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Antimicrobial Susceptibility of *Arcobacter Butzleri* Isolated from Environmental Samples in North of Iran

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ABSTRACT

Background: During the past decade *Arcobacter* has been shown to be responsible for gastrointestinal disease in human and animal. These organisms may enter the environment, including drinking water, through the feces of animals, birds or infected humans. **Objective:** The major purpose of this study was isolation, identification and antimicrobial susceptibility of *Arcobacter* spp. isolated from river water in north of Iran. *Arcobacter* spp. was isolated using standard method then identified by Phenotyping tests. Finally, the identification of strain was verified by PCR technique. Then antimicrobial susceptibility of the isolates against different antibiotics and Minimal Inhibitory Concentration (MIC) values were determined by disc diffusion and E-test respectively. **Results:** In general, 15 strains of *Arcobacter butzleri* were isolated. The results obtained indicated that frequency of occurrence of *Arcobacter butzleri* in environmental sample was high. All strain was resistant to Penicillin G, aztreonam, cephalothin, oxacillin and trimethoprim/sulfamethoxazole. In addition, the lowest values of MIC were found for Azithromycin, while the highest value was found for chloramphenicol. **Conclusion:** Overall, our observations, illustrated that pathogenic *Arcobacter* were existed in river water in north of Iran and therefore, the people who living in this area must respect to the personal hygiene in order to avoid from *Arcobacter* infection.

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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 polar flagella, they are motive severely in on end or each two ends [1]. These pseudo-campylobacterial organisms were isolated from the cows and pig's aborted-embryo by Ellis in 1977 and separated from the *Campylobacter* genus in 1992. *Arcobacter* is distinguished from the *Campylobacter* genus by the capability of growing in the presence of oxygen and low temperatures [2-3].

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. 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 [4].

There exists little information regarding the pathogenic mechanism, virulence factors and the manner of the transmission of *Arcobacter*, they are not present naturally in the human's intestine and have been isolated only from the patients infected with diarrhea, endocarditis and peritonitis [5]. The contaminated foods and waters consumption is the most important ways of transferring the disease to human [6]. Water plays an important role in the transmission of Arcobacters to animals and humans [7]. These bacteria are found from the soil and surface waters [8], sewage [9], water wells and rivers [10-11], ground waters [12], drinking water [13] and sea water [2].

However, antibiotic therapy in case of patients with acute *Arcobacter* enteritis involves treatment with Tetracycline and Fluoroquinolones but the resistant strains of *Arcobacter* to Clindamycin, Azithromycin, Cefoperazone, [14] Nalidixic Acid, Ciprofloxacin, [15] Novobiocin and Beta-lactams antibiotics from developed and developing countries were isolated [17]. These resistances in *Arcobacter* spp. are related to the

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antibiotic usage in veterinary medicine and prophylaxis. In such cases we will face to bacteria with increased levels of antibiotic resistances, by which the treatment process will be more difficult [18-19].

Therefore, based on forgoing evidence, the present study was undertaken to isolate *Arcobacter* spp. from environmental samples and determine their antimicrobial susceptibility in order to achieve maximum information concerning the same.

MATERIALS AND METHODS

2.1. Isolation of Arcobacter spp. from environmental samples:

In total, 180 water samples were obtained from Tonekabon area located in south coastal Caspian Sea in one year (2012). The river water samples were collected in 500 ml sterile bottles and transported to the laboratory at ambient temperature and stored at 4˚C before they were analyzed with in 2h. All the samples were subjected to detection of *Arcobacter* immediately upon arrival in the laboratory.

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 2 ml was used in order to isolate the bacterium. In the next step, water sample was transferred by the 0.45 µ filter to the priston media (Merck-Germany). 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 plates were placed in an incubator at temperature of 25^{O}C and under aerobic conditions for 72 h in order to isolate the species *Arcobacter*.

2.3. Identification 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 characterized by using standard phenotypic identification tests [2]. 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 by extraction kit (High Pure Viral Nucleic Acid, Roche Applied Science, Penzberg, Germany). The concentration and purity of the extracted DNA was assessed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths by biophotometer (Eppendrof- Germany).

Specific 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 13 µl of molecular biology-grade water (sigma aldrich company ltd.), 2.5 µl of 10×PCR buffer (Promega, USA), 1 µl of 10 pmol each forward and reverse PCR primers, 1 µl of a 10 mM dNTPs (Promega, USA), 0.5 µl of smar taq polymerase (Promega, USA), 1 µl of 50mM MgCl2 (Promega, USA) 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.

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 (figure 1).

2.5. Antibiotic susceptibility and Minimum Inhibitory Concentration:

In this study the antimicrobial susceptibility of *Arcobacter* spp. isolates were determined by disc diffusion test [20]. For disc diffusion test, Ampicillin (10 µg), Amikacin (10 µg), Amoxicillin (25 µg), Cephalothin (30 µg), Choloramphenicol (30 µg), Ciprofloxacin (5 µg), Amoxicillin/clavulanic acid (20+10 µg), Erythromycin (15 µg), Gentamicin (10 µg), Tetracycline (30 µg), Penicillin (10 µg), Tobramycin (10 µg), Streptomycin (10 µg), Norfloxacin (10 µg), Nitrofurantoin (300 µg),, Azithromycin (15µg) , Nalidixic Acid (30 µg), Oxacillin (1 µg), Trimethoprim/ sulfamethoxazole (25 µg) (PADTAN TEB - Iran) were used. The disc strengths and the zone size interpretation was in accordance with National Committee for Clinical Laboratory Standards.

To perform the disc diffusion test, each culture was grown in 5 ml of Muller-Hinton broth until the turbidity corresponded to 0.5 McFarland standard tubes (~1.5×10^{8} cfu/ml). This microbial suspension was spreaded out

on the surface of Muller-Hinton agar by strilled swap and various antibiotic discs were placed on it. After incubating the plates at 25^{o}C under aerophilic conditions for 48h the inhibition zone were recorded.

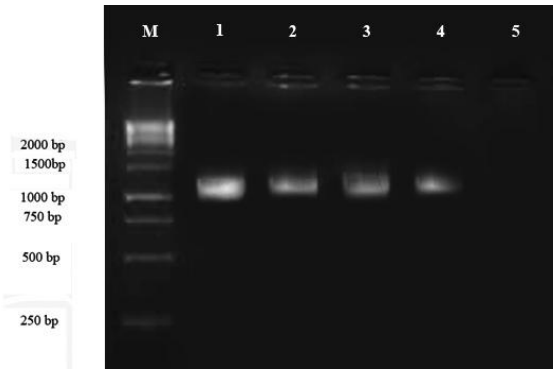

Fig. 1: Agarose gel (1.5%) analysis of a PCR diagnostic test. Lane M: size marker 1 kb, Lane 1: positive control, Lane 2-4: positive sample, Lane 5: Negative control.

To perform the E-test, ten different antibiotic E-test strips were applied on each plate. The plates were incubated at 25°C for 48 h under aerophilic conditions and inhibitory concentration of each antibiotic was read at the point where the elliptical zone of inhibition intersected the E-test strip.

Results:

3.1. Isolation and identification of Arcobacter spp.:

All the isolates were subjected for identification using standard phenotypic tests. Fifteen (8.33%) *Arcobacter butzleri* were isolated from environmental samples of river water in North of Iran. Our data regarding to comparison of Phenotyping and genotyping identification of the isolates indicated that all Molecular identification verified our Phenotyping identification.

3.2. Antibiotic susceptibility of Arcobacter butzleri isolates:

The results on antibiotic susceptibility of *Arcobacter* isolates from river water samples by disc diffusion method indicated that all strains were sensitive to Amikacin, Tobramycin, Choloramphenicol, Gentamicin, Azithromycin, Norfloxacin, Nitrofurantoin and Nalidixic acid while they showed different levels of susceptibility to other antibiotics including 93% to Erythromycin and Ciprofloxacin, 86% to Tetracycline, 53% to Streptomycin and cefotaxime, 40% to Amoxicillin/clavulanic acid and 33% to Ampicillin and Amoxicillin. All *Arcobacter butzleri* spp. isolates were resistant to Penicillin G, Aztreonam, Cephalothin, Oxacillin, and trimethoprim/sulphamethoxazol.

3.3. Minimal Inhibitory Concentration (MIC) of antibiotics against Arcobacter butzleri:

Minimal inhibitory concentration of ten important antibiotics against *Arcobacter butzleri* isolates from environmental samples were determined. As shown in table 1 varied range of MIC values were observed for different antibiotics due to varied responses of *Arcobacter butzleri* isolates. The lowest MIC values against the *Arcobacter* isolates from this area were found for Azithromycin (0.125 µg/ml) and highest MIC values was found for Choloramphenicol (64 µg/ml). Furthermore, the range of MIC values for Azithromycin was narrow while, for the other antibiotics tested was wide (table 1).

Table 1: Minimal inhibitory concentrations of antibiotics against Arcobacters isolates from river water samples in north of Iran.

Amikacin	2-4	2	4
Tobramycin	2-4	2	4
Chloramphenicol	16-64	16	64
Gentamicin	0.5-1	0.5	1
Ciprofloxacin	0.25-0.5	0.25	0.5
Azithromycin	0.125-0.5	0.125	0.5
nitrofurantoin	0.5-1	0.5	1
Nalidixic acid	4-8	4	8
Erythromycin	8-32	16	32
Norefloxacin	0.5-4	1	4

Discussion

At present, Arcobacters are not currently considered microorganisms of major public health concern, but, never the less, data increasingly suggest that their significance in human infections may be underestimated,

mainly because of inappropriate detection and identification methods [21-22]. One of the major pitfalls is that the optimum growth conditions for recovery of *Arcobacter* (25°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, this is the only temperature used for isolation of Campylobacters in the majority of laboratories [16, 23].

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 [24-25]. Continuous and severe diarrhea is the main symptom of the *Arcobacter butzleri* [5].

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 [26- 28]. The high prevalence of these bacteria in the environment suggests the contamination of the surface waters with feces [29].

On the basis of the information obtained in this research which was carried out in 2012, out of 180 collected samples, 15 strain of *Arcobacter butzleri* were isolated which this statistics suggests the existence of this bacterium in the environmental waters of the North of Iran. According to our data waters were major reservoirs of *Arcobacter* in investigated areas. Therefore, consumption of water can cause enteritis in human beings.

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 [30]. 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 [31].

The main treatment for most patients afflicted by *Arcobacter* is replacement of water and electrolyte. Patients who have lost water in a large quantity (greatly) must receive the intervessel liquids quickly. Although the Arcobacterial infection is usually self-limited, antibiotics treatment is suggested for patients suffered from gone up fever, bloody diarrhea, immune system deficiency and individuals with sever signs. If antibiotic treatment begging very quickly and after beginning of symptoms, duration of disease can be decreased almost from 10 days to 5 days [32]. However, antibiotic therapy in case of patients with acute enteritis involves treatment with Amikacin, Imipenem and Fluoroquinolones but the resistant strains of *Arcobacter* to Clindamycin, Azithromycin, Ciprofloxacin, Carbenicillin, Penicillin, Neomycin, Methicillin, Nalidixic Acid, Ciprofloxacin, Erythromycin and Tetracycline's from developed and developing countries were isolated. [5, 14, 32-33].

Our results regarding to antimicrobial susceptibility of the isolates illustrated that all the pathogenic Arcobacter isolates were sensitive to Amikacin, Tobramycin, Choloramphenicol, Gentamicin, Azithromycin, Clarithromycin, Nitrofurantoin and Nalidixic acid and resistant to Penicillin G, Cephalothin, Oxacillin, and Trimethoprim/sulfamethoxazole. These data were parallel to some reports obtained from developed and developing counties.

Conclusion:

In overall, due to high frequency of occurrence of beta lactams antibiotics, resistant *Arcobacter* spp. these antibiotics could not be considered as drugs of choice for treatment of gastrointestinal disease. Contrary to this, Ciprofloxacin and Amikacin would be drugs of choice and Tetracycline, Gentamicin and Erythromycin as alternative for treatment of gastrointestinal disease in this geographical area.

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