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Isolation of Arcobacter Butzleri from Caspian Sea's Water

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Abstract The major purpose of this study was isolation, identification and characterization of Arcobacter spp. from Caspian Sea's water in north of Iran. Sampling from the water was conducted within 4 seasons. Arcobacter spp. was isolated using standard method then identified by Phenotyping tests. Finally, the identification of strain was verified by PCR method. After conducting of the Phenotyping methods and their authentication with molecular technique, fourteen strains of Arcobacter butzleri were identified. With regard to the obtained results, prevalence of this bacterium in the coastal waters of the Caspian Sea's was evaluated as 5.32 percent. The result obtained from this study is, in fact, regarded as the first report from the isolation of Arcobacter butzleri from Caspian Sea. Knowledge of the state of the entry and time span of the survival of the Arcobacter in the water environments to control the water quality and prevention from the disease is of importance.

Keywords: *Arcobacter butzleri, isolation, Caspian Sea, water sample*

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1. Introduction

Arcobacters are the gram negative bacteria to be observed in the rod like, helical and curved shapes. Because of the existence of the single and polarflagella, they are motive severely in on end or each two ends [1]. These pseudo-campylobacterial organisms were isolated from the cow's and pig's aborted-embryo by Ellis in 1977 [2] and separated from the *Campylobacter* genus in 1992 [3]. *Arcobacter* is distinguished from the *Campylobacter* genus by the capability of growing in the presence of oxygen and low temperatures [3].

It's well-known and pathogenic species in human is *Arcobacter butzleri* which has been introduced as the most dangerous species for the health of human on the behalf of the International Commission on Microbiological Specifications for Foods and, recently, as important zoonotic pathogen [4]. The new evidences which are available show that arcobacters, particularly *Arcobacter butzleri* can cause the intestinal disease in the human and this disease has more prevalence in the developing countries because of low levels of the health and lack of access to drinking water [5,6].

There exists little information regarding the pathogenic mechanism, virulence factors and the manner of the transmission of arcobacters. They are not present naturally in the human's intestine and have been isolated only from the patients infected with diarrhea, endocarditis and peritonitis [7]. The contaminated foods and waters consumption is the most important ways of transferring the disease to human [8]. Water plays an important role in the transmission of arcobacters to animals and humans [9]. These bacteria are found from the soil and surface waters [10], sewage [11], water wells and rivers [12], ground waters [13] and drinking water [14].

The new species of the *Arcobacter* have been isolate from the different habitats. For example, they have been isolated from the active sludge [15], marine sediments, sea water and entrance of the rivers. Also, they have been found in the internal structure of the corals, tube worms, snakes and edible oyster [16,17].

On the basis of the available documents and where as the infections resulting from these bacteria are regarded as a big global complexity for the society's health and there were no information available regarding the identification and the rate of their frequency in the Caspian sea, the current study was designed in order to determine the prevalence of these bacteria.

2. Materials and Methods

2.1. Sample Collection

In total, 263 water samples were obtained from south coastal Caspian Sea in one year (2013). The water samples were collected in 50 ml sterile bottles (falcon tube), from one deep on surface sediment and transported to the laboratory at ambient temperature and stored at 4°C. In order to keep the temperature low, all test tubes containing the water samples were placed in the full-of-ice flask. Sampling time was from 8 a.m. to 2 p.m., and the

maximum time of transferring the samples to laboratory was one hour. The environmental features, including atmospheric condition, pH, temperature and salinity of water have been taken into consideration.

2.2. Sample Processing and Isolation

At first, the tubes containing water sample were centrifuged in 4000 rpm (ALC, Italy) within 10 minutes; then, supernatant was extracted and the remained quantity of 1 to 2ml was used in order to isolate the bacterium. In the next step, water sample was transferred to the brain heart infusion broth (Merck-Germany) by the 0.45 micrometer filter. The tubes were incubated at 25°C for 48 h under aerophilic conditions.

After this period a one loop was taken from the bacterial suspension and the spread culture was carried out on the CAMP medium (Merck-Germany), enriched by the defibrinated blood of sheep. All cultured plates were placed in an incubator at temperature of 25°C and under aerobic conditions for 72 hours in order to isolate the species *Arcobacter*.

2.3. Identification and Biotyping of *Arcobacter* Spp

Arcobacter identification was performed by subjecting of all the suspected colonies to microscopic examination of wet mount under dark field microscopy, gram staining, glucose fermentation, oxidase and catalase test. The isolates exhibiting Characteristic motility of *Arcobacter* were characterized by using standard *Arcobacter* phenotypic identification tests recommended by Atabay & Corry [18].These tests included H_2S by lead acetate strip, nitrate reduction, growth in 1% glycine and 3.5% NaCl, growth at different temperatures (25, 37 and 42°C), hippurate hydrolysis, urease production, resistance to nalidixic acid (30 µg) and cephalothin (30 µg).At the end, the PCR method was asked for in order to confirm the Phenotyping results.

2.4. DNA Extraction and PCR Method

DNA was extracted from suspected colony using phenol-cholorophorm method. The concentration and purity of the extracted DNA was assessed based on absorbance of the extracted DNA at 260 and 260/280 nm wavelengths respectively by biophotometer (Eppendrof-Germany).

Primers produced by TAG Copenhagen (Denmark) were used to amplify Arco gene. The sequence of forward and reverse primers were 5'-GGTGTAGGATGAGACTATATA -3' and 5'-GTCGTGCCAAGAAAAGCCA -3', respectively. Each reaction was performed in a total volume of 25 µl contained 14.5 µl of molecular biology-grade water (sigma aldrich company ltd.), 2.5 µl of 10×PCR buffer (cinagen-Iran), 1 µl of 10 pmol each forward and reverse PCR primers, 1 µl of a 10 mM dNTPs (cinagen-Iran), 0.5 µl of smar taq polymerase (cinagen-Iran), 1 µl of 50mM MgCl2 (cinagen-Iran) and 5 µl of DNA template. Non-template control (NTC) tube contained the same PCR reagents as above but had 5 µl of water substituted for template.

PCR amplification conditions on thermocycler (eppendrof -Germany) were as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 90 sec, with a final extension at 72°C for 10 min and storage at 4°C.

2.5. Agarose Gel Electrophoresis

All PCR products were run on a 1.5% (w/v) agarose gel with a 1kb DNA ladder (Fermntas-Russia). Aliquots of PCR products were electrophoresed at 75 V for 40 min; DNA was visualized using ethidium bromide and photographed after UV transillumination with Uvidoc (England).

3. Results

Sampling from the Caspian Sea's water was conducted within 4 seasons. Average water salinity of the Caspian Sea was 12.85 (ppt) in the various seasons. The minimum and maximum water temperature in the various seasons was reported 7 and 24°C, respectively. Also, water pH was ranged from 5 to 8 (Table 1).

After conducting of the Phenotyping methods and their confirmation with molecular technique, 14 strains of *Arcobacter butzleri* were identified.

The result obtained from molecular technique was clear (Figure 1).

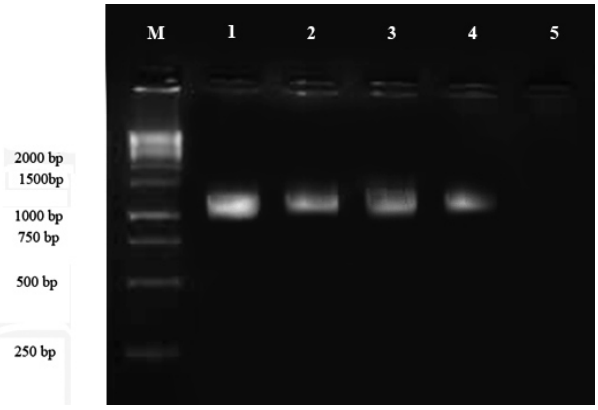

Figure 1. Agarose gel (1.5%) analysis of a PCR diagnostic test. Lane M: size marker 1 kb, Lane 1: positive control, Lane 2-4: positive sample, Lane 5: Negative control.

Our data regarding to comparison of phenotyping and molecular identification of the isolates indicated that all molecular identification verified our phenotyping identification.

With regard to the obtained results, prevalence of this bacterium in the coastal waters of the Caspian Sea's South of was evaluated as 5.32 percent (Table 2).

Table 1. Temperature, pH and Salinity of Caspian sea in 4 season sampling

Seasons	Temperature	pH	Salinity
Autumn	7-11	6/5-7	12/80
Winter	5-11	5-6/5	12/85
Spring	7-18	6/5-8	12/95
Summer	13-24	7	13/00

Table 2. frequency of occurrence of *Arcobacter* isolated from Caspian sea

seasons	No. sample collected	Isolated number (%)
autumn	41	0
Winter	61	0
Spring	67	8 (3.04%)
Summer	94	6 (2.28%)
Total	263	14 (5.32%)

Of 14 isolated strains, 8 strains in spring and 6 strains in summer were identified, respectively. It is required to mention that the isolation of bacterium was not performed in the autumn and winter seasons. In all cases which strains of species *Arcobacter* were isolated, the sea had the calm state so that the isolation of bacterium was not successful in the stormy and wavy state.

4. Discussion

At present, arcobacters are not currently considered microorganisms of major public health concern [19] but, never the less, data increasingly suggest that their significance in human infections may be underestimated, mainly because of inappropriate detection and identification methods [20]. One of the major pitfalls is that the optimum growth conditions for recovery of *Arcobacter* (30°C) are generally not applied with clinical specimens. In fact, despite the fact that only some *A. butzleri* and *A. skirrowii* strains are able to grow at 42°C [21], this is the only temperature used for isolation of campylobacters in the majority of laboratories [22].

The role of the species of the *Arcobacter* in the human diseases has not specified well, but, never the less, *Arcobacter butzleri* and *Arcobacter cryaerophilus* are related to the gastrointestinal diseases [23,24]. Continuous and severe diarrhea is the main symptom of the *Arcobacter butzleri* [7].

Water is the possible way of transferring the arcobacters to animals and humans. Members of this genus have been isolated from the varieties of the environmental waters, including surface waters, ground waters, rivers, lakes, sea water, sewage and from planktons [25,26]. The high prevalence of these bacteria in the environment suggests the contamination of the surface waters with feces [27]. In fact, *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* have more prevalence in the waters having the fecal contaminations. Isolation of a new species known as *Arcobacter defluvii* showed that the sewage is an important reservoir for arcobacters [26].

Van Driessche and Houf proved in 2008 that the capability of the *Arcobacter* species in order to survive is affected by the presence of the organic substances and temperature of or this reason, it is said that *Arcobacter* can survive for more than 250 days under temperature of 4°C [28].

As yet, isolation and identification of the *Arcobacter* species from the Caspian Sea's water has not reported. On the basis of the information obtained in this research which was carried out in four seasons of the year, out of 263 collected samples, 14 strain of *Arcobacter Butzleri* were isolated which this statistics suggests the existence of this bacterium in the coastal waters of the Caspian sea.

Within a research in Thailand, Dhamabutra *et al* could isolate the *Arcobacter* from water channels [29].

One year later, Jacob *et al* isolated 141 *Arcobacter* strains out of which 100 strains were *Arcobacter butzleri* within a research in the drinking water refinement factories in the Germany. In 2003, Fera *et* alconducted a research on the isolation of the species of Arcobacters in the coastal environment of the Mediterranean sea. In this research, they could identify only one of the *Arcobacter butzleri* from the sea water and the Plankton samples [30]. In another research which was implemented by the Maugeri *et al* in 2005 on the isolation and counting of the *Arcobacter* species on Messina coastal environment in Italy, they could isolate only *Arcobacter butzleri* from the sea water and Plankton samples [31].

In a research which was conducted by Collado et al in 2010 in the Liobregat river water (drinking water reservoir of Spain), species of the *Arcobacter butzleri* and *Arcobacter cryaerophilus* in the water river were isolated [32].

5. Conclusion

The results obtained from this research are regarded as the first report of isolating the *Arcobacter* species from the Caspian Sea in Iran. Awareness of the manner of entrance and time span of the survival of Arcobacters in the water environment is of importance to control the water quality and disease transfer.

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