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Distribution of *sul1* gene to sulfonamide resistance in *Escherichia coli* isolated from Zarjob River (Iran) in Guilan province

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ABSTRACT

Many antibiotics are released into urban wastewater due to incomplete metabolism in humans and ultimately make their path to various natural environments. The use of antibiotics produces a selective pressure that requires antibiotic-resistant bacteria and resistant genes. Sulfonamides are released in aqueous media, the remainder, depending on the chemical activity of the bacteria, is activated against the bacteria and can evolve antibiotic-resistant bacteria (ARB) in the environment Promote natural microbial contamination. The present study evaluates the resistance of *E. coli* isolated to common antibiotics and especially sulfonamides, and also, since transmission of resistant genes to other bacteria is important in this study, the *sul1* genes in *E. coli* was evaluated. During 5 months (September to December), 25 samples from 5 stations in Zarjob river, were collected. At the first, the MPN test was done, then the samples that were positive, were cultured in EMB and incubated for 24 hours. Antibiogram test was done according to disk diffusion method and CLSI. The serotyping, with multi antiserum kit of pathogenic *E. coli* was done. For extracting DNA, extraction kit, and PCR test was used to detect the *sul1* gene from isolated *E. coli*. Finally, it was found that 20% of samples were resistance to sulfamethoxazole and all of them were belong to Group III (O128, O125, O44) and according to PCR all of them had *sul1* gene. Antibiotic resistance has become a global issue. The most polluted river in the province of Giulan is Zarjob, which can cause pollution of various types of waters, aquatic organisms, fish and other marine organisms used by humans

1. Introduction

The widespread distribution of antimicrobial resistance among microbial communities is a problem that is increasing throughout the world. Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infections has increasingly led to the emergence of resistance to antimicrobials of the first line of treatment, which includes fluoroquinolones (Sabate et al., 2008). Antibiotic resistance has been identified in many aquatic environments that are considered as water resources including rivers, sewage, ocean water and drinking water. Increasing the emergence of antimicrobial agents in the environment through medical treatment, urban waste and wastewater, agriculture and animal husbandry, causes selective pressure on the bacterial population. In addition, water resources of rivers and lakes facilitate the growth and survival of water pathogens. Multiple international rivers are

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reservoirs for antibiotics resistant of microbes. In addition, antibiotics used in agriculture, cause antibiotic resistance in bacteria, which can be transmitted to humans. The resistance ratios of this type of antibiotics are high due to their use (Ash et al., 2002; Brunea et al., 2004; Col et al., 1987; Hermanson et al., 1987; Olapade et al., 2006; Pathak et al., 1993; Qadri et al., 2005; Ram et al., 2007 and Schwartz et al., 2003). The resistance of bacteria to antimicrobial agents that is increasing worldwide is often acquired through the last gene that has been mutated. An efficient tool for acquiring new genes are mobile genetic elements, including plasmids (R) and resistant transposons. Recently, a new class of the natural occurrence of mobile genetic elements, integrons, has been defined as a means of obtaining antimicrobial resistance genes. Horizontal and vertical displacement can easily occur, as is the wide acquisition of these genetic cassete among Enterobacteriaceae and pseudomonads (Hall and Collis, 1995). Among the antibiotics used in Asia, sulfonamides have been widely used in the treatment of humans and animals, and old studies have shown that the rate of bacterial resistance has increased compared to tetracycline and quinolone. A sulfonamide is released in aqueous media, the remainder, depending on the chemical activity of bacteria, is activated against bacteria and can evolve antibiotic-resistant bacteria (ARB) in the environment and Promote natural microbial contamination (Suzuki et al., 2013). The Zarjob river of Rasht is formed from the joining of the Siahrood and Gilrud rivers and the branch that goes out of the Sefidrood livestock breeding company and linked to Goharood in the Kamaklak area (north of Rasht) and from this location. Then the river Pirbazar goes to Anzali lagoon. During the wastewater path of the industrial town of Rasht, domestic and agricultural sewage has been added and has become the most polluted river in the province of Guilan. In the course of the movement of these rivers, farmers use water for irrigation of rice fields with the help of a pump or water storage of these rivers in waterways. Most waste shoppers enter the river's natural water directly, so *E. coli*, which is a typical and main indicator of water fecal contamination, can be used as a very powerful device for contaminating organic matter which is happening in this river (Ghannady, 2005). Antibiotic sulfamethoxazole with trimethoprime (Cotrimoxazole) is still commonly used to treat urinary tract infections, especially *E. coli* infections (Perreten et al., 2003). Resistance to at least two types of antimicrobial agents in *E. coli* today as a common finding in human and veterinary medicine has a growing influence on available therapeutic selections. Serotyping antigens is a very useful method for detection of *E. coli* strains in pathogens in clinical specimens, nutrients and environmental samples, as well as for understanding the epidemiology of pathogens (Wang et al., 2010).

The present study evaluates the resistance of *E. coli* isolated to common antibiotics and especially sulfonamides, and in this study, the *sul1* gene has been proved in isolated *E. coli*. Antimicrobial agents can be used in sewage effluents, especially where these drugs are extensively used, such as hospitals, drug production plants and farms that are used to breed animals with these drugs. (Ghannady, 2005).

2. Materials and Methods

Water samples were collected from 5 different stations of Zarjob River. Sampling was done during September to January, 2015, and a total of 25 water samples were collected. Water samples were collected in sterile plastic bottles and from a depth of 15-20 cm and were transferred to the laboratory in sterile condition and next to ice. Water temperature and pH in sampling locations were measured by thermometer and pH meter, respectively. For identification of E. *coli*, water samples were analyzed by MPN method (Chao et al., 2003). In these experiments, 9 tubes (3 series of triad tubes) containing lactose broth medium with Durham tubes were used, incubated for 48 h, and studied in terms of CO_2 production and growth. For *E. coli* isolates, a loop full of the culture media positive in terms of CO*2* production and growth was used and they were cultured in EMB medium. Then, plates were incubated for 24 h and 37°C. After incubation and colony observations, plates were studied with biochemical tests, pure test was prepared from the confirmed *E. coli*, and they were kept in the refrigerator at 4°C or in the freezer at -20°C for further testing. Antibiotic sensitivity test for *E. coli* isolates was done according to the disc diffusion method. All isolates of *E.coli* were tested for resistance to antibiotics including ampicillin (10 mcg), amikacin (30 mcg), ceftriaxone (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), gentamicin (10 mcg), kanamycin (30 mcg), nalidixic acid (30 mcg), tetracycline (30 mcg), trimethoprime (5 mcg) and sulfamethoxazole (SXT) (30 mcg). For serotyping, a polyvalent antiserum SIFIN kit of *E.coli* was used. Some colonies of *E.coli* isolated from fresh (18 hours) culture medium were mixed with anti-serum drops, and separately and completely dissolved. A droplet suspension was formed that was uniform and thick. Each slide was rotated and the reaction was carefully considered for agglutination before 30 seconds in front of a black and opaque plate. Specific clotting or complete agglutination during this period was considered as a positive reaction without observing the clot in the control droplet. For DNA extraction, the Cinnapure DNA Company kit with Cat: NO: PR881613 was used. To conduct PCR test, some primers with the following characteristics were provided from Sinagene Company (Table 1.) The PCR program is done with the following conditions (95°C: 1min, 59.5°C, 1:30 min, 72°C 2min) 35 cycles.

Table 1. Characteristic of primers used in research

1	2	3	4	5	6	7
Primer Name	OD (1000µl)	MW	Pmol	100µM	TM	Seq.(5-3)
*sul1:E.coli*a	13	6123	63694.27	636.94	55.2	TTCGGCATTCTGAATCTCAC
*sul1:E.*colib	10	6108	49115.91	491.16	51.9	ATGATCTAACCCTCGGTCTC

3. Results

According to the significance level of kruskal Wallis test, it was observed that pH rate has significant difference statistically in the studied months. It is observed that pH mean is maximum in Aug. (6.7) and it is minimum in Dec. and Jan. (pH: 5.5).Kolmogrov – smirnov test was used to examine the normality (Figure 2). In addition, it was identified that temperature has significant difference statistically in the studied months and tukey test was used to separate examination of different months. It is observed that maximum mean temperature is for Sep. and minimum mean temperature is for Nov. (Figure 1). According to the significance level of kruskal Wallis test, it was observed that there is no significant difference between the numbers of bacteria during the studied months. It is observed that mean number of bacteria is maximum in Oct. and is minimum in Sep. (Figure 2). The diameter of zone inhibition growth of *E. coli* isolated from river Zarjob against selected antibiotics with disk diffusion test showed in table1. Maximum resistance was associated to the antibiotics Penicillin (100%); Cephalexin (100%), Amoxicillin (100%), Ceftriaxone (100%) and minimum resistance was against Amikacin (12%). (Tables 2). There is significant difference between frequencies of *E.coli* serotypes isolated (Table 2.) Mann – whiteny test was used to separately examine the frequency of *E. coli* serotypes. According to Table 3, it is observed that maximum frequency is related to the group 1 and minimum frequency is related to the group 4 and untypeable. According to the antibiotic susceptibility results of 25 purified samples, it was found that only 5 samples were resistant to sulfanamides (sulfamethoxazole). As indicated, only 5 samples of 25 isolates were resistant to sulfamethoxazole (20%). After examining which resistant specimen from each station was isolated and comparing them with serotyping results, it was found that all 5 sulfamethoxazole-resistant specimens belonged to Group III (O44, O125, O128), and the PCR results for these five samples showed that all had the *sul* gene and the band of the tested specimens was completely equal to the line the third ladder 50 is about 150kbp (Figure 3).

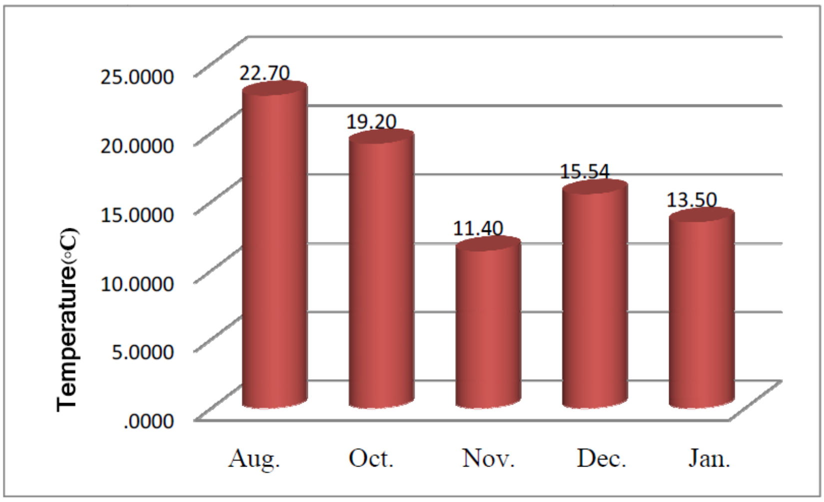

Figure 1. The average temperature of water samples collected from Zarjob River in 2015 based on month

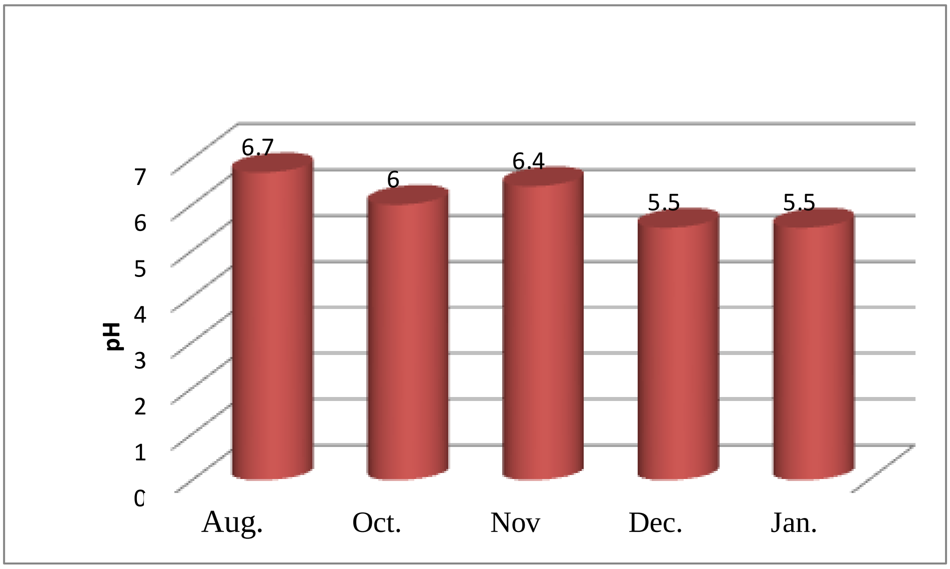

Figure 2. The average pH of water samples collected from Zarjob River in 2015 based on PH

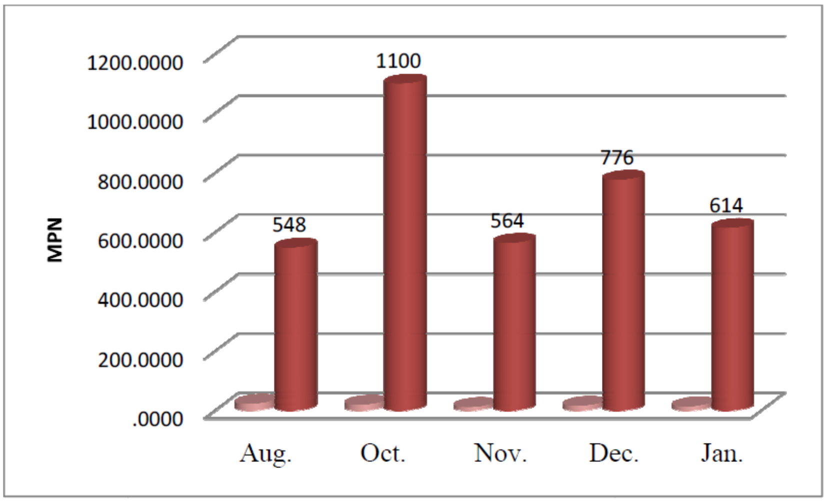

Figure 3. The average number of *E. coli* (MPN) isolated from Zarjob River in different months in 2015

Table 2. The antibiotic sensitivity of *E. coli* isolated from Zarjob River in 2015 against selected antibiotics

Antibiotic	Total number	Resistant		Intermediate		Sensitive	
		Percent	No	Percent	No.	Percent	No.
Ciprofloxacin	25	20	5	80	20	0	0
Ceftriaxone	25	100	25	0	0	0	0
Cefotaxime	25	0	0	12	3	88	22
Gentamicin	25	16	4	0	0	84	21
Amoxicillin	25	100	25	0	0	0	0
Penicillin	25	100	25	0	0	0	0
Ceftazidime	25	0	0	40	10	60	15
Cephalexin	25	100	25	0	0	0	0
Cefixime	25	0	0	0	-	100	25
Trimethoprim	25	84	21	0	0	16	4
Chloramphenicol	25	20	5	0	0	80	20
Tetracycline	25	84	21	0	0	16	4
Amikacin	25	12	3	8	2	80	20
Kanamycin	25	16	4	84	21	0	0
Nalidixic acid	25	64	16	20	5	16	4
Sulfamethoxazole	25	20	5	0	0	80	20

Table 3. *E. coli* serotypes isolated by polyvalent antisera kit

Isolate number	Serotype
1	Group III(O 44, O 125, O 128)
2	Group I(O 26, O 55, O 111)
3	Group I(O 26, O 55, O 111)
4	Group IV(O 20, O 114)
5	Group II (O 88, O 127)
6	Group I(O 26, O 55, O 117)
7	Group IV(020,0114)
8	Group III(044,0125,0128)
9	Group II (O 88, O 127)
10	Group II (O 88, O 127)
11	Group I(026,055,0111)
12	Group III(O 44, O 125, O 128)
13	Untypable
14	Group IV(O 20, O 114)
15	Group II (O 88, O 127)
16	Group II (O 88, O 127)
17	Group I(O 26, O 55, O 111)
18	Group I(O 26, O 55, O 111)
19	Group III(O 44, O 125, O 111)
20	Group I(O 26, O 55, O 111)
21	Group II (O 88, O 127)
22	Group I(O 26, O 55, O 111)
23	Untypable
24	Group III(O 44, O 125, O 128)
25	Group II (O 88, O 127)

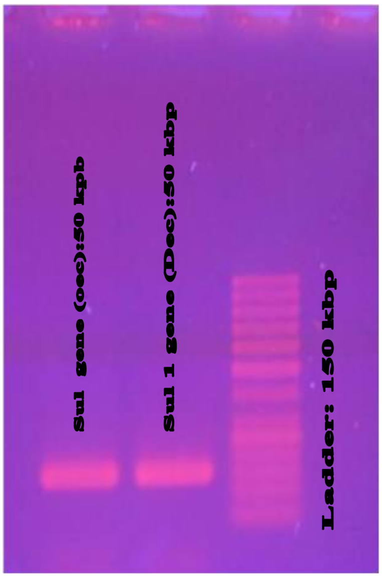

Figure 4. Representative PCR for the amplification of: *sul1* genes from culture of *E. coli* isolated from water samples

4. Discussion

Antibiotic resistant bacteria and their resistance genes are a global health issue. In the world, antibiotics are used to help improve health. The discovery of antibiotics in the twentieth century was a turning point in the treatment of infections. The ability to treat severe infections with primary antibiotics has led to advances in medical fields and a large range of medical assistance. Antimicrobial resistance is a specific problem in advanced countries. According to the World Health Organization, only in the United States millions of people every year suffer from infections caused by antibiotic-resistant pathogens, and hundreds of them this is the cause of many deaths. A large number of antibiotics are released into urban wastewater due to incomplete metabolism in humans and ultimately find their way to different natural environments (Lee, 2008; Levine and Cherian, 2007). Experiments conducted on isolated samples from the Zarjob River in Guilan province revealed that they were contaminated with various types of household, industrial and agricultural wastewater. The total coliforms and fecal forms are the indicators of water pollution that are found in it are found that *E. coli* bacteria are abundant. In this study, 25 samples of water were collected from the Zarjob River, which was collected over a period of 5 months. The pH of the samples was between 5.7 to 5 and temperatures between 11.5 and 23.5°C. All *E. coli* were isolated from all specimens (due to these temperatures and pH conditions). The average pH in September was the highest (6.7) and during the months of December and the lowest (5.5). The average temperature in the month of August was the highest (22.70°C) and the lowest in November (C11.40°C). This temperature difference can be attributed to the natural temperature change over the months and seasons. These samples were also evaluated for antibiotic susceptibility. The results showed that the highest number of bacteria is sensitive to gentamicin antibiotics, the antibiotic kanamycin has the highest number of semi sensitive antibiotics and ampicillin and ceftriaxone antibiotics have the most resistant number. More precisely, 80% were sensitive to gentamycin, Amikacin, chloramphenicol and sulfamethoxazole, 16% tetracycline, trimethoprime and Nalidixic acid, 84% and 8% to amikacin, 20% to Nalidixic acid, 80% to ciprofloxacin, 84% to kanamycin were semisensitive12% to amikacin, 16% to gentamicin and kanamycin, 20% to ciprofloxacin, chloramphenicol and sulfamethoxazole, 64% to nalidixic acid, 84% to tetracycline and trimethoprim, 100% to ampicillin and ceftriaxone, which looked like Studies in Toronto and India . In a study by Hu and colleagues in 2008 on isolated *E. coli* from the Beijing river, it was found to be the most resistant to sulfonamides, and 96% were resistant to sulfonamides, which resisted resistance genes test (A), test (B), tet (M) and their combination with *sul1, sul 2 and 3 sul* genes (Hu et al., 2008) while in our study, the highest resistance to ampicillin and ceftriaxone, So these two studies do not match each other. In a study conducted by sukumaran et al. In 2010-2011 on Cochin Gulf water samples in India to isolate *E. coli* and to investigate antibiotic resistance, it showed that the highest resistance of *E.coli* strains That is, 65.3% was ampicillin, about 29% were resistant to sulfonamides and in total 75 isolates of *E.coli*, and 13 were resistant to sulfonamides. Two strains were positive for the presence of *sul1* gene by PCR. Most of the different serotypes were isolated from stations near the city of Cochin, which suggests the probability of these organisms being released from hospital waste inside and outside the city. Hospital wastewaters often contain antimicrobial agents that sometimes cause the selection and survival of resistant strains (Sukumaran et al., 2012). In our study, it was found that the highest resistance of *E. coli* was 100% compared to ampicillin and ceftriaxone and 20% of all specimens were resistant to sulfonamides and all of them were positive for the presence of *sul1* gene by PCR, so these two studies are consistent. The bacteria carrying *sul1* genes with Class I and II introns often result from emptying the wastewater, so the swelling bacteria with sul1 genes are mainly from humans, animals and terrestrial regions that transfer genes to aquatic bacteria they give. The genes *sul1, sul2, sul3* were found in the seawater community, so they flow into the sea (Suzuki et al., 2013). In our study, bacteria carrying the *sul1* gene were found, and with regard to the disposal of hospital, industrial and agricultural wastewater into the Zarjob River and the connection of this river to the Mazandaran Sea, is similar to that of Suzuki et al. Study is the same. The pollution of the rivers is very important because of their flow over different distances and between different cities, and on the other hand, Antibiotic resistance has become a global issue. The Zarjob River also leads to the lagoon of Anzali and the Mazandaran Sea. The most polluted river in the province of Giulan is Zarjob, which can cause pollution of various types of waters, aquatic organisms, fish and other marine organisms used by humans. On the other hand, groundwater level in the province of Guilan is high, and the contaminated river could also pollute groundwater resources.

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Refereces

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