Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Detection of *Arcobacter* spp. in piggery effluent and effluent-irrigated soils in southeast Queensland

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Keywords
Arcobacter spp., effluent, MPN, pigs, soil.

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2006/1224: received 29 August 2006, revised and accepted 2 November 2006

doi:10.1111/j.1365-2672.2007.03275.x

Abstract

Aims: To investigate the occurrence and levels of *Arcobacter* spp. in pig effluent ponds and effluent-treated soil.
Methods and Results: A Most Probable Number (MPN) method was developed to assess the levels of *Arcobacter* spp. in seven pig effluent ponds and six effluent-treated soils, immediately after effluent irrigation. *Arcobacter* spp. levels in the effluent ponds varied from $6{\cdot}5 \times 10^5$ to $1{\cdot}1 \times 10^8$ MPN 100 ml^{-1} and in freshly irrigated soils from $9{\cdot}5 \times 10^2$ to $2{\cdot}8 \times 10^4$ MPN g^{-1} in all piggery environments tested. Eighty-three *Arcobacter* isolates were subjected to an abbreviated phenotypic test scheme and examined using a multiplex polymerase chain reaction (PCR). The PCR identified 35% of these isolates as *Arcobacter butzleri*, 49% as *Arcobacter cryaerophilus* while 16% gave no band. All 13 nonreactive isolates were subjected to partial 16S rDNA sequencing and showed a high similarity (>99%) to *Arcobacter cibarius*.
Conclusions: *A. butzleri*, *A. cryaerophilus* and *A. cibarius* were isolated from both piggery effluent and effluent-irrigated soil, at levels suggestive of good survival in the effluent pond.
Significance and Impact of the Study: This is the first study to provide quantitative information on *Arcobacter* spp. levels in piggery effluent and to associate *A. cibarius* with pigs and piggery effluent environments.

Introduction

The genus *Arcobacter* was originally created to house organisms that were initially regarded as aerotolerant *Campylobacter* species (Vandamme *et al.* 1991). The genus currently consists of five species – *Arcobacter butzleri*, *Arcobacter cibarius*, *Arcobacter cryaerophilus*, *Arcobacter nitrofrigilis* and *Arcobacter skirrowii* (Houf *et al.* 2005). *Arcobacter butzleri* has been found in human extraintestinal diseases but little is known about the organism's pathogenicity and virulence (Lehner *et al.* 2005). Even though *A. butzleri* has not been directly linked to food-borne illness, the fact that the organism is found on meats and causes diarrhoeal illness in humans suggests that it is a possible food-borne pathogen (Mansfield and Forsythe 2000).

Arcobacter spp. are found to survive in a wide range of environments such as the gut and faeces of pigs (Wesley *et al.* 1996; Van Driessche *et al.* 2004), poultry meat/carcass (Corry and Atabay 2001; Houf *et al.* 2002), poultry litter (Eifert *et al.* 2003), cattle (Kabeya *et al.* 2003), lamb meat (Rivas *et al.* 2004), drinking water (Jacob *et al.* 1998) and river water (Morita *et al.* 2004). Experimental infections of caesarean-derived, colostrum-deprived piglets showed that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* could all colonize the piglets but that severe gross pathology was absent (Wesley *et al.* 1996).

Arcobacter has been isolated from water treatment plants in Germany (Jacob *et al.* 1993), well water in United States (Rice *et al.* 1999), river and canal waters in Japan and Thailand, respectively (Morita *et al.* 2004) and sewage plants in Italy (Stampi *et al.* 1999). A laboratory

strain of *A. butzleri* (NCTC 12481) was able to maintain membrane integrity after 35 days of starvation in non-chlorinated drinking water (Moreno *et al.* 2004). Water and effluent clearly have the potential to play a role in the transmission of *Arcobacter* spp.

Pigs are a source of *Arcobacter* with the prevalence in faeces (16–85%) increasing with age and the dominant species being *A. butzleri* (Van Driessche *et al.* 2004). *Arcobacter* spp. have been isolated from nursing sows, grower pigs and market-age pigs at slaughter (Hume *et al.* 2001). *Arcobacter butzleri* is a routine contaminant of pork, with plants in the United States showing a prevalence that varied between 0% and 90% (Collins *et al.* 1996). Thus, pigs have been found to be a reservoir for *Arcobacter* spp. with the potential for the organism to transfer into the environment as result of effluent management practices. However, limited data are available on the role of piggery effluent in the survival and transfer of *Arcobacter* to the environment.

Being an organism of recent interest, no standard, widely accepted methodologies for the isolation and enumeration of levels of *Arcobacter* exist. Several studies have compared various media formulations as well as enrichment procedures for the recovery and isolation of *Arcobacter* spp. (Corry and Atabay 1997; Atabay and Corry 1998; Johnson and Murano 1999a,b; Houf *et al.* 2001). There is a need for suitable, optimal recovery media and conditions that can detect the levels of *Arcobacter* spp. in a range of different sources such as faeces, carcasses and the environment.

The present study was carried out to understand the presence, levels and species distribution of *Arcobacter* spp. in piggery effluent and soil using the Most Probable Number (MPN) technique. The study also evaluated the possibility of an abbreviated phenotypic testing scheme in comparison with a multiplex PCR for species identification.

Materials and methods

Samples for initial evaluation of direct plating and the development of a MPN method

Fresh pig faeces and pig effluent were collected and held on ice until arrival at the laboratory (4 h from collection), and processed on the day of collection. In addition, soil was placed in a plastic pot (pot volume of 200 ml) and the soil was then saturated with pig effluent.

Direct plating method

Direct plating involved the use of CAT agar which consisted of CCDA agar (Oxoid CM 739) with added C.A.T. supplement (Oxoid SR 174). *Arcobacter* agar was also used and consisted of *Arcobacter* broth (Oxoid CM 965) with added C.A.T. supplement (Oxoid SR 174) and bacteriological agar (Oxoid L11) (12 g l^{-1}). All media were prepared and supplemented as per the manufacturer's instructions. Direct plating was performed by mixing 10 g of faeces or 10 ml of effluent in 90 ml of 0·1% peptone. The mixture was shaken for 15 min. Serial 10-fold dilutions were prepared in 0·1% peptone by mixing 1 ml in 9 ml. Direct plating was performed by spreading 0·1 ml of the relevant dilution across the surface of the relevant medium. All inoculated plates were incubated at 28°C for 24–48 h.

MPN Methods A, B and C

All MPN methods were three tube methods and 0·1% peptone was used as diluent. All dilutions were prepared as an initial 10 g (faeces or soil) or 10 ml (effluent) sample in 90 ml of 0·1% peptone. All subsequent serial dilutions were performed as 1 ml in 9 ml of 0·1% peptone. If an undiluted sample was tested, then 1 ml of effluent or 1 g of soil was added directly to the enrichment broth.

Method A

The enrichment broth used was *Arcobacter* broth (Oxoid CM 965) with added C.A.T. supplement (Oxoid SR 174) (Atabay and Corry 1998). The inoculated broths were incubated at 25°C in tightly capped bottles under aerobic conditions for 48 h. The broths were then inoculated onto 5% sheep blood agar which was incubated for 48 h under aerobic conditions.

Method B

This method used the same basic broth for the enrichment stage, *Arcobacter* broth (Oxoid CM 965), as used in method A with the supplements used by Houf *et al.* (2001), i.e. 5% lysed horse blood, amphotericin B (10 mg l^{-1}), cefoperazone (16 mg l^{-1}), 5-fluorouracil (100 mg l^{-1}), novobiocin (32 mg l^{-1}) and trimethoprim (64 mg l^{-1}). The broths were incubated under microaerobic conditions, at 28°C, for 48 h. The plating medium consisted of *Arcobacter* broth (Oxoid CM 965), bacteriological agar (Oxoid L11) (12 g l^{-1}) and the same additives as the broth. The supplements were aseptically added just before the agar was poured. The inoculated plates were incubated at 28°C, under aerobic conditions, for 48 h.

Method C

The enrichment broth, termed JM broth (Johnson and Murano 1999a), contained special peptone (Oxoid L72) (10 g l^{-1}), yeast extract (5 g l^{-1}), beef extract (5 g l^{-1}),

NaCl (4 g l^{-1}), potassium phosphate (monobasic) (1·5 g l^{-1}), sodium phosphate (dibasic) (3·5 g l^{-1}), sodium pyruvate (0·5 g l^{-1}), sodium thioglycolate (0·5 g l^{-1}), charcoal (0·5 g l^{-1}), bile salts No 3 (Oxoid L56) (2 g l^{-1}) and bacteriological agar (Oxoid L11) (2 g l^{-1}). After sterilization by autoclaving, the following supplements were added: 5fluorouracil (200 mg l^{-1}) and cefoperazone (32 mg l^{-1}). The inoculated broths were incubated at 30°C for 48 h. After incubation, the JM broths were plated onto JM agar (Johnson and Murano 1999b). JM agar contained special peptone (Oxoid L72) (10 g l^{-1}), yeast extract (5 g l^{-1}), beef extract (5 g l^{-1}), NaCl (4 g l^{-1}), potassium phosphate (monobasic) (1·5 g l^{-1}), sodium phosphate (dibasic) (3·5 g l^{-1}), sodium pyruvate (0·5 g l^{-1}), sodium thioglycolate and bacteriological agar (Oxoid L11) (12 g l^{-1}). Immediately before pouring, JM agar was supplemented with cefoperazone (32 mg l^{-1}) and defibrinated sheep blood (50 ml l^{-1}). The inoculated JM agar plates were incubated at 30°C, under aerobic conditions, for 48 h.

Assessment of levels of *Arcobacter* spp. in effluent and soil

Effluent was collected from seven piggeries across south-east Queensland over a period of 3 years. About 1 l of effluent was collected from the final pond of each piggery and transported to the laboratory as previously mentioned. At five piggeries, effluent from the same pond was irrigated onto pastures near the piggery. Following this irrigation, samples of soil were collected aseptically using a stainless steel core to a depth of 4 cm within an hour after effluent application. The soil samples were composited and a 10-g sample was aseptically weighed. The sample was then shaken for 30 min in 90 ml of 0·1% peptone diluent. Appropriate 1 ml of serial dilutions from both soil and effluent (in 0·1% peptone) were then used in the Method C MPN. Selected typical isolates were picked for further identification. The results were expressed as MPN per 100 ml of effluent.

Confirmatory identification of presumptive isolates

Typical colonies (greyish yellow to grey moist) were subcultured, as a single colony pick, onto Abeyta-Hunt-Bark agar without antibiotics (AHB) (Hunt *et al.* 2001) which consists of heart infusion agar (Difco Cat # 244400) (40 g l^{-1}) and yeast extract (2 g l^{-1}). After overnight incubation at 30°C, subcultures on AHB agar were examined, under dark ground microscopy, for typical *Arcobacter* cell shape (slender, curved rods) and typical spiral motility. If the cell shape and motility were correct, the following tests were performed – catalase (using 3% H_20_2), and oxidase (using MVD strips – cat # BS210). The catalase reactions were termed as 'weak' catalase-positive meaning visible bubbles within10–15 s or 'rapid' catalase-positive, meaning instantaneous bubbling.

Cadmium chloride sensitivity (Kazmi *et al.* 1985) was carried out using sterile blank discs that were impregnated with 20 μl of a solution that contained 2·5 μg of cadmium chloride per 20 μl. The cadmium chloride sensitivity test was performed by placing the disc on the AHB subculture plate in the primary inoculum area. After 24 h of incubation, any zone of inhibition around the cadmium chloride disc was regarded as indicating a sensitive isolate. The indoxyl acetate reaction (On and Holmes 1992) was performed by preparing a 10% indoxyl acetate solution in ether and impregnating sterile blank discs with 25 μl of this solution. Dried indoxyl acetate discs were inoculated with a heavy smear of an overnight AHB agar culture and observed for 5 min. A dark blue colour under and around the growth was recorded as positive. To be regarded as *Arcobacter* spp, an isolate had to have the typical cell shape and motility, typical colony morphology on both JM agar and AHB agar and be oxidase- and indoxyl acetate-positive. Cadmium chloride and catalase reactions were used to assign isolates to a presumptive *Arcobacter* species.

Arcobacter isolates were selected from effluent and soil over the period of the study to represent the dominant colonial morphologies (large and small – on JM agar) and biochemical variations based on catalase reactions ('weak' and 'rapid') and cadmium chloride sensitivity.

Arcobacter Multiplex PCR

The type strains of *A. butzleri* (CCUG 30485^T), *A. cryaerophilus* (CCUG 17801^T) and *A. skirrowii* (CCUG 10374^T) were obtained from the Culture Collection of the University of Göteborg, Sweden.

The multiplex PCR for the identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* described by Houf *et al.* (2000) was used with some modifications. Template was prepared as follows. An overnight culture on JM agar was harvested into 100 μl of sterile water using a standardized 1-μl loop. The suspension was heated for 10 min at 98°C and then centrifuged. A 2-μl volume of this preparation was used as the template for the PCR. The PCR was performed using a PCR High Fidelity Master Mix (Roche Cat # 2 140 314) and consisted of 25 μl of Master Mix, 2 μl of template, 50 pmol of primers ARCO, BUTZ, CRY1 and CRY2 and 25 pmol of primer SKIR and sterile water sufficient to make a final volume of 50 μl. PCR consisted of an initial denaturation at 94°C for 2 min and 32 cycles of denaturation at 94°C for 45 s, primer annealing at 61°C for 45 s and chain extension at 72°C for 30 s

on a Hybaid Omnigene thermocycler (Thermo Hybaid Ltd, Middlesex, UK). A 10-μl sample of the assay was electrophoresed through a 1·5% agarose gel containing Tris-Acetate-EDTA (40 mmol l^{-1} of Tris-acetate, 2 mmol l^{-1} of EDTA, pH 7·5) and ethidium bromide (0·5 μg ml^{-1}) in TAE buffer at 5·5 V cm^{-1} for 1 h. The gel was viewed by ultraviolet illumination.

Amplification and sequencing of 16S rRNA

Arcobacter isolates that did not react in the multiplex PCR were subjected to partial 16S rDNA sequencing. DNA from 2-day-old cultures was extracted using the QIAamp DNA Mini Kit (QIAGEN Cat # 51306) as per manufacturer's instructions. The DNA concentration was estimated using a spectrophotometer (Biophotometer, Eppendorf) and 16–160 ng of DNA was used in each PCR reaction. The 100-μl PCR reaction mixture contained 10 μl of 10× PCR buffer (Roche Cat 11146173001), 200 mmol l^{-1} of each dNTP (Roche Cat #11814362001), 0·4 μmol l^{-1} of each of the forward (27f) and reverse (1525r) primers (Lane 1991) and 1·6 units of *Taq* DNA polymerase (Roche Cat # 11146173001). The PCR was performed using a Hybaid Express Thermal Cycler (Thermo Hybaid Ltd). Cycling consisted of an initial denaturation at 98°C for 2·5 min followed by 29 cycles of denaturation at 93°C for 1 min, annealing at 52°C for 45 s and extension at 72°C for 2 min. This was followed by a final cycle of denaturation at 93°C for 1 min, annealing at 52°C for 45 s and extension at 72°C for 10 min. Amplicons were purified from the PCR reaction using a Montage PCR column (Millipore, Cat # UFC7PCR50) as per the manufacturer's instructions. The resulting DNA was sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Cat # US81050) on an ABI Prism 377 DNA sequencer (PE Applied Biosystems Inc., Foster City, CA, USA). Both strands of the 16S rDNA were sequenced.

The resulting sequences were analysed using a FASTA search on the European Bioinformatics Institute website (http://www.ebi.ac.uk).

Results

Evaluation of direct plating media

When inoculated directly with dilutions of either pig faeces or pig effluent, both CAT agar and *Arcobacter* agar were overgrown with *Pseudomonas*-like organisms. There was evidence of colonies typical of *Arcobacter* present on some plates; however, the *Pseudomonas*-like organisms overwhelmed the slower growing *Arcobacter*-like organisms.

Table 1 *Arcobacter* spp. levels in pond effluent and soil freshly irrigated with effluent at six piggeries

		MPN (expressed as 100 ml^{-1} of effluent or gm^{-1} of soil)	
Piggery	Season	Effluent	Soil
C	Summer 2004	$4·3 \times 10^7$	ND*
	Winter 2003	$2·3 \times 10^6$;$1·1 \times 10^8$	ND
D	Summer 2004	$1·1 \times 10^8$	ND
G	Summer 2002	$4·3 \times 10^6$	$5·4 \times 10^3$
	Winter 2002	$2·5 \times 10^7$	$1·4 \times 10^4$
K	Summer 2003	$4·3 \times 10^6$	$4·1 \times 10^3$
	Winter 2003	$4·3 \times 10^6$	$2·8 \times 10^4$
R	Summer 2003	$1·4 \times 10^6$	$1·4 \times 10^4$
	Winter 2003	$6·5 \times 10^5$	$9·5 \times 10^2$
T	Summer 2004	$>1·1 \times 10^7$	$2·4 \times 10^4$
	Winter 2004	$4·3 \times 10^7$	$4·3 \times 10^3$
W	Summer 2002	$9·3 \times 10^5$	$3·3 \times 10^3$
	Winter 2002	$4·6 \times 10^6$	$2·5 \times 10^4$

*ND, not done; MPN, Most Probable Number.

Selection of a suitable MPN method for *Arcobacter* spp.

The MPN methods, A, B and C, were evaluated using the same sample of fresh piggery effluent. Method A did not yield typical *Arcobacter* spp. colonies at any of the three dilutions tested (0, 10^{-1} and 10^{-2}) meaning a count of <30 *Arcobacter* spp. MPN 100 ml^{-1}. Little or no growth following enrichment and plating was observed with method B (again yielding a count of <30 *Arcobacter* spp. MPN 100 ml^{-1}). However, method C yielded positives for *Arcobacter* spp. for all three dilutions tested (0, 10^{-1} and 10^{-2}) – a count of >11 000 *Arcobacter* spp. MPN 100 ml^{-1} of effluent. When tested using soil freshly irrigated with effluent, method C again yielded positives for *Arcobacter* spp. for all three dilutions tested (0, 10^{-1} and 10^{-2}) – a count of >110 *Arcobacter* spp. MPN gm^{-1} for treated soil. Thus, method C was selected as the MPN method for the enumeration of *Arcobacter* spp. in both piggery effluent and soil, in all further work in this study.

Levels of *Arcobacter* spp. in piggery effluent and effluent-treated soil

The levels of *Arcobacter* spp. in pig effluent and freshly irrigated soil are shown in Table 1. The effluent levels ranged from a minimum of $6·5 \times 10^5$ to a maximum of $1·1 \times 10^8$ MPN 100 ml^{-1} and did not vary much between winter (15°C–25°C) and summer (20°C–35°C). The mean level of *Arcobacter* spp. in the ponds was $2·7 \times 10^7$ MPN 100 ml^{-1}. The levels in soil varied from $9·5 \times 10^2$ to $2·8 \times 10^4$ MPN g^{-1}.

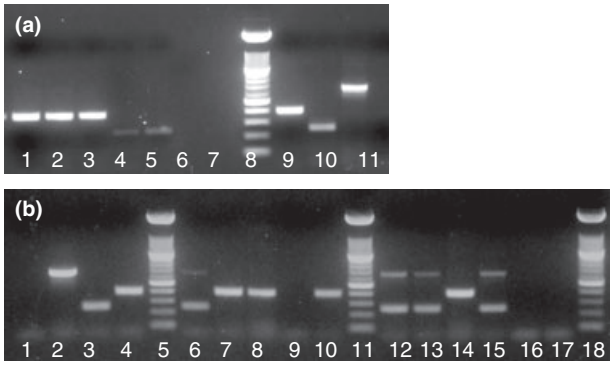

Figure 1 Example of multiplex *Arcobacter* polymerase chain reaction (PCR) results. (a) lanes 1, 2, 3: field isolates of *Arcobacter butzleri*; lanes 4, 5: field isolates of *Arcobacter cryaerophilus*; lanes 6, 7: negative control; lane 8: molecular weight marker; lane 9: *A. butzleri* CCUG 30485^T; lane 10: *A. cryaerophilus* CCUG 17801^T; lane 11: *Arcobacter skirrowii* CCUG 10374^T. (b) lane 1: negative control; lane 2: *A. skirrowii* CCUG 10374^T; lane 3: *A. cryaerophilus* CCUG 17801^T; lane 4: *A. butzleri* CCUG 30485^T; lanes 5, 11 and 18: molecular weight markers; lanes 6, 12, 13 and 15: field isolates of *A. cryaerophilus*; lanes 7, 8, 10 and 14: field isolates of *A. butzleri*; lanes 9, 16 and 17: field isolates that did not react in the multiplex PCR.

Validation of multiplex PCR

When used on the reference strains, the multiplex PCR of Houf *et al.* (2000) gave the expected bands of 641 bp for *A. skirrowii*, 401 bp for *A. butzleri* and 257 bp for *A. cryaerophilus* (see Fig. 1a,b). We intermittently observed nonspecific bands for both the *A. cryaerophilus* reference and field isolates at around 640 bp (Fig. 1b, lanes 6, 12, 13 and 15). These nonspecific bands were considerably fainter than the specific band and did not interfere with the ability of the PCR to correctly identify *A. cryaerophilus*.

Diversity of *Arcobacter* spp.

Over the 3-year course of the study, 83 isolates, 38 from effluent and 45 from soil treated with effluent, were selected to represent the phenotypic variance (colony morphology, catalase reaction and cadmium chloride sensitivity) seen during the confirmation of *Arcobacter* spp. All these isolates were examined by the multiplex PCR. The PCR confirmed 29 isolates (18 from soil, 11 from effluent) as *A. butzleri* and 41 isolates (17 from soil, 24 from effluent) as *A. cryaerophilus*. A further 13 isolates (10 from soil, 3 from effluent) did not give a band in the multiplex PCR. However, when subjected to partial 16S rDNA sequencing, all 13 multiplex PCR-negative *Arcobacter* isolates showed a high similarity (>99%) to *A. cibarius* as determined by the FASTA searches.

The distribution of three *Arcobacter* spp. detected in this work is shown in Table 2. *Arcobacter butzleri* and *A. cryaerophilus* were well distributed in both soil and effluent. *Arcobacter butzleri* was present in five of the six soil samples and four of the six effluent samples. *Arcobacter cryaerophilus* was present in five of the six effluent and soil samples. In contrast, *A. cibarius* was present in only one effluent sample and three soil samples.

Correlation between key phenotypic characteristics and PCR/sequencing results

Table 3 shows the results of the comparison between the key phenotypic characteristics, catalase reaction and cadmium chloride sensitivity and the species identification as confirmed by PCR or 16S rDNA sequencing. All 29 isolates that were 'weak' catalase-positive and cadmium chloride-resistant were confirmed as *A. butzleri*. All 33 isolates that were 'rapid' catalase-positive and cadmium chloride-sensitive were confirmed as *A. cryaerophilus*. Of the 17 isolates that had a 'weak' catalase reaction and were sensitive to cadmium chloride 13 were identified as *A. cibarius* and four as *A. cryaerophilus*. A further four confirmed that *A. cryaerophilus* isolates had the unique combination of being 'rapid' catalase-positive and resistant to cadmium chloride.

Discussion

Arcobacter species are present in the faeces of healthy pigs (Van Driessche *et al.* 2004) and thus can be expected to be present in stored effluent. Thus, effluent could be a source of transfer of this organism via the food process chain if the effluent is used within a food production context, e.g. for irrigation. The present study deals with the isolation, enumeration and species distribution of *Arcobacter* in Australian piggery effluent.

Our work has shown the presence of *A. butzleri*, *A. cryaerophilus* and *A. cibarius* in piggery effluent. The effluent ponds examined in this study differed in terms of their retention times and other physical parameters. Despite these variations, *Arcobacter* spp. were present in all seven ponds examined (across seasonal variations) and at levels of at least 10^6 MPN 100 ml^{-1}.

As indicated in Table 2, we established the simultaneous presence of two species of *Arcobacter* in four of the six effluent ponds. Van Driessche *et al.* (2004) have reported that healthy pigs can simultaneously be shedding two or three species of *Arcobacter*. We did not detect the presence of *A. skirrowii* – a species that has been found in the internal organs of aborted piglets (On *et al.* 2002) and the faeces of healthy pigs (Van Driessche *et al.* 2004). Van Driessche *et al.* (2004) reported that *A. skirrowii* was

Table 2 Distribution of *Arcobacter* species across different piggeries in effluent and soil samples*

Piggery	Substrate	Season	*Arcobacter butzleri*	*Arcobacter cryaerophilus*	*Arcobacter cibarius*
D	Effluent	Winter	4/12	8/12	0/12
G	Effluent	Winter	0/4	4/4	0/4
	Soil	Winter	0/5	5/5	0/5
K	Effluent	Winter	2/3	1/3	0/3
	Soil	Winter	6/12	2/12	4/12
R	Effluent	Winter	1/2	1/2	0/2
	Soil	Winter	2/6	4/6	0/6
T	Effluent	Summer	4/4	0/4	0/4
	Soil	Summer	7/12	4/12	1/12
	Soil	Winter	2/2	0/2	0/2
W	Effluent	Winter	0/13	10/13	3/13
	Soil	Winter	1/8	2/8	5/8

*Results are presented as number positive over number tested.

Table 3 Comparison of polymerase chain reaction (PCR) results with phenotypic characteristics

PCR identification	Number of isolates	Sensitivity to cadmium chloride*	Catalase Reaction†
Arcobacter butzleri	29	R	W
Arcobacter cibarius	13	S	W
Arcobacter cryaerophilus	33	S	P
	4	S	W
	4	R	P

*Any zone of inhibition recorded as sensitive (S); growth continuous to the disc was recorded as resistant (R).
†P, positive (frank bubbling immediately on mixing); W, weak (bubbling detected 10–15 s after mixing cells with reagent).

the least common of the three species present (*A. butzleri* 67%, *A. cryaerophilus* 23% and *A. skirrowii* 7%).

Arcobacter cibarius has only been recently recognized and only in association with broiler carcasses (Houf *et al.* 2005). Our study appears to be the first to associate this organism with pigs and the pig environment. The pathogenicity of *A. cibarius* is unknown and the significance of *A. cibarius* in piggery effluent is also unknown. It should be noted that On *et al.* (2002) reported that 6 of 27 *Arcobacter* isolates associated with porcine abortions could not be assigned to a recognized species. It is possible that a range of currently unrecognized species of *Arcobacter* may be present in pigs.

The multiplex PCR of Houf *et al.* (2000) gave some occasional nonspecific bands with both field isolates and the reference strain of *A. cryaerophilus*. This nonspecific reaction has not been reported by others. These nonspecific bands occurred at around the same molecular weight as the specific band for *A. skirrowii*. As we used the multiplex PCR only on pure cultures, this nonspecific band was not a problem. However, if this multiplex PCR was used on direct samples or on enrichment broths, it would be difficult to confidently conclude whether *A. cryaerophilus* or *A. cryaerophilus* and *A. skirrowii* were present.

We have shown that the enumeration of *Arcobacter* spp. from piggery effluent and soil treated with piggery effluent can be performed by an MPN method, the first such report of an MPN method. We developed the MPN approach by adopting an existing method for the selective isolation of *Arcobacter* spp. (Johnson and Murano 1999a,b). This MPN method resulted in a low level of competing bacteria under aerobic incubation thus allowing recognition of the typical *Arcobacter* spp. We found that the colony morphology of *Arcobacter* spp. was distinct, as originally reported (Johnson and Murano 1999a,b). Using this MPN method, we were able to isolate the faster growing *A. butzleri,* as well as the slower growing *A. cryaerophilus* and *A. cibarius,* after 48 h at 30°C under aerobic conditions. The distinct colony size difference between *A. butzleri* and *A. cibarius*/*A. cryaerophilus* makes it possible to recognize the presence of multiple species within the one sample.

Of the three methods initially trialled to enumerate *Arcobacter* levels, our work demonstrated that two alternative MPN methods, one based on CAT supplements and the other on the antimicrobials of Houf *et al.* (2001) were not suitable for piggery effluent. We found that the alternative method based on the use of antimicrobials described by Houf *et al.* (2001) was too selective when used with piggery effluent. The difficulty of overgrowth on isolation media containing CAT supplements that was observed in the present study has also been reported by others (Atabay and Corry 1997; Rivas *et al.* 2004). Atabay and Corry (1997) were able to overcome this problem by using either the Steele and McDermott (1984) or the Lammerding *et al.* (1996) filter methods on the enrichment before plating onto agar. In our view, the use of

Table 4 Suggested extended phenotypic scheme for the presumptive differentiation of *Arcobacter* species known to be associated with pigs*

Organism	Sensitivity to cadmium chloride†	Catalase reaction‡	Nitrate reduction	Growth in 4% sodium chloride
Arcobacter butzleri	R	W	+	–
Arcobacter cryaerophilus (80%)	S	P	+	–
(10%)	S	W	+	–
(10%)	R	P	+	–
Arcobacter cibarius	S	W	–	–
Arcobacter skirrowii	?	P	+	+

*Data for nitrate reduction and growth in 4% NaCl and all data for *A. skirrowii* from Houf *et al.* (2005). All other data from current study.
†Any zone of inhibition recorded as sensitive (S); growth continuous to the disc was recorded as resistant (R).
‡P, positive (frank bubbling immediately on mixing); W, weak (bubbling detected 10–15 s after mixing cells with the reagent).

filtration after enrichment and before plating is neither a convenient nor a suitable process within a context of an MPN method.

We used an abbreviated phenotypic testing scheme to screen the isolates of *Arcobacter* spp. The scheme proved useful and effective. By comparing the results of the testing with the PCR results, we have shown that all *A. butzleri* isolates are resistant to cadmium chloride and showed weak catalase activity (Table 3). The catalase test for *A. butzleri* has been ambiguously described in the literature, been reported as positive in 33% of the isolates (On *et al.* 1996, 2002) or in 100% of isolates that are weak positive (Vandamme *et al.* 1992; Schroeder-Tucker *et al.* 1996; Harrass *et al.* 1998; Atabay *et al.* 2006). Our results suggest that *A. butzleri* could be regarded as being uniformly weakly catalase-positive. It is possible that those studies that reported variable catalase activity were not aware of the need for a careful examination of the catalase reaction. Resistance to cadmium chloride has been consistently found in *A. butzleri* (Schroeder-Tucker *et al.* 1996).

We found that most, but not all, *A. cryaerophilus* isolates are strongly catalase-positive and are sensitive to cadmium chloride. While a number of other studies have reported that *A. cryaerophilus* are catalase-positive and sensitive to cadmium chloride, the occurrence of cadmium chloride resistance and weak catalase reaction has been reported by Kiehlbauch *et al.* (1991).

We found that our abbreviated phenotypic system resulted in a clear distinction of *A. butzleri* and *A. cryaerophilus* (Table 3). However, *A. cibarius* isolates produced a pattern that was not distinguishable from a small percentage of *A. cryaerophilus* isolates – being sensitive to cadmium chloride and showing a weak catalase reaction. Based on the work of Houf *et al.* (2005), *A. cibarius* is uniformly unable to reduce nitrates. As well, *A. skirrowii* has the ability to grow in 4% NaCl (Atabay *et al.* 1998). Hence, the addition of two tests would allow a quick phenotypic screening of suspect *Arcobacter* isolates and a presumptive allocation to the four species now recognized as being present in pigs (Table 4). While the original description of *A. cibarius,* which was based on 20 isolates, noted that the catalase reaction was variable (Houf *et al.* 2005), we found that all 13 of our isolates showed weak catalase activity. If isolates of *A. cibarius* do have strong catalase activity, the scheme in Table 4 should recognize the isolates provided that they also fail to reduce nitrates.

An understanding and appreciation of suitable isolation media by clinical microbiologists would increase the frequency of isolation of *Arcobacter* spp. in general and in particular *A. butzleri*, an emerging pathogen of concern, from clinical stool specimens. *Arcobacter* isolates are often obtained using *Campylobacter*-selective media – media which are recognized not to be optimal for *Arcobacter* (Prouzet-Mauleon *et al.* 2006). In France, *A. butzleri* formed 1% of the total *Campylobacter*-like isolations from a surveillance network using a specialized *Campylobacter*-selective medium – Campylosel (bioMérieux, Marcy l'Etoile, France) (Prouzet-Mauleon *et al.* 2006). The adoption of more appropriate isolation media such as that trialled in the present study may aid in better understanding the contribution of *A. butzleri* to human enteric infections as well as animal reservoirs.

There appear to be no previous reports on levels of *Arcobacter* spp. in effluent. However, levels of up to 10^4 CFU g^{-1} have been reported in pig faeces, with the dominant species being *A. butzleri* (Van Driessche *et al.* 2004). We have previously found that the mean level of *Escherichia coli* in 13 piggery effluent ponds in southeast Queensland is 1×10^5 MPN 100 ml^{-1} (Chinivasagam *et al.* 2004). Hence, it would appear that *Arcobacter* levels are around 100 times higher than *E. coli* levels in effluent ponds (10^7 compared with 10^5 MPN 100 ml^{-1}). However, the relative levels of the two organisms appear to be reversed in pig faeces – *Arcobacter* spp. being present at levels up to 10^4 CFU g^{-1} (Van Driessche *et al.* 2004) while *E. coli* has been reported to be typically present at levels of up to 10^8 CFU g^{-1} (Shuval 1991). This difference between *E. coli* and *Arcobacter* levels raises the possibility of *Arcobacter* spp. having the potential to grow in these anaerobic, nutrient-rich piggery effluent ponds. There is clearly then a potential

for the transfer and survival of these organisms within environments receiving effluent. We found that *Arcobacter* levels in freshly irrigated soil were around 10^4 MPN g^{-1}. There is a need to understand the survival of *Arcobacter* in soils receiving piggery effluent.

In conclusion, *Arcobacter* species were isolated from piggery effluent in high levels and can be enumerated using an MPN technique. Three species were identified – *A. butzleri*, *A. cryaerophilus* and *A. cibarius* – with the latter species being associated with pigs for the first time.

Acknowledgements

Australian Pork Limited provided funding for this study. The co-operation of the owners of the seven piggeries involved in this study is gratefully acknowledged.

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