Letter to the Editor

Genetic Characteristics and Antimicrobial Susceptibility of *Arcobacter butzleri* Isolates from Raw Chicken Meat and Patients with Diarrhea in China*

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Arcobacter is an emerging foodborne pathogen worldwide. In this study, the prevalence, antimicrobial susceptibility and genetic characteristics of *Arcobacter* from different sources were investigated. Eighteen *A. butzleri* isolates were obtained from 60 raw chicken meat samples (16/60, 27%) and 150 patients with diarrhea (2/150, 1.3%). The resistance ratios to nalidixic acid, ciprofloxacin, clindamycin, chloramphenicol, and florfenicol were 83.33% (15/18), 38.89% (7/18), 38.89% (7/18), 33.33% (6/18) and 33.33% (6/18), respectively. We performed whole genome sequencing of the 18 isolates, and we predicted antibiotic resistance genes and virulence factors by using assembled genomes through blastx analysis. Two resistance genes, $bla_{OXA-464}$ and *tet*(H), and the C254T mutation in *gyrA*, were identified in the genomes of some resistant isolates. Furthermore, virulence genes, such as *flgG*, *flhA*, *flhB*, *fliI*, *fliP*, *motA*, *cadF*, *cjl349*, *ciaB*, *mviN*, *pldA* and *tlyA*, were found in all strains, whereas *hecA*, *hecB* and *iroE* were found in only some strains. Phylogenetic tree analysis of *A. butzleri* isolates on the basis of the core-genome single nucleotide polymorphisms showed that two isolates from patients with diarrhea clustered together, separately from the isolates from raw chicken and the chicken strains. This study is the first comprehensive analysis of *Arcobacter* isolated in Beijing.

Key words: *Arcobacter*; *Arcobacter butzleri*; Prevalence; Whole genome sequence; Antimicrobial susceptibility; Genomic structure

Arcobacter spp. is a new foodborne pathogen and potential zoonotic agent that can also cause outbreaks of foodborne illness. *Arcobacter* spp. can cause diarrhea, mastitis, miscarriage and gastroenteritis in animals, and diarrhea, bacteremia, endocarditis and peritonitis in humans[1]. *Arcobacter* spp. is prevalent worldwide and has been identified in patients with diarrhea, and in the environment, including in water, animals and foods of animal origin, fish and seafoods, vegetables[2-4]. *Arcobacter* spp. can be transmitted through contaminated food and water sources and cause human diseases[4]. The *Arcobacter* genus comprises Gram-negative, curved rod-shaped bacteria 0.2–0.5 μm in diameter and 1–3 μm long, growing at temperatures of 15–42 °C, in contrast to the taxonomically related genus *Campylobacter*[4]. After 3 days of incubation on blood agar plates, the colonies are round and whitish with diameters of 2–4 mm.

Currently, the *Arcobacter* genus can be divided into 29 species, among which *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are considered most closely associated with human diseases, with *A. butzleri* and *A. cryaerophilus* considered hazardous to human health by the International Commission on Microbiological Specifications for Foods as early as 2002[1,2,4]. *A. butzleri* has been found to be the most prevalent species in a study of *Arcobacter* in patient feces, and has been reported to cause more long-lasting and aqueous, but less bloody and acute diarrhea, with little presence in the stools, among healthy asymptomatic people. In addition, *A. butzleri* is widely distributed in animal-derived food. It subsequently colonizes the intestines of animals (cattle, sheep, pigs and poultry) and contaminates the food supply during the slaughter process, thus

doi: 10.3967/bes2021.139

*This work was supported by National Key Research and Development Program of China [grant No.: 2021YFC2301000] and the Academic Commission of Shunyi District Center for Disease Control and Prevention, Beijing, China.

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indicating that these animals may be reservoir hosts[5]. The pathogenic mechanism of *A. butzleri* is unclear, but virulence genes have been studied through genome analysis and PCR, including *cadF* and *cjl349*, which encode fibronectin-binding proteins; *ciaB*, which encodes an invasion protein; virulence factors *mviN*; *pldA* and *tlyA*, which encode phospholipases; *hecA*, which encodes a member of the hemagglutinin family; *hecB*, which encodes a related hemolysin activation protein; *IroE*, which encodes an enterobactin hydrolase present in uropathogenic *E. coli*; and *irgA*, a homologue of the iron-regulated outer membrane protein in *Vibrio cholerae*[4].

In China, the distribution of *Arcobacter* in raw meat has been reported[6], but no report has addressed the prevalence of patients with diarrhea. In this study, the distribution and antimicrobial susceptibility of *Arcobacter* in market raw chicken samples and hospitalized patients with diarrhea were obtained. Next, the genetic characteristics of 18 *Arcobacter* isolates were investigated by whole genome sequencing to explore potential pathogenicity and drug resistance mechanisms, and to provide data for food safety assessment and *Arcobacter* disease prevention and treatment. This is the first comprehensive study of *Arcobacter* in China.

A total of 150 fecal samples from patients with acute diarrhea and 60 fresh raw chicken meat samples, comprising 44 samples from wholesale markets and 16 samples from retail stores, were examined in this study. The patients with diarrhea had three or more bouts of watery, loose, mucus-containing or bloody-stools during a 24 h period in hospitals in the Shunyi district, Beijing, between May 1, 2017 and October 31, 2017. Five milligrams of fresh stool samples were collected from each patient, kept at 4 °C and sent to the laboratory within 24 h. Questionnaire information from each patient was collected by the clinical doctor during hospital visits (Supplementary File S1, available in www.besjournal.com). The unfrozen chicken meat samples were collected from May to July (3 months, 20 samples per month) of 2018.

The *Arcobacter* isolation was performed with an *Arcobacter* isolation kit with the membrane filter method (ZC-ARCO-001, Qingdao Sinova Biotechnology Co., Ltd., Qingdao, China). The specific procedures were as follows: 200 mg stool specimens were transferred into 4 mL enrichment buffer which was provided in the kit. The principle component of the enrichment buffer was the modified preston broth which was described in the manual book. The raw chicken meat was fully washed with 500–1,000 mL buffered peptone water for 10 min, and then 2 mL buffered peptone water was added to 4 mL enrichment buffer. The enriched suspension from both stool and chicken samples was incubated for 24 h at 37 °C in a microaerophilic atmosphere consisting of 5% O_2, 10% CO_2, and 85% N_2. Subsequently, 300 μL cultured enrichment suspension was spotted onto Karmali and Columbia agar with a 0.45 μm cellulose membrane filter. After air-drying in a biological safety cabinet for 40 min, the filter membrane was removed, and the plates were incubated in a microaerophilic atmosphere at 37 °C for 48 h.

After incubation, small round and whitish colonies 2 mm in diameter were streaked on plates and confirmed by Gram staining, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and real-time PCR. The mass spectrometry analysis used Flexcontrol software, and the results were interpreted with IVD MALDI Biotyper 2.3 software (Bruker Daltonik GmbH, Bremen, Germany). The criteria for determining the genus and species of bacteria were as follows: 2.300 to 3.000 points indicated reliable species level identification and 2.000 to 2.299 points indicated reliable genus level and possible species level identification. In this study, scores ≥ 2.000 were considered credible. For PCR identification, a loop was used to collect suspicious pure culture colonies, which were re-suspended in 200 μL ultrapure water, boiled for 10 min and centrifuged for 10 min at 8,000 ×*g*. Subsequently, the supernatant was removed for PCR species identification with a Real-time PCR kit (ZC-ARCO-003, Qingdao Sinova Biotechnology Co., Ltd.). The PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min.

The minimum inhibitory concentrations (MICs) for 11 antibiotics (erythromycin, azithromycin, nalidixic acid, ciprofloxacin, gentamicin, streptomycin, chloramphenicol, florfenicol, tetracycline, telithromycin and clindamycin) were determined with an agar dilution method by using a commercial kit for fastidious bacteria (ZC-AST-010, Qingdao Sinova Biotechnology Co., Ltd.). The MIC was the lowest concentration without visible growth. The cut-offs for resistance used in this study were those with an MIC value greater than or equal to the standards used in the National Antimicrobial Resistance Monitoring System (NARMS-2014) for

Campylobacter in the United States: erythromycin (≥ 32 μg/mL), azithromycin (≥ 8 μg/mL), nalidixic acid (≥ 64 μg/mL), ciprofloxacin (≥ 4 μg/mL), gentamicin (≥ 8 μg/mL), streptomycin (≥ 16 μg/mL), chloramphenicol (≥ 32 μg/mL), florfenicol (≥ 8 μg/mL), tetracycline (≥ 16 μg/mL), telithromycin (≥ 16 μg/mL) and clindamycin (≥ 8 μg/mL). *C. jejuni* ATCC 33560, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 were used as controls.

The genomes of 18 *Arcobacter* isolates were sequenced on the Illumina NovaSeq 6000 platform at the Microbial Genome Research Center (Institute of Microbiology Chinese Academy of Sciences, Beijing, China), and general features of the *A. butzleri* genome sequences were examined (Table 1). To sequence the genome, we constructed a 500 bp paired-end library and generated 150 bp reads for each isolate. The sequencing reads were filtered with fastp (https://github.com/OpenGene/fastp, version 0.20.0), and low quality reads were removed. The FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, version 0.11.8) was used to evaluate the quality of the raw sequence data. The sequencing reads were trimmed with Trimmomatic (version 0.38) to remove sequencing adapters. High-quality reads were assembled with SPAdes genome assembler software (https://github.com/ablab/spades, version 3.11.0). Annotation was performed with Prokka (https://github.com/tappearann/prokka, version 1.13.3) with the recommended standard settings. Core-genome single nucleotide polymorphisms (SNPs) were called with Snippy4.3.6 software (https://github.com/tappearann/snippy) from the reference (RM4018 complete genome, isolated from a patient with gastroenteritis). Gubbins software (https://github.com/sanger-pathogens/gubbins, version 2.3.4) was used as the recombination-removal tool to determine the pure SNPs without recombination. Phylogeny reconstruction was performed with the maximum likelihood method in raxml software (https://github.com/stamatak/standard-RAxML, version 8.2.12) with 1000 bootstraps. *A. cryaerophilus* 16CS1285-4 (GenBank accession number CP060693) was used as the outgroup and root of the tree. Resistance genes were predicted with ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), Abricate (https://github.com/tappearann/abricate, version 0.9.8) and Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/?q=CARD/ontology/3550

Table 1. General features of the *A. butzleri* genome sequences studied

Genome	Contig	Full genome length	Maximum genome length	GC (%)	Coding DNA sequence	Gene	BioSample accessions
102-Ab	36	2298927	458361	26.79	2270	2318	SAMN13841214
103-Ab	32	2299734	458361	26.79	2271	2319	SAMN13841215
012-Ab	29	2109493	350195	26.97	2103	2151	SAMN12393772
026-Ab	36	2113416	315820	26.95	2109	2150	SAMN12393773
027-Ab	52	2068991	324478	26.96	2075	2115	SAMN12393774
029-Ab	39	2191289	317880	26.94	2183	2232	SAMN12393775
031-Ab	28	2153538	350195	26.98	2157	2205	SAMN12393776
033-Ab	30	2153538	350195	26.98	2157	2206	SAMN12393777
034-AB	58	2282271	448429	26.86	2242	2290	SAMN13841213
036-Ab	61	2159998	259114	26.96	2143	2191	SAMN12393778
037-Ab	30	2113458	322451	26.97	2098	2146	SAMN12393779
039-Ab	39	2155516	322451	26.94	2164	2212	SAMN12393780
051-Ab	23	2169363	350168	26.95	2175	2223	SAMN12393781
052-Ab	30	2185485	322263	26.87	2166	2214	SAMN12393782
055-Ab	29	2109493	350195	26.97	2103	2151	SAMN12393783
056-Ab	46	2112196	302739	26.94	2088	2129	SAMN12393784
058-Ab	61	2157334	257578	26.97	2139	2187	SAMN12393785
059-Ab	39	2112687	272651	27.02	2105	2153	SAMN12393786

6), with an E-value of at least 1×10^{-10} as the cut-off. The identity cut-off and query coverage values were kept > 80% and > 80%, respectively. Virulence factors were identified with VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer).

The detection rate of *Arcobacter* in raw chicken samples was 26.7% (16/60), and all isolates were identified as *A. butzleri*. However, one previous study on the contamination status of *Arcobacter* in retail chicken in Beijing has reported an isolation ratio of 78.21% (61/78), and most of the isolates were identified as *A. cryaerophilus* (91.20%, 114/125)[6]. These results indicate that the dominant contaminated species of *Arcobacter* in retail chicken meat might vary in different markets. In addition, this is the first study on *A. butzleri* infection in patients with diarrhea in China, and we found two positive cases among the 150 cases of diarrhea (1.3%). The isolation rate was consistent with those reported in studies from Portugal and India, where *A. butzleri* was detected with an isolation ratio of 1.30% and 2.67%, respectively[3,7], but was different from those in Belgium (0.7%, 49/6,774), Turkey (0.27%, 9/3,287) and New Zealand (0.50%, 7/1,380)[2,8,9].

A.*butzleri* is an opportunistic pathogen that can cause severe and persistent diarrhea and even bacteremia in immunocompromised people[10]. In our study, the two cases of diarrhea were in immunocompromised people, a post-cancer patient and a 71-year-old male, who were co-infected with other pathogens (one case with *V. parahaemolyticus*, and another case with both *V. parahaemolyticus* and enterotoxigenic *E. coli*). Immuno-compromised and older people with diarrhea should receive more attention for such infections. In the future, additional surveillance studies of patients with diarrhea are needed.

In our study, the resistance ratios of 18 isolates for 11 drugs were 83.33% (15/18) for nalidixic acid, 38.89% (7/18) for ciprofloxacin, 38.89% (7/18) for clindamycin, 33.33% (6/18) for florfenicol, 33.33% (6/18) for chloramphenicol, 22.22% (4/18) for tetracycline, 22.22% (4/18) for telithromycin, 22.22% (4/18) for azithromycin, 11.11% (2/18) for gentamicin and 5.56% (1/18) for streptomycin. In addition, six (33.33%) isolates were multi-drug resistant, and the dominant resistance pattern was nalidixic acid and ciprofloxacin combined resistance (33.33%, 6/18). The resistance ratios to macrolides, aminoglycosides and tetracyclines were similar to those in other reports, but the resistance ratios to nalidixic acid (83.33%) and ciprofloxacin (38.89%) were much higher than those in other reports[11]. Moreover, genetic determinants of antibiotic resistance were searched through whole genome sequencing analysis, and the C254T mutation, which causes a Thr-85-Ile substitution in the quinolone-resistance determining region of *gyrA*, was found in some both nalidixic acid and ciprofloxacin resistant isolates (027-Ab, 039-Ab, 051-Ab, and 052-Ab). This result was consistent with those of A. Khalil[12]. Furthermore, $bla_{OXA-464}$ or $bla_{OXA-491}$ and *tet(H)* were identified with both ResFinder and CARD. Fifteen isolates, except 029-Ab, 052-Ab and 059-Ab, had $bla_{OXA-464}$ or $bla_{OXA-491}$, and only two isolates, 027-Ab and 039-Ab, had *tet(H)*.

The assembled genome size for all 18 strains ranged from 2,068,991 to 2,299,734 bp, with an average of 2,163,707 bp assembled in an average of 39 contigs per genome. An average of 2,152 coding DNA sequences (CDS) and 2,199 genes were obtained from each genome. Two isolates from patients with diarrhea clustered together, apart from the isolates from raw chicken samples, and the chicken strains exhibited genetic diversity (Figure 1). The adhesion associated genes were present in 38.9% (7/18) of the isolates. Analysis of virulence genes indicated that all isolates contained genes involved in motion related flagellin synthesis, such as *flgG*, *flhA*, *flhB*, *fliI*, *fliP* and *motA*. In our study, *cadF*, *cj1349*, *ciaB*, *mviN*, *pldA* and *tlyA* were found in all

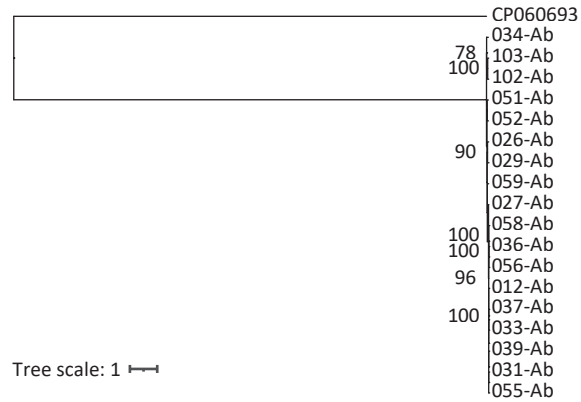

Figure 1. Phylogenetic tree of *A. butzleri* isolates based on the core-Genome SNPs. Phylogenetic tree was constructed based on core-SNPs of *A. butzleri* using raxml software. The scale bar represents substitution per site. The number before branching represents bootstrap. *A. cryaerophilus* 16CS1285-4 was used as outgroup and root of the tree. Only bootstrap values greater than 60 were displayed on the tree.

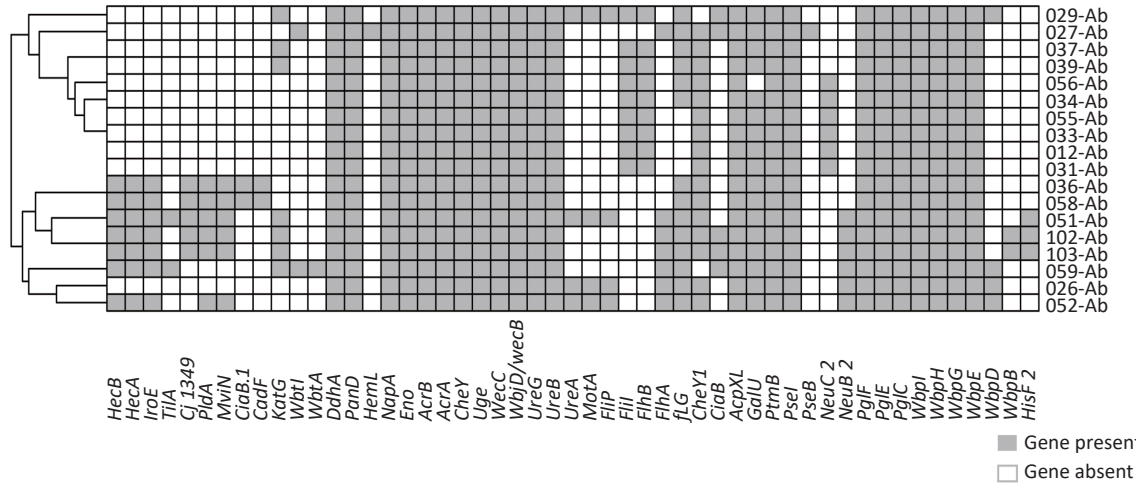

Figure 2. Heatmap of *A. butzleri* isolates based on virulence genes. The name of virulence genes is distributed horizontally and the name of *A. butzleri* isolates is distributed vertically in the heatmap. Gray indicates the presence of the gene and white indicates the absence of the gene.

strains, while *hecA*, *hecB* and *iroE* were found in only some strains, but *irgA* was not found in any strains. The presence of these virulence genes may play important roles in the pathogenicity of *A. butzleri*. The heatmap based on virulence gene clusters generated with the pheatmap package is presented in Figure 2.

In summary, we provide the first description of the prevalence and genetic characteristics of *Arcobacter* in patients with diarrhea in this study. The findings should aid in further investigation of this pathogen in China and worldwide.

Conflicts of Interest The authors have no conflicts of interest to declare.

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Received: March 2, 2021;

Accepted: July 16, 2021

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Supplementary File S1. Questionnaire for Stool Sample Collection

Case code:

Information provider:

(1) patient (2) family members or insiders (relationship:)

1. General information

1.1 Patient name:

1.2 Gender (1) male (2) female

If female, whether pregnant (1) yes, _____week(s) (2) no

1.3 Age (year)

1.4 Occupation

1.4.1 Employee in the catering industry (1) yes (2) no

1.4.2 Medical staff (1) doctors (2) nurses (3) care workers (4) Laboratory staff (5) administrative staff (6) others_____(please elaborate)

1.4.3 Other Occupation

(1) farmers (2) migrant worker (3) Industry blue collar Migrant worker (4) Cadre (5) teachers (6) students (7) Children in the kindergarten (8) Children not in the kindergarten (9) Housework or unemployed (10) Fisherman (11) herdsman (13)business services (16) others_____(please elaborate)

1.5 Current study or work place:

1.6 Address: ____Province _____City _____County (district) Township (street) _____Village (building) ____Team (unit)

1.7 Household registration: ____Province _____City _____County (district) Township (street) _____Village (building) ____Team (unit)

1.8 Nationality: (1) China (2) others

1.9 ID card or passport number:

1.10 Phone number:

2. Clinical manifestations

2.1 Date of onset: yy mm dd

2.2 Location of onset:

(1) China: ____Province _____City _____County

(2) Outside of China:

(3) On the transport: □ aircraft □ train □ ship □ car □ other

2.3 Clinical Symptoms:

Definition of dehydration: The physical sign include decreased skin elasticity, dry skin mucous membrane, accelerated or weakened pulse, collapsed superficial vein, frosty limbs, decreased urine volume, etc.

2.4 Outpatient/emergency treatment

2.4.1 Date of treatment:

2.4.2 Hospitals/departments:

2.4.3 whether used antibiotics in 3 days

If used, the name of the drugs:

2.5 Hospitalization and treatment

2.5.1 Whether hospitalization:

(1) yes (2) no (go to the part 3)

2.5.2 Date of admission: year month date

2.5.3 Admission hospital name:

2.5.4 Admission number:

2.5.5 Admission diagnosis: (1) diarrhea cases (2) fever cases (3) Other clinical diagnosis:

2.5.6 Treatment:

2.5.6.1 Drug treatment: (1) antibiotics (2) hormones (3) antiviral drugs (4) others:

2.5.6.2 Whether to stay in ICU:

(1) yes (Check in date: year month date) (2) no

3. Epidemiological information

3.1 daily diet 5 days before the onset

Food category:

(1) fruit and vegetables (2) meats and related products (3) grain and related products (4) eggs and related products (5) milk and related products (6) bean and related products (7) aquatic and related products (8) water, beverages and herbs (9) unknown

4. Specimen collection

Specimen Type:

(1) watery-stools (2) loose-stools (3) mucus-stools (4) bloody-stools (5) other (please elaborate):

Investigation units:

Investigation period:

Signature