Research Article

Genetic Diversity and Incidence of Virulence-Associated Genes of *Arcobacter butzleri* and *Arcobacter cryaerophilus* Isolates from Pork, Beef, and Chicken Meat in Poland

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Incidence of 9 virulence-associated genes and genetic diversity was determined in 79 *A. butzleri* and 6 *A. cryaerophilus* isolates from pork, beef, and chicken meat. All *A. butzleri* isolates harboured the *tlyA* gene, and most of them carried *ciaB*, *mviN*, *pldA*, *cadF*, and *cj1349* genes. *ciaB* was found to occur with higher frequency in poultry if compared with pork (*p* = 0.0007), while *irgA* was more frequent in poultry than in beef (*p* = 0.007). All 6 *A. cryaerophilus* isolates harboured the *ciaB* gene, while *mviN* and *tlyA* were detected in 3 out of these isolates. Only one isolate carried the *cadF* gene. All beef-derived *A. cryaerophilus* isolates carried *ciaB*, *mviN*, and *tlyA* genes. *A. cryaerophilus* isolates from chicken meat harboured *ciaB* gene only. The pork-derived isolate harboured *ciaB* and *cadF* genes. Seventy-four genotypes were distinguished within 79 *A. butzleri* isolates. Nineteen from 21 isolates derived from beef and pork were found to be closely related to *A. butzleri* from chicken meat. Each of the 6 *A. cryaerophilus* isolates was found to have unique genotype. We demonstrated that closely related genotypes can spread within pork, beef, and chicken meat populations of *A. butzleri* but not *A. cryaerophilus*.

1. Introduction

Genus *Arcobacter*, formerly known as “aerotolerant Campylobacter,” was included into the family Campylobacteraceae in 1991 [1] and currently consists of twenty species [2, 3]. Three *Arcobacter* species, that is, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, have been mainly associated with enteritis in humans and abortion in pigs [4–6]. *A. butzleri* has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods in 2002 [7]. The bacteria have been isolated from food, mainly from products of animal origin, with the highest prevalence in poultry, followed by pork and beef [8–10]. Consumption of *Arcobacter*-contaminated food is regarded as the most likely source of human infection [11]. *Arcobacter* spp. were also isolated from drinking water reservoirs and sewage [12]. Recent evidence suggests that *Arcobacter* spp., especially *A. butzleri* and *A. cryaerophilus*, may be involved in human enteric diseases [4, 13, 14]. Infections involving *Arcobacter* spp. may result in abdominal pain with acute diarrhoea or prolonged watery diarrhoea [15].

Analysis of complete genome of *A. butzleri* ATCC (American Type Culture Collection) 49616 revealed some homologues to virulence genes identified in *Campylobacter* and other bacteria, including *E. coli* and *Vibrio* spp. Already identified virulence-associated genes of *A. butzleri* include *cadF, cj1349, ciaB, mviN, pldA, tlyA, irgA, hecA*, and *hecB* [16]. *cadF* and *cj1349* encode fibronectin-binding proteins; *ciaB* encodes *Campylobacter* invasive antigen (CiaB), which may contribute to host cell invasion; *hecA* encodes HecA protein,
a member of the filamentous hemagglutinin; hecB encodes a hemolysin activation protein; tlyA encodes hemolysin; mviN encodes essential protein MVIN required for peptidoglycan biosynthesis; pldA encodes outer membrane phospholipase PldA associated with lysis of erythrocytes; and irgA encodes iron-regulated outer membrane protein IrgA. Although some authors reported on abundance of virulence factors, pathogenicity, and genetic diversity of food-derived Arcobacter isolates throughout the world, characteristics of Arcobacter spp. in Poland are still unknown [17–21].

The aim of this work was to determine the incidence of virulence-associated genes and genetic diversity of A. butzleri and A. cryaerophilus isolates from pork, beef, and chicken meat in Poland.

2. Materials and Methods

2.1. Bacterial Strains. Seventy-nine A. butzleri and 6 A. cryaerophilus cultures were isolated from meat samples, purchased from retail market in Lower Silesia region (Poland) as described previously [22]. Among 79 A. butzleri isolates, 58, 11, and 10 were obtained from chicken, beef, and pork samples, respectively. Within A. cryaerophilus cultures, 3 isolates were from beef, 2 were from chicken, and one was from pork meat. Arcobacter spp. isolates were cultured according to Houf et al. [23] with modifications, in Arcobacter broth (Oxoid) with selective supplement containing cefoperazone, amphotericin B, and teicoplanin (CAT, Oxoid). Additionally, novobiocin (32 mg/L), 5-fluorouracil (100 mg/L), and trimethoprim (64 mg/L) (Sigma) were added to the broth. After 48-hour incubation in aerobic atmosphere, at 30°C, the bacteria were subcultured on Arcobacter agar plates (supplemented with chemotherapeutics mentioned above) and in parallel on agar plates with defibrinated sheep blood (supplemented with chemotherapeutics mentioned above). Phenotypically suspected colonies were transferred to blood agar plates and incubated in aerobic conditions for 48 h at 30°C. One Arcobacter spp. isolate per sample was taken for further characterization. The isolates were preserved by freezing in Cryobank (Mast Diagnostics) at −80°C.

2.2. Preparation of Bacterial DNA. Total DNA was isolated as described by Agersborg et al. [24]. Briefly, the bacteria from 1 mL overnight culture were pelleted by centrifugation and suspended in 200 μL of distilled water containing 1% Triton X-100. The mixture was boiled for 10 min, and then the tubes were centrifuged for 5 min at 13,000 × g. The supernatant containing DNA was used in PCR.

2.3. Species Identification of Arcobacter Isolates. The isolates were identified at species level using multiplex PCR according to Houf et al. [25]. Amplification products were resolved in 1.5% agarose containing 0.5 μg/mL ethidium bromide and documented using GelDocXR System (BioRad, Hercules, CA). Each PCR run was performed using DNA from the reference CCM (Czech Collection of Microorganisms) 4826 A. butzleri and CCM 3933 A. cryaerophilus strains as positive controls and ATCC 33560 Campylobacter jejuni as a negative control.

2.4. Identification of Virulence-Associated Genes. A. butzleri and A. cryaerophilus isolates were PCR-screened for the presence of nine virulence-associated genes, such as cadF, cj1349, ciaB, mviN, pldA, tlyA, irgA, hecA, and hecB, as described by Douidah et al. [17]. The 10 μL aliquots of PCR products were resolved on 1.5% agarose gel and documented with a GelDoc XR documentation system (BioRad, Hercules, USA).

2.5. Genotyping of A. butzleri and A. cryaerophilus Isolates. A survey of the genetic diversity of the A. butzleri and A. cryaerophilus isolates was performed using ERIC PCR primers ERIC2 5'-AAGTAAGTGAGGGTGAGCG-3' and ERICIR 5'-ATGTAAGCTCCTGGGGATTAC-3' as described by Houf et al. [26]. The PCR was performed in a mixture containing 20 mmol/L Tris-HCl, pH 8.4, 3 mmol/L MgCl2, 50 mmol/L KCl, 0.2 mmol/L of each deoxyribonucleotide, 1.25 U of Taq DNA polymerase (Thermo Scientific, Poland), 50 pmole of each primer (Genomed, Warsaw, Poland), and 2 μL of DNA solution. Thirty-six PCR cycles were carried out according to the following scheme: denaturation at 94°C for 30 s, annealing at 35°C for 30 s, and elongation at 72°C for 120 s. The denaturation/annealing and annealing/elongation ramping rates were set to 5 minutes [27]. Amplicons were separated on 3% agarose gel at 170 V for 3 h. The gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed. The resulting profiles were analyzed using the software available on http://insilico.ehu.es/dice_upgma/ to generate dendograms by UPGMA clustering, using Dice correlation.

2.6. Statistical Analysis. Mann-Whitney U test was applied to assess statistical significance of obtained data. Calculations were performed using Statistica 9.1 (StatSoft, Poland). p values below 0.05 were considered to be significant.

3. Results

3.1. Prevalence of Virulence-Associated Genes in A. butzleri and A. cryaerophilus Isolates. Screening of 79 A. butzleri isolates revealed that all of them harboured the tlyA gene. Most of the A. butzleri isolates carried ciaB (97%), mviN (97%), pldA (92%), cadF (89%), and cj1349 (66%), while hecB (48%), irgA (46%), and hecA (30%) genes were less frequent (Table 1). ciaB was found to occur with higher frequency in poultry if compared with pork (p = 0.0007), while irgA was more frequent in poultry than in beef (p = 0.007).

Ten percent (8 out of 79) of the A. butzleri isolates harboured all nine genes, and most of them (7 isolates) derived from chicken meat. The carriage of 8 and 7 virulence-associated genes was reported in 44% (35 out of 79) of A. butzleri isolates. Most of them were found in chicken meat (28 isolates), followed by pork (4 isolates) and beef (3 isolates). Seventeen (22%), 14 (18%), and 2 (3%) A. butzleri isolates possessed 6, 5, and 4 virulence-associated genes, respectively (Figure 1). The isolates harboured 3 (2 isolates) and 2 genes (1 isolate) were only reported in A. butzleri from beef and pork, respectively.
and chicken meat. The populations recovered from retail pork, beef, and chicken meat harboured mviN while those from molluscs harboured ciaB and cadF genes. Two A. cryaerophilus isolates from chicken meat harboured ciaB gene only. The pork-derived isolate harboured ciaB and cadF genes (Table 1).

3.2. Genotypes of A. butzleri and A. cryaerophilus. Seventy-four genotypes were distinguished within 79 A. butzleri isolates (Figure 1). These isolates could be grouped into 8 clusters using 90% cut-off similarity. Nineteen from 21 isolates derived from beef and pork were found to be closely related to A. butzleri from chicken meat. Genotypes of 6 isolates from beef and 6 from pork shared >90% similarity to chicken isolates, when >80% similarity to chicken genotypes was found in 4 isolates from beef and 3 isolates from pork. Only two genotypes, one shared by beef (isolate 204) and one by pork isolate (isolate 60), were found to be distant from chicken genotypes. No relation between A. butzleri genotype and incidence of virulence genes could be found.

Each of the 6 A. cryaerophilus isolates was found to have unique genotype (Figure 2). Over 80% genotype similarity was only found within two beef and two pork A. cryaerophilus isolates but not between isolates from beef, pork, and chicken meat.

4. Discussion

A. butzleri and A. cryaerophilus are considered as potential food-borne pathogens [28], but the knowledge on the presence of virulence-associated factors and genetic diversity of food-derived isolates is still limited. Thus, we examined the prevalence of virulence-associated genes in A. butzleri and A. cryaerophilus populations recovered from retail pork, beef, and chicken meat. The tlyA, ciaB, mviN, pldA, cadF, and ciaB genes were found to occur most frequently in A. butzleri isolates. Similar results were obtained by Collado et al. [18], who showed that at least 73% of A. butzleri isolates from shellfish in Chile harboured cadF, ciaB, mviN, pldA, and tlyA genes, although only 31% of the isolates possessed ciaB gene. In turn, in Belgium [17], Germany [29], and Iran [21], these six genes were found in all A. butzleri isolates recovered from various sources, including meat. A high occurrence of these genes was also reported in Spain, where all isolates from meat, mussels, and sewage possessed ciaB gene, when 92% of them harbored cadF and ciaB genes [30]. Consistent to other reports, we showed hcaA, hcbB, and irgA genes to occur most rarely within A. butzleri population [17, 18, 29, 30].

Our results indicate that 14% of A. butzleri isolates harboured all nine virulence-associated genes. These results are similar to that obtained by Douidah et al. [17], who found all these genes in 15% of A. butzleri isolates, and Karadas et al. [29], who reported 13% of A. butzleri isolates carrying all mentioned virulence-associated genes.

Consistent with previous reports [18, 21, 30], we noted a higher incidence of virulence-associated genes in A. butzleri as compared to A. cryaerophilus. In our study, only ciaB gene was detected in all the six A. cryaerophilus isolates, mviN and tlyA were detected in three isolates, and cadF gene was detected in one isolate. A high frequency of virulence genes was observed in beef-derived isolates, since all the three isolates possessed ciaB, mviN, and tlyA genes. A high incidence of mviN (100%) and tlyA (62%) genes was already reported in A. cryaerophilus derived from cattle [21]. Collado et al. [18] showed that mviN and tlyA genes commonly occur in A. cryaerophilus from molluscs, when Douidah et al. [17] reported mviN gene to dominate in A. cryaerophilus from human and animals. In contrast to A. butzleri, no A. cryaerophilus isolates harboured multiple virulence genes, which is consistent with results obtained by other authors [17, 18, 21]. According to Douidah et al. [17], this difference could be explained by a different pathogenic behaviour of these species or due to a high heterogeneity of their genomes.

A high diversity of virulence genes pattern was found in A. butzleri isolates from each source, except ciaB and irgA genes found to be more frequent in A. butzleri from poultry.

Relatively little is known on genetic structure of Arcobacter spp. population and even less on impact of genetic background on distribution of virulence-associated genes. Data obtained in this study demonstrate no relation between genotype and virulence genes repertoire in studied Arcobacter species. According to our data, even closely related isolates may carry different virulence genes. Similar observation was already made by Lehmann et al. [19] studying Arcobacter spp. genotypes from food in Germany.

| Species          | Origin/number of isolates | Percent of isolates harbouring virulence genes possessing specific gene |
|------------------|--------------------------|---------------------------------------------------------------------|
|                  |                          | cadF | ciaB | mviN | tlyA |
| A. butzleri      | Chicken meat/58          | 90   | 71   | 100  | 53   |
|                  | Pork/10                  | 90   | 60   | 80   | 90   |
|                  | Beef/11                  | 82   | 45   | 100  | 9    |
|                  | Total/79                 | 89   | 66   | 97   | 46   |
| A. cryaerophilus | Chicken meat/2            | 0    | 0    | 100  | 0    |
|                  | Pork/1                   | 100  | 0    | 100  | 0    |
|                  | Beef/3                   | 0    | 0    | 100  | 0    |
|                  | Total/6                  | 17   | 0    | 100  | 0    |

| Species          | Origin/number of isolates | Percent of isolates harbouring virulence genes possessing specific gene |
|------------------|--------------------------|---------------------------------------------------------------------|
|                  |                          | ciaB | mviN | tlyA |
| A. butzleri      | Pork/10                  | 90   | 60   | 100  |
|                  | Beef/11                  | 82   | 45   | 100  |
|                  | Total/79                 | 89   | 66   | 97   |
| A. cryaerophilus | Chicken meat/2            | 0    | 0    | 100  |
|                  | Pork/1                   | 100  | 0    | 100  |
|                  | Beef/3                   | 0    | 0    | 100  |
|                  | Total/6                  | 17   | 0    | 100  |
Figure 1: Dendrogram generated by UPGMA clustering based on PCR fingerprinting using ERIC primers performed on 79 *A. butzleri* isolates. Repertoire of virulence-associated genes of each isolate was presented in left panel. Isolate origin was indicated as C, P, and B for chicken, pork, and beef meat, respectively.
Irrespective of genotypic method used, a large genetic heterogeneity of *Arcobacter* spp. population is frequently reported [20, 31–33]. However, some reports point out that closely related *Arcobacter* spp. genotypes can be isolated from some nonoverlapping sources like beef, lamb, pork, poultry, and fish meat [9, 19]. Our data also argue that genotypes closely related to *A. butzleri* from poultry meat can also be found within populations from pork and beef meat. In *A. cryaerophilus* isolates studied here, low genetic relatedness between pork, beef, and chicken meat isolates was noted, but general conclusions cannot be drawn here due to limited number of isolates.

### 5. Conclusions

Reertoire of virulence-associated genes in *A. butzleri* and *A. cryaerophilus* isolates from retail meat in Poland is similar to that reported in other countries. It seems that closely related genotypes can spread within pork, beef, and chicken meat populations of *A. butzleri* but not *A. cryaerophilus*.

### Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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