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First isolation report of *Arcobacter cryaerophilus* from a human diarrhea sample in Costa Rica

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ABSTRACT

Arcobacter cryaerophilus is an emerging enteropathogen and potential zoonotic agent transmitted by food and water. In Costa Rica, this bacterium has not been associated with cases of human gastroenteritis, even though it has been isolated from farm animals, especially poultry. This paper reports the first isolation of *A. cryaerophilus* from a human case of bloody watery diarrhea and the virulence genes associated with this isolate.

KEYWORDS: *Arcobacter cryaerophilus*. First isolation. Chronic diarrhea.

Arcobacter cryaerophilus is an emerging enteropathogen and potential zoonotic agent that can be transmitted by food and water[1-3]. It is a Gram-negative curved rod recognized as a potential food and waterborne pathogen[3]. This bacterium was formerly known as aerotolerant *Campylobacter*-like bacteria[4] and the *Arcobacter* genus was proposed by Vandamme and De Ley more than twenty years ago[5]. *Arcobacter* species have been isolated worldwide. Reports of their presence in diverse products of animal origin include poultry[6], beef[7], pork[8], shellfish[9] and milk[10]. Similarly, isolation from drinking and fecal polluted water has been reported[5,10].

Distinct *Arcobacter* species can produce different pathologies in animals and humans and these bacteria are to be considered enteric pathogens according to the World Health Organization[11]. The ability of this group of agents to produce disease, such as human and animal enteritis has been proved. Nevertheless, there is a limited knowledge about their pathogenesis and infecting dose[12].

Four species, including *A. butzleri*, A. *cryaerophilus*, *A. skirrowii* and *A. thereius*, have been isolated from human infectious processes, especially diarrhea[13,14].

Infection can occur through cross-contamination during food handling, consumption of contaminated food of animal origin, contaminated drinking water or by direct contamination with stools[1,15,16].

A. butzleri, A. thereius, A. skirrowii and *A. cryaerophyilus* have been isolated from different animal sources in Costa Rica, especially from poultry[17-20]. Nevertheless, human diarrhea associated to *Arcobacter* sp. has not yet been described. This paper reports the first isolation of *A. cryaerophilus* from a human case of bloody watery diarrhea and the virulence genes associated with this isolate.

A 27-year-old female patient, living in the capital city and referring a previous chronic diarrhea for two months, sought a clinical laboratory because of a sudden change to a bloody watery diarrhea. She referred acute stomach pain and a diet with a strong intake of chicken meat in the last days.

The patient's stools were studied for common enteropathogens, including virus and parasites, but all tests were negative. Because of the presence of a comma-like

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rod in a smear coming from a colony isolated in blood agar aerobically cultured, and since the culture of *Arcobacter* is not part of common clinical microbiology laboratory routine in Costa Rica, the sample was sent to the Food and Water Microbiology Laboratory, University of Costa Rica, where the detection and identification of this microorganism was carried out. During the period of analysis, the patient did not receive any antibiotic and, when contacted again, referred to be healthy and without any further problems.

For analysis, 1 g of the sample was transferred into tubes containing 10 mL of *Arcobacter* enrichment broth developed by Houf that was aerobically incubated at 30 °C for 48 h[21]. After this time, *Arcobacter* isolation was performed using the membrane filtration technique[21]. Briefly, 100 mL of enrichment media were inoculated onto a sterile 0.45 µm pore size nitrocellulose membrane filter placed on the surface of non-selective blood agar plates, based on the Cape Town protocol[22]. Plates were left at room temperature for one hour. After removing the filter, plates were aerobically incubated at 30 °C for up to 5 days. Suspicious colonies were selected, checked by Gram staining and oxidase test. Gram-negative isolates showing a positive oxidase test and a curved shape were seeded on blood agar plates in order to have a pure culture before performing PCR identification.

Arcobacter isolates were identified by PCR. DNA extraction was performed using the boiling lysis method. PCR technique described by Harmon and Wesley[23] was employed for identification at the genus level. Briefly, PCR amplification was performed in a reaction mixture (50 µL) containing Tris-HCl buffer (pH 7.4), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 µM of each primer, 1.5 U- of *Taq* polymerase (Oxoid) and 5 µL of template DNA. The *Arcobacter* genus-specific 16S rRNA fragment was amplified using the forward primer Arco I (5´-AGAGATTAGCCTGTATTGTATC-3´) and the reverse primer Arco II (5´-TAGCATCCCCGCTTCGAATGA-3´) (*19*). The thermocycling program was as follows: 94 °C for 4 min; 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min (25 cycles); and 72 °C for 7 min. Analysis of PCR products was done by electrophoresis (60 V, 1.5 h) in 1.5% agarose gels (w/v) with Mass Ruler (100-1000 bp) and GelRed staining. Positive *Arcobacter* identification was reported when a product of 1,223 bp was obtained.

To differentiate *Arcobacter* isolates at the species level, the multiplex-PCR assay described by Douidah *et al*.[24] was used. Briefly, amplification of the specific fragments was done with the forward primer Arco F (5´-GCTAGAGGAAGAGAAATCAA-3´) and the reverse primers ButR (5´-TCCTGATACAAGATAATTGTACG-3´), TherR (5´-GCAACCTCTTTGGCTTACGAA-3´), CibR (5´-CGAACAGGATTCTCACCTGT-3´), and SkiR (5´-TCAGGATACCATTAAAGTTATTGATG-3´) for *A. butzleri* (2,061 bp), *A. thereius* (1,590 bp), *A. cibarius* (1,125 bp), and *A. skirrowii* (198 bp), respectively. Amplification of the 395-bp fragment from *A. cryaerophilus* was done with the primers CriF (5´-CAGAGGAAGAGAAATCAAAT-3´) and CriR (5´-CCCACTATTCCATCAGTGAG-3´). Template preparation and reaction mixture were prepared as described above. Amplification was performed using the Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, USA). The thermocycling program was as follows: 94 °C for 4 min; 94 °C for 45 s, 58 °C for 45 s and 72 °C for 2 min (30 cycles); and 72 °C for 7 min. PCR products were separated by electrophoresis in 2% GelRed-stained (Sigma) agarose. Gels and UV light was used for visualization. The reference strain *A. butzleri* UACH001 was used as positive control, as well as a previously identified isolate of *A. cryaerophilus.*

Virulence genes were identified using the protocol described by Douidah *et al.*[25], which includes 9 different virulence genes. Primers used are described in Table 1.

PCR amplification was performed in a reaction mixture (50 µL) containing Tris-HCl buffer (pH 7.4), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 2 µM of each primer, 1.5 U/µL of *Taq* polymerase (Oxoid) and 2 µL of template DNA.

The thermocycling program was: 94 °C for 3 min; 94 °C for 145 s, 56 °C for 45 s and 72 °C for 45 s. (32 cycles); and 72 °C for 3 min. Analysis of PCR products was done by electrophoresis (60 V, 1.5 h) in 1.5% agarose gels (w/v) with Mass Ruler (100-1000 bp) and GelRed staining.

Isolation of *Arcobacter* from Costa Rican food products has been well documented. In poultry, Villalobos *et al.*[17] reported a 17.3% frequency from chicken viscera used for human consumption and Fallas-Padilla *et al.*[18] have reported a 56% frequency from chicken breast samples. Also, a 6.5% isolation frequency has been described for the whole poultry production chain. From minced meat, a 48% frequency has been reported by Córdoba *et al.*[20]. However, despite the fact that *Arcobacter* has been isolated from food products of animal origin in Costa Rica, there are no reports about the presence of this bacterium in stool samples from humans suffering from diarrhea.

Arcobacter has been reported as an organism associated with intestinal infections in humans since 1987, and *A. butzleri* and *A. cryaerophilus* have been associated with abortion and enteritis in animals and with diarrhea and bacteremia in adults and children[26].

In this study, *Arcobacter* was isolated from a human stool sample of a diarrheic patient and identified as *A. cryaerophilus.* This is the first case in Costa Rica. To our knowledge, few cases of *A. cryaerophilus* infection have

Table 1 - Sequence of nucleotides used as primers for detection of *Arcobacter* virulence genes

Primer	Target gen	Sequence	Size (bp)
cadF-F	cadF	TTACTCCTACACCGTAGT	283
cadF-R		AAACTATGCTAACGCTGGTT	
ciaB-F	ciaB	TGGGCAGATGTGGATAGAGCTTGGA	284
ciaB-R		TAGTGCTGGTCGTCCCACATAAAG	
cj1349-F	cj1349	CCAGAAATCACTGGCTTTTGAG	659
cj1349-R		GGGCATAAGTTAGATGAGGTTCC	
mviN-F	mviN	TGCACTTGTTGCAAAACGGTG	294
mviN-R		TGCTGATGGAGCTTTTACGCAAGC	
pldA-F	pldA	TTGACGAGACAATAAGTGCAGC	293
pldA-R		CGTCTTTATCTTTGCTTTCAGGGA	
tlyA-F	tlyA	CAAAGTCGAAACAAAGCGACTG	230
tlyA-R		TCCACCAGTGCTACTTCCTATA	
irgA-F	irgA	TGCAGAGGATACTTGGAGCGTAACT	437
irgA-R		GTATAACCCCATTGATGAGGAGCA	
hecA-F	hecA	GTGGAAGTACAACGATAGCAGGCTC	537
hecA-R		GTCTGTTTTAGTTGCTCTGCACTC	
hecB-F	hecB	CTAAACTCTACAAATCGTGC	528
hecB-R		CTTTTGAGTGTTGACCTC	

been reported so far[27-30]; the latest one being reported by Figueras *et al.*[30] in 2014 from a 26-year-old Spanish male that presented bloody diarrhea and had no comorbidities. Low incidence of human disease from *A. cryaerophilus* may be due to the difficulty in recognizing or identifying this bacteria, because of their slow growth rate and the use of specific multiplex PCR methods for its identification[26,31].

Virulence genes are denominated as putatives due to the amino acid structures and functions corresponding to other genomic structures already characterized in other bacterial species like Campylobacter[32]. Nine different putative virulence genes were searched in the *A. cryaerophilus* strain isolated. From these, four were detected, including cadF, cj1349, plda and ciaB. The cadF and cj1349 genes encode fibronectin-binding proteins that promote the bacterial adherence to intestinal cells. The cadF also induces the bacterial internalization through the ATPase activation. The plda gene encodes an A phospholipase of the external membrane and it is also associated with reticulocyte lysis[33]. The ciaB gene translates invasion proteins that are injected into host cell through a T-type III secretion system and translocates virulence factors to the interior of the cell[34]. All these virulence genes were present in the isolate obtained from the human stool sample in Costa Rica, strongly suggesting a potential role of this organism as the causative agent of to the disease.

Earlier papers have suggested that the presence of *Arcobacter* species in poultry and minced meat from Costa Rica may represent a public health risk, and control measures should be developed by both industry and consumers to minimize this risk. This first isolation of *A. cryaerophilus* from a human stool sample demonstrates that virulent strains of this bacterium have the potential to colonize susceptible individuals producing diarrheal disease and should be considered during differential diagnosis of human gastroenteritis. Isolation and characterization of this bacterium might be cumbersome, but it is feasible, especially in modern times in which selective media might be used and filtration method has proved to be effective. Nevertheless, further studies are needed to establish a correlation between the presence of genes and their expression *in vitro*.

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