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Arcobacter species in diarrhoeal faeces from humans in New Zealand

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Abstract

Aim To determine the prevalence, genetic diversity and antimicrobial susceptibility of *Arcobacter* spp in faecal samples from humans with diarrhoea in New Zealand.

Methods An enrichment method was used to isolate *Arcobacter* spp from diarrhoeal human faeces submitted to a community laboratory in Hawke's Bay. The identity of isolates was confirmed by PCR and their diversity was determined by pulsed field gel electrophoresis (PFGE). Antibiotic susceptibility was established with E test strips.

Results *Arcobacter* spp were isolated from 12 of 1380 diarrhoeal faecal samples examined (0.9%), including 7 *A. butzleri* and 5 *A. cryaerophilus*. Additional enteric pathogens were detected in four of these diarrhoeal faecal samples. All the *Arcobacter* isolates were genetically distinct and susceptible to ciprofloxacin. Most were also susceptible to erythromycin (92%) but fewer to tetracycline (67%) and ampicillin(50%).

Conclusion *A. butzleri* and *A. cryaerophilus* cause a small proportion of cases of diarrhoea in humans resident in New Zealand.

Arcobacter species, formerly classified as aerotolerant *Campylobacter species*, are widely distributed in production animals, pets, wild animals, and the environment. Colonised animals, particularly poultry, frequently show no symptoms but, on occasions, *Arcobacter* spp. have been implicated in abortions, mastitis and diarrhoea.[1,2] *Arcobacter* spp are also common in foods such as meats and shell fish, and fresh water.[1,2]

Three of the 12 species, *A. butzleri, A. cryaerophilus* and *A. skirowii* have been isolated from humans with diarrhoea or other gastrointestinal symptoms,[1,3] in particular, watery or persistent diarrhoea.[4–7] *A. butzleri* was the only pathogen detected in an outbreak of recurrent abdominal cramps in 10 children aged 3 to 7 years in an Italian school.[8]

Occasionally *A. butzleri*[9-11] and *A. cryaerophilus*[12,13] have been isolated from patients with bacteraemia but *Arcobacter* species have also been isolated from faecal samples from healthy humans.[7,14-16]

Arcobacter spp. have recently been detected in a high proportion of chicken meat samples purchased in Palmerston North, New Zealand[17] so the aim of this study was to investigate their prevalence in the faeces of humans with diarrhoea in one region of New Zealand.

Materials and Methods

All faecal samples sent to a community laboratory in Hawke's Bay, New Zealand, for diagnosis of gastrointestinal infection, between October, 2007 and June, 2008, were cultured for *Arcobacter* spp. after they had had been sampled for routine screening of pathogens.

For the initial enrichment, 1 g of faeces was emulsified in 9 mL of *Arcobacter* broth (Oxoid Ltd, UK) and incubated at 28°C for 48 hrs in a microaerobic atmosphere with gas packs (AnaeroPack System™, Mitsubishi Gas Chemicals, Japan). This was then subcultured onto *Arcobacter* selective agar, containing *Arcobacter* broth (28g L^{-1}), Oxoid No. 1 agar (12g L^{-1}), plus the following antimicrobial agents supplied by Aldrich Sigma NZ: cefoperazone (16mg L^{-1}), trimethoprim (64mg L^{-1}), novobiocin (32mg L^{-1}). amphotericin B (10mg L^{-1}), 5-fluorouracil (100mg L^{-1}).

Agar plates were incubated for 48 hrs in a microaerobic atmosphere. Preliminary identification was based on colony morphology and Gram reaction of the isolates from pure culture, by oxidase test using oxidase strips (Oxoid Ltd, UK), and by dark-field microscopy for darting motility. Presumptive isolates of *Arcobacter* spp, subcultured onto 5% sheep blood agar, were preserved on Microbank porous beads system (Pro-Lab Diagnostic) and stored at -80°C for later molecular characterization.

Routine faecal screening included culture for *Salmonella, Shigella, Campylobacter*, *Yersinia* and *Aeromonas* species. Selected stools were also examined for *E. coli* O157 and/or rotavirus. If requested, a *Helicobacter pylori* faecal antigen test, a *Cryptosporidium* plus *Giardia* species antigen test and microscopic examination for parasites were also done. Clinical data on positive samples was derived from laboratory records. Reference strains of *A. butzleri* (ATCC 49616) and *A. cryaerophilus* (ATCC 43158 and ATCC49942) were obtained from the Institute of Environmental Science and Research Limited (ESR), Wellington, New Zealand. Ethical approval was provided by the Central Ethical Committee (HDEC CEN/07/04/026).

The minimum inhibitory concentration (MIC) of ampicillin, tetracycline, ciprofloxacin and erythromycin was determined for *Arcobacter* spp. grown for 48 hrs on blood agar and suspended in saline to a density equivalent to 1.0 McFarland standard. For each antibiotic-isolate combination, a Mueller Hinton agar plate enriched with 5% sheep blood (Fort Richard, NZ) was spread with 100µL of the suspension, overlaid with an MIC Evaluator strip (Oxoid, UK) and incubated at 28°C for 48 hrs in a microaerobic environment. The MICs were classified as susceptible, intermediate or resistant according to the criteria used in the 1997-2006 NARMS report for *Campylobacter* for tetracycline, erythromycin and ciprofloxacin and for *Salmonella, Shigella* and *E. coli* O157 for ampicillin.[18]

Multiplex polymerase chain reaction (m-PCR) was performed as described by Houf et al (2000),[19] except that loading buffer was omitted, the $MgCl_2$ concentration was increased from 1.3 to 1.5 mmol L^{-1} and one to two colonies of suspected *Arcobacter*, grown for 48 h on 5% sheep blood agar plates at 27±2°C microaerobically, were added directly to the reaction mix which was then heated to 94°C for 3 min prior to amplification in a GeneAmp PCR System 2400 (Biosystems, Singapore) Amplified products were separated by electrophoresis in 1.5% agarose. Gels were stained with ethidium bromide and inspected visually under UV light. DNA from *A. butzleri* (ATCC 49616), and *A. cryaerophilus* (ATCC 43158) type strains were included as positive controls.

For PFGE, frozen-stored isolates of *Arcobacter* were streaked onto 5% sheep blood agar plates and grown microaerobically for 48–72 hours at 27±2°C. Colonies were suspended in 2 mL of phosphate buffered saline (PBS) to a final optical density (OD) of 1.00 ± 0.20. Suspended cells (400 mcL) were mixed with 20 mcL of proteinase K (20 mg mL^{-1}) (Amresco, USA) and equal volumes of 1% Seakem Gold agarose (Cambrex Bioscience, USA) prepared in 0.5× TBE buffer. The mixture was transferred to Chef disposable plug moulds (Bio-Rad, USA) and allowed to solidify at room temperature. Plugs were incubated at 55°C in 5 mL of lysis buffer (50 mM Tris, 50 mM EDTA and 1% Sarcosyl) and 25 mcL of proteinase K for 3 hours.

Treated plugs were washed once with 10-15 mL of MilliQ (MQ) water and four times with 10-15 mL of TE buffer (10 mM Tris and 1 mM EDTA) for 10-15 min at 55°C. About 2 mm of the plug was digested with *Eag*I (New England Biolabs, USA) at 37°C for four hours. The restriction fragments were separated by electrophoresis in 1% of Seakem Gold agarose (Cambrex Bioscience, USA) using a CHEF Mapper (Bio-Rad, USA).

The gels were run using the following conditions: Initial switch time 0.1 seconds, final switch time 90 seconds, run time 20 hours, angle 120°, gradient 6V/cm, temperature 14°C and ramping factor linear.

The gels were stained for 10 minutes in ethidium bromide solution, destained with sterile water and visualised using the Gel-DOC 2000 software (Bio-Rad, USA).

Results

From 1380 diarrhoeal faecal samples, 16 isolates were presumptively identified as *Arcobacter* spp. but only 12 (0.9%) were positive by multiplex PCR.

Table 1. Details of patients whose faeces yielded *Arcobacter* spp

Isolate	Age (yrs)	Sex	Symptoms	Appearance of faeces	*Arcobacter* spp isolated	Other enteric pathogens detected
1	32	M	Diarrhoea lasting 1 week	Diarrhoeic	*butzleri*	None
2	32	M	NR[1]	NR	*butzleri*	None
3	46	F	Persistent diarrhoea lasting 1 week	Loose	*butzleri*	None
4	53	M	Persistent diarrhoea	Loose	*butzleri*	*Helicobacter pylori* antigen positive
5	72	M	NR	Semi-formed	*butzleri*	None
6	76	M	Diarrhoea and vomiting	Soft	*butzleri*	*Aeromonas hydrophila*
7	78	M	?Diarrhoea	Loose	*butzleri*	None
8	2	F	Diarrhoea	Loose	*cryaerophilus*	None
9	31	F	NR	Watery	*cryaerophilus*	None
10	40	F	NR	Loose	*cryaerophilus*	*Blastocystis hominis*
11	56	F	?Diarrhoea	Semi-formed	*cryaerophilus*	*Helicobacter pylori* antigen positive
12	71	F	?Diarrhoea	Semi-formed	*cryaerophilus*	None

[1]NR: none recorded

A. butzleri was cultured mainly from males and *A. cryaerophilus* from females (Table 1) and the difference between the two sexes is statistically significant (p=0.015). Four patients had an additional pathogen detected, namely *Helicobacter pylori* (two), *Blastocystis hominis* and *Aeromonas hydrophila.* All except one of the patients were adults, with ages ranging from 31 to 78 years. Three patients had persistent diarrhoea but, information was not provided for another four.

PFGE indicated that the *Arcobacter* isolates from diarrhoeal faeces were different from each other (data not shown) and also from those from poultry meat previously isolated in Palmerston North.[17]

All of the *Arcobacter* isolates were susceptible to ciprofloxacin and all but one susceptible to erythromycin. That *A. butzleri* isolate was resistant to ampicillin and tetracycline with intermediate resistance to erythromycin (Table 2). Three additional *Arcobacter* isolates showed intermediate resistance to tetracycline. Only half the isolates were susceptible to ampicillin.

Table 2. Antimicrobial susceptibility of Arcobacter spp. isolated from the faeces of patients with diarrhoea

Isolate	*Arcobacter* species	Ciprofloxacin (mg/L) Sens≤1[1]	Erythromycin (mg/L) Sens≤8[1]	Tetracycline (mg/L) Sens≤4[1]	Ampicillin (mg/L) Sens≤8[1]
1	*butzleri*	0.12	4	4	4
2	*butzleri*	0.06	4	4	8
3	*butzleri*	0.06	2	2	32
4	*butzleri*	0.25	8	8	8
5	*butzleri*	0.12	8	8	64
6	*butzleri*	0.25	16	16	32
7	*butzleri*	0.25	4	4	64
8	*cryaerophilus*	0.12	8	8	8
9	*cryaerophilus*	0.06	1	1	64
10	*cryaerophilus*	0.12	2	2	8
11	*cryaerophilus*	0.12	1	1	4
12	*cryaerophilus*	0.25	2	2	16

[1] The resistance break points were ≥4 mg/L for ciprofloxacin, ≥32 mg/L for erythromycin, ≥16 mg/L for tetracycline and ≥32 mg/L for ampicillin.[18]

Discussion

The isolation of *A. butzleri* and *A. cryaerophilus* from 0.9% of diarrhoeal faecal samples collected in Hawke's Bay, New Zealand is consistent with the 1% isolation rate of *A. butzleri* reported for diarrhoeal stools in France[11] but higher than the 0.14% reported for *A. butzleri* and *A. cryaerophilus* in both Belgium[7] and Denmark.[20]

Culture-based methods yielded *A. butzleri* from 2.4% of faecal samples collected from Thai children with diarrhoea[21] but the use of PCR to detect *Arcobacter* spp. directly from faeces has generally yielded a higher proportion of positive results, e.g. 7.5% for *A. butzleri* , 3.5% for *A. cryaerophilus* and 2% for *Arcobacter skirowii* for patients hospitalised with diarrhoea or other gastrointestinal disorders in South Africa[15] and 8% for *A. butzleri* from patients with travellers' diarrhoea who had visited Mexico, Guatemala or India.[22] By contrast, *A. butzleri* was detected in only 1.2% of diarrhoeal stools by means of PCR in another study in France.[23]

However the real significance of *Arcobacter* isolation is difficult to determine since several pathogens have been detected in a number of these patients. In the present study, one third had a second pathogen detected (Table 1) which is comparable with the 20% of patients with *A. butzleri* plus another enteric pathogen reported by Vandenberg et al (2004).[7] The latter group also found that 16% of patients with *A. butzleri* in their faeces had an underlying disease and 20% of the *A. butzleri* isolates were from asymptomatic patients. Of 16 patients with travellers' diarrhoea with *A. butzleri* detected, 15 also harboured either enterotoxigenic *Escherichia coli* (ETEC) or *Campylobacter* sp.[22]

Likewise 20 of 33 patients with *Arcobacter* spp. hospitalised in South Africa had one to three other gastrointestinal pathogens detected.[15] *Arcobacter* spp. have also been detected in faeces collected from asymptomatic patients, including 7 abattoir workers in Switzerland[14] and 26% of healthy subjects in Italy.[16] Interestingly the latter group

found an increased carriage rate of *Arcobacter* spp. (79%) in older people with type 2 diabetes but no gastrointestinal disorders.

Other bacterial species isolated from the 1380 diarrhoeal faecal samples examined for *Arcobacter* spp. in the current study were: *Campylobacter* (15.1%), *Salmonella* (2.6%), *Aeromonas* (2.2%), *Yersinia* (1.9%) and *Shigella* (0.1%) (S. Wallace, personal communication). Thus *Arcobacter* spp. (0.9%) were more common than *Shigella*, much less common than *Campylobacter* spp and roughly similar in frequency to the other enteric bacterial pathogens.

Two studies found that *A. butzleri* was more common in the faeces of females than males[7,8] and one found the opposite[15] but the differences in all studies were small. Another group isolated *A. cryaerophilus* from the faeces of 1.4% of healthy men who worked in abattoirs.[14] Thus it is likely that the unequal distribution of the two *Arcobacter* species across the sexes shown in Table 1, although statistically significant, is not biologically meaningful.

Based on results from single isolates, *Arcobacter* spp. have been described as antibiotic resistant.[9,24] However, the observation that all the isolates in this study were susceptible to ciprofloxacin (Table 2) is consistent with reports that 89 to 100% are susceptible to ciprofloxacin.[11,25–27] Likewise, erythromycin susceptibility (92%, Table 2) and 87 to 100%[27-29] is common among *Arcobacter* spp. By contrast, the relatively low proportion of isolates susceptible to tetracycline in this study (67%, Table 2) differs from the 100% susceptibility reported for isolates from the USA,[27] Japan,[29] and Thailand[26] but resistance to ampicillin is common worldwide.[9,28,30]

We conclude that *A. butzleri* and *A. cryaerophilus* do occasionally cause diarrhoea in New Zealanders which may be persistent or watery. However their real significance as emerging enteric pathogens, both in New Zealand and overseas,[1] is unclear. Their ability to colonise healthy animals and survive on meats[1,17] and in the environment does mean human exposure is likely to be common but further studies would be useful to better establish the virulence of *Arcobacter* spp. for humans before recommending that laboratories routinely test for these bacteria.

Competing interests: None declared.

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