 1999).

Using primers and an oligonucleotide probe derived from the sequences of the *Arcobacter* spp. and *A. butzleri*-specific 16S rRNA-based DNA probes (Wesley *et al.*, 1995), a PCR assay has been developed which has

Table 2. Molecular typing methods used to identify *Arcobacter*

Method	Samples tested	Advantages	Disadvantages	Reference
Ribotyping	Type and reference strains	Discriminatory between *Arcobacter* strains	Minimum number of cells required	Kiehlbauch *et al.* (1994)
Hybridization with 16S rRNA probes for *Arcobacter* spp. and *A. butzleri*	Field isolates from aborted livestock foetuses; reference strains	No cross reaction of *Arcobacter* spp. probe with *Helicobacter*/*Campylobacter* spp. or of *A. butzleri* probe with *A. skirrowii* or *A. cryaerophilus*; differentiates between *A. cryaerophilus* 1A and 1B	Does not distinguish *A. skirrowii*	Wesley *et al.* (1995); de Oliveria *et al.* (1999)
PCR using 16SrRNA DNA probes specific for *Arcobacter* (as above)	Porcine faeces; water	*A. butzleri*, *A. cryaerophilus* and *A. skirrowii* differentiated; no cross reaction with *Helicobacter* or *Campylobacter*; results within 8 h	None described	Harmon and Wesley (1996); Rice *et al.* (1999)
PCR-RFLP analysis 16SrRNA gene	Clinical isolates; reference strains	Differentiates all *Arcobacter* spp.	Developed for differentiation between *Campylobacter*, *Helicobacter* and *Arcobacter* strains but does not differentiate between *C. jejuni* and *C. coli*	Marshall *et al.* (1999)
PCR-RFLP analysis 23SrRNA gene	Field isolates; reference strains	Relatively inexpensive; differentiates most *Campylobacter* spp.	Does not distinguish *A. cryaerophilus* and *A. skirrowii*	Hurtado and Owen (1997)
m-PCR using species-specific primers for *A. butzleri* and *C. jejuni*	Artificially contaminated meat, fruit and dairy products	Identifies *C. jejuni* and *A. butzleri* in one assay within 8 h	Not tested for *A. cryaerophilus*	Winters and Slavik (2000)
m-PCR using primers for 16SrRNA genes of *Arcobacter* spp. and a *A. butzleri* specific 23SrRNA gene portion	Reference strains, poultry isolates, porcine isolates	Rapid; specific for *A. butzleri*	Does not differentiate non-butzleri species	Harmon and Wesley (1997)
m-PCR-culture using species specific primers	Reference strains; poultry isolates	Sensitive; rapid; distinguishes *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*	None described	Houf *et al.* (2000)
PCR-culture using *Arcobacter*-specific primers	Reference strains and chicken isolates	Sensitive, rapid	Only identifies *Arcobacter* spp. with no differentiation	Gonzalez *et al.* (2000)
PCR-hybridization based on *gly*A gene	Type and reference strains	Low levels of detection of *A. butzleri* (50 pg DNA or 23,000 copies)	Only *A. butzleri* or *A. butzleri*-like strains identified	Al Rashid *et al.* (2000)
AFLP profiling	Type, reference and field strains	Highly discriminatory for *A. butzleri* and *A. cryaerophilus*	Large database required; some *A. skirrowii* isolates produce aberrant results	Scullion *et al.* (2001)

proved robust. It allows detection of *Arcobacter* spp. without the need for ribotyping and, because the method may be used with crude cell lysate as well as purified DNA, reduces the time of identification to approximately 8 h compared with several days with conventional culturing techniques (Harmon & Wesley, 1996). A combined PCR-culture technique consisting of pre-enrichment in AB with CAT supplement for 16 h, followed by a PCR targeting 16 rRNA genes, has been suggested as a reliable and rapid method for assessing *Arcobacter* contamination of poultry meat. The PCR appears to be as consistent as sub-culturing onto mCIN/CAT agar but reduces the total detection time by 48 h (Gonzalez, Garcia, Antolin, Hernandez, & Martin, 2000).

The 23S rRNA gene is larger with more variable regions than the 16S rRNA gene and so provides the basis for an identification scheme that is potentially more discriminating and specific than one based on the 16S rRNA gene. A protocol using three restriction endonucleases *Hpa*II, *Cfo*I and *Hin*fI to digest 23S rRNA gene PCR amplicons, results in the 23S rRNA gene-based PCR-RFLP identification generating conserved restriction profiles, allowing differentiation of *A. butzleri* and *A. nitrofigilis* as separate species. However, it does not distinguish between *A. cryaerophilus* and *A. skirrowii* because they have identical patterns with these restriction enzymes. When evaluated as a means of identifying 21 field strains that had proved difficult to characterize by phenotypic tests 17 (86%) were easily assigned to species level, indicating the reliability of the method. Of the remainder, one isolate was either *A. cryaerophilus* or *A. skirrowii* (not differentiated in this method) and the other three shared similar patterns but did not match any reference known strain and were therefore presumably *Arcobacter*-type strains (Hurtado & Owen, 1997).

Although modified methods have been suggested for use in clinical laboratories (Kiehlbauch *et al.*, 1994) ribotyping methods are resource laden and time-consuming. PCR-mediated detection of DNA polymorphisms with primers aimed at variable sequence motifs is faster, just as reliable and may be used to differentiate between strains within species which may be important in epidemiological studies where common sources of infection and spread need to be identified (Vandamme *et al.*, 1993).

Genus-specific PCR and three species-specific PCR assays, based on a target sequence comprising the most variable region of 23S rDNA, have been shown to be reliable in identifying reference strains and field isolates of *Arcobacter* spp. This specific PCR may be used either for classification at the genus level using the primer combination ARCO1-ARCO2 or, together with the species-specific primers ARCO1-BUTZ, ARCO1-CRYAE and ARCO1-SKIR, for identification to species level (Bastyns, Cartuyvels, Chapelle, Vandamme, Goossens, & DeWachter, 1995).

Multiplex-PCR (m-PCR) techniques based on multiple primer sets for the detection of *Arcobacter* spp. have been developed (Table 2). One protocol used two primers sets: one targeting a section of the 16S rRNA genes (ARCO1 and ARCO2) (Harmon & Wesley, 1996) and the other amplifying a portion of the 23S rRNA genes unique to *A. butzleri* (ARCO-BUTZ) (Bastyns *et al.*, 1995). PCR amplification resulted in all of the *Arcobacter* isolates tested yielding a 1223 bp product, whereas *A. butzleri* yielded both a 1223 and a 686 bp product. The reliability of this method was demonstrated by analysis of 108 field strains of *Arcobacter* spp., previously characterized to species level by either DNA–DNA hybridization, dot blot hybridization, ribotyping or by serology. The 1223 bp multiplex PCR product identified all of the isolates as *Arcobacter* spp. while the presence of both the 1223 and 686 bp amplicons identified 66 strains as *A. butzleri* agreeing with results obtained by other methods (Harmon & Wesley, 1997).

Another multiplex PCR assay also targeting the 16S and 23S rRNA genes, but using five primers, has been developed for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. The selected primers amplify a 257-bp fragment from *A. cryaerophilus*, a 401-bp fragment from *A. butzleri* and a 641-bp fragment from *A. skirrowii*. Using reference strains, this m-PCR proved specific for these three *Arcobacter* species. After enrichment of spiked chicken samples in either Arcobacter broth (Atabay & Corry, 1998) or ASB (DeBoer *et al.*, 1996) the detection limit was 10^3 cfu g^{-1} for *A. butzleri* and *A. cryaerophilus* and 10^2 cfu g^{-1} for *A. skirrowii*. An evaluation of this m-PCR combined with either enrichment broth for 24 or 48 h in order to identify *Arcobacter* spp. from poultry samples, indicated that, combined with a 24-h pre-enrichment in Arcobacter broth with CAT supplement, the method was a species specific and rapid technique for detecting and identifying *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Houf, Tutenel, De Zutter, Van Hoof, & Vandamme, 2000). Combined with the fact that m-PCR is a robust method would make it the technique of choice in a routine laboratory. The primer sequences for 16S rRNA, 23S rRNA, nested PCR and PCR fingerprinting have been reviewed recently by Mansfield and Forsythe (2000).

Isolates from nursing sows and developing pigs on three farms of a farrow-to-finish swine operation, identified by PCR and their genotypic fragment patterns examined by pulsed-field gel electrophoresis (PFGE) showed that the level of genotypic variation revealed suggested that pigs in this farrow-to-finish operation were colonised by multiple *Arcobacter* parent genotypes that may have undergone genomic rearrangement,

common to members of *Campylobacteraceae*, during successive passages through the animals. Additionally, the level of genotypic diversity seen among *Arcobacter* isolates from individual farms suggested an important role for genotypic phenotyping as a source identification and monitoring tool during outbreaks (Hume, Harvey, Stanker, Droleskey, Poole, & Zhang, 2001).

Whole-genomic fingerprinting by the determination of amplified fragment length polymorphism (AFLP) is a high resolution genotyping method. The digestion of target DNA with two restriction enzymes of differing cutting frequencies, ligation of half-site specific adaptor nucleotides, selective amplification of adapted genomic fragments and detection of subsequent PCR products has been used for genotyping a range of bacteria (Savelkoul *et al*., 1999), including *C. jejuni* and *C. coli* (Duim, Wassenaar, Rigter, & Wagenaar, 1999; Kokotovic & On, 1999). On and co-workers have shown that numerical analysis of AFLP profiles based on *Bg*111-*Csp*61 polymorphisms is useful in identifying taxonomic (species and sub-species) and epidemiological (clone and strain) relationships in wider range of *Campylobacter* species (On & Harrington, 2001a). Further they have established that AFLP may be used for concurrent species identification of the family *Campylobacteraceae,* including four species of *Arcobacter* (On and Harrington, 2001b) and that the method is effective in identifying genetic diversity among different clonal types from distinct geographical areas (On *et al*., 2001). A comparison of m-PCR (Houf *et al*., 2000) and AFLP- profiling for speciation of *Arcobacter* spp. by these researchers (Scullion, On, Madden, & Harrington, 2001) resulted in a good correlation between the two methods although three *A. skirrowii* isolates tested gave 'atypical' AFLP profiles (i.e. not similar to the type strain) whereas a PCR method (Harmon & Wesley, 1996) confirmed as them as *Arcobacter* spp. AFLP is a demanding technique and requires a large reference database, although once adopted in a routine laboratory it has the benefits of being robust and highly discriminatory.

The occurrence of *Arcobacter* with *Campylobacter* in the same food, human or environmental sample is a great possibility. A PCR-oligo hybridization strategy using PCR amplification of the partial *gly*A gene with three degenerate primers based on conserved *gly*A region, followed by species-specific oligo probe hybridizations to identify *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *A. butzleri* and *A. butzleri*-like strains, may provide an useful diagnostic tool for testing of such samples for these species. Although oligo probes have a high specificity they are not as sensitive as probes based on DNA fragment hybridizations (Al Rashid *et al*., 2000). An m-PCR has been designed to detect both *A. butzleri* and *C. jejuni* in the same specimen using one PCR tube and to be performed within an 8-h day. Although only tested with spiked samples the method appears robust with a wide range of foods (Winters & Slavik, 2000). The usefulness of rapid and specific methods for these two pathogens may prove to be important in screening samples for *Arcobacter* concurrently with *Campylobacter* so gaining important information of the occurrence of *Arcobacter* in food and human specimens.

Occurrence in animals and food

'Aerotolerant *Campylobacter*-like' organisms have been associated with reproductive problems in pigs since their first isolation from aborted foetuses and sows with reproductive problems, although they have also been isolated from normal foetuses and pigs (Ellis, Neill, & O'Brien, 1977; de Oliveira, Baetz, Wesley, & Harmon, 1997; de Oliveria *et al*., 1999; Neill *et al*., 1978, 1979; Wesley, 1994; Wesley *et al*., 1995). Strains from normal and reproductively impaired sows may be antigenically similar, suggesting that the strains associated with infertility are opportunistic pathogens colonising the foetus following placental damage (Wesley, 1994).

In Brazil, *A. cryaerophilus* has been isolated from sows with reproductive problems and from aborted porcine foetuses with *A. cryaerophilus* 1B being the most common isolate (de Oliveira *et al*., 1997). However, using RLFP analysis for isolates from male pigs, *A. cryaerophilus* 1A and 1B seem to be equally distributed and more common than *A. butzleri* (de Oliveria *et al*., 1999). Several studies in the USA have isolated *A. butzleri* and *A cryaerophilus* from aborted porcine foetal tissues using ribotyping techniques (SchroederTucker, Wesley, Kiehlbauch, Laron, Thomas, & Erickson, 1996; Wesley *et al*., 1995).

Since *Helicobacter pylori* has a role in the aetiology of human gastric ulceration, it has been suggested that *Arcobacter* may have a role in gastric ulceration in pigs, which is an economically important disease in livestock husbandry. However, in a study using a nested PCR combined with hybridization with a species-specific DNA probe, no statistical difference was demonstrated comparing isolation rates from specimens with gross gastric pathology with those without. However, there was a statistically more likelihood of isolating the organism from the non-glandular region than the glandular region making a definitive association difficult to determine (Suarez *et al*., 1997). Using an enrichment culturing technique only 4/71 samples were positive for *Arcobacter* compared with 44/86 for the nested PCR. Similarly *Arcobacter* could not be isolated using culturing techniques from artificially infected piglets which develop lesions in the gastric mucosa (Wesley, Baetz, & Larson, 1996).

Although pork is contaminated by *Arcobacter* spp., particularly *A. butzleri*, the recovery rate varies. This may be an indication of the actual incidence or be a reflection of the isolation methods used in various stud-

ies (Table 1). In Italy one out of 27 (3.7%) pork samples were positive for *A. butzleri* (Zanetti, Varoli, Stampi, & Luca, 1996), while in The Netherlands one out of 194 (0.5%) were positive (DeBoer *et al.*, 1996). However, in a study of ground pork from five processing plants in Iowa the isolation rates varied from 90% in one plant to 13.3% in another, 3.3% in two and 0% in the fifth. The original primary source of the animals or the hygiene practices at the abattoir itself or both may contribute to this variation (Collins *et al.*, 1996). A similar variation in isolation rate has been reported from a long-term (9-month) study of market age pigs at slaughter from two farms in the USA where the mean prevalence rate was 5% (range 0–20%) (Harvey *et al.*, 1999).

Similarly, *Arcobacter* spp. has been isolated from poultry, particularly chicken, with varying recovery rates which again may be an indication of the actual incidence in the animal population or a reflection of the isolation techniques (Table 1). Eggs, however, do not appear to be infected (Zanetti *et al.*, 1996). In a study in The Netherlands 24.1% of 220 poultry meat products were positive for *Arcobacter* (DeBoer *et al.*, 1996). In Canada, *A. butzleri* was recovered from 97% of poultry carcasses from five different processing plants (Lammerding *et al.*, 1996) and another in France produced an 81% isolation rate (Marinescu *et al.*, 1996b). Isolates from poultry both at the abattoir and on retail sale include *A. skirrowii* and *A. cryaerophilus* as well as *A. butzleri* (Atabay, Corry, & On, 1998). In Spain 53% poultry samples were positive for *Arcobacter* spp. using a combined PCR-culture technique (Gonzalez *et al.*, 2000). Using a m-PCR-culture technique a study in Belgium showed 76% of poultry samples were positive for *A. butzleri* and *A. cryaerophilus* (Houf *et al.*, 2000) whereas 100% (30/30) of chicken carcasses, seven out of 10 duck carcasses and 24% (4/17) of turkey flocks were positive for *Arcobacter* spp. (*A. butzleri*, *A. skirrowii* and *A. cryaerophilus)* in a study in Denmark using the same technique (Atabay, Waino, & Madsen, 2001). Thus m-PCR is a valuable tool for identification and differentiation of *Arcobacter* isolates.

Although *A. butzleri* is routinely isolated from poultry carcasses, it is infrequently detected in caecal samples suggesting post-slaughter rather than primary contamination of the animal (Atabay & Corry, 1997; Harrab, Schwarz, & Wenzel, 1998). In a study in Iowa, *Arcobacter* spp. was isolated from 14.3% of 405 cloacal swabs, of which 1% were *A. butzleri* indicating natural infections do occur. As with pigs, variations in isolation rate from poultry occurred which is dependent on the environmental conditions of the originating flock. However, unlike piglets, experimental infections with *A. butzleri* were not established in orally challenged poultry only in inoculated birds and only in the highly inbred Beltsville White strain of turkeys (Wesley & Baetz, 1999; Wesley *et al.*, 1996).

The results of a survey of mechanically separated turkey samples from different processing plants suggest that this meat may be highly contaminated by *Arcobacter* spp. or *A. butzleri* with rates of 77 and 56%, respectively, being reported. However, similar to the studies on pig processing plants, there was variation in prevalence. One plant yielded 96% of samples contaminated with *Arcobacter* species (80% of which were *A. butzleri*) whereas samples from another yielded 44% of samples positive for *Arcobacter* spp. of which 59% were *A. butzleri*. Using PCR-based fingerprinting for identification a diversity in DNA patterns was found within all plants sampled suggesting a multiplicity of contaminating sources rather than one major source (Manke, Wesley, Dickson, & Harmon, 1998).

Using selective enrichment and confirmation by SDS-PAGE to isolate *Arcobacter* spp. from a small sample (10) duck carcasses at the abattoir, *A. butzleri* was isolated from two, *A. skirrowii* from one and *A. cryaerophilus* from five. One flock out of four flocks investigated was positive for *A. butzleri* and one for *A. cryaerophilus*. This study emphasized the influence of isolation and identification techniques over the actual prevalence rates achieved and therefore reported (Ridsdale *et al.*, 1998).

Infections in cattle have been less reported than either pigs or poultry. Beef is probably not contaminated to such an extent as pork or chicken. In one study, only 1.5% (1 out of 68) minced beef and 4.9% (3 out of 61) samples of minced mixed pork/beef samples were positive for *Arcobacter* compared with 24.1% (53 out of 220) samples of poultry (DeBoer *et al.*, 1996). *Arcobacter* spp. has also been isolated from bovine foetuses (Neill *et al.*, 1978, 1979) and cattle with mastitis (Wesley, 1994). In 1995 the first isolation of *A. cryaerophilus* was reported in Chile from a bovine abortion using phenotypic characteristics to speciate which, as discussed previously is notoriously difficult (Fernandez, Rojas, & Gajardo, 1995). The organism has also been isolated from aborted bovine foetuses from two cattle herds in Germany and an aetiological association between *A. cryaerophilus* and serial abortion was been established in one of the herds (Parvanta, 1999). Using a highly specific m-PCR assay healthy cows have been shown to shed *Arcobacter* spp. in their faeces with feeding of alfalfa and the use of individual waterers being protective (Wesley *et al.*, 2000).

This latter finding suggests that water probably has a role in the transmission of *Arcobacter* spp. to animals. *A. butzleri* has been isolated from canal water in Thailand (Dhamabutra, Kamol-Rathanakul, & Pienthaweechai, 1992), a drinking water reservoir in Germany (Jacob *et al.*, 1993), brackish water (Maugeri, Gugliandolo, Carbone, & Fera, 2000) and a well-water source in USA (Rice, Rodgers, Wesley, Johnson, & Tanner, 1999). In a 2-year study of water treatment plants in

Germany *A. butzleri* was isolated more frequently from raw compared with treated water but was present at all stages of the processing. The predominant serotype was serotype 1 although serotypes 17, 19 and 2 were also isolated (Jacob, Woodward, Feuerpfeil, & Johnson, 1998). This pattern corresponds to studies in poultry (Lammerding *et al.*, 1996; Marinescu *et al.*, 1996b) and clinical samples (Vandamme *et al.*, 1992b). A study in Italy found that oxygen-activated sludge treatment followed by tertiary treatment with 2 ppm chlorine dioxide is effective in reducing the loading of *Arcobacter* spp (specifically *A. cryaerophilus*) by 99.9% although the same treatment completely eliminated thermophilic campylobacters. This suggests that *A. cryaerophilus* may show some resistance to chlorine treatment (Stampi, Varoli, Zanetti, & Deluca, 1993) although *A. butzleri* has been reported as being sensitive to chlorine inactivation (Rice *et al.*, 1999). *A. butzleri* shows a higher sensitivity to BrCl than other micro-organisms (Zanetti, Stampi, Luca, Varoli, & Tonelli, 1996). Significant numbers of activated sludge samples (4%) have been demonstrated to contain *Arcobacter* (Snaidr, Amann, Huber, Ludwig, & Schleifer, 1997). Another Italian study reported an isolation frequency of *A. butzleri* from sewage sludge of 41% in digested sludges (values of 7649 MPN/g) to 80% in thickened and activated sludges. In comparison, the thermophilic campylobacters (*C. jejuni* and *C. coli*) were only isolated from primary sludge and at substantially lower counts of 278 and 1403 MPN/g, respectively (Stampi, Luca, Varoli, & Zanetti, 1998), perhaps indicating that the land application of sludge may provide a means of *Arcobacter* spp. infection in animals and humans via run-off into the water course as has been suggested for other pathogens (Keene *et al.*, 1994).

Control

Campylobacter spp. is a well-described human pathogen present in many food products and, during the last two decades, many existing and newly designed treatments have been evaluated for their effectiveness in eliminating this organism. However, these may not be as effective inactivating or removing *Arcobacter* spp., even though *Arcobacter* and *Campylobacter* are closely related. For example, *A. butzleri* is more resistant than *C. jejuni* to irradiation treatment. The average D_{10} value is 0.27 kGy compared with 0.19 kGy for *C. jejuni*. Therefore radiation doses currently allowed for pork in USA (0.3–1.0 kGy) do provide an effective method of reducing, if not completely eliminating, *A. butzleri* as well as *C. jejuni* from pork (Collins, Murano, & Wesley, 1996). In foods liable to be contaminated by *Arcobacter* spp. such as pork and pork products, various treatments have been demonstrated to be effective in reducing contamination by other pathogens. Organic acids sprays reduce *Salmonella* and *Campylobacter* spp. on pork carcasses (Epling, Carpenter, & Blankenship, 1993) and incorporating nisin and/or sodium lactate into fresh pork sausage has been suggested to reduce contamination by another gram-negative bacterium, *Salmonella kentucky* (Scannell, Hill, Buckley, & Arendt, 1997). Although continuous exposure to sodium lactate in pure culture is not effective against *A. butzleri*, both citric acid and lactic acid eliminate growth, while nisin (at 500 IU/ml) reduces survival by approximately 50% (Phillips, 1999). Short-term treatment with both trisodium phosphate and EDTA (alone and in combination with nisin) are effective in reducing survival of *A. butzleri* in pure culture (Phillips & Duggan, 2001). Decreasing temperature appears to have an effect in reducing growth but *A. butzleri* is still able to survive both in culture and in chicken at low temperatures (Phillips & Duggan, in press; Phillips & Long, 2001).

Studies with other gram-negative bacteria suggest that nisin may be less effective in food systems than in pure cultures (Boziaris & Adams, 1999, 2000; Carneir-odeMelo, Cassar, & Miles, 1998; Cutter & Siragusa, 1995a,b) and this is also the case for *A. butzleri* (Phillips & Long, 2001). If *Arcobacter* spp. are demonstrated to be a major cause of foodborne illness, then each control treatment should be re-evaluated for its effectiveness against the organism in order to ensure reduction of contamination is actual and not merely based on extrapolation from studies with other pathogens.

Arcobacter and human infections

The common symptoms of *Arcobacter* infection are persistent diarrhoea accompanied by abdominal pain and stomach cramps (Lerner *et al.*, 1994; Vandamme *et al.*, 1992b). These symptoms are similar to campylobacteriosis and may be transient in nature making infection rates difficult to assess.

The first description of a clinical case of *A. cryaerophilus* 1B human infection was in a homosexual male with diarrhoea (Tee *et al.*, 1988). Clinical isolates of *Arcobacter* spp. have been reported in children and adults with diarrhoea (Taylor & Kiehlbauch, 1991; Marinescu *et al.*, 1996). *A. butzleri* has isolated from cases of bacteremia, including a case of neonatal patient (On *et al.*, 1995) and one with liver cirrhosis (Yan *et al.*, 2000), as well as from two patients with severe diarrhoea, both of whom were suffering underlying chronic disease (Lerner *et al.*, 1994). A case of an uremic patient with bacteremia caused by *A. cryaerophilus* 1B suggests that the organism is able to produce invasive infections although, once again, the patient concerned had an underlying illness which may have contributed to the progress of *A. cryaerophilus* infection (Hsueh *et al.*, 1997).

Biotypes and serotypes of *A. butzleri* isolates from poultry in France are similar to those isolated from human diarrhoeal cases indicating that infection may

arise from a food reservoir (Marinescu *et al*., 1996b). Molecular typing techniques are useful in epidemiological settings as they are able to discriminate between different strains resulting in the resolution of the spread and source or sources of the outbreak. Using PCR-mediated DNA fingerprinting a single clone was found to be the cause of an outbreak in an Italian school, indicating person to person spread may be a feature of outbreaks (Vandamme *et al*., 1992b, 1993), while ribotyping identified several different strains in a non-human primate colony suggesting multiple sources of infection rather than a common one (Anderson, Kiehlbauch, Anderson, McClure, & Wachsmuth, 1993).

The significance of *Arcobacter* spp. as a cause of human illness is, as yet, not determined. This may be because standard primary screening procedures used for *Campylobacter* spp. do not allow recovery of *Arcobacter* spp. (Marinescu *et al*., 1996a) and clinical specimens are not routinely tested for *Arcobacter* spp. *per se*. A Belgium study which screened all routine stool specimens submitted for detection of enteropathogens between 1991 and 1994 found that less than 0.1% were positive for *Arcobacter* spp. (8 out of 21,527 samples). However, the isolation technique used involved a non-selective medium together with microaerobic conditions, which are probably not the optimum conditions for recovery. Using the same protocol, no *Arcobacter* isolates were obtained from 879 samples from 468 elderly patients during a one-year study (Lauwers *et al*., 1996).

A 5-year study in Belgium between 1995 and 2000 isolated 336 non jejuni/coli *Campylobacter* species from 179 patients of which 24.6% were *A. butzleri*. The isolation method used included enrichment in Brucella broth (containing antibiotic supplement and 5% laked horse blood) for 24 h at 24°C microaerobically followed by incubation on Arcobacter selective medium for 3 days at 25°C also microaerobically (Vandenberg *et al*., 2001). These conditions may not give optimal isolation rates since the filter method onto mCCDA or CAT agar has been shown to give the best rates as shown by a recent re-evaluation of isolation methods for *Campylobacte*r and related organisms, including *Arcobacter*, carried out in Denmark which suggest that the prevalence in human faecal samples has been under-estimated. Therefore isolation techniques must be carefully designed to ensure maximal recovery so that the true occurrence and epidemiology of each pathogen may be determined (Engberg, On, Harrington, & Gerner-Smith, 2000).

The *Arcobacter* haemagglutinin has been described as an immunogenic protein of about 20 kDa, the activity of which is sensitive to heat and proteolytic digestion. It is a lectin-like molecule, which may bind to erythrocytes via a glycan receptor (Tsang, Luk, Woodward, & Johnson, 1996). *A. butzleri* produces cytotoxins or cytolethal distending factors (dependant on strain) which affect Hela, CHO and Intestine 407 cells *in vitro* (Musmanno, Russi, Lior, & Figura, 1997). Invasion was not observed in this study although cell adherence was demonstrated. However, in Hep-2 cells and a rat ileal loop assay, Fernandez, Eller, Paillacar, Gajardo, and Riquelme (1995) proposed invasion as a means of pathogenicity. The likely mechanism of virulence of this organism *in vivo* therefore remains a matter for controversy.

Arcobacter spp. are able to colonise neonatal piglets as demonstrated by their recovery from faeces and from tissues such as liver, kidney, ileum and brain although no gross pathology is observed (Wesley *et al*., 1996). The use of a porcine model for investigating the pathogenesis of *Arcobacter* may prove to be as valuable as have those evaluating the pathogenicity of *Campylobacter* spp. (Babakhani, Bradley, & Joens, 1993) and *Helicobacter* spp. (Eaton, Brooks, Morgan, & Krakowka, 1991; Eaton, Morgan, & Krakowa, 1992).

Conclusion

The significance of *Arcobacter* spp. as a human pathogen is not fully evaluated at present although, considering its isolation in cases of human and animal illness and from foods of animal origin, it certainly may be added to the ranks of emerging foodborne pathogens. With the development and use of specific and robust isolation, detection, and identification techniques, accurate information will be accumulated on its epidemiology and occurrence, whether in the environment and/or in food.

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