Antibiogram of *Escherichia coli* Isolated from different Hospitals Wastewater in Erbil City, Iraq

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Abstract

Three hospitals were chosen for the present (Maternity hospital, Raperin hospital and Rhizgari hospital) survey within Erbil city, 36 water samples were collected at regular monthly interval periods beginning at January to December 2012. Microbial analysis was done by selective medium and biochemical tests and the isolated bacteria from those hospitals were *Eshcerichia coli*, *Acinetobacter lowffii*, *Klebsiella pneumoniae*, *Moraxilla* spp., *Salmonella* Typhi, *Citrbtobacter freundii*, *Vibrio fluvials*, *Acinetobacter haemolyticus*, *Weeksella zoohelcum*, *Pasteurella multicida*, and *Pseudomonas aeroginosa*. *E. coli* isolates were subjected to antimicrobial susceptibility testing. *In vitro* activities of 10 different antibiotics against *E. coli* isolates were showed a high resistance rate observed against ampicillin (80%) while the lowest resistance was to pipercillin/ tazobactam (22%). A high proportion of the *E. coli* isolates from the three hospitals revealed resistance to more than one antibiotic.

نمط المقاومة لمضادات الحياة للاشيريكة القولونية المعزولة من مياه فضلة عدة مستشفيات في مدينة اربيل، العراق

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الخلاصة

في هذه الدراسة جمعت عينات المياه العادمة من ثلاث مستشفيات في مدينة اربيل هي مستشفى رزكاري ومستشفى رابرين ومستشفى الولادة، حيث تم جمع 36 عينة في فترات فاصلة منتظمة شهريا ابتداء من كانون الثاني 2012 إلى كانون الاول 2012. تم اجراء التحليل البكتيريولوجي باستخدام الاوساط الانتقائية والاختبارات البايوكيميائية وكانت البكتيريا المعزولة كالتالي : *Escherichia coli, Acinetobacter lowffii*, *Klebsiella pneumoniae*, *Moraxilla* spp., *Salmonella* Typhi, *Citrbtobacter zoohelcum*, *Weeksella haemolyticus*, *Acinetobacter freundii*, *Vibrio fluvials*, *Pasteurella multicida*, and *Pseudomonas aeroginosa*. وقد اجري اختبار الحساسية لعزلات *E. coli* باستعمال عشرة مضادات حياتية وكانت اعلى نسبة مقاومة للبكتيريا (80%) ضد ampicillin، في حين كان أدنى مقاومة ضد pipercillin/ tazobactam (22%) . وكانت هناك نسبة عالية من عزلات *E. coli* التي تم عزلها من المستشفيات الثلاثة مقاومة لأكثر من مضاد حياتي واحد .

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Introduction

One of the main environmental problems putting by the hospital effluents is their discharge, in the same way as the urban classic effluents, towards the urban sewer network without preliminary treatment. Indeed, since the 1980s. Data about the occurrence of pharmaceuticals in natural surface waters and the effluent of sewage treatment plants have been reported [1-3].

Hospitals consume an important volume of water a day. Undeniably the consumption of domestic water is on average 100 liters/person/day, while the value generally admitted for hospitals varies from 400 to 1200 liters/day/bed [3- 5]. The water consumed in different units such as inpatient wards, operating rooms, laboratories, laundries, kitchens, health services and administrative units decreases it's physical, chemical and biological quality and is converted to wastewater [6]. Considering the volume of wastewater generated by the hospitals and of the extreme diversity of physical, chemical and biological evolutions that these effluents can know it seems important to make their ecotoxicological and sanitary risk assessment [3].

The increasing incidence of resistance to a wide range of antibiotics by microorganisms, clinical infections, disease and death caused by resistant bacteria are increasingly common and is a major concern facing modern medicine. There is increasing concern about the growing resistance of pathogenic bacteria in the environment, and their ecotoxic effects. This includes both the ecology of resistance genes and that of the resistant bacteria themselves [7].

β-Lactam antibiotics are bacteriostatic, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity being the outermost and primary component of the wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs vary in their affinity for binding penicillin or other β-lactam antibiotics. The amount of PBPs varies among bacterial species [8].

The most common response of the cell to antibiotics is to cease growing (bacteriostasis), but for certain classes of compounds such as b-lactams, Bacteria often develop resistance to β-lactam antibiotics by synthesizing a β-lactamase, an enzyme that attacks the β-lactam ring, continued growth is permitted, with inhibition of the target in the organism leading indirectly to cell death. Such augmentation is typically absent in the environment. In this respect, there is little knowledge of environments such as waste water, sludge, surface water, and soil compared with the medical use and effectiveness of antibacterials. Concentrations are normally some orders of magnitude lower in the environment than for therapeutic use [9-11].

Beta-lactamases are enzymes produced by some bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem) (Carbapenems are relatively resistant to beta-lactamase). The lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties [12]. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer (plasmid mediated resistance), and β-lactamase gene expression may be induced by exposure to β-lactams [13].

Materials and Methods

Sample collection

36 samples were performed from three sites, effluents of hospitals in Erbil city; Rhizgari (general hospital), Raperin pediatrics hospital and maternity hospital at monthly intervals during January 2012 to December 2012.

Sampling usually started at 9 am and was completed at 12pm. Water samples were collected from surface water (30-40 cm depth) using autoclaved amber bottles pre-washed by water sample twice before filling.

HPLC test

HPLC system with detector was used to analyse cefotaxime and ceftazidime in comparison to tap water and purified water, the mobile phase was included solution which contained 40 mL of acetonitrile and 200 mL of pH 7 of HPLC buffer, and diluted with water to obtain 2000 mL of solution. The flow rate set at 2 mL / min with an injection volume of 20 µL. The detection of ceftazidime was conducted at wavelength of 254 nm and of 235 nm wavelength for cefotaxime [14].

Isolation *E. coli* from effluent

Escherichia coli were isolated from the hospitals effluent samples by processed Eijkman test [15]. After collection of samples in autoclaved bottles, aliquot of 100 µl direct from the effluent sample was added to three test tubes one was contained 10 mL of buffer peptone, another test tube contained phenol red with 10% Dextrose (2-3- 4 B) and Durham tube, the third one contained MacConkey broth with Durham tube. All tubes were incubated in water bath 46°C for 24hr. After that, Kovac`s Indole reagent was added to tubes with buffer peptone to indicate positive results. The change of phenol red with dextrose tubes and MacConkey broth tubes to yellowish color with gas production were indicated as positive results. *E. coli* ATCC 8739 was used as a positive control.

The tubes with positive results were subclutured on MacConkey agar, Endo agar and Eosin Methelyne and incubated at 37°C for 24hr, then proceed to identification methods.

Identification of bacteria

In order to identify *E. coli* isolates and other bacterial species; samples with positive Eijkman test results and from filtration method, respectively, were subjected to Microgen test, Gram stainability, Biochemical tests and cultural characteristics.

Antibiotic susceptibility test

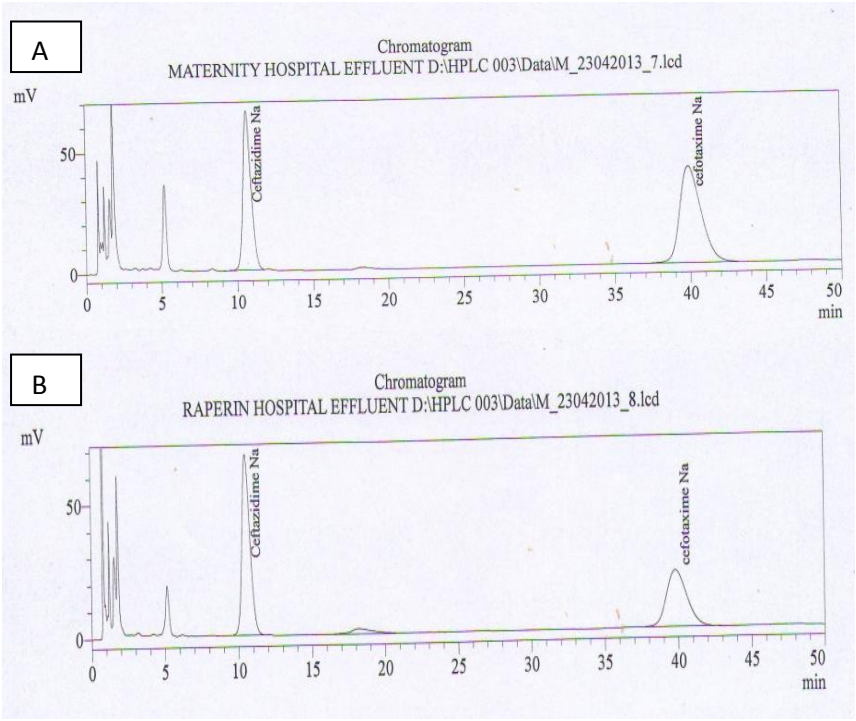
This test was done using standardized Kirby Bauer Disc Diffusion Test [16]. All *E. coli* isolates were tested against several commonly used antibiotics (Amoxicillin/Clavulanic acid 20/10 µg, Ampicillin10 µg , Aztreonam30 µg ,Cefepim30 µg, Cefotaxime30 µg, Ceftazidime30 µg, Imipenem10 µg, Meropenem10 µg, Piperacillin100µg, Piperacillin/Tazobactam(100/10) µg).

Inhibition zones were measured and recorded during the period of between 16-24hr incubation with the use of Electronic Digital Caliper. These zones were compared with zone diameter interpretation standards approved by the Clinical and Laboratory Standards Institute [17]. *E. coli* ATCC 25922 was employed as quality control isolate.

Results and Discussion

HPLC test for Cefotaxime and Ceftazidime detection in hospital`s eflluents

The qualitative determination of Cefotaxime and Ceftazidime in the effluents of the study hospitals showed that these two antibiotics were estimated in that effluents in comparison to tap water and purified water. The recoveries were 50.6%, 86.3% and 93.8% for Ceftazidime, figure-1, in Rhizgari, Raperin and Maternity hospitals, respectively. For Cefotaxime the recoveries were 18%, 30.34% and 57% in Rhizgari, Raperin and Maternity hospitals, respectively, figure-1.



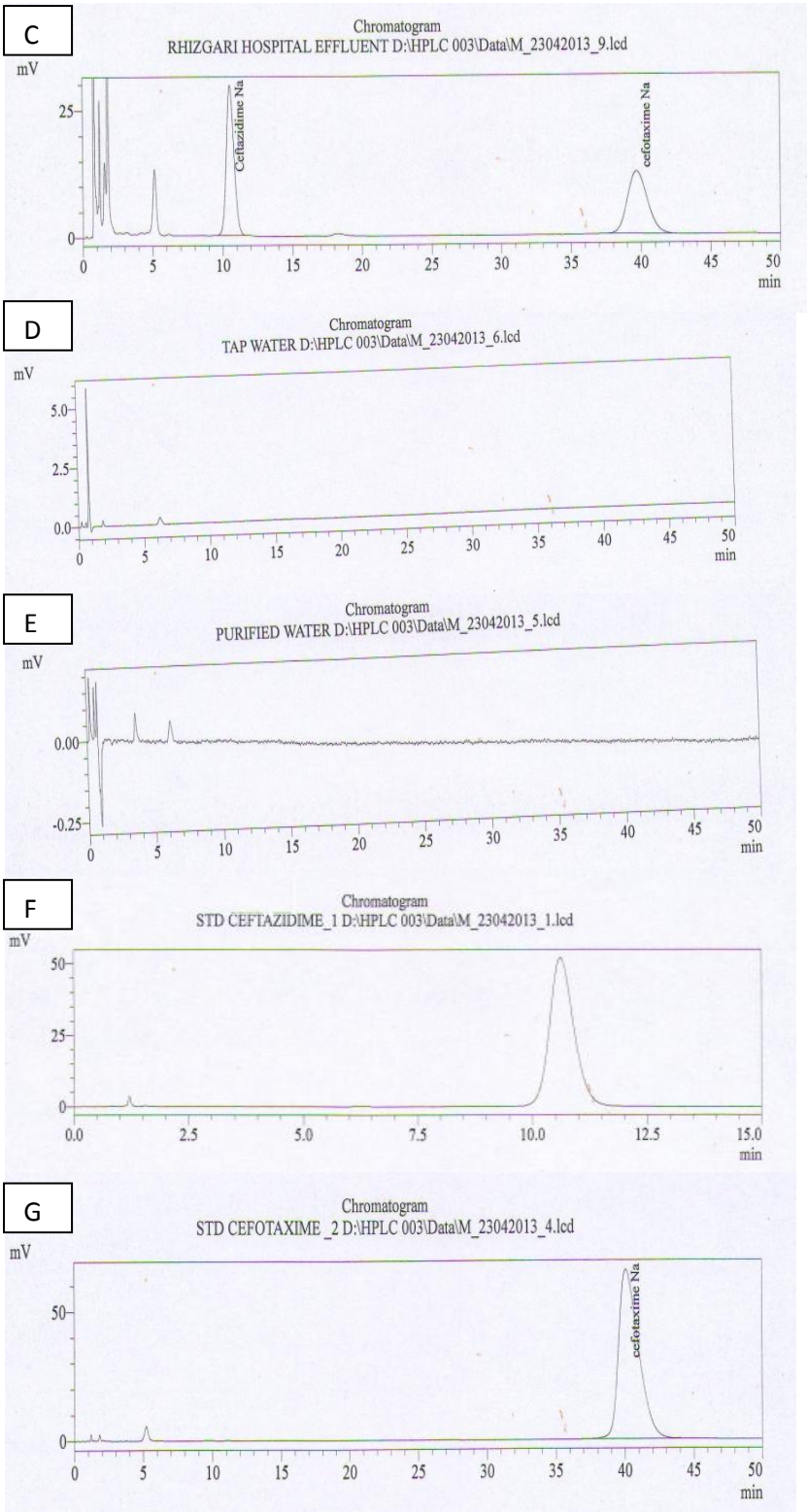


Figure 1- Chromatogram of HPLC showing the recoveries of Beta lactams in hospital effluents: A: Maternity hospital, B: Raperin hospital, C: Rhizgari hospital, D: Tap water, E: Purified water, F: Ceftazidime Standard solution, G; Cefotaxime Standard solution.

The β -Lactam antibiotics account for the most antibiotics usage in human therapy [18]. The previous studies indicate that β -Lactam antibiotics were not detected in most environmental waters [19] and that class of antibiotics generally undergo hydrolysis fairly quickly under mild acidic and base conditions [20], while Al-Ahmad *et al*. (1999) showed that β -Lactam antibiotics have low biodegradation rate [21].

β-lactam antibiotics, foremost penicillins and cephalosporins, have been described to occur in untreated and treated sewage from WWTPs (Waste Water Treatment plant) [22-24], but concentrations were generally low, measured in ng - μg per litre. One study focusing on WWTPs in Sweden, showed that penicillins and cephalosporins could not be detected in a majority of samples [25] Furthermore, the β-lactams were reduced in the treatment process at WWTPs by up to 100 % and detected with far lower frequency in treated sewage [23, 24]. However, the removal varies and the cephalosporin Cefalexin varied in reduction between 9 and 89 % between different WWTPs [23]. In addition, the cephalosporins were generally found in higher concentrations compared to the other groups, and appeared to be more resistant to the wastewater treatment, i.e. found in higher degree and concentration in outlet [24]. The penicillins on the other hand were only occasionally detected, and in very low concentrations [23, 24]. In surface waters, β-lactams were rarely detected and when they occurred it was at concentrations well below those in wastewater [26]. The reason why β-lactams are not persistent in aquatic environments is hydrolysis of the chemically unstable β-lactam ring [22]

A study by Kuch *et al*. [27] for β-Lactams in HWW (hospital wastewater) by using liquid chromatography showed that Penicillins, cephalosporins low stability and persistence. As for other pharmaceuticals, it has been found that the concentrations of antibiotics measured in different countries are in the same range of concentrations in the different compartments such as sewage and surface water, respectively [28]. Xu *et al*.[29] showed the antibiotics concentrations were in the higher l g-per-litre range in hospital effluent, in the lower l g-per-litre range in municipal waste water, and in the higher and lower l g-per-litre range in different surface waters, ground water and sea water in a harbor if found at all in the latter.

Wang *et al*. [30] showed that cephalosporin antibiotics were identified in the influent and effluent of a local WWTP at $ng.L^{-1}$-$\mu g.L^{-1}$ level.

Huang *et al.*[18] showed that β -Lactam are least likely to persist in the environment but served indicators for antibiotic contamination. Furthermore, the antibiotic use patterns change rapidly and new compounds are being developed and introduced continually and lead to a more rapid development of bacterial resistance toward these drugs and their risks for human health if they are present in water and that means the pseudo-persistent contamination of cephalosporin antibiotics in the water environment could not be neglected.

Identification of Bacteria

This study was conducted during the period of January, 2012 to December, 2012. There was a variation in bacterial population, eleven frequently bacterial isolates were identified and characterized (table-1 and table-2) and these include *E.coli* (100%), *Acinetobacter lowffii* (41.6%), *Klebsiella pneumoniae* (33.3%), *Moraxilla* spp. (25%), *Salmonella typhi* (25%), *Citrbtobacter freundii* (25%), *Vibrio fluvials* (16.7%), *Acinetobacter haemolyticus* (16.7%), *Weeksella zoohelcum* (16.7%), *Pasteurella multicida* (8.3%), *Pseudomonas aeroginosa* (8.3%).

Isolated bacteria	***E. coli***	***Vibrio fluvialis***	***Salmonella Typhi***	***Moraxilla* spp.**	***Acinetobacter lowffii***	***Acinetobacter haemolyticus***	***Weeksella zoohelcum***	***Pasteurella multocida***	***Klebsiella pneumoniae***	***Citrbtobacter freundii***	***Pseudomonas aeroginosa***
%	100	16.7	25	25	41.6	16.7	16.7	8.3	33.3	25	8.3

Table 1- Results of biochemical tests for isolated bacteria

Reaction		***E. coli***	***Vibrio fluvialis***	***Salmonella Typhi***	***Moraxilla* spp.**	***Acinetobacter lowffii***	***Acinetobacter haemolyticus***	***Weeksella zoohelcum***	***Pasteurella multocida***	***Klebsiella pneumoniae***	***Citrbtobacter freundii***	***Pseudomonas aeroginosa***
Oxidase		-	+	-	+	-	-	+	+	-	-	+
Nitrate		+	+	+	+	-	-	-	+	-	+	+
Urea hydrolysis		-	-	-	+	+	-	+	-	+	+	+
Motility		+	+	+	-	-	-	-	-	-	+	+
H_2S Production		-	+	+	-	-	-	-	-	-	+	-
Indole		+	-	-	-	-	-	+	+	-	+	-
Sugar fermentation	Slant (Glucose fermentation)	+	+	+	-	+	-	-	+	+	+	+
	Butt (Sucrose or lactose fermentation)	+	+	+	-	-	-	-	+	+	+	-
	Gas	+	-	+	-	-	-	-	-	+	-	-
MR		+	+	+	+	-	-	-	-	-	+	-
VP		-	-	-	-	-	-	-	-	+	-	-
Citrate, Simmons		-	+	+	+	-	+	-	-	+	+	+
Catalase		+	-	+	+	+	+	+	+	+	+	+
Gram stain		-	-	-	-	-	-	-	-	-	-	-

Table 2- Results of identification by Microgen test kit

		Identified Microorganisms											
	Reaction	Well Number	***E.coli***	***Vibrio fluvialis***	***Salmonella Typhi***	***Moraxilla* spp.**	***Acinetobacter lowffii***	***Acinetobacter haemolyticus***	***Weeksella zoohelcum***	***Pasteurella multicida***	***Klebsiella pneumoniae***	***Citrbtobacter freundii***	***Pseudomonas aeroginosa***
	Oxidase		-	+	-	+	-	-	+	+	-	-	+
	Motility		+	+	+	-	-	-	-	-	-	+	+
	Nitrate		+	+	+	+	-	-	-	+	-	+	+
GN A wells	Lysine	1	+	-	+	+	+	+	-	-	+	-	+
	Ornithine	2	+	-	-	+	-	-	-	+	-	-	-
	H_2S	3	-	+	+	-	-	-	-	-	-	+	-
	Glucose	4	+	+	+	-	+	-	-	+	+	+	+
	Mannitol	5	+	+	+	-	-	-	-	+	+	+	+
	Xylose	6	+	-	+	-	-	-	-	+	+	+	+
	ONPG	7	+	+	-	-	-	-	-	+	+	+	-
	Indole	8	+	-	-	-	-	-	+	+	-	+	-
	Urease	9	-	-	-	+	+	-	+	-	+	+	+
	V.P.	10	-	-	-	-	-	-	-	-	+	-	-
	Citrate	11	-	+	-	+	-	+	-	-	+	+	+
	TDA	12	-	-	-	-	-	-	-	-	-	-	-
GN B wells	Gelatin	13	-	+	-	+	-	-	-	-	-	-	+
	Malonate	14	-	-	-	-	-	-	-	-	+	+	-
	Inositol	15	+	-	-	-	-	-	-	-	+	-	-
	Sorbitol	16	+	-	+	-	-	-	-	+	+	+	-
	Rhamnose	17	+	-	-	-	-	-	-	-	+	+	-
	Sucrose	18	+	+	-	-	-	-	-	+	+	+	-
	Lactose	19	+	-	+	-	-	-	-	+	+	+	-
	Arabinose	20	-	+	+	-	-	-	-	-	+	+	+
	Adonitol	21	-	-	-	-	-	-	-	-	+	-	-
	Raffinose	22	+	-	-	-	-	-	-	-	+	+	-
	Salicin	23	+	+	-	-	-	-	-	-	+	-	-
	Arginine	24	+	+	+	-	+	+	-	-	-	+	+

The present work showed the various effects of hospital wastewater on the bacteriological parameters on the receiving environment. The hospital wastewater was observed to play a significant role in the influence on the qualities of the parameters studies. The introduction of wastewater in the environment brings about increased amount of organic matter and essential nutrient, which influence the changes in the microflora [41]. Aluyi *et al*. [42] noted that the high counts of bacterial load reflected the level of pollution in the environment that is an indication of the amount of organic matter present. When evaluating the effects of hospital wastes on microbial communities, it is important to note that, target organisms vary between hospital wastes. Indigenous communities of bacterial populations are very complex and they have the important task of cycling nutrients [43].

Antibiotic susceptibility test

The in vitro activities of 10 different antibiotics against the isolated *E. coli* were illustrated in table-3. A high resistance rate was observed against ampicillin (80%) followed by amoxicillin/clavulanate (77%), cefotaxime (60%), pipercillin (58 %), ceftazidime (46 %), azetreonam (34%), imipenem (31%), cefepime (26%). The lowest resistance was to pipercillin/ tazobactam (22%).

Table 3- Antibiogram of 10 different antibiotics against the isolated *E. coli* from the studied hospitals

Antibiotics	Susceptibility percentage%		
	Resistance%	Intermediate%	Sensitive%
Pipercillin	58	8	34
Amoxicillin/Clavulanate	77	16	7
Cefotaxime	60	17	23
Meropenem	28	0	72
Imipenem	31	3	66
Ampicillin	80	4	16
Azetreonam	34	23	34
Ceftazidime	46	13	41
Pipercillin/Tazobactam	22	6	72
Cefepime	26	15	59

A high proportion of the isolated *E. coli* from the three hospitals was revealed resistance to more than one antibiotic. The isolates were resistant to one antibiotic (6.9%), resistance to two (16.2%), resistance to three (5.8%) , resistance to four (10.4%) resistance to five (18.6%) , resistance to six (12.7%), resistance to seven (6.7%) , resistance to eight(3.4%), resistance to nine (2.3%), resistance to ten (3.4%) and these resistance pattern of multidrug resistant bacteria (MRD) to 3 or more antibiotics were illustrated in table-4.

Table 4- Multiple resistance pattern of the *E. coli* isolates

Resistance	*E. coli* N=86	
	No.	%
Not resistant	11	12.7
To one antibiotic	6	6.9
To two antibiotics	14	16.2
To three antibiotics	5	5.8
To four antibiotics	9	10.4
more than 4	41	47.6

The results indicated a high incidence of antibiotic resistance among all *E. coli* isolates. The same result was reported in previous study in Al-Shifa hospital wastewater in Gaza, Palestine [31]. The highest percentage (more than 50%) of multiple drug resistant bacteria was found to be even greater than that of a similar studies carried out in India [32].

It was showed that the hospital wastewater could increase the number of resistant bacteria in the recipient sewer by both mechanisms of introduction and selection for resistant bacteria [33]. A study carried out in Bangladesh found out that the resistance development was directly related to the use of antibiotics [34]. A study in Nepal showed such higher MDR percentage could be attributed to the absent or nonfunctioning operating status of the treatment plant in addition to excessive use of antimicrobial to treat higher number of patients in these hospitals resulting in increased selective pressure for the bacteria, that was supported by the fact of the significantly higher number of MDR bacteria in the hospitals with treatment plants than those of not having such plants and the possible explanation for this difference is that bacteria are not killed, get enough time to mix, proliferate and drug resistant plasmids from MDR bacteria get transferred to otherwise sensitive bacterial population inside the effluents [35]. In a study carried out in Madrid, Spain, the spread of *E. coli* strains with high level of Cefotaxime and Ceftazidime resistance between community, long term care facilities and hospital institutions showed that epidemic or occasional isolates of ceftazidime and cefotaxime resistant *E. coli* can spread between distinct health facilities including hospital, community health centers and long term care centers [36].

Antimicrobial –resistant bacteria may be discharged into the environment from human sources (hospitals and municipal effluent) and agricultural sources and there is considerable potential for dissemination of antimicrobial-resistant organisms and resistant determinations from such sources through contamination of food and water [37, 38].

The origin of such MDR bacterial strains appears to be the hospital environment and the selective pressure responsible for expanding such bacterial populations in hospitals must have been through the use of drugs in humans and not from their use in the veterinary and agriculture field [39].

Patchanee *et al*. [40] attributed the increase of antibiotic resistant isolated *E. coli* in the hospitals effluents to 1) selection of antibiotic resistant strains originated from the effluent in presence of the antibiotics. 2) Genetic mutation which makes them resistant to the antibiotics. 3) Horizontal transfer of antibiotic resistance genes from other bacteria existing in the effluents.

Conclusions

From the present investigation we can conclude that the release of wastewater from the hospitals under study was associated with an increase in the prevalence of antibiotic resistance, and hospitals liquid wastes were laden with MDR bacteria and seemed to pose a huge public health threat in the transfer of such resistance to the bacterial pathogens causing community acquired infections, thereby limiting our antibiotic pool.

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