Comparative detection and quantification of *Arcobacter butzleri* in stools from diarrheic and non-diarrheic human beings in southwestern Alberta, Canada

Webb AL¹,², Boras VF³, Kruczkiewicz P⁴, Selinger LB³, Taboada EN⁴#, and Inglis GD¹#

Agriculture and Agri-Food Canada, 5403 – 1st Avenue S, Lethbridge, AB, Canada¹; Department of Biological Sciences, University of Lethbridge, Lethbridge, AB, Canada²; Department of Laboratory Medicine, Chinook Regional Hospital, 960-19th Street S, Lethbridge, AB, Canada³; Public Health Agency of Canada, Township Rd. 9-1, Lethbridge, AB, Canada⁴

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#Address correspondence to: G Douglas Inglis, douglas.inglis@agr.gc.ca, or Eduardo Taboada, eduardo.taboada@phac-aspc.gc.ca
Arcobacter butzleri has been linked to enteric disease in human beings, but its pathogenicity and epidemiology remain poorly understood. The lack of suitable detection methods is a major limitation. Using comparative genome analysis, we developed PCR primers for direct detection and quantification of A. butzleri DNA in microbiologically-complex matrices. These primers, along with existing molecular and culture-based methods, were used to detect A. butzleri and enteric pathogens in stools of diarrheic and non-diarrheic people (n=1596) living in southwestern Alberta from May to November 2008. In addition, real-time quantitative PCR was used to compare A. butzleri densities in diarrheic and non-diarrheic stools. Arcobacter butzleri was detected more often by PCR (59.6%) than by isolation methods (0.8%). Comparison by PCR-based detection found no difference in prevalence of A. butzleri between diarrheic (56.7%) and non-diarrheic (45.5%) individuals. Rates of detection in diarrheic stools peaked in June (71.1%) and October (68.7%), but there was no statistically significant correlation between the presence of A. butzleri and patient age, sex, or place of habitation. Densities of A. butzleri DNA in diarrheic stools (1.6 log_{10} ± 0.59 copies mg^{-1}) were higher (P=0.007) than in non-diarrheic stools (1.3 log_{10} ± 0.63 copies mg^{-1}). Of the 892 diarrheic samples that were positive for A. butzleri, 74.1% were not positive for other bacterial and/or viral pathogens. The current study supports previous work suggesting that A. butzleri pathogenicity is strain-specific, and/or dependent on other factors such as the level of host resistance.
[INTRODUCTION]

Nearly 1.7 billion cases of diarrheal disease are reported globally each year (1), although this is an underestimation of true rates of enteritis as many afflicted individuals do not have access to or choose not to pursue medical assistance (2). For those seeking diagnosis, the majority of cases of acute enteritis are not linked to an identified etiological agent (3, 4). Ascertaining the etiology of enteric disease is essential for the development of effective therapeutics and preventative mitigation strategies. Direct contact with animals and ingestion of untreated water and/or undercooked animal products are recognized risk factors for acute enteritis (3), which suggests that a significant number of cases of enteritis are incited by unidentified biotic pathogens of human or zoonotic origin. Critical components of the epidemiology of arcobacteriosis and the population structure of *A. butzleri* have yet to be resolved, in large part because effective culture and/or molecular-based detection methods for this bacterium have yet to be developed. *Arcobacter butzleri* is ubiquitous in the environment (e.g. river water contaminated with human and/or non-human animal feces) (5-7). That the bacterium is detected in such a variety of sources suggests that pathways for transmission among animals and environmental sources exist, but accurate source tracking of *A. butzleri* is hampered by a lack of standard detection and isolation methods. Most methods for the isolation of *A. butzleri* from microbiologically-complex matrices rely on selective enrichments and/or antibiotics to inhibit the growth of non-target microorganisms (8, 9). In addition, the incubation temperature and atmosphere utilized for isolation have been inconsistent; temperatures vary from 25°C (10) to 37°C (11), and atmospheres range from aerobic (9, 12) to microaerobic (5-6% O₂, 6-10% CO₂, 0-7% H₂, and 79-85% N₂) and anaerobic (10, 13-15). Accumulated evidence indicates that no single medium, temperature, or atmosphere will isolate all strains of *A. butzleri*. For example, Merga et al. (16) recently compared five media and plating techniques and found that the most effective strategy only detected *A. butzleri* in 70.7% of positive samples.

A number of researchers have utilized primers to detect *A. butzleri* in non-selective enrichment (17, 18). However, no primers have been specifically designed to detect and quantify *A. butzleri* DNA extracted directly from complex matrices without an intermediate enrichment step. Primer development for the detection of microorganisms can be divided into two broad steps: (i) the *in silico* design of primers targeting taxon-specific gene sequences ascertained from comparative analysis of genome data; and (ii) the *in vitro* validation of primer sensitivity (i.e. the minimum detectable amount of target DNA), specificity (i.e. the lack of detection of non-target taxa), and inclusivity (i.e. the detection of all subtypes within a target taxon). During primer design, potential gene targets must be identified and compared to a sequence database to identify marker sites that have conserved nucleotide length, composition, and presence within the target species while being absent from non-target species. As genomic databases cannot contain the entirety of genetic diversity of bacteria, and data are particularly lacking for the genetically diverse *A. butzleri*, developed primers must also be carefully evaluated to ensure sensitivity, specificity, and inclusivity. This is
especially true for development of primers to detect DNA in complex matrices such as feces.

We hypothesized that *A. butzleri* is a significant enteric pathogen that is underdiagnosed because of the limitations of culture-based detection. Thus, *A. butzleri* DNA will be more prevalent in stools from diarrheic than from non-diarrheic individuals (i.e. cohorts in the same space and time). Furthermore, *A. butzleri* loads will be higher in diarrheic stools, and the bacterium will be present in diarrheic stools in the absence of other recognized bacterial and viral pathogens. To test these hypotheses, the following objectives were established: (i) use comparative whole genome sequence analysis to select unique, highly conserved, non-variable loci to develop direct detection and quantification primers for *A. butzleri*; (ii) evaluate the sensitivity, specificity, and inclusivity of the developed primers; (iii) contrast isolation and PCR detection frequency of *A. butzleri* in stools of diarrheic and non-diarrheic people (*n* ≈ 1600) living in southwestern Alberta as a model health region; (iv) use quantitative PCR to contrast *A. butzleri* DNA load in stools from diarrheic and non-diarrheic people; and (v) determine the frequency to which *A. butzleri* occurs with other recognized bacterial and viral pathogens.

**MATERIALS AND METHODS**

**Primer design and in silico evaluation.** The online tool Rapid Annotation Using Subsystem Technology (RAST) (19) was used to identify open reading frames (ORF) for genomic sequences from 12 *A. butzleri* strains available in the NCBI database (PRJNA233527, PRJNA58557, PRJNA158699, PRJNA61483, PRJNA200766), including eight sequenced by our research group (7), along with whole genome sequences from ten additional *A. butzleri* strains (PRJNA309088) provided by Catherine Carrillo (Canadian Food Inspection Agency). The Basic Local Alignment Search Tool (BLAST) (20) and a program developed in-house (Concatenator) were used to compare ORFs between *A. butzleri* strains; those that were redundant or missing from any strains, or that varied in terms of their length or sequence were removed from consideration. The RAST and BLAST tools were also used to compare the *A. butzleri* genomic sequences to those of four *Arcobacter skirrowii* (PRJNA307998) and six *Arcobacter cryaerophilus* (PRJNA307600) strains that were sequenced as part of the current project; any *A. butzleri* ORFs that were detected in *A. skirrowii* or *A. cryaerophilus* were removed from consideration. The program Geneious (version 5.3.6, Biomatters Ltd, Auckland NZ) was used to concatenate and align the remaining sequences, and to identify sites for PCR primer design. Primers for endpoint and real-time PCR were designed for optimal use with HotStar Taq Plus DNA polymerase (Qiagen Inc., Toronto ON) and QuantiTect SYBR® Green (Qiagen Inc.).

**Primer evaluation.** (i) **Primer specificity.** Selected PCR primers were tested for specificity against genomic DNA from 22 type strain taxa within the order *Campylobacterales*, including *Arcobacter* spp. (i.e. *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*), *Campylobacter* spp. (i.e. *C. coli*, *C. concisus*, *C. curvus*, *C. fetus* subspecies *fetus*, *C. hominis*, *C. hyointestinalis* subspecies *hyointestinalis*, *C. insulae* *insulae*, *C. jejuni*, *C. jejuni* subspecies *doyleri*, *C. laniennes*, *C. lari*, *C. mucosalis*, *C. showae*, *C. sputorum* subspecies *sputorum*, and *C. upsaliensis*), and *Helicobacter* spp. (i.e. *H. canadensis*, *H. pullorum*, *H. pylori*). Amplification reactions...
consisted of 2.0 µl 10X PCR Buffer containing 15 mM MgCl₂ (Qiagen Inc.), 2.0 µl UltraPure BSA (1.0 mg ml⁻¹; Ambion, Life Technologies Inc., Burlington ON), 0.4 µl dNTP mix (10 mM; Bio Basic Canada Inc., Markham, ON), 0.1 µl HotStar Taq Plus DNA Polymerase (5.0 U µl⁻¹; Qiagen Inc.), 1.0 µl ddAbutzF (10 µM; Integrated DNA Technologies, Coralville, IA), 1.0 µl ddAbutzR (10 µM; Integrated DNA Technologies), 2.0 µl DNA template, and 11.5 µl Nuclease-Free Water (Qiagen Inc.). The PCR reaction consisted of activation at 95°C for 5.0 m, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 90 s and elongation at 72°C for 60 s, followed by a final elongation at 72°C for 5 m and storage at 4°C. Amplicons were visualized on a QIAxcel capillary electrophoresis machine (Qiagen Inc.) using the AM320 separation and resolution method, with 15-3000 bp alignment marker and 100-2500 bp size marker.

(ii) Primer inclusivity. Primers were evaluated for their ability to amplify DNA from 130 A. butzleri isolates representing 92 different subtypes. The PCR reagents and conditions used for primer evaluation were the same as described for primer specificity. The identity of isolates was confirmed by sequencing the near complete 16S rRNA gene (21). Isolate subtypes were identified using a comparative genomic fingerprinting method (CGF₄₀) specific to A. butzleri (7).

(iii) Primer sensitivity. To determine the limit of detection of developed primers, DNA extracted from porcine feces seeded with A. butzleri was tested; pigs were selected as a monogastric model for human beings. Multiple fresh samples of feces were collected from three pigs obtained from the University of Alberta Swine Unit (Edmonton, AB), and were stored at -20°C. No antibiotics were administered to the pigs.

To produce cells for incorporation into feces, A. butzleri ATCC49616 was cultured in triplicate on Columbia Agar (DF0944-17-0, Difco) containing 10% sheep blood (CBA) in microaerobic atmosphere (i.e. 5% O₂, 3% H₂, 10% CO₂, and 82% N₂) at 37°C for 48 h. Biomass from the three cultures was removed from the surface of the medium and combined in Columbia broth (Difco; CB). The absorbance (A₆₀₀) was adjusted to 0.5, which contained approximately 2.0 x 10⁸ cells ml⁻¹. The suspension was diluted with CB in a ten-fold dilution series. Feces was thawed and 1.0 ml from each dilution of A. butzleri cells was thoroughly mixed into a 10 g subsample of the feces. The control treatment consisted of 10 g of feces mixed with 1.0 ml of sterile CB. Three 0.2 ± 0.02 g subsamples were removed from the seeded feces and stored at -20°C for later DNA extraction. To enumerate A. butzleri cells by culture, a 1.0 g subsample of the seeded feces was suspended in 9.0 ml of CB and diluted in a ten-fold dilution series, and 100 µl of each dilution was spread on CBA in duplicate, cultures were incubated in a microaerobic atmosphere (i.e. 5% O₂, 3% H₂, 10% CO₂, and 82% N₂) at 37°C, and colonies were enumerated at the dilution yielding 30 to 300 CFU after 48 and 96 h. The experiment was conducted two times on separate occasions.

DNA was extracted from the frozen feces subsamples using a QIAamp DNA Stool Mini Kit (Qiagen Inc.) according to the manufacturer’s specifications for pathogen detection. As an internal amplification control (IAC), 2 µl of DNA (1 x 10⁸ copies µl⁻¹) from a synthesized gene designed using the Pyrococcus yayanosii genome (22) was added to the feces subsamples prior to extraction; this bacterium is an obligate
piezophilic hyperthermophilic archaeon isolated from deep-sea hydrothermal sites (23). The IAC targets a 268 bp sequence in a putative carbohydrate kinase (PfkB family; AEH23732.1) using the primers IAC-f (3’- GGTATGCTAGCCCCGCTTAGGGT-5’) and IAC-r (3’-TGCTCCAGAAAGATGTCCAGCGG-5’), and was synthesized by Integrated DNA Technologies. The presence and quantities of the IAC was confirmed by real-time PCR amplification on a Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara CA) using the following reagents: 10 µl 2X Quantitect SYBR Green (Qiagen Inc.), 2.0 µl UltraPure BSA (1.0 mg ml⁻¹; Ambion), 1.0 µl primer IAC-f (10 µM; Integrated DNA Technologies), 1.0 µl primer IAC-r (10 µM; Integrated DNA Technologies), 2.0 µl DNA template, and 4.0 µl Nuclease-Free Water (Qiagen Inc.). Samples were quantified in duplicate reactions. The amplification conditions were one cycle at 95°C for 15 m, followed by 40 cycles of 15 s at 94°C, 30 s at 64°C, and 30 s at 72°C for data acquisition. Direct endpoint detection of A. butzleri DNA was carried out as described above for primer specificity. Real-time PCR detection of A. butzleri was carried out on a Stratagene Mx3005P qPCR System (Agilent Technologies) using the following reagents: 10 µl 2X Quantitect SYBR Green mastermix (Qiagen Inc.), 2.0 µl UltraPure BSA (1.0 mg ml⁻¹; Ambion), 1.0 µl ddAbutzF (10 µM; Integrated DNA Technologies), 1.0 µl ddAbutzR (10 µM; Integrated DNA Technologies), 2.0 µl DNA template, and 4.0 µl Nuclease-Free Water (Qiagen Inc.). Samples were quantified in duplicate reactions. The amplification conditions were one cycle at 95°C for 15 m, followed by 40 cycles of 30 s at 94°C, 90 s at 65°C, and 60 s at 72°C for data acquisition. At the end of amplification, melt curve analysis was conducted. The quantitative PCR data were analysed using MxPro (Version 4.10, Agilent Technologies Inc.).

Detection and quantification of A. butzleri in diarrheic and non-diarrheic stools. (i) Ethics approval. Scientific and ethics approval to isolate, detect, and quantify A. butzleri from diarrheic and non-diarrheic human beings (i.e. healthy volunteers) was obtained from the Regional Ethics Committee of the former Chinook Health Region and from the University of Lethbridge Human Subject Research Committee. (ii) Acquisition of stool samples. A total of 1506 stool samples were obtained from diarrheic individuals submitting samples to the Chinook Regional Hospital between May 1 and November 25, 2008. Stool samples from diarrheic people were suspended in Cary-Blair medium (24) for transportation to the Chinook Regional Hospital in Lethbridge, AB. In addition, stool samples were obtained from 90 non-diarrheic volunteers from October 27, 2008 to November 12, 2008. Samples were kept at 4°C for no longer than 24 h. Information provided with the samples included stool collection date, along with the age, sex, and place of habitation (i.e. postal code) of the submitting individual. Using the same method as described for seeded porcine feces, 0.2 ± 0.02 g subsamples were taken from stools and stored at -20°C for later DNA extraction. (iii) Isolation of A. butzleri. Media for isolation of A. butzleri were CBA, Karmali agar (CM0935, Oxoid) with Karmali supplement (KS; SR0167, Oxoid), Karmali agar (CM0935, Oxoid) with Bolton supplement (KB; SR0183E, Oxoid), Arcobacter Selection and Isolation Agar (ASIA) (25), and Johnson Murano Agar (JMA) (26). The isolation method varied by medium: membrane filtration (13) was used for CBA; direct plating of 100 µl
of the processed sample was used for KS, KB, and ASIA; and Bolton broth (CM0983, Oxoid) with Bolton supplement (SR0183E, Oxoid) (BBS) was used for enrichment culture with subsequent isolation on KS, KB, ASIA, and JMA. The CBA cultures were incubated at 37°C for up to ten days, and all other agar media were incubated at both 30°C and 37°C for 72 h. All cultures were maintained in a high hydrogen atmosphere (i.e. 5% O₂, 30% H₂, 10% CO₂, and 55% N₂). For enrichment cultures, 25 µl of each sample was added to 2.0 ml of BBS and incubated at both 30°C and 37°C. At 24 and 48 h, 10 µl of the enrichment was streaked on the KS, KB, ASIA, and JMA.

Two colonies per morphology per medium per sample were collected and streaked for purity on CBA, and examined microscopically for cell size, shape, and motility. Genomic DNA was extracted from isolates using the DNeasy Blood and Tissue Kit (Qiagen Inc.) according to manufacturer specifications and an automated system (Model 740, Autogen, Holliston, MA). *Arcobacter butzleri* DNA was identified by taxon-specific PCR using the same reagents and conditions as specified for primer specificity, and sequencing of the near complete 16S rRNA gene (21). All recovered *A. butzleri* isolates were subtyped using CGF40 (7).

(iv) Extraction of total DNA from feces and direct detection of *A. butzleri* DNA. The IAC was added to all stool subsamples, and genomic DNA was extracted using the QiAamp DNA Stool Mini Kit (Qiagen Inc.). Real-time PCR for the IAC and endpoint PCR for *A. butzleri* were conducted as described for seeded porcine feces. Amplifications were scored as positive or negative, and only samples that were positive for the IAC in the absence of *A. butzleri* amplification were considered to be true negatives.

(v) Specificity of primers in stools by sequencing of direct PCR amplicons. To confirm the specificity of amplification, 90 arbitrarily-selected amplicons were sequenced. In order generate enough product for sequence analysis, the *A. butzleri* PCR reaction volume was doubled to 40 µl, containing 4.0 µl 10X PCR Buffer with 15 mM MgCl₂ (Qiagen Inc.), 4.0 µl UltraPure BSA (1.0 mg ml⁻¹; Ambion), 0.8 µl dNTP mix (10 mM; Bio Basic), 0.2 µl HotStar Taq Plus (5.0 U µl⁻¹; Qiagen Inc.), 2.0 µl ddAbutzF (10 µM; Integrated DNA Technologies), 2.0 µl ddAbutzR (10 µM; Integrated DNA Technologies), 4.0 µl DNA template, and 23 µl Nuclease-Free Water (Qiagen Inc.). The PCR reaction mix was activated at 95°C for 5 m, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 60 s, and elongation at 72°C for 30 s, a final elongation at 72°C for 5 m, and storage at 4°C. Products were purified with a MinElute 96 UF Purification Kit (Qiagen Inc.), and rehydrated to 20.0 µl. Sequencing was conducted by Eurofins MWG Operon, and sequences were aligned in Geneious (Version 5.3.6, Biomatters) and identified using the BLAST program in NCBI.

(vi) Quantification of *A. butzleri* DNA extracted from stools. DNA from human diarrheic (n=69) and non-diarrheic (n=50) stools collected during the same time period (i.e. October 27 to November 11, 2008) that tested positive for *A. butzleri* by direct detection PCR was quantified by real-time PCR using the same conditions as for seeded porcine feces.

(vii) Comparison of *A. butzleri* prevalence to known pathogens. The current study was part of a larger...
study examining the prevalence of bacterial and viral pathogens in stools from diarrheic and non-diarrheic people living in southwestern Alberta. All samples were processed by staff at the Chinook Regional Hospital for *Aeromonas* spp. (i.e. *A. caviae*, *A. hydrophilia*, *A. salmonicida*, *A. sobria*, and *A. veronii*) (27), *Edwardsiella* spp. (*E. hoshinae* and *E. tarda*) (28), *Campylobacter* spp. (*C. coli*, *C. fetus*, *C. lari*, *C. jejuni*) (29), *Escherichia coli* 0157:H7 (30), *Plesiomonas shigelloides* (28), *Salmonella enterica enterica* (30), *Shigella* spp. (*S. boydii*, *S. dysenteriae*, *S. flexneri*, *S. sonnei*) (30), *Staphylococcus aureus* (31), *Vibrio* spp. (*V. alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. metschnikovii*, *V. mimicus*, *V. parahemolyticus*, and *V. vulnificus*) (32), and *Yersinia* spp. (*Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. ruckeri*) (33). In addition, RNA viruses (Norovirus GI, GII, GIII, GIV, Sapovirus, Rotavirus, Astrovirus) were detected using Taqman PCR (34) (D. Leblanc, G. D. Inglis, V. F. Boras, J. Brassard, and A. Houde, submitted for publication).

(viii) Data analysis. All statistical analyses were carried out using SigmaPlot (version 12.0, Systat Software, San Jose CA). The chi-square test of independence was used to calculate significant differences in prevalence of *A. butzleri* between diarrheic and non-diarrheic people by culture-based isolation and by PCR-based detection, as well as for calculating significant differences in prevalence of *A. butzleri* in diarrheic humans by age, sex and location. The chi-square test of independence was also used to ascertain possible difference in rates of coinfection of *A. butzleri* with known pathogens in diarrheic human beings. In order to determine if significant differences existed in the rate of coinfection of *A. butzleri* with more than two tested pathogens, the rate of coinfection for each pathogen was compared to the mean coinfection of all other pathogens. The Mann-Whitney Rank Sum test was used to calculate significant difference between abundance of *A. butzleri* in stools from diarrheic and non-diarrheic human beings.

**RESULTS**

Primer design and *in silico* evaluation. Comparative whole genome sequence analysis of *Arcobacter* species revealed 1906 conserved ORFs. Of the 66 ORFs that were not present in *A. skirrowii* or *A. cryaerophilus*, 48 did not contain sufficient length or sequence variation, and 42 were also longer than 300 bp. These 42 ORFs were concatenated for further analysis. The gene sequence for PCR amplification was required to be no more than 200 bp long, with a primer length between 19 and 23 nucleotides, a GC content of 35% to 65%, a melting temperature of 60°C to 68°C, and self-annealing or cross-annealing stretches less than four bp in length. The designed primers (ddAbutzF: 5'-AGTGATGGTGGAGTTGCTAGTC-3'; ddAbutzR: 5'-GTTGCAGGAGCTTTTTCACTCC-3') targeted a sequence that was identified as part of a putative gene encoding the gamma subunit of quinohemoprotein amine dehydrogenase (WP_004510536.1). *In silico* analysis of 22 *A. butzleri* strains (PRJNA233527, PRJNA58557, PRJNA158699, PRJNA61483, PRJNA200766, and PRJNA309088) identified a single copy of the target sequence per genome. The predicted PCR product was 137 bp, and was unique to *A. butzleri* by BLAST analysis (35). In addition, the primer target sequences were identical to all available *A. butzleri* genomes, and the closest non-target match possessed 79% query coverage.
Primer evaluation. (i) Primer specificity. Of the 22 taxa within *Campylobacterales* that were evaluated, only *A. butzleri* produced a detectable PCR amplification product when tested with the ddAbutz primers.

(ii) Primer inclusivity. All 130 isolates (100%) were amplified by endpoint PCR using the ddAbutz primers.

(iii) Primer sensitivity. The ddAbutz primers amplified *A. butzleri* DNA at concentrations as low as 0.6 Log_{10} copies mg^{-1} by endpoint PCR and RTQ-PCR. This equated to a minimum detection limit of 1.1 copies per reaction.

Detection and quantification of *A. butzleri* in diarrheic and non-diarrheic stools. (i) Isolation of *A. butzleri*. The overall rate of detection of *A. butzleri* by culture-based isolation using a variety of media and plating methods was low (0.8%), and there was no difference (P=0.81) in detection between diarrheic and non-diarrheic individuals (Table S1). For culture positive samples, 8 of 13 were positive by a single method, and membrane filtration on CBA was the most inclusive (46%). No *A. butzleri* isolates were obtained by direct plating of processed stools onto KS. No medium and plating technique was specific to *A. butzleri*; each selected for at least one non-target bacterium (Table S2). There were too few *A. butzleri* positive stools to compare the effectiveness of direct plating compared to enrichment techniques.

(ii) Total DNA extraction and detection of *A. butzleri* DNA. Of the 1596 human stool samples tested, an IAC and/or *A. butzleri* amplicon were not observed in extracted DNA from 26 samples (1.6%). Of the remaining 1570 stools, 1482 samples were obtained from diarrheic people and 88 were obtained from non-diarrheic people. The overall prevalence of *A. butzleri* was 60%, and there was no difference (P=0.13) in prevalence of *A. butzleri* DNA between diarrheic (57%) and non-diarrheic (46%) stools. The rate of detection of *A. butzleri* in diarrheic individuals varied throughout the sample period with peaks at the beginning and the end of the summer (Fig.1). No correlation was observed between *A. butzleri* prevalence in diarrheic stools with sex (P=0.37), age (P≥0.26), or place of habitation (P=0.15) (Table 1).

(iii) Specificity of PCR primers in diarrheic stools by PCR amplification. All 90 (100%) of the amplicons from human stools that were sequenced were identified as *A. butzleri* by BLAST analysis. Trimmed sequences were 93 bp to 95 bp in length. Because the sequences were identical, a single consensus sequence was compared to the NCBI database.

(iv) Quantification of *A. butzleri*. Overall cell density in human stool samples was 1.4 ± 0.62 Log_{10} cells mg^{-1}, but quantities of DNA were higher (P=0.007) in stools of diarrheic (1.6 Log_{10} ± 0.59 copies mg^{-1}) than non-diarrheic (1.3 Log_{10} ± 0.63 copies mg^{-1}) people.

(v) Comparison of *A. butzleri* prevalence to known pathogens. Of the 1482 diarrheic samples examined, 390 (26%) were positive for recognized bacterial and/or viral pathogens. Of the samples positive for *A. butzleri*, 661 (74%) were not positive for other bacterial and/or viral pathogens. None of the recognized pathogens were more likely to be co-detected with *A. butzleri* (P≥0.26) (Table 2).
DISCUSSION

Efficiency of *A. butzleri* detection methods. In the current study we compared the detection of *A. butzleri* by isolation to detection by PCR. We found the rate of detection of *A. butzleri* in human stools by isolation was low (0.8%) compared to PCR-based detection (60%). Others have found that PCR was more effective than culturing for detection of *A. butzleri* in human stools (12), seawater (36), and wastewater and chicken carcasses (37). Fera et al. (12) suggested that the decreased rate of detection observed in selective and enrichment media may be the result of competition by non-target members of the source microbiota, along with difficulty replicating source conditions for growth in a controlled system. In addition, the use of enrichment culture has been shown to reduce the diversity of other enteric pathogens (38, 39), and antimicrobial agents in *A. butzleri* selective media may also reduce diversity (40). This is problematic because antimicrobial agents are often required to inhibit growth of non-target taxa that could exclude *A. butzleri*. We frequently isolated presumptive *A. butzleri* based on colony morphology that turned out to be *Alistipes spp.*, *Bacteroides spp.*, *Catabacter spp.*, *Citrobacter spp.*, *Helicobacter spp.*, and particularly *Campylobacter spp.*, which were very commonly recovered. Previous studies have noted a similar lack of specificity for culture isolation of *A. butzleri* from fecal samples (10, 16).

Prevalence of *A. butzleri* in human stools. We observed that the overall prevalence of *A. butzleri* in human stools was 60%, which is much higher than rates of 25% or less reported by others (9, 12, 41, 42). We attribute the high rate of *A. butzleri* detection in the current study to our use of primers designed and validated for maximum efficiency in complex matrices. While previous studies evaluated primer sensitivity and/or specificity, they typically did not examine inclusivity. In contrast, we emphasized inclusivity as a critical component of our primer design and evaluation. PCR inclusivity is the ability of primers to amplify all subtypes of the target taxon, and it is reduced as a result of poor binding efficiency at the primer binding site. It is therefore important to select a target site that lacks sequence variation within the targeted bacterium so that it is not susceptible to competitive binding by non-target taxa. The PCR primers used in previous studies target universal gene sequences such as 16S rRNA (42), 235 rRNA (9), hsp60 (17), and gyrA (41). The strategy that we employed identified non-universal gene sequences that were conserved within *A. butzleri*, thereby circumventing the potential pitfalls of PCR amplification of universal genes. To validate primer inclusivity, we examined 130 *A. butzleri* isolates representing 92 different CGF subtypes, and the primers successfully amplified the gamma subunit of the quinohemoprotein amine dehydrogenase gene (WP_004510536.1) for all 130 isolates. In comparison, previous studies have evaluated inclusivity of their primers against a relatively small number (one to seven) of *A. butzleri* isolates (17, 43-45).

Detection of *A. butzleri* in diarrheic and non-diarrheic stools. *Arcobacter butzleri* is the fourth most commonly isolated Campylobacter-like organism from diarrheic humans (10), but few studies have compared the prevalence of *A. butzleri* in diarrheic and non-diarrheic humans. We hypothesized that if *A. butzleri* is an emerging pathogen, it would be significantly more prevalent in stools from diarrheic than non-
diarrheic people. Even though we detected a much higher prevalence of *A. butzleri* in stools than previous studies, we found no significant difference between diarrheic and non-diarrheic groups. Collado et al. (9) also found no difference in prevalence between stools from diarrheic and non-diarrheic people in Chile, although there were too few *A. butzleri* positive stools for statistical comparison. In South Africa, Samie et al. (46) used PCR to compare prevalence of *A. butzleri* in stools from diarrheic and non-diarrheic individuals and found no significant difference. These findings contrast with those of recognized enteric pathogens, which are more prevalent in diarrheic than non-diarrheic individuals (47).

**Quantification of *A. butzleri* in diarrheic and non-diarrheic stools.** In situations where the pathogenicity of enteric bacteria is uncertain (48, 49), quantification of microorganism density can provide evidence in support of pathogenicity (i.e. an increase in density of a microorganism in diseased individuals). For example, Phillips et al. (50) observed that viral loads of the recognized pathogen, Norovirus GII were much greater in diarrheic than non-diarrheic individuals, and Brassard et al. (51) observed that viral loads of the emerging pathogen, Torque teno virus were much greater in diarrheic than non-diarrheic people. To our knowledge, the current study is the first to compare densities of *A. butzleri* in diarrheic and non-diarrheic people. Although we observed that *A. butzleri* DNA loads were low in both diarrheic and non-diarrheic individuals, the density of *A. butzleri* DNA in stools from diarrheic people was slightly higher than in stools from non-diarrheic individuals. It is uncertain whether the difference in DNA loads between the two groups is biologically relevant (i.e. that pathogenic subtypes exist and contribute to the differential density), or is confounded by the diseased status of the diarrheic group. This warrants further investigation.

**Epidemiology of diarrheic individuals infected with *A. butzleri*.** The prevalence of *A. butzleri* in diarrheic human stools increased with the onset of summer, and it remained relatively high throughout the sample period, but there was no correlation between rate of detection of *A. butzleri* and patient age or sex. Previous studies also found no correlation between *A. butzleri* infection and patient age or sex (41, 46). In comparison, host infection by pathogenic campylobacters is influenced by both age and sex (46, 52, 53), as is infection by other emerging pathogens such as *H. pylori* (46) and Torque teno virus (51). We found no correlation between *A. butzleri* infection and place of habitation (i.e. whether patients lived in an urban or rural area). However, we were unable to ascertain the degree to which people living in urban versus rural locations interacted with livestock (e.g. through occupational exposure). Thus, it is not possible to determine the degree to which occupation influenced intestinal colonization by *A. butzleri* in the current study.

**Co-isolation of *A. butzleri* with recognized pathogens.** We examined whether *A. butzleri* was present in diarrheic human beings in the absence of recognized pathogens. We found that 74% of *A. butzleri* positive samples were not positive for recognized pathogens. The most commonly detected bacterial pathogen was *C. jejuni*, but the rate of co-infection with *A. butzleri* was not significantly greater than with other pathogens. Although it is difficult to directly compare our results with previous studies (i.e. because the
pathogens detected varied, as did the methods of detection), others reported that significant numbers of
samples ranging from 16% (46) to 60% (41) were positive for *A. butzleri* and not for recognized pathogens.
Considering that most cases of enteritis are not attributed to a single pathogenic species (3, 4), and that the
majority of cases of enteritis are not linked to an etiological agent (2), the isolation of *A. butzleri* in the
absence of other pathogens does not necessarily indicate that *A. butzleri* incites disease. Furthermore, our
observation that *A. butzleri* is equally and highly prevalent in diarrheic and non-diarrheic individuals
supports the conclusion that *A. butzleri* does not possess species-wide pathogenicity.

**Conclusions.** We examined the prevalence and abundance of *A. butzleri* in stools from diarrheic and
non-diarrheic people living in southwestern Alberta. We tested the hypothesis that, as an emerging enteric
pathogen, the prevalence and abundance of *A. butzleri* will be greater in diarrheic than in non-diarrheic
people. Culture-based isolation and novel direct detection PCR primers were used to detect *A. butzleri* in
1596 human stools. We found that the vast majority of *A. butzleri* infections were not detected by culture-
based isolation, that there was no difference in prevalence of *A. butzleri* between diarrheic and non-
diarrheic cohorts, and that *A. butzleri* DNA loads were only slightly greater in diarrheic stools. Thus, we
conclude that either *A. butzleri* is not a pathogen, or the strain of *A. butzleri* and/or the status of the host
regulates pathogenicity (e.g. *A. butzleri* is an opportunistic pathogen in a similar manner to *H. pylori* (54)).

The application of high-throughput subtyping methods such as CGF40 (7) is necessary to ascertain whether
specific strains of *A. butzleri* are associated with disease in human beings, with confirmation using models
of pathogenicity/virulence.

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*
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REFERENCES


Table 1. Direct PCR detection of *A. butzleri* in diarrheic stools.

<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory</th>
<th>Samples (n)</th>
<th>Rate of infection (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>599</td>
<td>61.8</td>
<td>0.37</td>
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<tr>
<td></td>
<td>Female</td>
<td>873</td>
<td>59.5</td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>0-4</td>
<td>215</td>
<td>62.3</td>
<td>0.53</td>
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<tr>
<td></td>
<td>5-18</td>
<td>112</td>
<td>55.4</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>19-64</td>
<td>747</td>
<td>61.3</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>65+</td>
<td>398</td>
<td>59.0</td>
<td>0.52</td>
</tr>
<tr>
<td>Habitation*</td>
<td>Rural</td>
<td>560</td>
<td>57.7</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Urban</td>
<td>887</td>
<td>61.4</td>
<td></td>
</tr>
</tbody>
</table>

* Rural or urban location of habitation was ascertained from postal codes submitted by diarrheic individuals.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Positive samples (n)</th>
<th>Coinfections with A. butzleri (n)</th>
<th>Rate of coinfection (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas spp. a</td>
<td>9</td>
<td>6</td>
<td>66.7</td>
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</tr>
<tr>
<td>C. coli</td>
<td>16</td>
<td>9</td>
<td>56.3</td>
<td>0.94</td>
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<td>C. difficile a</td>
<td>7</td>
<td>5</td>
<td>71.4</td>
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<tr>
<td>C. jejuni</td>
<td>183</td>
<td>103</td>
<td>56.3</td>
<td>0.68</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>17</td>
<td>11</td>
<td>64.7</td>
<td>0.54</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>25</td>
<td>15</td>
<td>60.0</td>
<td>0.79</td>
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<tr>
<td>Astrovirus</td>
<td>20</td>
<td>10</td>
<td>50.0</td>
<td>0.49</td>
</tr>
<tr>
<td>Norovirus GI</td>
<td>16</td>
<td>7</td>
<td>43.8</td>
<td>0.26</td>
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<tr>
<td>Norovirus GII</td>
<td>110</td>
<td>66</td>
<td>60.0</td>
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<tr>
<td>Norovirus GIII a</td>
<td>0</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Norovirus GIV a</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>---</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>14</td>
<td>6</td>
<td>42.9</td>
<td>0.26</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>26</td>
<td>16</td>
<td>61.5</td>
<td>0.66</td>
</tr>
<tr>
<td>Total</td>
<td>444</td>
<td>255</td>
<td>57.4</td>
<td>---</td>
</tr>
</tbody>
</table>

*Pathogen was not detected in enough samples to be statistically viable.
Fig 1. Rate of infection (%) of *A. butzleri* in stools from diarrheic humans by targeting the single-copy quinohemoprotein amine dehydrogenase gene with novel ddAbutz primers using direct endpoint PCR.

The total number of human stools processed by month were 209 (May), 232 (June), 199 (July), 228 (August), 225 (September), 198 (October), and 191 (November).