International Journal of Molecular and Clinical Microbiology 6(2) (2016) 715-724

International Journal of
Molecular and Clinical Microbiology

Evaluation of multi-drug resistant pathogenic Escherichia coli in Zarjob River located in the state of Guilan, Iran

Khosro Issazadeh*, Sadaf Alsadat Seyedi and Nafiseh Ghorbani

Department of Microbiology, College of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran

ARTICLE INFO

Article history:
Received 26 august 2016
Accepted 8 october 2016
Available online 1 December 2016

Keywords:
TEM,
SHV,
E. coli,
Zarjob River,
Serotyping

ABSTRACT

In modern medical processes, new antimicrobial drugs are being widely used in order to rapid and essential eradication of resistant bacterial strains. Broad-spectrum beta-lactamases are enzymes originated from mutations in the common plasmid genes mediators for beta-lactamases such as TEM-1, TEM-2, and SHV-1 are transferred among bacterial species. The genes coding the production of ESBL are often connected to other resistant genes causing resistance to drugs with broad spectrums. Therefore, there are different experimental techniques to detect $ESBL_s$. This study aimed to investigate the presence of bla*SHV* and bla*TEM* genes coding beta-lactamase enzymes in *Escherichia coli* isolated from Zarjob River in Gilan Province, their resistance to different antibiotics, and their stereotyping. In order to isolate and identify *E. coli*, 25 water samples were taken from Zarjob River and analyzed by MPN test and biochemical tests. Antibiotic sensitivity testing with Kirby-Bauer method, i.e. disk diffusion on 25 *E. coli* isolates, and also serotyping with agglutination on a slide were performed. To detect $_{bla}$SHV and $_{bla}$TEM genes in *E. coli*, PCR technique was used and all results were analyzed by using the software SPSS. Among the 25 E. *coli* isolates, 18 cases (72%) had the gene $_{bla}$TEM and 15 cases (60%) had the gene $_{bla}$SHV. The results of polyvalent antiserums serotyping were observed as Group I (O26, O55, O111), Group II (O88, O127), Group III (O44, O125, O128), and Group IV (O20, O114).

1. Introduction

Among various provinces and regions of our country, Gilan province has a special status in environmental terms. The population of this province is over 2250000. In this province, the risk of permeation of different kinds of effluents and, as a result, contamination of soil and water is higher due to higher groundwater levels. In more than 80 rivers in Gilan, contaminants flow through municipal and domestic sewage. Until three decades ago, these rivers had clear water full of different kinds of fish and aquatic organisms (Clark et al. 1991). *Escherichia coli* have their origin in warm-blooded animals. The examined levels of *E. coli* in water can represent the presence of fecal contamination entered by animals or human waste. In the past, *E. coli* has been identified based on biochemical or cultural methods. Recently, this is done based on PCR for different pathogenic and non-pathogenic genes (Bush et al.,1995). PCR is a major advance in molecular diagnostics of pathogenic microorganisms, including *E. coli*. PCR primers have been developed successfully for several of

*Corresponding author: Dr. Issazadeh
E-mail address: issa_kaam@yahoo.com

the categories of diarrheagenic *E. coli* (Stacy-Phipps et al.1995).

Beta-lactamases are a group of enzymes capable of hydrolyzing the quadrilateral loop of beta-lactam antibiotics. Broad-spectrum beta-lactamases are enzymes originated from mutations in the common plasmid genes mediators for beta-lactamases such as TEM-1, TEM-2, and SHV-1 and are transferred among bacterial species. *Klebsiella* and *E. coli* species are two bacteria producing the widespread and universal Extended-spectrum beta-lactamases which is found in different countries with different levels of resistance (Salmanzadeh et al., 2005).

Zarjob River, also known as Siahrud, originates from the low-height mountains of Hezar Marz, Neyze Sar, Chakul Bandan, and Kachador at about 25 km from the south of the city of Rasht with a maximum altitude of 810 m, travels an 8-km path along the south to the north after passing the villages Bahdan, Chumacha, Donahre Siyavash, Sangar, Bijarpas, Rudberah, Gol Paresar, Kabkh and so on, joins Garmrud River (Pir Bazar River) in Busar inside the city of Rasht, and finally enters Anzali Wetland. This river is 41 kilometers long together with the length of main tributaries to the place it joins Garmrud and has a water capacity of 173.4 million m^2. Gilan Province and Rasht City as its center and an important tourism hub of the country are faced with the problem of waste and agricultural, industrial, and municipal wastewater. Most importantly, Siahrud River that is known as Zarjob River passes through Rasht, has many houses around itself, and brings along all pollutants into Anzali Wetland which is one of the most beautiful wetlands (Monavary 1990). It is clear that the increase in population is associated with lots of environmental, social, and economic problems. In the assessment of sources of contamination, we can refer to municipal and domestic sewage, industrial sewage, rural and urban solid waste (garbage), agricultural sewage, and slaughterhouses.

Zarjob water pollution is caused by the discharge of different kinds of sewage, urban sewage, and waste in the environment which are produced by sanitary sewage, domestic sewage, sewage of public places such as hospitals, hotels, and bathrooms, and surface sewage washed off by rain and can influence the river water as temporary contaminants. Eighty percent of the 90-liter per capita consumption of water in the city of Rasht is disposed as sewage and this sewage flows into the downstream of agricultural and rural areas and Anzali Wetland which is the final place of discharge for this river, thus causing environmental pollution. Along the Zarjob River, it is polluted with several sources such as factory and agricultural effluents in the region and industrial, urban, and domestic sewage (Edelberg et al., 1988).

2. Materials and Methods

Water samples were collected from 5 different stations of Zarjob River. Sampling was done during September to January 2015 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 conditions and in the surrounding ice. Water temperature and pH in sampling locations were measured by a thermometer and pH meter, respectively. In order to isolate and identify *E. coli*, water samples were analyzed by Most Probable Number method. 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 linearly 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 and pure culture was prepared from the confirmed *E. coli*, and then 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. Double disc synergy test (DDST) was performed in order for the confirmatory testing to produce ESBL according to the standard method S22CLCIM100. The strains showing resistance or sensitivity to cephalosporins were tested in terms of the presence of ESBL by using the double disc synergy test (DDST). DDST was carried out on Mueller Hinton-agar with discs containing 30 μg ceftazidime, cefotaxime, or aztreonam at a distance of 15 mm (center to center) from a disc containing clavulanic acid-amoxicillin (10 μg/20 μg) placed in the center of

the plane. For *E. coli* isolates serotyping, the pathogen *E. coli* antiserums kit, a product of the Bahar Afshan Company, was used. For DNA extraction, the Cinna pure 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 test was used to detect the presence of the genes blu*sh* that encodes the beta-lactamase enzyme. A 147 kb area of the encoding genes blaTEM and blaSHV in *E. coli* were amplified by PCR using the afore mentioned primers (Table 2). Kruskal Wallis test was used for determinate significant difference pH rate on months. The Kruskal-Wallis test is a nonparametric (distribution free) test, and is used when the assumptions of ANOVA are not met. They both assess for significant differences on a continuous dependent variable by a grouping independent variable (with three or more groups). In the ANOVA, we assume that distribution of each group is normally distributed and there is approximately equal variance on the scores for each group. However, in the Kruskal-Wallis Test, we do not have any of these assumptions. Like all non-parametric tests, the Kruskal-Wallis Test is not as powerful as the ANOVA. Kolmogorov–Smirnov test was used for determinate significant difference temperatures of samples. The Kolmogorov-Smirnov test is a hypothesis test procedure for determining if two samples of data are from the same distribution. The test is non-parametric and entirely agnostic to what this distribution actually is. The fact that we never have to know the distribution the samples come from is incredibly useful, especially in software and operations where the distributions are hard to express and difficult to calculate with.

Table1. Characteristic of primers used in research

Primer Name	OD (1000µl)	MW	pmol	100µM	TM	Seq.(5-3)	Reference
blaSHV*:E. coli1*	11	5904	55894.31	558.94	51.7	AGGATTGACTGCCTTTTTG	Colom et al.(2003)
blaSHV*:E. coli2*	10	5551	54044.32	540.44	53.9	ATTTGCTGATTTCGCTCG	Colom et al.(2003)
blaTEM*:E. coli1*	9	5236	51566.08	515.66	45.6	ATCAGCAATAAACCAGC	Colom et al.(2003)
blaTEM*:E. coli2*	10	5210	57581.57	575.82	53	CCCCGAAGAACGTTTTC	Colom et al.(2003)

Table 1. PCR cycling in research

Primer Name	Red master mix amplicon	Primer F	Primer R	WD	DNA	Total	PCR Program	Electrophoreses
blaTEM:*E. coli*	10 (MgCL2=2.5)	1.5	1.5	5	2	20µL	94° 5min,(95° 1min,59.5° 1:30 min ,72° 2min)35 cycle,72 °5 min	Gel 2.5% ,80 Voltage, time:60 min
blaSHV:*E. coli*	10 (MgCL2=2.5)	1.5	1.5	5.5	1.5	20µL	94° 5min,(95° 1min,59.5° 1:30 min ,72° 2min)35 cycle,72° 7 min	Gel 3% ,80 Voltage, time:60 min

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. Based on data, it is observed that pH mean is maximum in Shah River (7.6) and it is minimum in Dec. and Jan. (pH: 5.5). Kolmogorov–Smirnov test was used to examine the normality. In addition, it was identified that temperature has significant difference statistically in the studied months and Turkey's test was used to separate examination of different months. It is observed that maximum mean temperature is for Sep. and the average minimum 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 the mean number of bacteria are maximum in Oct. and are minimum in Sep. (Figure 2.). Zone of inhibition's size of E. coli isolated from river Zarjob against selected antibiotics and in double disk diffusion test showed in figures 3 and 4. Maximum resistance was associated to the antibiotics penicillin (100%); cephalexin (100%), amoxicillin (100%), ceftriaxon (100%) and minimum resistance were against amikcin (12%). Most sensitive of cefixime (100%), maximum number of semi-sensitive to kanamycin (84%), respectively (84%) (Tables 3 and 4). The most resistant of bacteria were to Gentamicin, amikacin, kanamycin, nalidixic acid, Tetracyclin, chloramphenicol, trimetoprim, cephalexim, penicillin and amoxicillin. That is, there was significant difference between frequencies serotypes of *E.coli* (Table 5.). Mann-Whitney test was used to determine differences in the prevalence of *E. coli* serotypes. According to the Table 5, the highest frequency and lowest were in group 1 to group four respectively. During PCR, it was found that amongst 25 purified samples, 18 samples (72%) had bla- TEM gene and 15 samples (60%) had bla- SHV gene. Samples are completely in the same sized as 50 ladders third line i.e. about the size 150. Flowingly, two figures, one of them by Document gel Camera and the other by a digital camera, are observed (Figure 5).

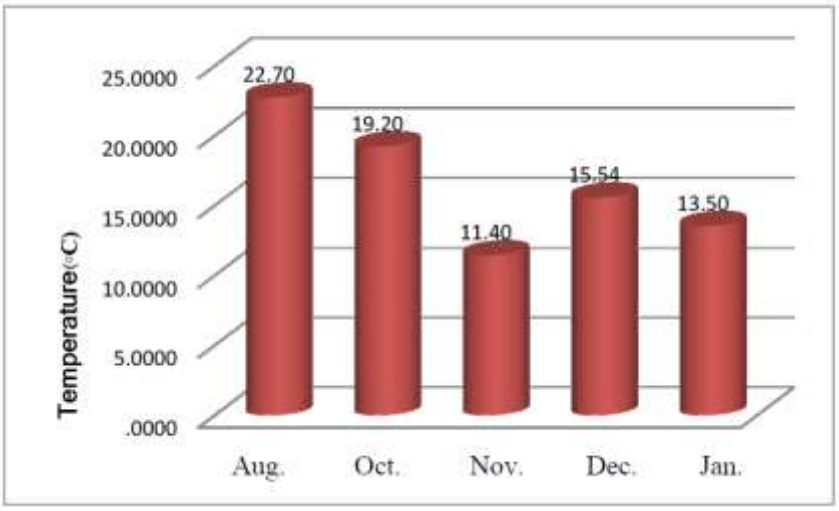


Figure1.The average temperature of water samples collected from Zarjob River in 2015 based on month

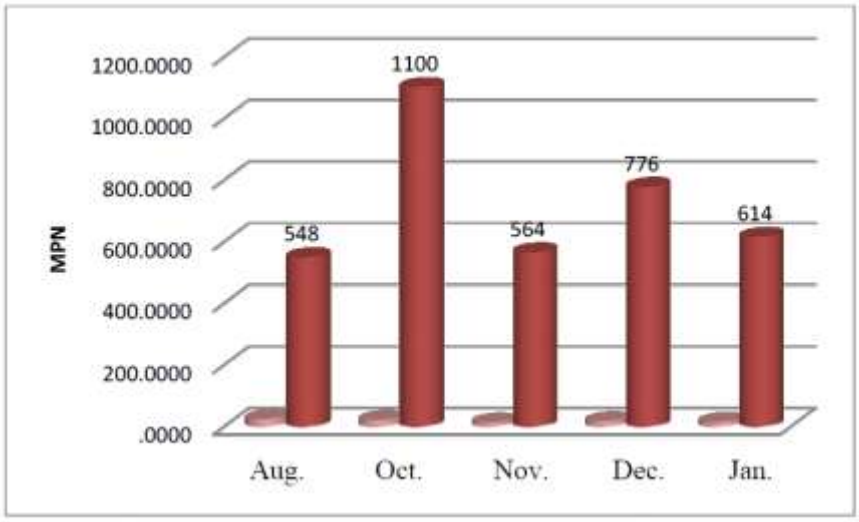


Figure 2. The average number of *E. coli* (MPN) isolated from Zarjob River in different months in 2015

Figure 3.The diameter of zone inhibition growth of *E. coli* isolated from river Zarjob against selected antibiotics

Figure 4.The diameter of zone inhibition growth of *E. coli* isolated from river Zarjob in double disk diffusion test against selected antibiotics

Table 3.The antibiotic sensitivity of *E. coli* isolated from Zarjob River in 1393 against selected antibiotics

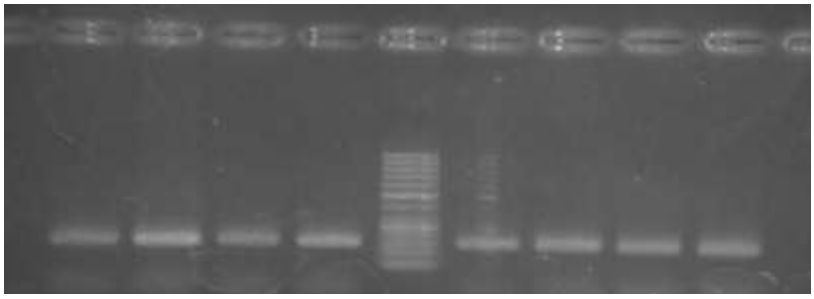
Resistant		Intermediate		Sensitive		Total number	Antibiotic
Percent	No	Percent	No.	Percent	No.		
٪20	5	٪80	20	-	-	25	CP
٪100	25	-	-	-	-	25	CRO
-	-	٪12	3	٪88	22	25	CTX
٪16	4	-	-	٪84	21	25	GM
٪100	25	-	-	-	-	25	AMX
٪100	25	-	-	-	-	25	p
-	-	٪40	10	٪60	15	25	CAZ
٪100	25	-	-	-	-	25	CN
-	-	-	-	٪100	25	25	CFM
٪84	21	-	-	16	4	25	TMP
٪20	5	-	-	٪80	20	25	C
٪84	21	-	-	٪16	4	25	TE
٪12	3	٪8	2	٪80	20	25	AN
٪16	4	٪84	21	-	-	25	KA
٪64	16	٪20	5	٪16	4	25	NA

Table 4. The antibiotic sensitivity of *E. coli* isolated from Zarjob River in double disk diffusion test

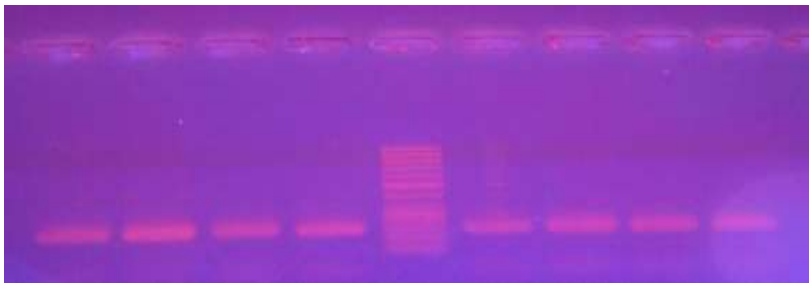
Resistant		Intermediate		Total number	Double disk with AMC
Percent	No.	Percent	No.		
50	10	50	10	20	CRO
100	25	–	–	25	AMX
100	25	–	–	25	P
–	–	100	10	10	CAZ
100	20	–	–	20	CN
100	25	–	–	25	TMP
100	5	–	–	5	C
100	25	–	–	25	TE
100	10	–	–	10	NA
100	5	–	–	5	KA
100	5	–	–	5	AN
100	10	–	–	10	CM

Table 5. *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)



A



B

Figure 5. Representative PCR for the amplification of bla_{TEM} and *blaSHV* genes from culture of *E. coli* isolated from water samples (A: Document gel Camera and B: digital camera) from right to left. 1. *(Sep.) bla-SHV: E. coli, 2. (Oct.) bla-SHV: E. coli, 3. (Nov.) bla-SHV: E. coli, 4. (Jan.) bla-SHV: E. coli, 5. Ladder 50 bp, 6. (Jan.) bla-TEM: E. coli, 7. (NOV.) bla-TEM: E. coli, 8. (Oct.) bla-TEM: E. coli*

4. Discussion

Among the contamination indications of this water are total coliforms and fecal coli forms, among them *E.coli* is found abundantly. This typical organism exists with high frequency in the stool; it should be survived in the water for longer period than other pathogens and should be resistant against the antiseptics such as chloride. Among 25 samples of isolated *E.coli* 18 samples (72%) had bla - TEM gene and 15 samples (60%) had bla - SHV gene. This fact indicated that this gene is active in pH 5.5-7 and temperature about 11-23.5°C and exists in the most samples of *E.coli*. Statistical results indicated a significant relationship between the number of sensitive, semi- indicative and resistant samples. In addition, by studying on the frequency of *E.coli* serotypes, it was defined that maximum frequency was associated the group I (O26, O55, and O111) (32%) and minimum frequency was related to group IV (020, 0144) (12%). Among them, group IV appeared as 100% agglutination with completely transparent background and distinct particles, group III was appeared as 75% agglutination with relatively transport, group II appeared as 50% agglutination with relatively opaque background and group I appeared as 25% agglutination with opaque background. In a study by Sukumaran, et al. during 2010-2011 on the samples from cochin gulf's water located in India to study the patterns of antibiotic resistance in the isolated *E.coli,* they concluded that most *E.coli* strains (33.65%) were resistant to ampicillin, followed by resistance to antibiotics Nalidixic acid (37.33%), tetracycline (33.33%), co-trimoxazole (17%), trimetoprim (17%), kanamycin (14%) and ciprofloxacin (12%) (Edberg1988). Ribot et al.(2006), studied 233 samples, 57% (n=133) were resistant to ampicillin, followed by 45% (n=105) were resistant to tetracyclin, 37%(n=87)were resistant to acid nalidixic, 37% (n=83) to trimethoprim , sulfamethoxazole, 17% (n=31) to ciprofloxacin, 9% (n=22) to ceftriaxon, 9% (n=20) to mecilinam, 8% (n=18) to chloramphenicol and 1%(n=2) to gentamicin. More than 73% (n=171) of isolates were resistant against to one antibiotic and 36% of isolates (n=84) were resistant to 3 class or more antibiotics. So they were identified as multidrug resistant (MDR). Further examination on 22 ceftriaxon resistant isolates revealed that all of them were ESBL producer which was approved through the double disk cooperation test. All 22 isolates were resistant to cefotaxim and cefixim 82% were resistant to erythromycin, 64% to aztereonam, 55% to ciprofloxacin and norfloxacin, 32% to kanamycin and ceftazidime, 14% to piperacillin-tazobactam, and 9% to cefoxitin. No isolate was resistant to carbapenern antibiotics including imipenem and meropenem (Espy 2016). In our study, results of the double disk test were as follows: ceftazidim had the maximum semi-sensitive cases, gentamicin, amikacin, kanamycin, nalidixic acid, tetracycline, chloramphenicol, trimethoprim, cephalexin, penicillin and amoxicillin had maximum resistant number (100%). Marta Alva, et al (2014) studied on the sea water which is one of multi drug resistant *E.coli* resources including strains carrying the resistant to plasmid mediate quinolon and wide-spread Beta-lactamase genes and expansion of antibiotic resistance in the coast waters. Samples were collected in Berlenga which is a non-residential island classified as a natural reservoir and visited by tourists for water recreational activities (Marta et al. 2014). Based on the genetic classification, 414 strains were identified. Distributions of *E. coli* phylogenic groups among the isolates of all resources were similar. Resistant to streptomycin, tetracycline, cefalotin and amoxicillin had the maximum frequency. Higher antibiotic resistance was observed among the sea water and stool samples, except for final level of antibiotics used in human medicine. Nucleotide sequences adjusted to resistance genes *bla -TEM*, *sul 1*, *sul* 2, *tet A* and *tet B* existed in all sources. Genes conferring the resistance to third generation cephalosporins, were identified in the stools of water fowls (blacmy-2) and sea water (blaCTX=M-1, blaSHV-12). Resistance patterns of isolates against 16 antibiotics were indicated. Resistance (or medium resistance) against all the studied antibiotics was observed and 94% of isolates were resistant against to at least one antibiotic. Most definite resistance to antibiotic was against streptomycin (83-100%), followed by cefalotin and tetracycline in the stool sources (34% and 35% in the waterfowl stool and 23% for both antibiotic in the swage). Isolates were more sensitive against imipenem, third generation cephalosporins, gentamicin and combination of piperacillin/ tazobactam. For

most studied antibiotics (such as penicillin, cefalotin, tetracycline), sensitivity rates in the isolates from sea water and waterfowl stool were higher. Resistance against to imipenem, third generation cephalosporin and ciprofloxacin had a maximum outbreak in the sewage. Multi resistance rates (that is, resistance against the antibiotics of at least 3 classes) were higher amongst isolates from waterfowl stool (32%) and sewage (295). In total, in 126 phenotype, various resistance (or medium resistance) was observed. But most of them (n=86) were observed only with one isolate. Resistance to streptomycin was most common phonotype detected in 90 isolates, followed by resistance to streptomycin and tetracycline (30 isolates), streptomycin and cephalotin 927 isolates). Several multiresistance phonotypes were detected (Yu and kapper 1992).In our study, most typical resistance was against to penicillin, cephalexin, amoxicillin and ceftriaxone (100%). Sengul Alpay-karaoglu, et al studied the antibiotic resistance pattern and TEM type Beta-lactamase gene in ampicilin resistant *E.coli* strains from potable water. Fifty five ampicillin resistant *E.coli* strain (Ampr) were isolated during 2000-2002 and from January to February 2004 from 51 potable water bodies in the Rize area in Turkey with abundant freshwater resources (Sengul Alpay-karaoglu et al., 2007). They found that a lot of organisms indicated the resistance to 3 or more antibiotics used in human and veterinary. These strains indicated a multi –resistance phenotype. About half of the strains (27%) indicated ceftazidim resistance, but according to the results of double disk co operation test, these strains are not wide –spread B-lactamase producer. Then, all the isolates were screened in respect of TEM type B-lactamase gene (blaTEM) through polymerase chain reaction (PCR). TEM_s type B-lactamase genes were found in 6 isolates (11%). Sequence analysis indicated TEM-1 type genes. However, is electric focus analysis didn't prove TEM-1 type B-lactamase production except for one strain. Conjugation tests indicated that ampicilin, tetracycline or trimetoprim/ sulfa metoxasol resistances were transmittable in 6 isolates (11%). The Appearance of transmittable antibiotic resistance and blaTEM-1 gene in *E.coli* strain from public potable waters had significant public health risk. Compared to our study, ceftazidim sensitivity was (60%) in double disk cooperation test results accounted for maximum semi-sensitive number (100%). TEM type B-lactamase genes were found in 18 samples.

Acknowledgements

We are grateful to Islamic Azad University, Lahijan Branch Authorities, for their useful collaboration.

Refereces

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