

Antimicrobial Susceptibility Profiles of Environmental Enterobacteriaceae Isolates From Karun River, Iran

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Received: December 12, 2013; **Revised:** February 19, 2014; **Accepted:** March 2, 2014

Background: Antibiotic resistance among bacteria is a worldwide problem. Enterobacteriaceae resistance to third-generation cephalosporins is typically caused by the production of β -lactamases.

Objectives: The aim of this study was to determine antimicrobial susceptibility of environmental Enterobacteriaceae isolates from Karun River in Iran.

Materials and Methods: A total of 600 water samples were collected from nine stations along Karun River in Iran, during spring and summer of 2012. In this research, different waterborne bacterial pathogens were isolated and identified using the membrane filtration technique and analytical profile index system for Enterobacteriaceae (API 20E). Then, disk diffusion method (CLSI, 2010; M2-A9) was used for testing the antibiotic resistance susceptibility. *Enterobacteriaceae* genera were tested against sixteen antibiotics: ampicillin, carbencillin, methicillin, cephalothin, cefotaxime, vancomycin, amikacin, ofloxacin, kanamycin, tetracycline, erythromycin, clindamycin, norfloxacin, nitrofurantoin, chloramphenicol, and amoxicillin.

Results: The results of this study suggested that the level of fecal contamination in Karun water was very high. Among the isolated Enterobacteriaceae, there were 287 strains of (65%) *Escherichia coli*, 162 (27%) *Enterobacter aeogenes*, 73 (12.16%) *Citrobacter freundii*, 58 (9.66%) *Proteus vulgaris*, and 20 (3.3%) *Salmonella typhi*. All Enterobacteriaceae isolates showed 100% resistance to ampicillin, carbencillin, methicillin, vancomycin, erythromycin, clindamycin, and tetracycline. They failed to exhibit resistance to norfloxacin and ofloxacin. Other antibiotics showed intermediate activity, and some isolates were resistant.

Conclusions: Detection of fecal indicator bacteria (*E. coli*) in more than 75% of water samples indicates the possible presence of other bacteria causing infectious diseases.

Keywords: Microbial Sensitivity Tests; Enterobacteriaceae; Iran

1. Background

Resistance to broad-spectrum antibiotics (β -lactamases) has been known among Enterobacteriaceae family as a health problem (1). This resistance is a major public health problem worldwide, and affects the veterinary field too (2). Antibiotic-resistant bacteria are responsible for increased mortality (3). Bacterial resistance to antibiotics may be arises for several reasons particularly by mutations (4).

2. Objectives

The objective of our study was to determine antimicrobial status of Enterobacteriaceae isolates from Karun River in Iran.

3. Materials and Methods

3.1. Sampling Sites

Samples from different regions of the Karun River were collected during spring and summer 2012. For conducting this study, Forty-five sites were chosen (Table 1).

Water sampling was carried out (season summer and winter 2012) according to standard methods for examination of pathogenic microorganism (4). The water samples were collected from the depth of 1m in sterilized 250-mL stopper polypropylene bottles. The samples were stored in cold box at 4°C and delivered immediately to the laboratory for analyses.

Implication for health policy/practice/research/medical education:

The aim of this study was to determine antimicrobial susceptibility of environmental *Enterobacteriaceae* isolates form Karun River in Iran.

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Table 1. Location of the Samples From Different Parts of the Karun River

Sample Collection	Geographical Coordinates		Depth Line, m
Aghili (Gotvand junction)	32.25°20'80"N	48.82°36'71"E	1
Gotvand City	32.25°07'00"N	48.81°50'02"E	1
Shushtar (Jam Kanar)	32.04°97'71"N	48.83°77'48"E	1
Shushtar (Islan park)	31.98°31'81"N	48.88°46'54"E	1
Shushtar (SikaPark)	32.04°98'43"N	48.83°75'76"E	1
Molasani (Ahvaz junction)	31.57°97'05"N	48.88°71'00"E	1
Zergan (Ahvaz functions)	31.37°00'54"N	48.76°28'17"E	1
Karoun river in Ahvaz (Naderi)	31.30°78'15"N	48.66°74'59"E	1
Karoun river in Ahvaz (Slaughter house)	31.24°93'33"N	48.55°23'18"E	1

3.2. Study of Isolated Enterobacteriaceae

Enterobacteriaceae isolation was performed by the standard plating technique. The first step was done on the MacConkey agar (Conda, Spanish) for *Enterobacteriaceae* genera. Incubation period was 24 hours at 37°C. After incubation, we used Chromogenic coliform agar (Merck, Germany), XLD agar (Conda, Spanish) and Salmonella/Shigella agar (Conda, Spanish). Then, we selected the streak plate (four-ways) methodology for getting the pure colonies. Incubation period was conducted for 24 hours at 37°C.

3.3. Multiple-tube Fermentation Technique

Fecal (thermotolerant) coliforms represent a set of total coliforms. The method consists of a series of tubes inoculated with the acceptable decimal dilution of samples of water. Based on production of gas, acid formation and after the 48 hrs incubation at 35-37°C the bacteria were identified based on standard procedures. The formation of gas in a brilliant green lactose bile broth fermentation tube within 48h at 35°C constitutes a positive confirmation check. The results of the MTF technique were expressed in terms of the most probable number (MPN) of microorganisms present. A complementary test, API 20 E kit (bio-Merieux, Marcy L'Etoile, France) was used for the biochemical identification of all Enterobacteriaceae (Figure 1).

Using a sterile Pasteur pipette, the tube section of the microtubule was filled with bacterial suspension; the tube and cupules' section of the CIT, VP and GEL tubes were also filled with bacterial suspension. After inoculation, the cupules' section of the ADH, LDC, ODC, H2S and URE tubes were filled with sterile mineral oil in order to make an anaerobic environment (Table 2). The basis of API 20E in classical microbiology, is clinically proven and accepted. For, fecal coliform (*E. coli*) the colony forming units (CFU) were counted as below.



Figure 1. Negative and Positive Test Results on API 20E Test

Table 2. Dehydrated Substrates in API 20E System

S. No.	Substrate	Full Name
1	OPNG	Ortho-NitroPhenyl-βD-Galctopyranoside
2	ADH	Arginine DiHydrolase
3	LDC	Lysine DeCarboxylase
4	ODC	Omithine DeCarboxylate
5	CIT	Citrate
6	H2S	Hydrogenase
7	URE	UREase
8	TDA	L-Tryptophane De Aminase
9	IND	Indole
10	VP	Voges-Proskauer
11	GEL	Gelatinase
12	GLU	Glucose
13	MAN	Mannitol
14	INO	Inositol
15	SOR	D-Sorbitol
16	RHA	L- Rhamnose
17	SAC	D-Saccharose
18	MEL	L-Melibiose
19	AMY	Amygdalin
20	ARA	L- Arabinose

3.4. Antibiotic Resistance Profile of Enterobacteriaceae

The standard Kirby-Bauer disk diffusion technique according to Clinical and Laboratory Standard Institute (CLSI) in 2010 (5) was used for the determination of antibiotic susceptibility of microorganism isolates. They were tested against sixteen antimicrobial antibiotics: amoxicillin, ampicillin, carbenicillin, methicillin, cephalothin, cefotaxime, vancomycin, amikacin, kanamycin, tetracycline, erythromycin, clindamycin, norfloxacin, ofloxacin, nitrofurantoin, and chloramphenicol.

The colonies from the growth plate were transferred aseptically to saline solution. Then, a sterile cotton swab immersed into the culture suspension and then used for inoculating the surface of Mueller Hinton agar (Merck, Germany) plates. Afterwards, the plates were incubated

at 37°C for 24 hours. The inhibition zone around the antibiotic disks was measured to the nearest whole number (mm) and interpreted according to protocols standardized for the test of antibiotic compounds by CLSI approved standards; M2-A9-Performance Standards for Antimicrobial Disk Susceptibility Test; and Approved Standard-Ninth Edition. The results were classified as R (resistant), I (intermediate sensitive) and S (sensitive).

4. Results

The results showed that Enterobacteriaceae family grew rapidly in the spring. As shown in Table 3, out of 600 water samples collected from Karun River (Iran), 287 (47.8%) samples contained *Escherichia coli*, 162 (27%) *Enterobacter aerogenes*, 73 (12.1%) *Citrobacter freundii*, 58 (9.6%) *Proteus vulgaris* and 20 (3.3%) of *Salmonella typhi*, during spring and summer of 2012 (Figure 2).

Table 3. Frequency of Potential Bacterial Pathogens in Karun River (Iran)^a

Sampling Area	Microorganisms					Total
	Enterobacteriaceae genes	<i>Escherichia coli</i>	<i>Citrobacter freundii</i>	<i>Proteus vulgaris</i>	<i>Salmonella typhi</i> (%)	
Aghili (Gotvand functions)	2 (0.3)	5 (0.8)	0	0	0	7 (1.1)
Gotvand River City	5 (0.8)	8 (1.3)	0	0	0	13 (2.1)
Shushtar (Jam Kanar)	1 (0.16)	5 (0.8)	1 (0.16)	5 (0.8)	1 (0.8)	13 (1.92)
Shushtar (Islan park)	3 (0.5)	9 (1.5)	2 (0.3)	2 (0.3)	0	16 (2.6)
Shushtar (SikaPark)	15 (2.5)	25 (4.1)	5 (0.8)	8 (1.3)	1 (0.16)	54 (8.86)
Molasani (Ahvaz functions)	21 (3.5)	46 (7.6)	6 (1)	2 (0.3)	5 (0.8)	80 (13.2)
Zergan (Ahvaz functions)	25 (4.1)	52 (8.6)	2 (0.3)	5 (0.8)	3 (0.5)	87 (14.3)
Karoun river in Ahvaz (Naderi)	38 (6.3)	65 (10.8)	25 (4.1)	1 (0.16)	4	133 (21.36)
Karoun river in Ahvaz (Slaughterhouse)	52 (8.6)	72 (12)	32 (5.3)	25 (4.1)	6 (1)	187 (31)

^a Abbreviations: Data are presented as No. (%).

4.1. Antibiotic Results

All *Proteus vulgaris* isolates showed 98% resistance against 12 antibiotics: amoxicillin, ampicillin, carbenicillin, methicillin, amikacin, kanamycin, vancomycin, tetracycline, erythromycin, clindamycin, nitrofurantoin

and chloramphenicol. Environmental *Citrobacter freundii* and *Salmonella typhi* showed 95% resistance against tested antibiotics. Finally, the highest resistance was seen against ampicillin, carbenicillin, methicillin, vancomycin, erythromycin, clindamycin, and tetracycline (Table 4).

Table 4. Enterobacteriaceae M2-A9 Disk Diffusion

Antimicrobial Agent	Disk Content	Resistance of Bacteria			
		<i>Citrobacter freundii</i>	<i>E. coli</i>	<i>P. vulgaris</i>	<i>S. typhi</i>
Amoxicillin	10 µg	S	S	R	S
Ampicillin	10 µg	R	R	R	R
Carbenicillin	100 µg	R	R	R	R
Methicillin	20 µg	R	R	R	R
Cephalothin	30 µg	R	S	I	S
Cefotaxime	30 µg	S	S	I	S
Amikacin	30 µg	S	S	R	S
Kanamycin	30 µg	R	S	R	I
Vancomycin	30 µg	R	R	R	R
Tetracycline	30 µg	R	I	R	R
Erythromycin	15 µg	R	R	R	R
Clindamycin	30 µg	R	R	R	R
Norfloxacin	10 µg	S	S	S	R
Ofloxacin	5 µg	S	S	S	R
Nitrofurantoin	300 µg	R	S	R	R
Chloramphenicol	30 µg	R	S	R	R

5. Discussion

Data obtained in this work about the frequencies of resistant enterobacteria in water sources are in agreement with those reported in Brazil (6), and in South Africa (7), where *E. coli* was the most frequent species followed by *Citrobacter*, *Enterobacter* and *Klebsiella*. During the summer of 2005, water samples collected from environmental sites (raw water source in the Cree Community of Mistissini) showed a wide range of total coliforms, *E. coli*, and enterococci organisms.

Like isolation of antibiotic resistant bacteria from the clinical samples is very important, the isolation of bacteria recovered from the environmental specimens is also very critical; because these bacteria may have a great role in high prevalence of and transport of resistant genes to other bacteria. According to studies carried out in vari-

ous countries, a number of antibiotics in the low concentrations (microgram per liter or nanograms per liter) have been identified in different parts of the environment (8). The clinically important observed Enterobacteriaceae were: *Klebsiella*, *Proteus*, *Enterobacter*, *Morganella*, and *Serratia*. The high percentage of resistance has been observed for freshwater enterobacteria to at least one antimicrobial agent (93%) and to multiple-antimicrobial agents (61%). The same resistance has also been found in bacteria isolated from rural groundwater supplies and wild mammals (9). For example, classification of known tetracycline resistance genes is presented (3, 10) in Table 5.

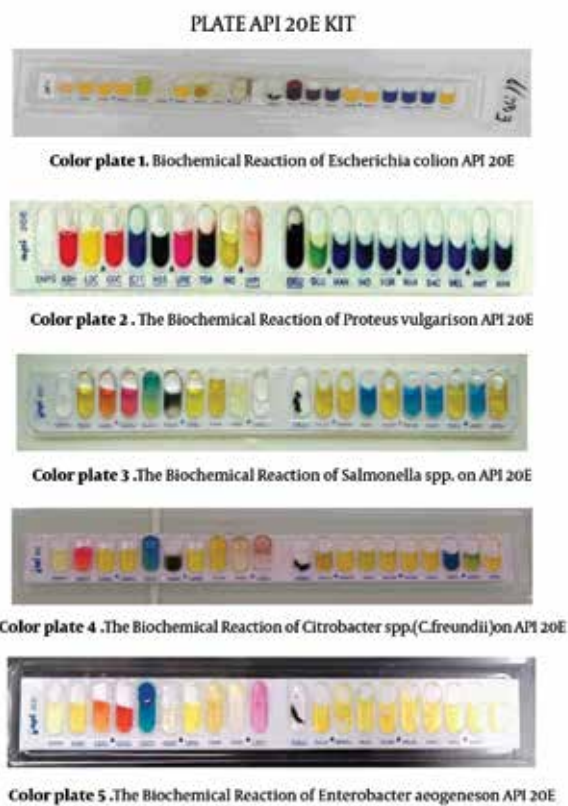
In recent years, human actions directly and indirectly influenced the aquatic ecosystems. The results of this study recommend that understanding ecology of family Enterobacteriaceae, and estimation of microorganism

Table 5. Classification of Known Tetracycline Resistance Genes (9, 10)

Class	Localisation	Mechanism	Distribution
A	plasmid/transposon	efflux	Gram-negative: <i>Aeromonas</i> , <i>Citrobacter</i> , <i>Edwardsiella</i> , <i>Escherichia</i> , <i>Klebsiella</i> , <i>Plesiomonas</i> , <i>Proteus</i> , <i>Pseudomonas</i> , <i>Salmonella</i> , <i>Serratia</i> , <i>Shigella</i> , <i>Vibrio</i>
C	plasmid	efflux	Gram-negative: <i>Citrobacter</i> , <i>Enterobacter</i> , <i>Escherichia</i> , <i>Proteus</i> , <i>Pseudomonas</i> , <i>Salmonella</i> , <i>Serratia</i> , <i>Shigella</i> , <i>Vibrio</i>
I	?	efflux?	Enterobacteriaceae
D	plasmid/chromosome		Gram-negative: <i>Aeromonas</i> , <i>Citrobacter</i> , <i>Edwardsiella</i> , <i>Enterobacter</i> , <i>Escherichia</i> , <i>Klebsiella</i> , <i>Pasteurella</i> , <i>Plesiomonas</i> , <i>Salmonella</i> , <i>Shigella</i> , <i>Vibrio</i> , <i>Yersinia</i>

antibiotic resistance profiles are important tools for public health authorities. Therefore, in our study, the family Enterobacteriaceae was selected as an indicator of fecal contamination. In subsequent studies, evaluating the risk of transmission of resistant organisms to humans via the food chain is recommended. The presence of drug-resistant bacteria in surface waters is a major concern for public health (as the drug-resistant bacteria by gene resistance transposon, plasmid, and conjugative transposon/plasmid/chromosome) can be transmitted to humans through consumption of contaminated water.

Figure 2. Plate API 20E KIT



Color plate 1, Biochemical Reaction of *Escherichia coli* on API 20E, Color plate 2, Biochemical Reaction of *Proteus vulgaris* on API 20E, Color Plate 3, Biochemical Reaction of *Salmonella* spp. on API 20E, Color Plate 4, Biochemical Reaction of *Citrobacter* spp. (*C. freundii*) on API 20E, Color plate 5, Biochemical Reaction of *Enterobacter aegogenes* on API 20E

Acknowledgements

This research was approved in the Student Research Committee at Ahvaz Jundishapur University of Medical Sciences with the research proposal No. 89S.72. Special thanks to research affairs, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran for the financial support.

Authors' Contribution

All authors participated equally in this study.

Financial Disclosure

There is no conflict of interest.

Funding/Support

The study was by Ahvaz Jundishapur University of Medical Sciences, Iran

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