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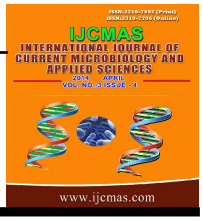

Original Research Article

DNA Sequence Analysis of *gyrA* provides a Rapid and Specific Assay to Identify *Arcobacter butzleri* Isolates from the Environment

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Keywords

Arcobacter; gyrA; PCR RFLP; waterborne diseases; Phylogenetic; 16s rRNA

A B S T R A C T

Arcobacter is an emerging human pathogen, which belongs to the epsilon proteobacteria. Rapid and cost effective classification of these organisms has proved challenging because of the morphological, biochemical, and genetic similarity between *Campylobacter* and *Arcobacter*. This study investigated of a disease outbreak on South Bass Island, OH, *Campylobacter*-like isolates from groundwater in 5 of 16 wells sampled (31%) were recovered. The relationship between the South Bass Island (SBI) isolates and A. *butzleri* and *Campylobacter* spp. of public health concern was assessed. A rapid PCR-RFLP (Restriction Fragment Length Polymorphism) assay classified one group of SBI isolates as *Arcobacter butzleri* and another group as *Arcobacter*-like bacteria. Subsequently, 16S rRNA and *gyrA* sequence analysis supported the *Arcobacter butzleri* group designation but both the *gyrA* and 16S rRNA sequence data demonstrated that the group of *Arcobacter*-like organisms belongs to a different bacterial family. Sequence analysis of a 300 bp *gyr*A fragment from each isolate provided better identification of *Arcobacter* spp compared to a PCR RFLP assay. This study illustrates that gyrA phylogenetic analysis provides a rapid, accurate and effective method for identification of species within the *Campylobacteraceae* family and may use as a molecular tool in future studies in the surveillance of *Campylobacter*-like organisms.

Introduction

Infectious diseases continue to stimulate global public health concern because they account for more than one-quarter of all human deaths annually and a similar fraction of morbidity. The genus *Arcobacter* is considered as an emerging

infectious food-borne and waterborne pathogen. Members of this genus have been recovered from a number of water (Rice *et al*, 1999 and Musmanno *et al*, 1997) and food sources (Wisley and Baetz 1999). Center for Disease Control and Prevention (CDC) reported a number of *Arcobacter* isolates from a wide range of nosocomial sources. Vandamme *et al* (1992) reported one potential cause of gastrointestinal illness is associated with the presence of this organism. However, there is little direct evidence that *Arcobacter* spp. are involved in human enteric disease (Engberg *et al*., 2000 and Wybo *et al.* 2004). Occasionally, *A. butzleri* is reported in cases of human extra-intestinal diseases (Yan *et al.* 2000). Despite the acknowledged importance of *A. butzleri* as a nosocomial pathogen, little is known about potential virulence factors or epidemiology. Also unclear is the reservoir for *A. butzleri* transmission (Ho *et al*, 2006). The presence of this organism in groundwater and commercial livestock populations has suggested its potential as a food-borne (Mansfield and Forsythe, 2000) and waterborne disease agent garnering increased public awareness.

Arcobacter is a member of the family *Campylobacteracae;* like *Campylobacter*, the organism is spirochete in shape, highly motile, microaerophilic, and exhibits low G+C chromosomal content. Because of the phenotypic, biochemical, and genetic similarity (Vandamme and De Ley, 1991) between the two genera, classification of these organisms has proved challenging. Therefore, a rapid and accurate identification technique was needed to distinguish *Arcobacter* from other members of the Campylobacteriaceae.

In the present study, *Campylobacter*-like isolates from groundwater in the vicinity of a waterborne disease outbreak on South Bass Island, Ohio were obtained. In this outbreak, gastrointestinal illness was reported by the Ohio Public Health Department in small number patients that visited South Bass Island. *Campylobacter jejuni* was implicated based on its recovery from five patients with enteritis, however, in many instances the causative agent of enteritis remained unidentified. In related work, Fong *et al* (2007) analyzed for the presence of pathogenic organisms and indicator microorganisms in the ground water; they reported that *Arcobacter* may be a source of contamination of ground water. This observation fueled interest in development of molecular approaches to identify the causative agent of this outbreak.

The objective of this current study was to identify potential causative agents of disease in the groundwater of South Bass Island associated with the enteritis outbreak of 2004. It was therefore proposed to accomplish this by analyzing the genetic relationship between the SBI groundwater isolates and strains recovered from the patients in the same geographic area. The second objective was to classify *Campylobacter*-like bacteria recovered from the groundwater and to demonstrate the potential of *gyr*A sequence analysis for discerning species within the *Campylobacteraceae* family. 16s rRNA sequence analysis is an effective tool for the speciation of *Arcobacter* spp. and this method was successfully applied by Houf *et al*, (2000). However, sequence divergence within 16s rRNA is often insufficient to discriminate between closely related taxa (Logan *et al*., 2002 and Dewhirst *et al*., 2005). Therefore a panel of molecular techniques was conducted to obtain high-quality taxonomic resolution of the SBI isolates.

16S rRNA and *gyrA* DNA sequence analyses as well as a PCR-RFLP-based diagnostic method on SBI isolates and on *Arcobacter* and *Campylobacter* isolates of human health significance was performed.

Materials and Methods

Environmental sample collection, Enrichment and Culture conditions

Ground water samples were collected from 16 wells near Put-In Bay, Ohio. This location was associated with several cases of enteritis as part of an outbreak that occurred between July and Sepetember, 2004. Four liter grab samples were collected from each well for analysis. Concentrated Maximum Recovery Diluent (2x; Oxoid) was added to each water sample in a ratio of 1:10. The samples were transported to the laboratory at Michigan State University on ice and processed within 24 h of collection. Samples were filtered through 0.45 μm membrane filters (Gelman Sciences). The membranes were then placed on Bolton Selective Enrichment Agar - BSEA (Oxoid) and flooded with 10 ml of Bolton broth supplemented 5% sheep blood (Cleveland Scientific, Bath, Ohio) with cefoperazone (20 μg/ml), vancomycin (10 μg/ml), and amphotericin B (2 μg/ml). The plates were placed in an anaerobic jar and incubated at 37 °C, with 40 rpm agitation under an atmosphere of 10 % CO_2, 10 % H_2, and 80 % N_2. After 48h of incubation, the broth culture was diluted using Bolton broth and inoculated onto BSEA to isolate colonies. Plates were incubated at 37°C for 48 h, under an atmosphere of 10 % CO_2, 10 % H_2, and 80 % N_2. After incubation, five isolated colonies from each sample were selected and transferred to fresh BSEA plates to achieve single colony isolation. The plates then incubated at 37°C, under an atmosphere of 10 % CO_2, 10 % H_2, and 80 % N_2.

Bacterial strains and genomic DNA isolation

Different bacterial strains were used in this assay (Table 1). *Arcobacter* spp. was grown microaerobically on Bolton Agar supplemented with 5% sheep blood for 48h at 37°C. Cells (from pure culture) were harvested and suspended in TSB (Trypticase soy broth) for chromosomal DNA extraction. Bacterial cultures were pelleted by centrifugation at 10000 x g at 4°C, and DNA was extracted from cell pellets using a DNA extraction kit from Invitrogn. Quantification of DNA was conducted using a Nanodrop spectrophotometer (ND-1000, Wilmington, DE, USA).

Restriction Fragment Length Polymorphism Analysis of 16s rDNA

A common 1004bp fragment carrying a conserved region of the 16S rDNA gene in *Campylobacter*, *Arcobacter* and *Helicobacter* (CAH) spp. was amplified with primers CAH 16S1a (5'-AAT ACA TGC AAG TCG AAC GA-'3) and CAH 16S1b (5'-TTA ACC CAA CAT CTC ACG AC-3'). The resulting fragments were analyzed by PCR restriction fragment length polymorphism (PCR-RFLP) using the protocol of Marshall et al. (1999). Briefly, PCR products were purified with Qiagen's Qiaquick PCR purification kit (Qiagen, Valencia, CA) and subjected to restriction endonuclease digestion in a 20 μl reaction mixture which included 15 μl of the purified PCR amplicon (approximately 1000 ng), 2 μl of sterile water, 2 μl of 10 x restriction buffer and 1 μl (10 U) of restriction

endonuclease *DdeI* and *TaqI* (Invitrogen Inc.) following the conditions recommended by the manufacturer. The reaction mixture (20 µl) was analyzed by gel electrophoresis on a 2 % agarose gel in 0.5x Tris-botate-EDTA running buffer (ICN Biomedicals, Aurora, Ohio) and stained with ethidium bromide after electrophoresis. *Dde*I and *TaqI* restriction digestion products were identified by matching RFLP pattern obtained with a database produced by Marshall *et al.* (1999).

16S rRNA Sequencing

Approximately 1492bp of the 16S rRNA was amplified and subjected to nucleotide sequence analysis based on methodology supported by the CDC (Centers for Disease Control and Prevention; Houf *et al* , 2000). Three primer sets were used to amplify overlapping fragments of 16S rRNA; the same primer sets allowed complete sequencing of the target (8F: AGTTGATCCTGGCTCAG and R503: TATTACCGCGGCTGCTGG, F495:GCGGTAATACGGAGGGTGC and R940: GCGTATCTTCGAAT TAAA CCACA and F900: AGCGGTGGA GCATGTGGTTTA and R1492: ACCTTGTTACGACCT. The resulting sequence data were analyzed by CDC using their 16S rRNA database which contains 16S rRNA sequences from *Campylobacters, Arcobacters, Helicobacters* and related organisms. The sequence data were also analyzed by the RDP database (Ribosomal Database Project II) at Michigan State University.

PCR amplification and sequence analysis of *gyrA*

We amplified a 420bp fragment in the quinolone resistance determining region (QRDR) of *gyrA* in the SBI isolates using the protocol and primer sets developed by Wilson *et al* (2000)**.** PCR was conducted in a 50 µl reaction mixture that contained10 to 15 ng of template/µl, 0.2mM (each) deoxynucleoside triphosphate (dNTP), 0.5pmole of each primer/µl, 1.5mM $MgCl_2$, 1 x PCR buffer and 0.05U of Pfu Turbo polymerase (Stratagene, La Jolla,CA)/µl. The thermocycling conditions were described as a earlier (Wilson *et al* 2000). The PCR products of expected sizes were isolated in a 1.75% low-melting-temperature agarose gel (SeaPlaque GTG; Cambrex, Baltimore, Maryland) and purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA). Then the Nanodrop apparatus quantified the concentration of purified product. Nucleotide sequence analysis was conducted on purified amplicons using the same primers in a *Taq* dye terminator cycle. Automated sequencing was accomplished with an ABI 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.) at the Michigan State University (MSU) Research Technology Support Facility.

gyrA sequence data from *Campylobacter* spp.

Nucleotide sequence data from the *gyrA* gene of *Campylobacter jejuni, C. coli, C. lari, C. fetus, C. hyointestinalis, C. upsaliensis, Helicobacter pylori, Acenetobacter* spp. and *Escherichia coli* were compared to SBI isolate data in the current study. These sequences were utilized and cited in Wilson *et al* (2000)**.** The *gyrA* gene sequence of *A. cryaerophilus, A. cibarius* and *A. skirrowii* (gene bank accession numbers DQ464336, DQ464337, DQ464338 respectively) were also incorporated in the analysis**.** The partial *gyrA* gene sequences were analyzed with Laser Gene Software

Package (DNA Star, Madison, WI). Multiple sequence alignment and phylogram analysis of *gyrA* was performed using Clustal W and phylogenetic tree analysis in the MegAlign program. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Distances were corrected for multiple base changes by the method of Jukes and Cantor,(1969). The topology of the tree was evaluated by 1,000 trials of bootstrap analysis.

Nucleotide sequence accession numbers and Human isolates analysis

GenBank accession numbers of the QRDR of the gyrA gene of *A. butzleri* strains ANDL01, ANDL02, ANDL03, ANDL04 and ANDL05 (Table 1) are KF690263–KF690267, respectively. The GenBank accession numbers of the 16S rRNA gene of *A. butzleri* strains ANDL01, ANDL02, ANDL03, ANDL04 and ANDL05 are KF690262– KF690258, respectively. Selected strains were highlighted in the legends of figures and tables which included in the respective assays and analysis.

Genus - Specific PCR analysis

The SBI groundwater isolates were tested to identify *Campylobacter* by PCR the method of Wilson et al., (2000). Individual colonies were picked from *Campylobacter* selective media. The primers used for identification of *C. jejuni* were JL 238 (5′-TGG GTG CTG TTA TAG GTC GT-3′) and JL239 (5′-GCT CAT GAG AAA GTT TAC TC-3′) as described by Wilson et al., (2000). Similar analyses were conducted with genus specific primers to amplify a 439bp 16S rRNA fragment from *Campylobacter* as described previously (Moreno et al., 2003).

Results and Discussion

The water samples were isolated from 16 wells. Sample from each well generated 5 individual clones for Arcobacter ssp culture methods but only twelve groundwater samples (Table 2) were recovered in pure cultures as *Arcobacter spp*. on the *Campylobacter*-selective media which morphologically resembles *Campylobacter*-like colony. The morphology of these cells was confirmed under a darkfield microscope. *Arcobacter* isolates from clinical sources (Human isolates from CDC and Public Health Canada) were successfully recovered and tested positive growth (Table 1), which were collected from different geographical area around the Great Lake.

All twelve were positively identified to be members of the Campylobacteriaceae (*Arcobacter*, *Camylobacter* and *Helicobacter* spp) in 16S rDNA PCR RFLP assay (Table 2**).** The results of PCR-RFLP patterns from the environmental isolates are shown in figures 1A and 1B. The digestion of PCR purified amplicon with *Dde I* involves initial typing scheme which generates five isolates (Sample serial number 1-5, Table 2) *Arcobacter* specific banding pattern (421bp,353bp,128bp) and six isolates (50%) produce *Arcobacter butzleri*-like banding pattern (421bp,353,125bp) which is not Dde I *Arcobacter* specific banding pattern (Sample serial number 6-11, Table 2) .To discriminate *A.butzleri*, *A.cryaerophilus* and *A. skirrowi*, the enzyme Taq I, which produced five (41%) unique *A.butzleri* fingerprints (795bp, 135bp, 120 bp) (Figure1B and Figure 5) from the all five *Arcobacter* isolates which is confirmed in Dde I assay (Table 1:strains ANDL01, ANDL02, ANDL03,

ANDL04 and ANDL05). One isolate generated a different banding pattern, which fails to differentiate in restriction digestion assay (Sample serial number 12, Table 2). The results of PCR RFLP assay for environmental isolates with respect to different well of groundwater were recorded (Table.2). Five human isolates (Table.1 strain D4310921, D4310939, D4311140, D4306217 and D4306289) acquired from CDC in the same outbreak area were also positive to 16sDNA of Arcobacter, *Camylobacter* and *Helicobacter* spp. These human isolates from the outbreak produced *Campylobacter*-like banding pattern with Dde I digestion (Figure 1A: cj SBI patient 1 and 2).All five human isolates were positive to *Campylobacter jejuni* specific PCR assay from the outbreak (data not shown). The isolates from the wells of groundwater were negative to species specific screening of *C.jejuni* PCR assay (data not shown).

For further examination of the same twelve groundwater isolates were confirmed by using 16S rRNA data base for *Arcobacter* and *Campylobacter*. Results of 16s rRNA sequence analysis for environmental groundwater samples were shown in Table 2 and Figure 5. Five, out of twelve isolates were confirmed as *A.butzleri* (Table 1: strains ANDL01, ANDL02, ANDL03, ANDL04 and ANDL05) and six isolates were confirmed as *Acinetobacter s*pp. which belongs to gamma Proteobacteria (Sample serial number 6-11, Table 2) . One isolate was confirmed as *Sphingobacterium* sps (Sample serial number 12, Table 2). In addition to this, the analyzed sequences of 16S rRNA results were confirmed in RDP data base at MSU.

All the environmental isolates and human isolates were PCR amplified the QRDR of the gyrA gene with the expected product of 420bp (Table 1 and Table 2). Multiple sequence alignment of selected *Arcobacter* isolates were also presented (Figure 3)**.** Alignment of gyrA gene was observed as 97-100% sequence identity among the *A. butzleri* isolates collected from human and environmental origin (Table 3). Seven isolates from environmental sample were observed very close to Enterobacteriace group and observed as negative to *A.butzleri* in gyrA assay (Table 2, Sample serial no: 6-12). The confirmed five *A.butzleri* from environmental isolates (Strain ANDL01, ANDL02, ANDL03, ANDL04 and ANDL05) were shown sequence divergence of one to three nucleotide bases between isolates in the gyrA sequences. Two isolates from environmental water (SBI Airport and SBI Skyway) were observed 100% sequence identity with human isolates.

A phlyogram based on the 300bp of gyrA gene is constructed (Figure 2). All the isolates from the environment and selected human isolates of *A. bulzleri* sequence were clustered together and have a common ancestor. Phylogram was made known clear divergence of *A.butzleri* from *Campylobacter*, *Helicobacter* and Enterobacteriaceae group (Figure 2). Six *Arcobacter* like organisms confirmed from PCR RFLP assay were illustrated as different family in gyrA phylogram analysis (Figure 2 and Table 2). Within the genus *Arcobacter, A. butzleri* shown distinct node and internode from *A. skirrowii, A. cryaerophilus, A. cibarius* (Figure 2).The percent identity in terms of nucleotide bases gyrA shows apparent variation of 72-78% between *Campylobacter* species and *A. butzleri.* In addition, the sequence identity was found 71% between *Helicobacter* and

Arcobacter. Within the *Arcobacter* species, nucleotide sequence of the *A.skirrowoii* gyrA gene was the most closely linked with *A. butzleri* with 88 to 89 % identity, whereas the sequence of *A. cryaerophilus* and *A. cibarius* showed only 87.5 % and 85 % identity respectively (Table.3).

A phylogenetic analysis was conducted on the nucleotide sequences of gyrA gene and nucleotide sequences of 16S rRNA gene from *Arcobacter* strains and from other closely related bacterial strains. The 16S rRNA gene phylogenetic tree showed two distinct clusters with *A. butzleri* isolates clearly separated from the other *Arcobacter* species (Figure 4A), whereas the gyrA phylogenetic tree showed a paraphyletic organization, i.e. the common ancestor of *A. butzleri* is also of ancestor *A. cryaerophilus, A. cibarius* and *A. skirrowii* (Figure 4B) as well as other bacterial strains. Previous phylogenetic analysis matches similar results of the gyrA gene and 16S rRNA genes demonstrated that Arcobacter isolates from human and animal (Abdelbaqi *et al.*, 2007) form two distinct clusters as observed for the 16S rRNA phylogenetic tree. Possibly the paraphyletic organization of gyrA gene within *Arcobacter* strains and non *Arcobacter* strains is due to more distinct potential of gyrA gene discrimination in closely related bacterial strains . Clearly, phylogenetic analyses of gyrA genes (and not 16S rRNA gene sequences) will be useful and specific in *A. butzleri* differentiation.

As it is known 16S rRNA gene sequence analysis is the hallmark basis for the classification of bacterial taxonomy and has provided evidence to be highly conserved. However, it shows to have insufficient discrimination in the case of *Epsilonproteobacteria* (Dewhirst *et al* , 2005). Owen (2004) was demonstrated that the gyrA gene encoding a subunit of DNA gyrase is an important tool for bacterial taxonomical identification. Moreover, analysis of gyrA was considered as a potential technique for the discrimination of *Campylobacter* at the species level (Husmann *et al*., 1997; Wilson *et al*., 2000). Essentially, concerning *Arcobacter* spp only one study (Abdelbaqi *et al* , 2007) have focused on the importance of the use of gyrA gene as a taxonomic tool for human and animal isolates. However, they used full sequence of gyrA gene. In this study, the gyrA sequence of *Arcobacter* strains is established based on the 300bp fragment of gyrA and to our knowledge the analysis is performed first time for environmental and human isolates. This study also demonstrated the comparative evaluation for the three molecular methods.

In the present study, PCR RFLP, 16S rRNA and *gyrA* sequence analysis confirmed that *A.butzleri* was present in five of the sixteen wells of groundwater (Figure 5). Our finding of PCR RFLP results for the species identification was supported by Marshall *et al*. (1999). However six isolates confirmed as *A. butzleri* like organism by PCR-RFLP, which doesn't agree with gyrA and 16S rRNA sequence analysis. So it was found that, PCR RFLP assay is a useful assay to the differentiation and identification of organisms from these closely related genera but fails to differentiate within species.

Twelve environmental isolates that were positive for *Arcobacter* spp by culture method were discriminated differently into species level by PCR RFLP analysis

(Table 2). Only five isolates turned out as *A.butzleri* in genotyping analysis (strain ANDL01, ANDL02, ANDL03, ANDL04 and ANDL05). This may indicate that culture method is not selective or specific for the identification *A.butzleri*. The culture method may confound their correct identification due to morphological similarity between *Campylobacter* and *Arcobacter* (Wesley I.V.1997). Six isolates which observed *A.butzleri* like organism with PCR RFLP analysis were demonstrated negative to *A.butzleri* in gyrA phylogenetic analysis. Examination of the PCR RFLP was not useful for the specific identification of closely relatated *A.butzleri* like organisms. The same difficulty for the analysis of PCR RFLP was reported by Gonzalez *et al* (2006) for differentiation of species specific identification of *Campylobacter* and *Arcobacter*. The gyrA sequence analysis was confirmed 97-100% sequence identity of *A.butzleri* from environmental water strains with human isolates *A.butzleri*. Therefore, gyrA sequencing is potentially useful tools for detection of *Arcobacter* spp. from environmental samples. These results demonstrated the prevalence of *Arcobacter* in water and also demonstrated the lack of available methods for its selective identification. The association of *A.butzleri* with the ground water distribution system could significantly result as one of the potential role of water-borne diseases. All five human isolates in the same outbreak region was found *C.jejuni*,(data not shown) which demonstrated that there is no relationship between human and environmental isolates in the outbreak region. Another possible explanation is that it may be due to *Arcobacter* infection since information of isolates from all the patients who had gastrointestinal illness in that outbreak region was not available.

Detection of *Arcobacter* from water has been reported previously (Rice *et al* , 1999) but to our best knowledge, this study represents the first report of establishing the gyrA sequence relationship between the environmental and human isolates of *Arcobacter* spp. Phylogenetic analysis and multiple sequence alignment supported the gyrA sequence comparison and recognition of *A. butzleri* from human and environmental sources. The gyrA gene encoding a subunit of DNA gyrase was reported to be an important tool for bacteria phylogeny (Owen, 2004). In fact, the use of gyrA sequence comparison for identification of C*ampylobacter* at the species level were illustrated through phylogram analysis of the multiple sequence alignment (Husmann *et al*.,1997 ; Wilson *et al*.,2000). In the phylogenetic analysis of gyrA gene of five environmental and 34 human isolates (all data not shown) of *Arcobacter* strains, it was demonstrated high sequence similarity and representative of common ancestral provides the evidence that all belongs to the *A. butzleri* strain.

The gyrA gene allowed a good quality discrimination of these species from related species belongs to *Campylobacter*, *Helicobacter* genera and *Enterbacteriace* group by phylogenetic analysis. Moreover, the phylogenetic analysis of gyrA encoded gene was reported a specific differentiation among *Arcobacter* strains in contrast to 16S rRNA gene tree (Abdelbaqi *et al*., 2007).The gyrA gene of sequence analysis in our study between and within the *Arcobacter* genus and related species gives substantial taxonomic tool which supported by the previous observation reported by Wilson et al., (2000) in *Campylobacter.*

Table.1 Different bacterial isolates, their source and time of isolation

Species	Strain	State or province	Source	Year of Isolation
Arcobacter butzleri [a]	ANDL01	Ohio,USA	SBI Airport, Groundwater	2004
A. butzleri [a]	ANDL05	Ohio,USA	SBI Clinster, Groundwater	2004
A. butzleri [a]	ANDL02	Ohio,USA	SBI Horny Toad, Groundwater	2004
A. butzleri [a]	ANDL03	Ohio,USA	SBI Island Club17,Groundwater	2004
A. butzleri[a]	ANDL04	Ohio,USA	SBI Skyway, Groundwater	2004
A. butzleri [b]	D2989	Kentucky, USA	Human	1990
A. butzleri [b]	D6075	Ohio, USA	Humn	2002
A. butzleri [b]	D5353	Michigan, USA	Human	1997
A. butzleri [b]	D4317	Kentucky, USA	Human	1992
A. butzleri [b]	D5006	Indiana, USA	Human	1994
A. butzleri (2)[d]	ER4982,ER4387	Ontario, Canada	Human	2001
A. butzleri (2) [d]	ER1341, ER263	Ontario, Canad	Human	2002
A. butzleri [d]	ER 4119	Ontario, Canada	Human	2003
A. butzleri (3) [d]	ER 174, ER1390,ER1169	Ontario, Canada	Human	2004
A. butzleri [f]	042012	Monitoba,Canada	Human	2004
A. butzleri (11 [d]	ER3958,ER5041,ER4024 ER4580,ER4416,ER1933 ER3267,ER180,ER5042 ER6177.ER3902	Ontario, Canada	Human	2005
A. butzleri [f]	056595	Monitoba,Canada	Human	2005
A. butzleri (8) [d]	ER5255,ER4460,ER5551 ER768,ER4682,ER631 ER4604,ER4603	Ontario, Canada	Human	2006
A. butzleri [c]	ATCC49616	California,USA	Human	-----
Campylobacter jejuni (2) [b]	D4310921,D4310939	Ohio,USA	Human	2004
Campylobacter sps (3) [b]	D4311140,D4306217 D4306289	Ohio,USA	Human	2004

[a] Strains characterized in this study, [b] Strains collected from CDC (Center for Disease Control and Prevention),[c] American typle culture collection.[d] Collected from Ontario Public Health laboratories, Canada. [f] Collected from Canadian science center for Human and Animal Health.

Table.2 Comparison of different detection techniques for confirmation of *Arcobacter utzleri* in environmental well water sample

Sl. No.	Sample ID	PCR RFLP	gyrA Assay	16S r DNA Assay	Culture Assay
1	SBI Airport	+	+	+	+
2	SBI Clinster	+	+	+	+
3	SBI Horny Toad	+	+	+	+
4	SBI Island Club	+	+	+	+
5	SBI Skyway	+	+	+	+
6	SBI IC11	- (*)	-	- (#)	+
7	SBI IC16	- (*)	-	- (#)	+
8	SBI Sounder's South	- (*)	-	- (#)	+
9	SBI ODNR Oak Point	- (*)	-	- (#)	+
10	SBI South Bass State Park	- (*)	-	- (#)	+
11	Kens Place	- (*)	-	- (#)	+
12	Bird's Nest	-	-	- (@)	+

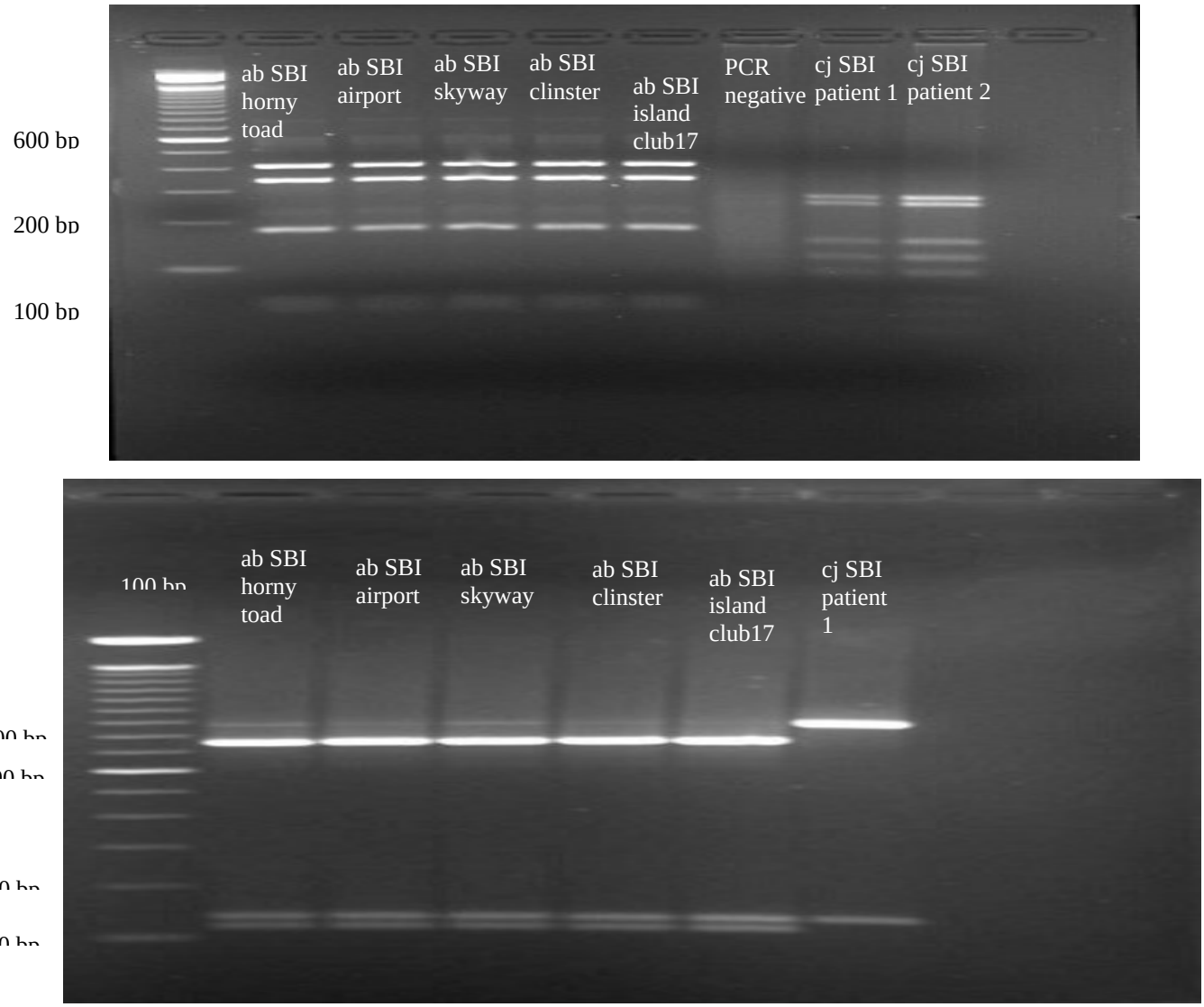
+ implies detection of *A.butzleri* - implies absence of *A.butzleri* (*) Confirmed as *Arcobacter* like organism
(#) Confirmed as *Acinetobacter sp*. (@) Confirmed as *Sphingobacterium sp*.

Table.3 Sequence pair uniqueness in terms of percent identity of gyrA gene analyzed by clastal W and sequence distance program of Megalign (DNA Star)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
1		70.2	73.2	71.5	72.2	72.2	71.2	71.5	71.2	72.2	80.1	73.2	72.2	73.8	79.8	72.5	61.3	68.9	25.5	72.2	**1**
2	70.2		90.7	87.7	87.7	88.1	87.1	87.1	87.1	87.7	77.5	75.8	76.2	88.4	74.8	73.5	62.9	71.5	24.5	88.1	**2**
3	73.2	90.7		88.4	89.1	89.4	88.1	88.4	88.1	89.1	77.8	77.2	77.2	86.8	76.5	76.2	61.6	71.9	24.8	89.4	**3**
4	71.5	87.7	88.4		97.4	97.7	99.0	99.3	99	97.4	77.2	73.5	75.8	85.4	74.8	74.2	63.2	70.9	23.2	97.7	**4**
5	72.2	87.7	89.1	97.4		99.7	96.4	96.7	96.4	100	77.2	74.5	75.2	85.4	74.8	74.5	62.9	71.9	22.8	99.7	**5**
6	72.2	88.1	89.4	97.7	99.7		96.7	97	96.7	99.7	76.8	74.2	75.5	85.8	74.5	74.2	63.2	71.9	22.8	100	**6**
7	71.2	87.1	88.1	99.0	96.4	96.7		99	100	96.4	77.8	73.5	75.2	84.8	74.8	73.5	62.9	70.9	23.2	96.7	**7**
8	71.5	87.1	88.4	99.3	96.7	97	99		99	96.7	77.2	73.5	75.2	85.1	75.5	73.5	62.9	71.2	23.2	97	**8**
9	71.2	87.1	88.1	99	96.4	96.7	100	99		96.4	77.8	73.5	75.2	84.8	74.8	73.5	62.9	70.9	23.2	96.7	**9**
10	72.2	87.7	89.1	97.4	100	99.7	96.4	96.7	96.4		77.2	74.5	75.2	85.4	89.4	74.5	62.9	71.9	22.8	99.7	**10**
11	80.1	77.5	77.8	77.2	77.2	76.8	77.8	77.2	77.8	77.2		81.1	80.5	75.8	80.4	80.5	63.2	71.9	25.5	76.8	**11**
12	73.2	75.8	77.2	73.5	74.5	47.2	73.5	73.5	73.5	74.5	81.1		89.4	75.2	80.8	78.1	61.9	72.2	22.8	74.2	**12**
13	72.2	76.2	77.2	75.8	75.2	75.5	75.2	75.2	75.2	75.2	80.5	89.5		75.8	78.8	79.8	61.6	71.2	24.8	75.5	**13**
14	73.8	88.4	86.8	85.4	85.4	85.8	84.8	85.1	84.8	85.4	75.8	75.2	75.8		75.8	71.9	61.6	71.9	24.2	85.8	**14**
15	79.8	74.8	76.5	74.8	74.8	74.5	74.8	75.5	74.8	89.4	80.4	80.8	78.8	75.8		80.5	61.9	73.2	25.8	74.5	**15**
16	72.5	73.5	76.2	74.2	74.5	74.2	73.5	73.5	73.5	74.5	80.5	78.1	79.8	71.9	80.5		63.6	75.8	26.2	74.2	**16**
17	61.3	62.9	61.6	63.2	62.9	63.2	62.9	62.9	62.9	62.9	63.2	61.9	61.6	61.6	61.9	63.6		62.3	21.5	63.2	**17**
18	68.9	71.5	71.9	70.9	71.9	71.9	70.9	71.2	70.9	71.9	71.9	72.2	71.2	71.9	73.2	75.8	62.3		22.5	71.9	**18**
19	25.5	24.5	24.8	23.2	22.8	22.8	23.2	23.2	23.2	22.8	25.5	22.8	24.8	24.2	25.8	26.2	21.5	22.5		22.8	**19**
20	72.2	88.1	89.4	97.7	99.7	100	96.7	97	96.7	99.7	76.8	74.2	75.5	85.8	74.5	74.2	63.2	71.9	22.8		**20**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	

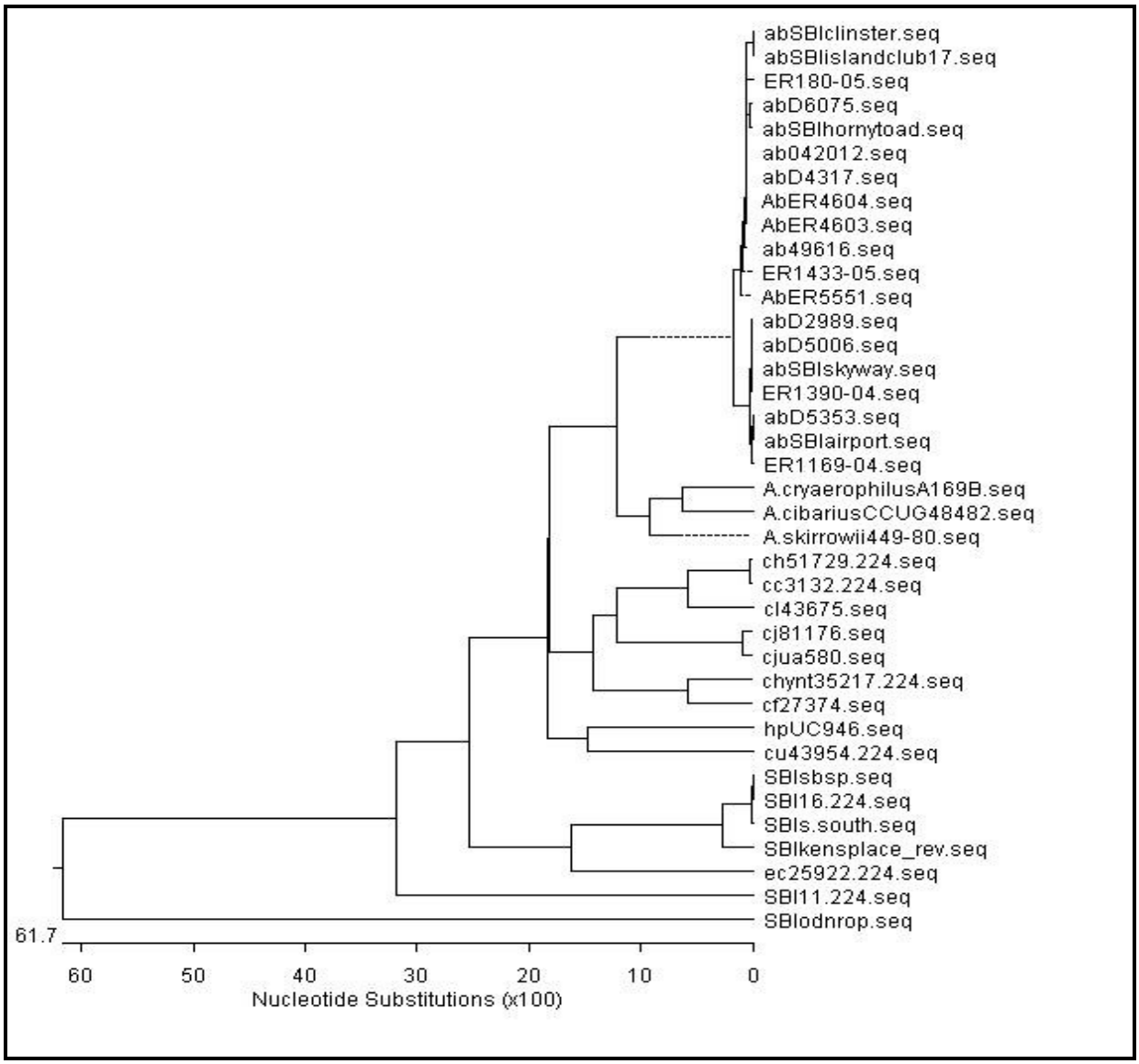
*Campylobacter jejuni*81176; 2, *Arcobacter cryaerophilus*A169/B; 3, *Arcobacter skirrowii*449/80; 4, *Arcobacter.butzleri* ATCC49616; 5, *Arcobacter butzleri*D2989 (CDC clinical strain), abSBI, *Arcobacter butzleri* South Bass Island (6, airport; 7, Clinster; 8, hornytoad; 9, Islandclub17; 10,skyway); 11, *Campylobacter coli1*679368; 12, *Campylobacter fetus*27374;13,*Campylobacter hyointestinalis*35217;14,*Arcobacter cibarius*CCUG48482;15,*Campylobacter lari*43675;16, *Campylobacter upsaliensis*43954; 17, *Escherichia coli*25922;18, *Helicobacter pyloriI*946; 19, *Pseudomonas aeruginosa*01; 20, *Arcobacter butzleri*ER1169-04 (Human isolates from Canada).

Figure.1 (A &B) 16S rDNA PCR-RFLP patterns of chromosomal extracts from bacterial isolates recovered on South Bass Island**.** A) *Dde*I digest of 16S rDNA PCR from chromosomes of island isolates



Lane 1 is a 100 bp marker, Lanes 2 – 6 isolates from island ground water, Lanes 7 – 9 isolates recovered from individual patients. *Dde* I restriction fragments of 421 bp, 353 bp, and 183 bp are indicative of *Arcobacter* and *Dde* I fragments of 272 bp, 247 bp, 153 bp, 120 bp, and 95 bp are representative of *Campylobacter* as specified by Marshall *et al*. 1999. B) *Taq* I digest of 16S rDNA PCR from chromosomes of island isolates. *Taq* I restriction fragments of 795 bp, 135 bp, and 120 bp are indicative of *A. butzleri.* Lanes 2 – 6 isolates from island ground water, Lane 7 isolate from patient 1

Figure.2 Phylogram is based on the same 300 bp DNA fragment of the *gyrA*. Nodes indicate a common ancestor



The lengths of the horizontal lines represent the degree of relatedness between individual strains. Multiple sequence alignment and phylogram analysis were performed using the clustal W and Phylogenetic Tree programs of Laser gene DNA STAR Sequencing (Madison, WI).). **abSBI**, South Bass Island isolates; a**bD**, *Arcobacter butzleri* CDC; **ER** and **abER** *Arcobacter butzleri* from Canada; *A.skirrowii*; *A.cryaerophilus*; *A.cibarius*;**cc**, *Campylobacter coli*; **ch**, *Campylobacter hyoilei*; **chynt**, *Campylobacter hyointestinalis*; **cf**, *Campylobacter fetus*; **cj**, *Campylobacter jejuni*; **cl**, *Campylobacter lari*; **cu**, *Campylobacter upsaliensis;* **ec**, *Escherichia coli*; **hp**, *Helicobacter pylori*; **pa**, *Pseudomonas aeruginosa*.

Figure.3 Multiple Sequence Alignment of Selected *Arcobacter* isolates recovered from South Bass Island and Human. The alignment represents a 300 bp fragment of *gyrA* sequences. abSBI, *Arcobacter butzleri* South Bass Island, *Arcobacter butzleri*D2989 (CDC clinical strain), *Arcobacter butzleri*ER1169-04 (Human isolates from Canada).

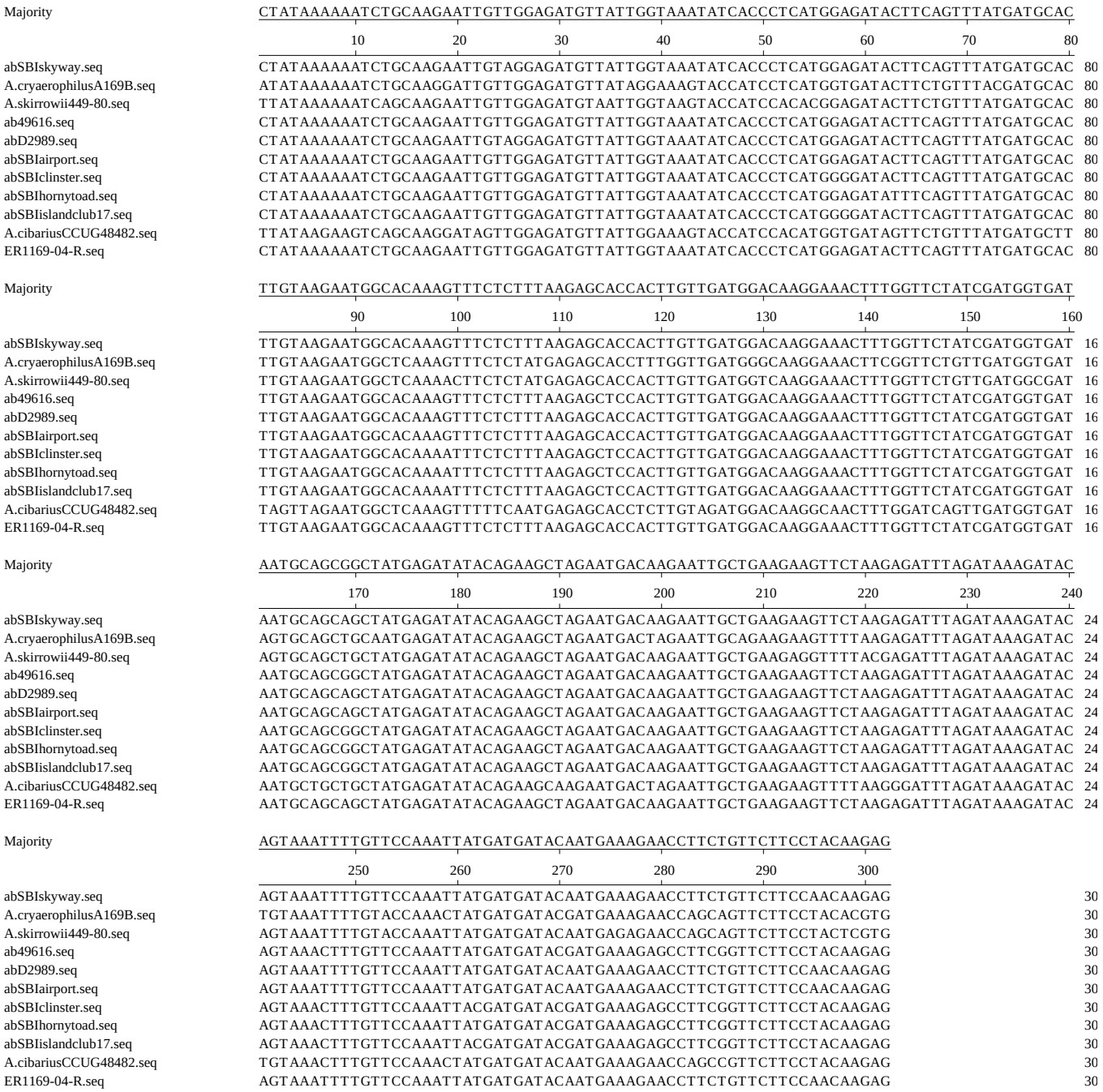
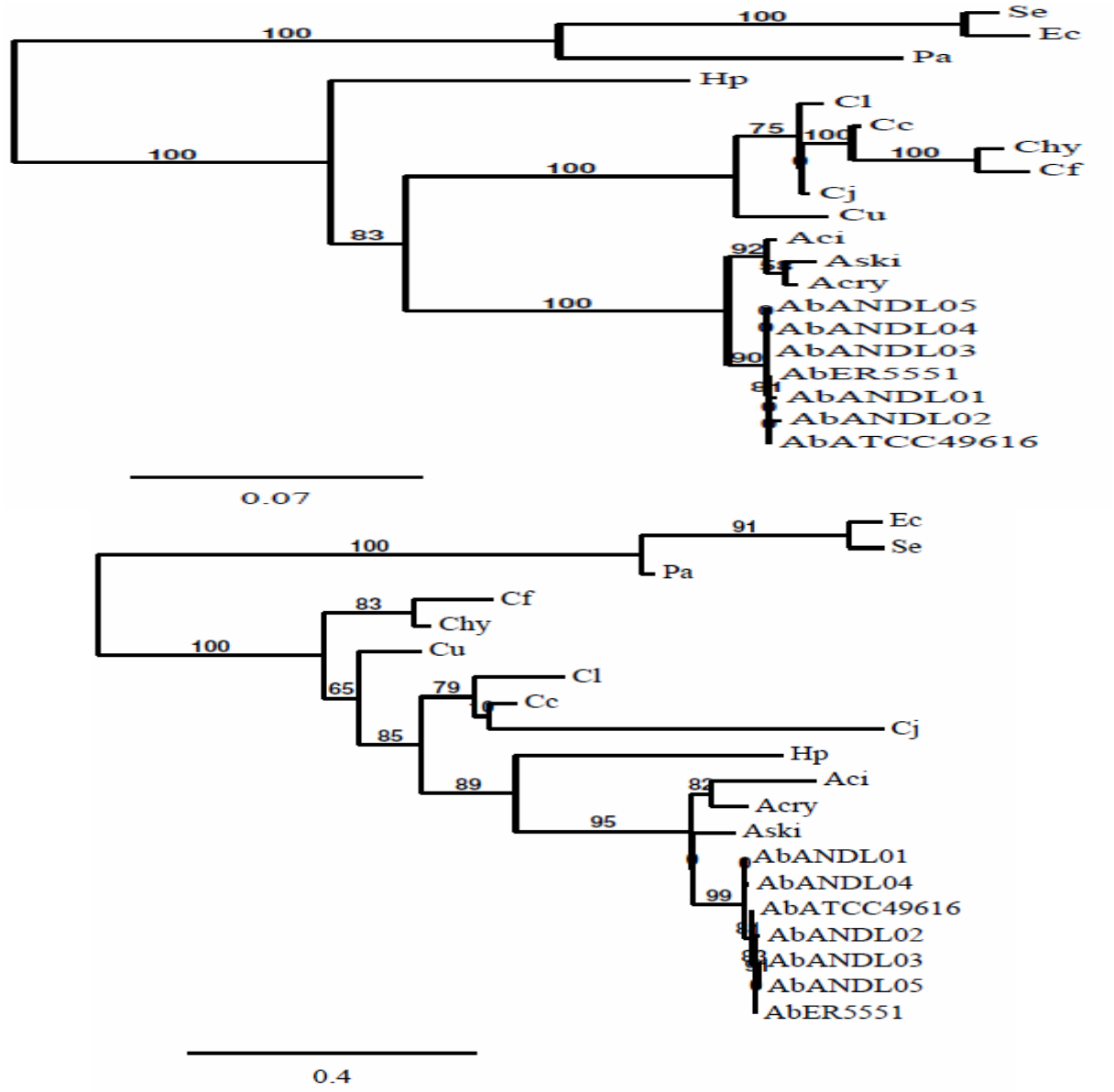
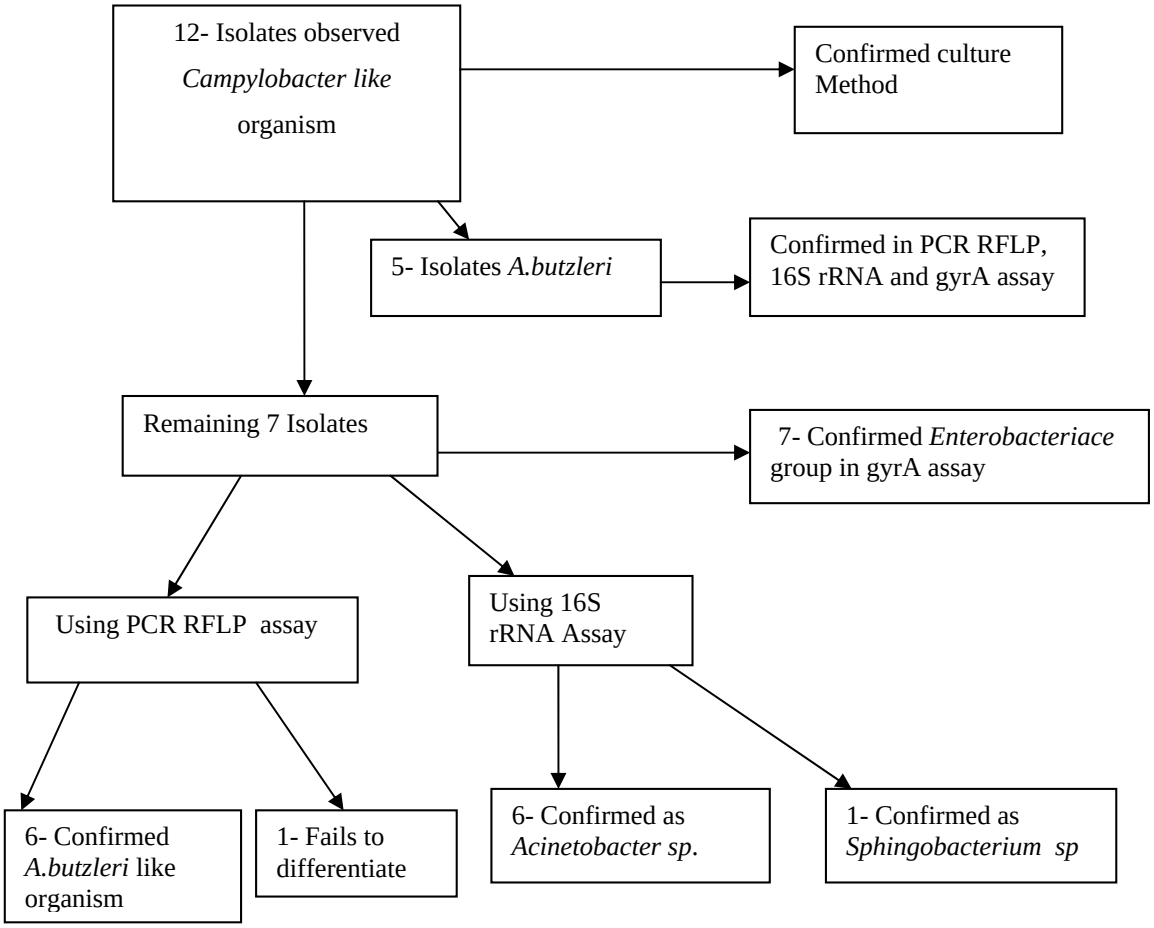


Figure.4 Comparative phylogenetic analysis of gyrA and 16S rRNA gene generated with the neighbor-joining method. The phylogeny presented is based on the alignment of the16S rRNA gene (Figure 4A) and gyrA gene (Figure 4B) sequences. The analysis was performed on the Phylogeny.fr platform. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT).Collapse branches having branch support value smaller than 50%.Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Dereeper *et al*, 2008)



ANDL01-05 : *Arcobacter butzleri* strains from South Bass Island (abSBI) ; abER: *Arcobacter butzleri* from Canada; abATCC:*Arcobacter butzleri* from ATCC; Aski: *A.skirrowii*; Acry: *A.cryaerophilus*; Aci: *A.cibarius*; Cc:*Campylobacter coli*; Chy: *Campylobacter hyointestinalis*; Cf: *Campylobacter fetus*; Cj:*Campylobacter jejuni*; Cl:*Campylobacter lari*; Cu:*Campylobacter upsaliensis;* Hp: *Helicobacter pylori* ; Se: Salmonella enteric; Ec: *Escherichia coli;* Pa: *Pseudomonas aeruginosa*

Figure.5 Flow chart for analysis results of environmental isolates using different Methods



Some of the *A.butzleri* isolates from the environment were recognized as 100% sequence similarity with human isolates, which established the most attention-grabbing evidence of relationships, which concern to public health risk. This substantiates that of *A. butzleri* may cause potential water borne diseases.

It was found that gyrA sequence analysis is the potential tools compared to 16S r RNA sequence analysis and PCR RFLP analysis for the identification of *A.butzleri*. The molecular tools as its own benefit to analyze the *Arcobacter* isolates. However, advantage of gyrA target size is small (300bp) which is very small and simple for the sequence analysis. This study demonstrated that PCR RFLP assay fails to differentiate species level discrimination of *Arcobacter*. Although 16S rRNA analysis is illustrated the identification of *A.butzleri* but not as better as gyrA assay in terms of species and sub-species level discrimination. Another reason is some of the isolates were confirmed in 16S rRNA as *Acinetobacter* spp which belongs to *gamma Proteobacteria*. However, gyrA assay confirmed those isolates as completely distance group of family and it gives a strong evidence of species level discrimination within the *Arcobacter* species. To our knowledge this is the first

report established the relationships of gyrA sequence of *Arcobacter* spp. between environmental and human isolates and assumed that *Arcobacter* may be associated with waterborne disease transmission.

The *gyrA* gene encodes one subunit of DNA gyrase and is highly conserved between species. The *gyr*A sequencing analysis continues to be a relatively simple, specific and accurate method for identification of species within the Campylobacteraceae family and should prove useful in future surveillance studies of *Campylobacter*-like organisms. The assessment of results obtained from using the different molecular methods suggested in this study that gyrA sequencing is suitable tools for the identification of *Arcobacter* from water samples. The gyrA sequence analysis is proved to be a efficient sequencing tools for classification of species within the Campylobacteraceae family and established a important methods in future studies of *Campylobacter*-like organisms. The evidence in ground water may be the potential to cause waterborne disease and is an impending risk for public health. Additionally, these were similar to clinical isolates from Canada and CDC, which bring to a close possibility of root of infection.

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