Prevalence and Distribution of *Arcobacter Butzleri* Virulence Genes in Poultry Slaughterhouse Effluent Samples in Tonekabon

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Abstract

*Arcobacter butzleri* is known as the cause of enteritis, abdominal cramps, bacteremia, and appendicitis in humans, it is also the cause of enteritis and abdominal pain in animals. It has been introduced as the most dangerous species for human health by the International Committee on Food Microbiology and recently as an important zoonotic pathogen. The aim of this study was isolation and characterization of *Arcobacter butzleri* based on pathogenic genes (*tlyA*, *ciaB*, *mviN*) in different seasons. Therefore, 238 samples were collected from the effluent of poultry slaughterhouse in Tonekabon, Iran. The suspected colonies were identified using biochemical test and then confirmed using the Polymerase chain reaction (PCR) technique. Out of 42 isolated of *A. butzleri*, the virulence genes were detected in 15 isolates. The results showed that *ciaB* gene was present in 13 strains with a frequency of 30.9%, *mviN* in 11 samples with a frequency of 26.2%, and *tlyA* gene in 9 samples with a frequency of 21.4%. The results also revealed that the highest frequency of *A. butzleri* was 8.4% in spring and the lowest rate was 1.2% in winter.

Keywords

*Arcobacter butzleri*

Virulence gene

Polymerase chain reaction

Introduction

*Arcobacter* genus is a member of *Campylobacter* family and belongs to the class of *Epsilon proteobacteria* (Ellis, Neill, O'Brien, Ferguson, & Hanna, 1977). *Arcobacteria* can cause infertility, miscarriage, and digestive disorders in animals, and bacterial gastroenteritis, endocarditis, and peritonitis in humans (Badilla-Ramirez, Fallas-Padilla, Fernandez-Jaramillo, & Arias-Echandi, 2016). They are widely found in water and the sources of animal foods. *Arcobacter butzleri* is the most common species which is usually isolated from a variety of environments (Figueras et al., 2014; Jyothsna, Rahul, Ramaprasad, Sasikala, & Ramana, 2013; Levican, Collado, Yustes, Aguilar, & Figueras, 2014) Most studies on the prevalence of *Arcobacter* in foods are related to the poultry that have the highest prevalence, followed by pork and raw milk products (Collado, Guaro, & Figueras, 2009; Figueras et al., 2014) The genus *Arcobacter* has become very important in recent years because its members are
considered as a potential zoonotic agent. The global prevalence of \textit{Arcobacter} infection is unclear since the usual diagnostic method is not applicable to this bacterium. There is no doubt that the \textit{Arcobacter} species can cause disease in humans but more clinical observations are essential to determine their pathogenicity in human (Levican, Alkeskas, Günter, Forsythe, & Figueras, 2013). Preliminary studies on \textit{A. butzleri} have shown that these bacteria have clinical and microbial characteristics similar to \textit{Campylobacter jejuni} strain but they are more associated with severe and persistent diarrhea and less associated with \textit{C. jejuni} (Vandenberg et al., 2004; Wesley & Miller, 2010). In the recent decade, sequencing of the RM4018 genome of \textit{A. butzleri} was an important step in the development of research on \textit{Arcobacter}, in which 10 pathogenic genes were identified (Douidah et al., 2012; Ferreira, Queiroz, Oleastro, & Domingues, 2016; Miller et al., 2007). The pathogenicity of an organism is characterized according to the presence of virulence genes (Ghaju Shrestha, Tanaka, Sherchand, & Haramoto, 2019). The \textit{cadF} and \textit{cj1349} genes encode outer membrane proteins, which promote bacteria to cell contact by adherence to fibronectin (Dasti, Tareen, Lugert, Zautner, & Groß, 2010). \textit{CiaB} gene promotes host cell invasion through the secretion system (Tabatabaeei, Aski, Shayegh, & Khoshbakht, 2014). \textit{MviN} gene is a vital protein required for peptidoglycan biosynthesis (Inoue et al., 2008). \textit{PldA} encodes the outer membrane phospholipase A (Grant, Belandia, Dekker, Richardson, & Park, 1997). \textit{TlyA} gene encodes hemolysin which plays an important role of \textit{C. jejuni} in adhesion to Caco-2 cells (Slater, Bailey, Tett, & Turner, 2008). The \textit{hecA} gene encodes a protein belonging to a group of adhesives (Filamentous haemagglutinin family). The \textit{hecB} encodes a related haemolysin activation protein (Miller et al., 2007; Rojas, Ham, Deng, Doyle, & Collmer, 2002). \textit{IrgA} gene encodes an outer membrane protein and the periplasmic enzyme of the \textit{iroE} gene, both of which regulate iron (Mey et al., 2002; Rashid, Tarr, & Moseley, 2006; Zhu, Valdebenito, Winkelmann, & Hantke, 2005). Although it remains unclear whether these virulence genes have similar functions to their homologs in different pathogenic bacteria, showed little or no invasion of cells by \textit{Arcobacter} strains that lack virulence genes. Therefore, the detection and quantification of virulence genes of \textit{Arcobacter} may elucidate its role in waterborne infections (Ghaju Shrestha et al., 2019). \textit{Arcobacter} can be considered as a relatively resistant bacteria which can survive in adverse conditions and affect the pathogenicity process in human (Ferreira et al., 2016). Thus, the aim of this work was to isolate and identify this bacteria from wastewater of poultry slaughterhouse in Tonekabon to examine the presence of genes.

Materials and methods

Sample Collection and screening of the isolates

238 samples were collected from wastewater of poultry slaughterhouse in Tonekabon located in the geographical position of \(x=0494258\) and \(y=4069580\) (\(N=36.752036,\ E=50.929654\)) in different seasons. To isolate \textit{A. butzleri}, 50 mL of the samples were transferred to sterile screw cap tubes and transferred to the microbiology laboratory. Then they were cultivated onto the Preston medium Broth enriched with supplements (Preston supplement, biomark) and incubated at 25 °C for 24 h. After enrichment, the membrane filtration technique was applied using 0.45 \(\mu\)m pore size nitrocellulose membrane filters. Then the samples transferred to Campylobacter Selective Agar enriched with defibrillated sheep blood and Campylobacter supplement, and
the plates were incubated at 25 °C for 48 to 72 h. Finally, the suspected colonies were identified using biochemical tests (Atabay & Corry, 1997).

Identification of Arcobacter butzleri and its pathogenic genes

All isolates were identified using polymerase chain reaction (PCR) techniques. For this purpose, DNA was extracted using Gene all kit (GeneAll Biotechnology Co., LTD.Korea, Cat.No. 106-152). The 16S r-RNA gene and virulence genes of mviN, ciaB, and tlyA of A. butzleri were amplified using their special primers. Moreover, all PCRs were prepared with a final volume of 20 µL containing 10 µl of Mastermix (Hot Start Taq Polymerase, dNTP, buffer, MgCl), 1 µL of forward primer, 1 µl of reverse primer, 3 µl of distilled water, and 5 µl of DNA. To begin the process of polymerization of the 16S r-RNA gene, the thermocycler was set at 94 °C for 4 min for initial denaturation. Afterward, 25 cycles of PCR were performed including 94, 56, and 72 °C for 60s. The final extension was carried out at 72 °C for 7 min. In addition, the cycling condition for mviN and ciaB genes were placed in a thermocycler device at 94 °C for 3 min for initial denaturation. In the next stage, 35 PCR cycles were performed including 94, 56, and 72 °C for 45 s. The final extension was carried out at 72 °C for 10 min. To carry out the polymerization process of the tlyA gene, the thermocycler device was set on 94 °C for 3 min for initial denaturation. Then, 35 PCR cycles were performed including 94, 55, and 72 °C for 45 s. Finally, the final extension was carried out at 72 °C for 10 min (Douidah et al., 2012; Tabatabaei et al., 2014).

Results and discussion

Isolation of A. butzleri from wastewater of a poultry slaughterhouse in Tonekabon

Out of 238 collected samples in 4 different seasons, 42 samples were positive for A. butzleri with a frequency of 17.6%. In this regard, the highest rate of isolation of A. butzleri was 8.4% in spring and the lowest rate was 1.2% in winter. In spring, 20 strains with a frequency of 8.4% in summer 8 strains with a frequency of 3.3% in fall, 11 strains with a frequency of 4.6%, and in winter, 3 strains of Arcobacter with a frequency of 1.2% were isolated from the slaughterhouse wastewater.

Identification of A. butzleri

The results of PCR and sequencing of 16S r-RNA specific genes confirmed the phenotypic results obtained in 42 isolated strains (Fig. 1).

Evaluation of the presence of A. butzleri pathogenic genes

The presence of pathogenic genes of tlyA, ciaB, mviN, in A. butzleri was evaluated using polymerase chain reaction. According to the results obtained from this study indicated that 13 strains showed the presence of ciaB gene (frequency of 30.9%) (Fig. 2), 11 strains, mviN gene (frequency of 26.2%) (Fig. 3), and 9 strains showed tlyA gene (frequency of 21.4%) (Fig. 4). The results also revealed that out of 15 strains of A. butzleri, 9 strains with 60% frequency had all 3 pathogenic genes (Table 1).
**A. butzleri** is listed as one of the most dangerous bacteria that threatens human health (Collado & Figueras, 2011). There are many reports on the presence of *Arcobacter* and its pathogenicity in animals as well as in human (Tabatabaei et al., 2014). In 2002, the International Commission on Microbial Specification for Foods included *A. butzleri* in the list of microorganisms posing a serious risk to human health (Tompkin, 2002). However, many other *Arcobacter* species have been described in recent years. Although the number of diseases caused by *Arcobacter* spp. has increased in recent years, very little is known about their pathogenesis (Waite et al., 2017). Moreover, the mechanisms of pathogenicity of the *A. butzleri* are still unknown (Ferreira et al., 2016). Adhesion, invasion, and cytotoxicity have been previously examined in vitro using various cell lines (e.g., Caco-2, HeLa, HT-29, Hep-2, and IPEC-J2) (Ho et al., 2007; Karadas et al., 2013).

*Arcobacter* can be considered as an organism with a high ability to survive in adverse conditions, and it has shown to be highly antibiotic-resistant in multidrug therapy (Chieffi, Fanelli, & Fusco, 2020; Ferreira et al., 2016).

Most studies on the prevalence of *Arcobacter* in foods are related to the poultry that has the highest prevalence (Collado et al., 2009; Jribi et al., 2020). There is still debate about the source of the contamination because some researchers believe that the slaughterhouse
environment was not the main source of excrement as *Arcobacter* could not be isolated from the feces. However, some researchers have shown that the bacterium can reside in chicken's intestines, suggesting that the age of the animals, the specimen, and the method used for its collection and identification affects the prevalence of the bacterium (Collado & Figueras, 2011). Consumption of contaminated food or water with *Arcobacter* is a route of transmission to humans and animals (Gobbi et al., 2018). The association between *Arcobacter* species and their pathogenicity in human, in addition to their detection and prevalence in food and water, have shown that further studies should be performed on the survival of this microorganism. Thus, new therapies need to be explored to control and eliminate this bacterium.

In the present study, 42 samples of *A. butzleri* were isolated and identified by culture standard and based on 16S rRNA gene analyses. Eifert, Castle, Pierson, Larsen, & Hackney (2003) and Ghane, Moein, & Massoudian (2012) isolated *Arcobacter* species using culture and PCR techniques (Houf, De Zutter, Van Hoof, & Vandamme, 2002).

Snelling, Matsuda, Moore, & Dooley (2006) presented a technique enrichment step with filtration for isolation of *Arcobacter*, this method was used in this study and showed to be useful in the isolation of *Arcobacteria*. In our study, using the mentioned method was proved to be the most appropriate technique for isolation.

Although there are different types of specimen for isolation of *Arcobacter*, in this study, we attempted to isolate the organism from wastewater treatment of poultry slaughterhouse in Tonekabon, through which spreading bacteria into the environment and transmission to human can be prevented.

For this purpose, we considered seasonal changes for the prevalence of this bacterium and showed that the highest rate of isolation of *A. butzleri* was in spring with a frequency of 8.4%. Due to the high existing amount of organic matter in the effluent, the rate of isolation of *A. butzleri* was higher in slaughterhouse effluent. Rasmussen, Kjeldgaard, Christensen, & Ingmer (2013) suggested that the high prevalence of *Arcobacter* in slaughterhouses can be due to inadequate detoxification processes. The organism can potentially survive in 5% ethanol on culture and steel surfaces (Rasmussen et al., 2013).

Furthermore, the results presented that the highest and the lowest prevalence of virulence genes belonged to *ciaB* (30.9%) and *tlyA* genes (21.4%), respectively. The invasive *ciaB* gene attacks the host cell through the secretion system (Tabatabaei et al., 2014). It is an infection caused by *Arcobacteria* as a result of adhesion of this bacterium to the mucosal layers that protect epithelial cells (Wilson, Abner, Newman, Mansfield, & Linz, 2000). This phenomenon is often observed in infections caused by *Arcobacter* and the presence of *ciaB* gene. Hence, the invasive *ciaB* gene is one of the important pathogenic factors of this bacterium, which can increase its ability to survive in adverse environmental conditions, such as in poultry slaughter effluents and human transmission.

Collado, Jara, Vásquez, & Telsaint (2014), examined the presence of 9 pathogenic genes of *Arcobacter* using ERIC-PCR, PCR-RFLP, and PCR techniques in 106 strains of isolated *Arcobacter* from oysters. The results suggested that 62% of the isolated strains were *A. butzleri*. They identified *mviN* gene with the frequency of 83.8% *ciaB* gene with 82.8% and *tlyA* gene with 72.7% frequency in different strains of *Arcobacter*. They also found that the frequency of these 3 genes was higher than *cadF, pldA, hecB, hecA, irgA, cj1349* genes (Collado et al., 2014), which is similar to our study. However, Girbau, Guerra, Martínez-Malaxetxebarria, Alonso, & Fernández-Astorga (2015) reported the presence of *ciaB* and *mviN* genes with
100% frequency, \textit{tlyA} gene with 89.7%. Šilha, Vacková, & Šilhová (2018) showed the presence of \textit{ciaB} gene with 98.8\%, \textit{mviN} gene with 97.5\% and the \textit{tlyA} gene with 95.0\% frequency in 80 strains of \textit{A. butzleri} isolated from water and clinical specimens using PCR technique, which is more than our results.

As virulence genes are associated with the pathogenic characteristics of \textit{Arcobacter}, such as adhesion, invasion, and cytotoxicity in different cell lines. The detection of these genes in surface water and groundwater needs to be taken seriously (Ferreira et al., 2016). Thus, the detection of virulence-associated genes along with \textit{Arcobacter} in water samples may represent a high risk of waterborne disease transmission (Ghaju Shrestha et al., 2019). More comprehensive studies on the survival of this microorganism are recommended considering the relationship between \textit{Arcobacter} species and their pathogenicity in humans. Their detection and occurrence in food and water are also very important. Novel therapies should be developed for controlling and eliminating this bacterium (Cervenka et al., 2008).

Conclusions
The results displayed that poultry slaughterhouse effluents are the source of \textit{A. butzleri} isolates. According to these findings, pathogenic genes of \textit{tlyA, mviN, ciaB} are present in all isolates of \textit{A. butzleri} and the highest prevalence belonged to \textit{ciaB} gene. The presence of pathogenic genes in the genome of \textit{A. butzleri} can cause disease in humans. They also increase the environmental survival and the growth potential of this bacterium, increasing the likelihood of its transmission to humans and animals. Furthermore, blood infection caused by \textit{A. butzleri} can be a serious threat to individuals with the impaired immune system and they could be life-threatening. Therefore, it is necessary to develop diverse methods to control or prevent the transmission of this bacterium.

Since most of the bacteria spend a big part of their whole life struggling with environmental tensions, more information about these tensions and responses to them are necessary to perceive the physiology of bacteria. Exploring the effects of environmental stress on the expression of \textit{A. butzleri} virulence genes can be a new topic for biological and applied research. Also, with the introduction of new antimicrobial agents, appropriate strategies can be found to control and transmit this bacterium.

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References


شیوع و توزیع زندهای ویرولانس آرکوپاکتر بوتزلری در نمونه‌های بسیار کشتارگاه مرج تنگابن

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چکیده

ارکوپاکتر بوتزلری در ارتباط با انتی‌بیوتیک‌ها، کارامپین، باکتریمی، فاکتور واکنشی در انسان و انتی‌بیوتیک و درد شکمی در حیوانات شناخته می‌شود. این باکتری به‌عنوان خطرناکترین گونه برای سلامت انسان براساس شاخص‌های کمپیوتر موبایلیزیت غذایی اعلام شده است. همچنین به‌عنوان پاتوزن مهم زنانیک معرفی شده است. هدف از این مطالعه جداسازی، شناسایی، آرکوپاکتر بوتزلری براساس زندهای بیماری (ciaB) در حوزه مختلف بود. از این رو، 238 نمونه از بسیار کشتارگاه مرگ شهروندی تنکابن جمعآوری شدند. سپس ژن ciaB در 12 نمونه با فراوانی 2/3 درصد، ZnN در 11 نمونه با فراوانی 2/9 درصد، ZnN در 9 نمونه با فراوانی 2/4 درصد و tlyA در 11 نمونه با فراوانی 2/3 درصد وجود دارد. همچنین نتایج نشان داد که بالاترین میزان جداسازی آرکوپاکتر بوتزلری ۹/۸ درصد در بخار و کمترین میزان آن ۲/۷ درصد در زمان بود. همچنین نتایج نشان داد که بالاترین میزان جداسازی آرکوپاکتر بوتزلری در فصل بهار با فراوانی ۷/۲ درصد و کمترین میزان جداسازی در فصل زمستان با فراوانی ۱/۲ درصد بوده است.

واژه‌های کلیدی: آرکوپاکتر بوتزلری، زندهای ویرولانس، واکنش زنجیره‌ای پلی‌مراز