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GENETIC SITE DETERMINATION OF ANTIBIOTIC RESISTANCE GENES IN ESCHERICHIA COLI ISOLATED FROM DIFFERENT SOURCES OF HUMAN INFECTION

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SUMMARY: This study includes isolation of Escherichia coli *from different sources of human infections (urine, stool, burns, wounds, and cerebrospinal fluid). In addition to these, a few samples were taken from sewage water. Eighty-three isolates of* E. coli *were obtained from 264 samples. According to the resistance to antibiotics, isolates were classified into 41 groups. The isolates varied in their resistance to tested antimicrobials. Isolate E48 was resistant to all antimicrobials under study, while isolate E37 was resistant only to three antimicrobials. All isolates showed resistance of 97.59% to Chm and less sensitivity to Amk (2.40%). The transformation was conducted successfully by plasmid DNA of isolate E48 and failed by plasmid isolate E38. The results cleared that the genes responsible for resistance to Amk, Chm, Cln, Dox, Kan, Lin, Pac, Tet, Tob, and Tri were located on the plasmid DNA, while the genes responsible for resistance to Cef, Cph, Cip, Gen, Gul, Nal, Nit, Pip, and Rif were located on the chromosome. It appeared from the electrophoresis run DNA samples on gel that* E. coli *K12JM83 strain obtained two plasmids through the transformation process.*

Keywords: Escherichia coli, Antibiotic Resistance, Transformation, Gel Electophoresis.

INTRODUCTION

Since the introduction of antibiotics, there has been tremendous increase in the resistance of diverse bacterial pathogens. This shift in susceptibility greatly affects our ability to successfully treat patients empirically (1) Antibiotic resistance has long been a significant problem in the treatment of bacterial infection since several types of bacteria have been found to be able to resist more than one type of antibiotics. Resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs that are utilized as therapeutic agents. This results in the need either for higher doses of antibiotics,increasing the risk of drug toxicity, or for the consideration to change the regimen. Moreover, excessive budget is currently spent on import of antibiotics manufactured abroad. Therefore, the antibacterial activity of local medicinal plants should be studied to provide alternative and locally available antibacterial regimens (2,3).

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Plasmids are most replicating circular pieces of DNA, smaller than the bacterial genome, which encoded their transfer by replication into another bacterial strain or species. They can carry and transfer multiple resistance genes, which may be located on the section of DNA capable of transfer from one plasmid to another or to the genome—transposon or jumping gene (4).

Plasmid in *E. coli* is of the pathogenic nature and it is resistant to most antibiotics. The success of *E. coli* as common hospitalized pathogen organisms leads to many studies on this organism including their plasmids, which are responsible for the inactivation of antibiotics (5). Researchers had extracted many R plasmids that confer resistance to a number of antibiotics such as penicillin, rifampicin, and so on (6). It was found that most of these plasmids are transferable to either other bacteria or other genus, with different molecular weight.

MATERIALS AND METHODS

Bacterial isolates

The bacterial strains of *E. coli* were isolated and collected from 264 samples and from different sources of human infections (cerebrospinal fluid, urine, stool, wounds, and burns), from Hawleri Ferkari, Rezgary, Al-amal, and Raparine hospitals, from Khanzad lab, and from sewage water in Erbil city, Iraq, during July 1, 2005, to November 20, 2005, using nutrient, MacConkey, and EMB media. The isolates were identified depending on cultural, morphological, and biochemical tests (catalase, oxidase, IMViC, gelatenase, and urease), and also API20E system was performed (7–9).

Bacterial strains

The standard strain of *E. coli* K12JM83 serotype obtained from Dr Adel Kamal Khider, Salahaddin University /Erbil,-Iraq, was used in this study. The organism was maintained on nutrient agar slants at 4°C. The bacterial isolates were preserved at −20°C after suspension in 20% (V/V) glycerol (10).

Determination of antibiotic susceptibility

The antimicrobial resistance phenotypes of all isolates were determined using dilution method in agar plate (7). The final concentration of antibiotics (Amikacin (Amk), Canamycin (Kan), Cifixime (Cef), Cefalothin (Cph), Clindamycin (Cln), Doxicycllin (Dox), Gentamycin (Gm), Gulmentin (Gul), Lincomycin (Lin), Trimethoprim (Tri), Tetracycline (Tet), Tobramycin (Tob), Pipracillin (Pip), Pan-cloxacin (Pac), Chloramphenicol (Chm), Nitrofuration (Nit), Ciprofloxacin (Cip), Nalidixic acid (Nal), and Rifampicin (Rif)) was added to the medium after sterilization and cooled to 50°C. The media were mixed and poured into Petri-dishes. After solidification, the plates were inoculated with isolates using streaking method, and then incubated at 37°C for 24 hours. The results were recorded the next day.

Plasmid preparation

This method, used for the screening of a large number of transformants, was described by Birnboim and Doly and Kochonic *et al.* (11,12). A 105 mL aliquot of a bacterial culture grown in selective media was pelleted at 4000 x g. The pellet was suspended by vortexing in 200 μL of solution No. 1, and then left for 10 min at room temperature. Then 200 μL of solution No. 2 was added and the tube was mixed by inversion and placed on ice for 5 min. Then 200 μL of solution No. 3 was added and the tube was mixed by inversion and placed on ice for 10 min. The supernatant was placed into fresh tube and 400 μL of phenol–chloroform–isoamyl alcohol (25:24:1) was added and mixed. After centrifugation at 13,000 x g for 5 min, the aqueous layer was removed to fresh tube and an equal volume of isopropanol was added. After 10 min at room temperature, the tube was centrifuged for 10 min at 13,000 x g to pellet the plasmid DNA. The pellet was washed with 70% ethanol, dried briefly in a vacuum dryer, and suspended in 50 μL of TE buffer. Plasmid prepared was stored at 4°C.

Preparation of competent cells

A modified method of Sambrook *et al.* (13) was used for the preparation of competent cells for transforming *E. coli* JM83 cells. An overnight culture of *E. coli* was prepared by suspending a colony from a fresh nutrient agar plate in 100 ml nutrient broth. The culture

Table 1: Distribution of E. coli isolates according to the source of isolations.

Source of Isolation	Isolates number	No. of bacterial isolates
Body fluid	66	1
Burns	48	1
Cerebrospinal fluid	1	1
Sewage Water	4, 32	2
Urine	2, 6, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 27, 28, 29, 31, 33, 34, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 49, 50, 51, 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77, 81, 82, 83	60
Stool	5, 8, 9, 10, 25, 26, 78, 79, 80	9
Wound	3, 7, 17, 30, 35, 36, 53, 65, 75 9	
Total	**83**	**83**

was incubated in a shaking incubator at 100 rpm at 37°C for 24 h. A total of 10 ml of this culture was then suspended in 90 ml fresh nutrient broth and was allowed to grow for 90 min to an OD 600 nm of approximately 0.3<Author: Check the intended meaning.>. A total of 10 ml aliquots were centrifuged at 4000 x g for 10 min to pellet the cells. The supernatant was discarded and the cells were washed in 5 ml ice-cold 10 mM NaCl and recentrifuged. The supernatant was discarded and cells were suspended in $CaCl_2$ and recenterifuged twice. Following a third washing with 5 ml 30 mM $CaCl_2$, cells were then recenterfuged and resuspended in 1 ml of ice-cold 30 mM $CaCl_2$ + 15% glycerol. The cells could be used immediately for transformation or stored at −20°C.

Transformation

A total of 200 μL of *E. coli* competent cells were mixed with 2 μL of extracted plasmid, and the tubes were left on ice for 30 min. After that the tubes were incubated for 30 sec at 42°C in a water bath and then placed on ice for 5 min. To the tubes was added 800 μl of nutrient broth followed by their incubation at 37°C. The cells were then centrifuged at 6000 x g for 1 min; the pellet was resuspended in 200 μL nutrient broth. A total of 100 μl of the cells were plated out on nutrient agar containing appropriate antibiotic markers. Post-incubation, the plates were screened for recombination, which were selected and streaked onto fresh agar plates for plasmid purification (14).

Agarose gel preparation

Agarose gel (0.8%) was prepared in 100 ml 1 x TE buffer and boiled. This was allowed to cool to 50°C, and 2 μl (10 mg/ml) of ethidium bromide was added to it. The gel was poured and a comb was inserted to make wells. When the gel was set, the comb was removed and the gel was placed in the gel box and immersed in 1 x TAE buffer. Samples were prepared by taking 10 μl of DNA and 2 μL of loading dye. The gel was run for 90 min at 75 Volt, and was viewed and photographed (14).

RESULTS

It was of interest to study the clinical isolates of *E. coli* that were obtained from different hospitals in Erbil city, Iraq. Out of 264 specimens, 83 *E. coli* were isolated (Table 1). Preliminary identification tests were performed on all isolates (Gram stain, oxidase, catalase and motility). The isolates were identified using a variety of techniques, which included morphological characteristics and, biochemical tests; the API 20E system was also performed. The cells and verity of biochemical tests of the isolates indicated that all isolates

Table 2: Resistance of E. coli isolates to antimicrobials.

Antimicrobials	Symbol	No. of resistant isolate	Resistant %
Amikacin	Amk	2	2.40
Cifixime	Cef	30	36.14
Cephalothin	Cph	51	61.44
Chloramphenicol	Chm	81	97.59
Ciprofloxacin	Cip	22	26.50
Clindamycin	Cln	80	96.38
Doxicillin	Dox	37	44.57
Gentamycin	Gen	41	49.39
Glumentin	Gul	21	25.30
Kanamycin	Kan	27	32.53
Lincomycin	Lin	76	95.18
Nalidixic acid	Nal	41	49.39
Nitrofurantion	Nit	76	91.56
Pan-cloxacillin	Pac	24	28.91
Pipracillin	Pip	56	67.46
Rifampcin	Rif	3	3.61
Tetracyclin	Tet	57	68.67
Tobramycin	Tob	7	8.43
Trimethoprim	Tri	49	59.03

were identified as being Gram negative, non-pore forming, motile rods. The isolates were all oxidase negative, and catalase, tryptophanase, methyl red positive. The isolates were lactose and glucose fermenter, producing acid and gas, but were negative for citrate utilization, vogas proskawer, gelatenase liquefaction, H_2S production, and urease production (7).

Eighty-three *E. coli* isolates were screened for their resistance to 19 widely used antibiotics in medicine. Table 2 illustrates that all isolates varied in their response to the use of the antimicrobials agents. It also shows that all of the examined isolates were resistant at least to three antibiotics, and the highest percent was 100% for one isolate that was resistant to all the tested antibiotics, in contrast with the lowest percent 2.40% for 5 isolates. According to the susceptibility results the bacterial isolates were grouped to 41 antibiogram groups (Table 3).

To determine if the antibiotic resistance in isolated *E. coli* is encoded by plasmid DNA or chromosomal DNA, the transformation process was performed to the most resistant isolate of *E. coli* (E48) in group No. 1 and more sensitive isolate (E38) in group No. 41 and *E. coli* K12JM83 strain. The extracted plasmid DNA from *E. coli* E48 transferred successfully to *E. coli* K12JM83 strain, while E38 isolate plasmids failed to enter the host by transformation process after repeating the process several times. The results revealed that the genes responsible for resistance in isolate E48 to Amk, Chm, Cln, Dox, Kan, Lin, Pan, Tet, Tob and Tri are located on plasmid DNA, and others are located on chromosomal DNA.

The number of transformant colonies (Table 4) varied when grew on plates supplemented with different antibiotics. A total of 245 transformant colonies were obtained for E48 isolate. The number of these colonies decreased to 47 to 100 when subcultured on plates supplemented with different antibiotics, with the transformation frequency of 1×10^{-9}.

DISCUSSION

E. coli is an opportunistic pathogen that causes human infection; it can be isolated from different environments (Table 1) depending on cultural, morphological, and biochemical tests. The synonyms number obtained for tested samples by API 20E system ranged 1164552–7574552, which indicates that all were *E. coli* isolates. Isolated bacteria exhibited high resistance to the most widely used antibiotics; 81 isolates (Table 2) were resistant to Chm and 80 isolates to Cln. All isolates showed resistance to more than three antibiotics, and this phenomenon is called multiple drug resistant (MDR). MDR *E. coli* has been previously reported (15–17). In this study all 81 isolates (97.59%) were resistant to Chm and 80 isolates (96.38%) were resistant to Cln (Table 2). Ineffectiveness of these antibiotics against *E. coli* has been reported (18–21).

The results of the transformation process (Table 4) help us conclude that the genes responsible for antibiotic resistance to Amk, Chm, Cln, Dox, Kan, Lin, Pan, Tet, Tob and Tri in *E. coli* E48 isolate are not chromosomally coded; this increases the possibility of distribution of resistance for these antibiotics among pathogenic bacteria by transformation, conjugation, and transduction (22)

Table 3: Antibiogram groups and percent of resistance to antimicrobials.

Antibiogram groups	No. of isalates	% of Resistance	Antimicrobials at final concentrations																		
			Amk	Cef	Cph	Chm	Cip	Cln	Dox	Gen	Gul	Kan	Lin	Nal	Nit	Pac	Pip	Rif	Tet	Tob	Tri
1	1	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	1	89.47	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
3	2	89.47	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
4	2	84.31	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
5	1	73.68	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+
6	1	73.68	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	+
7	1	73.68	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+
8	2	73.68	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+
9	4	73.68	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	+
10	1	68.42	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+
11	1	68.42	-	-	+	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+
12	7	68.42	-	-	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-	+
13	1	63.15	-	+	+	+	-	+	+	+	-	-	+	+	+	-	+	-	+	-	+
14	1	63.15	-	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+
15	1	63.15	-	-	+	+	-	+	+	+	-	+	-	+	+	+	+	-	+	-	+
16	1	63.15	-	+	+	+	+	+	-	+	-	+	+	+	+	-	+	-	-	-	+
17	6	63.15	-	+	+	+	-	+	+	+	-	-	+	+	+	-	+	-	+	-	+
18	1	57.89	-	-	+	+	-	+	+	+	-	+	+	-	+	-	+	-	+	-	+
19	1	57.89	-	-	+	+	-	+	+	+	-	-	+	+	+	-	+	-	+	-	+
20	1	57.89	-	+	+	+	+	+	-	-	-	+	+	+	-	-	+	-	+	-	+
21	1	57.89	-	+	+	+	+	-	-	+	+	-	+	-	+	-	+	-	+	-	+
22	1	52.63	-	-	+	+	-	+	+	-	+	-	+	-	+	-	+	-	+	-	+
23	1	52.63	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+
24	1	47.36	-	-	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+
25	1	47.36	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+	-	+	-	-
26	1	42.10	-	+	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+
27	1	42.10	-	+	-	+	-	+	-	-	+	-	+	+	+	-	+	-	-	-	-
28	1	42.10	-	-	+	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
29	2	42.10	-	-	+	+	-	+	+	-	-	-	+	-	+	-	+	-	+	-	-
30	1	36.84	-	-	-	+	-	+	-	-	+	-	+	-	+	+	+	-	-	-	-
31	2	36.84	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-
32	2	36.84	-	+	-	+	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+
33	4	36.84	-	-	+	+	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-
34	2	31.57	-	-	+	+	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+
35	2	31.57	-	-	-	+	-	+	-	-	+	-	+	-	+	-	+	-	-	-	-
36	1	26.31	-	-	-	+	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+
37	1	26.31	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+
38	1	26.31	-	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
39	15	26.31	-	-	-	+	-	+	-	-	-	-	+	-	+	-	-	-	+	-	-
40	1	15.78	-	-	-	+	-	+	-	-	-	-	+	-	+	-	+	-	-	-	+
41	4	15.87	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
E. coli JM 83			-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+

+: Resistant, -: Sensitive

Table 4: Number of transformant colonies and transformation frequency of *E.coli* K 12 JM83.

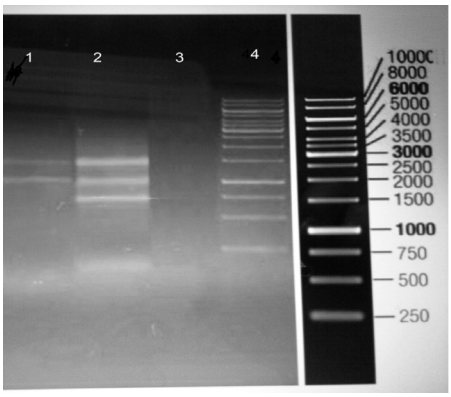
Isolate	No. of transformat colonies	No. of colonies grow on nutrient agar containing antimicrobials in μg/ml																			Trans-forma-tion
		Amk	Cef	Cph	Chm	Cip	Cln	Dox	Gen	Gul	Kan	Lin	Nal	Nit	Pac	Pip	Rif	Tet	Tob	Tri	
48	245	47	S	S	93	S	93	87	S	S	100	77	S	S	77	S	S	87	93	87	$1*10^{-9}$
38	Did not success after repeating the process several times.																				

S: Sensitivity of bacterial isolate to antimicrobials.

than the other tested antibiotic resistance genes that are chromosomally coded. The number of purified transformant colonies produced by the plasmid DNA of the tested isolates decreased than the number they started from; such as for E48 isolate, the number of transformant colonies obtained was 245; however, the number of colonies obtained after subculturing on agar plates containing tested antibiotics was lesser (Table 4). This means that the antibiotic-resistant genes are located on different fragments of R-plasmid (4); one of these fragments is called RTF (resistance transfer factor), as it comprises all the genes necessary for conjugation and is of large size. Other fragment is called the r-determinant. It contains all the genes conferring antibiotic resistance except the tetracycline-resistance determinant that is on RTF (23–25). These two fragments of R-plasmid may enter the JM83 host with different efficiency because of their variation in size (5). In addition, irregular segregation of plasmid DNA species may result difference in the transformant colonies for other antibiotics. The transformant colonies failed to grow on nutrient agar supplemented with Cph, Cip, Gen, Gul, Nal, Nit, Pip, and Rif. It can be inferred from this finding that the genes which are responsible for resistance to these antibiotics either are located on the chromosomal DNA or on the large plasmid that cannot enter the *E. coli* k12JM83 strain acting as a host. From the findings of this study, it is evident that there were relatively high incidences of MDR *E. coli* in Erbil city, and relatively low effects of the tested antibiotics on these MDR bacteria. This has been attributed to the ability of these organisms to produce resistance mechanisms; for this the characterization of plasmid DNA for *E. coli* isolates indicated that this isolate transfer to another bacteria, in present study to *E. coli*.

K12JM83 strain (plasmid-less strain Figure 1 lane 3) was obtained from two plasmids through the transformation process (Figure 1 lane 1). Moreover the antibiotic resistance genes can be distributed naturally among bacteria through various reasons: conjugation and transduction process (26), poor quality of antibiotics available in the city, lesser concentration of antibiotics incorporated, and antibiotics are available to the public. These factors could enhance the high incidence rate of resistant pathogenic bacteria in hospitals; moreover, the antibiotics are often used in the case of animals and the antibiotics are sold over the counter without a prescription that compounds the problem. Misuse and overuse of antibiotics by doctors as well as patients should not be neglected. Therefore, our study recommends that more restrictions should be imposed on the irrational use of antibiotics, and public awareness activities should be undertaken to alert the public to the risks of the unnecessary use of antibiotics.

Figure 1: Plasmid profile of E. coli E48 isolate and transformant cells.



Lane 1: Transformant cells (E. coli K12 JM83). Lane 2: E. coli E48 isolate. Lane 3: E. coli K12 JM83 (plasmid-less strain). Lane 4: DNA marker 10000 bp.

CONCLUSION

According to the results of antibiotic sensitivity test, it was concluded that some isolates of *E. coli* were the most resistant bacteria, resisted to all used antimicrobials under study, and the genes that were responsible for (Amk, Chm, Cln, Dox, Kan, Lin, Pan, Tet, Tob, and Tri) resistance in *E. coli* were located on plasmid DNA, while the others were located on chromosomal DNA. These resistance genes were successfully transferred to laboratory *E. coli* K12 JM83 strain through the transformation process, and documented by gel electrophoresis.

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