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Antibiotic Resistance Pattern and Detection of Some ESBL Genes amongDiarrheagenic *E. coli* O157:H7

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Abstract

During the period of 20th of May to1st of November 2013, twenty five isolatesof *E. coli* O157:H7 were identified among 200 samples taken from children under ten years old suffer from diarrhea (68 males and 132 females) admitted to Rapareen Pediatric hospitals in Erbil City. Isolates were identified from stool sample by using cultural, morphological, biochemical characteristics and serological test. Antibiotic sensitivity testing was done for all isolates by using fourteen antibiotic types The resistance rate of the isolates toward these antibiotics were 100 % for AMP, 96% for MET, 76% for CTX, 72% for TE and KF, 68% for TMP, 56% for FEP, 52% for C and SXT, 48% for CRO, 28% for AK, 20% for CIP, while the lowest percent 16% was for CN, and all isolates were sensitive for IPM. Extended spectrum ß-lactamase (ESBL) production was done for all isolates .Out of 25 *E. coli* O157:H7isolates, 8(32%) were ESBL producers, while17(68%) were non ESBL producers. The plasmid profile of 25 isolates conducted by using gel electrophoresis, the results revealed different bands with molecular weight ranging between (2kbp- more than 10kbp) in size. The transformation process conducted successfully for (E1 and E15) and the results showed that the resistance genes for ampicillin, Cephalothin, Cefepime, metronidazole and trimethoprimwere located on plasmid DNA for E1 isolate, while for E15 isolate, the ampicillin, Cephalothin, Cefepime, ceftriaxone, Cefotaxime, metronidazole and trimethoprimlocated on plasmid. The results of transformation confirmed by gel electrophoresis, and showed that one plasmids of E1 and two plasmid E15 had been transformed successfully with molecular weight of more than 10 Kbp. All *E. coli* O157:H7isolates were screened for the presence of ESBL genes including (*bla*CTX-M - *bla*SHV-and *bla*TEM) on plasmid DNA using polymerase chain reaction (PCR) assay. Results showed that all isolates were positive for the existence of (*bla*SHV-and *bla*TEM), while 88% of the isolates contained(*bla*CTX-M) .

Introduction

Infection diarrhea is one of world's causes of morbidity and mortality, resulting in about two million deaths per year. The majority of cases of serious diarrhea occur among children in developing countries ***[1]***. In children , diarrhea caused by certain strain of *E. coli* may lead to destruction of red blood *c*ells and kidney failure (hemolytic-uremic syndrome) ***[2]***.*Escherichia coli* O157:H7 is an emerging public health concern in most countries of the world ***[3]***. Complications related to infection include diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome ***[4]***.*E. coli 0157:H7* produced very elevated levels of a Shiga-like cytotoxin (also called Vero cell toxin), in addition to Shiga-like toxin as virulence factor for *E.coli* 0157:H7 strains ***[5]***. Themajor problem in the world now "antibiotics resistance" , so the medicine manufactures which yearly produced new generations of antibiotic to solute this problem , specially there are some bacteria considereda multiple antibiotic resistance as *Escherichia coli* ,*Klebsiella* and others

[6,7]. Plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera ***[8]***. The location of virulence factors on genetic mobile elements may facilitate the spread of virulence properties within bacterial communities ***[9]***. The risk of *E. coli* O157:H7occurs in its plasmid, because of its ability to transfer plasmids to other bacteria or other strains of *E. coli* by transformation, conjugation and transduction processes***[10]***.Determination of ESBL genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections ***[11]***. At present, there are more than 300 different ESBL variants. *TEM* (Temoniera) and *SHV* (sulphydryl variable) were the major types. However, *CTX-M* type (predominantly hydrolyze Cefotaxime) is increasingly becoming important ***[12]***.For this reasons this study concerned with the isolation and identification of *E.coli* 0157:H7 from children suffering from diarrhea,study the antimicrobial resistance patterns of the isolated *E.coli* 0157:H7 to different antibiotics,determination of the frequency of ESBLs production among the *E.coli* 0157:H7 isolates, characterization of the plasmid DNA profile pattern of two isolates using gel electrophoresis technique, determination of the site of genes encoding resistance to antibiotics of the most resistant isolates by performing genetic transformation, and determination of the presence of some ESBLs genes using polymerase chain reaction (PCR) assay.

Materials and methods

A. Specimens collection

The samples were collected during the period 20st May 2013 to 1th November 2013. A total of two hundred (200) stool specimens were collected from infants and children, aged one day- 10 years attending Rapareen Teaching Hospital for Children in Erbil City and the relevant information were recorded from each patient including age and sex . All stool samples were collected in clean disposable plastic containers from diarrheal patient**.** The specimens were transferred to the laboratory and processed within half an hour of collection.

B. Biochemical tests

The biochemical tests of suspected bacteria that are isolated from the different specimens, was detected according to H2S production, catalase, oxidase, urea hydrolysis and motility test ***[13]***.

C. Antibiotic susceptibility test

Disc diffusion method, also known as the Kirby- Bauer-method was carried out according to the Clinical and Laboratory Standard institute guidelines [CLSI , formerly the National Committee for Clinical Laboratory Standards (NCCLS)] ***[14]***.

D. Detection of Extended spectrum beta lactamase (ESBLs) activity

The ESBLs activity was investigated by double disc synergy method; the tested inoculums (adjusted to 0.5 McFarland turbidity) were spread onto Muller-Hinton agar using sterile cotton swabs. A disc of Augmentin AMC was placed on Muller-Hinton agar, then discs of CTX, CAZ and AT was kept around it, at distance ranging between 16 and 20 mm from the Augmentin disc (center to center). The plate was incubated at 37°C overnight and the results recorded***[15]***.

E. Plasmid DNA Extraction

Laboratory Protocol

Plasmid DNA was extracted and purified from 5ml overnight culture of the selected isolates of the *E.coli* O157:H7 grown in LB broth medium containing 100µg/ml Ampicillin(Dar-Alhikma,Jordan) using a plasmid DNA purification kit, according to the manufacturer's instructions(Geneaid,Taiwan).

Plasmid profile

The extracted plasmids were electrophoresed in 0.7% Agarose gel with Tris-borate ethylene diamine tetra-acetic acid (TBE), Agarose gel electrophoresis was used to separate DNA fragments according to size ***[16]***.

Determination of location of genes conferring antibiotic resistance

Determining the location of the antibiotic resistant gene in *E.coli* carried out by isolation of the plasmid DNA content from the bacterial isolates, then transformed into standard strains, which used as bacterial hosts for DNA uptake. Transformation process includes:

Preparation of competent cells

To make the cured cultures competent, 5 ml of LB broth was inoculated with a single colony of *E. coli* DH5α (whose plasmid is manipulated genetically, plasmidless), incubated with shaking (100 rpm) for 24 hours at 37° C,then 1 ml of bacterial culture was added to 50 ml LB broth, incubated with shaking at 37°C for 3-4 hrs. The bacterial growth was monitored by measuring the optical density (OD_{650}) with spectrophotometer. Logarithmic-phase cultures of *E. coli* typically have an (OD_{650} of 0.5–0.7). The cells were harvested by centrifugation at 5,000 rpm for 10 min, the supernatant discarded, and then resuspended. One ml of ice-cooled 0.1 M $CaCl_2$ added to thepellet, and then 39 ml of the same solution was added. The resuspended cells left on ice for 30 min, centrifuged for 10 minutes at same velocity, the supernatant discarded, and the bacterial pellet resuspended in 2.5 ml of ice-cold $CaCl_2$.

Transformation of Competent Cells

Ten µl of prepared plasmid DNA from *E. coli O157* isolates plated on LB agar plates and incubated at 37°Cfor 24 hours to ensure that they are not contaminated with the bacterial cells. Two-hundred µl of bacterial suspension transferred into a sterile eppendorf tube containing 5µl of plasmid DNA and mixed gently. For each set of transformations, negative controls prepared that consist of competent cells without DNA were plated on LB agar plate with AMP. The transformation mixture placed on ice for 30 min, and then placed in water bath at 42°C for 90 second without shack the tubes. After heat shock, the transformation mixture was put on ice for 5 min. The transformation mixture transferred to a suitably sized tube containing one and a half ml of LB broth, and incubated with shaking at 37°C for 1 hour. Following incubation, the mixture aseptically transferred to eppendorf tube and centrifuged at 14,000 rpm for 1min. Then, the supernatant discarded, and the pellet resuspended in small amount of remaining supernatant. Finally, the resuspended pellet spread on selective LB agar plates. All the plates were incubated at 37 ^{o} C for 24 hours ***[17]***.

F. Polymerase chain reaction assay (PCR) for detection of ESBLs genes

The reagent of master mix is an optimized ready to use 2 × PCR mixtures of Taq DNA polymerase, PCR buffer, dNTPs, gel loading dyes and Novel Green.It contains the fluorescence dye, which is directly detected on the blue-light transilluminator or UV epi-illuminator after the DNA electrophoresis. Master Mix contains all components for PCR, except DNA template and primer.

G. Protocol of PCR technique

Plasmid DNA extracts was used as a template in the PCR technique. PCR was performed in a 25µl of reaction volume as Master Mix22 µl, Forward Primer 1 µl, Reverse Primer 1 µl, Template DNA 1 µl.

H. PCR Technique Procedure

PCR was used to detect theTEM (Temoniera), SHV (sulphydrylvariable) and CTX-M type (predominantly hydrolyze Cefotaxime) gene in the plasmid DNA of the *E. coli* O157:H7 isolates.The forward and reverse primer (Designed for this study) used was as follow:TEM 1(Forward)

5'TGATAACACTGCGGCCAACT '3, TEM 1(Reverse) 5'TGACTCCCCGTCGTGTAGAT'3. CTX-M(Forward) 5'AAGCACGTCAATGGGACGAT'3, CTX-M (Reverse) 5'TATCCCCCACAACCCAGGAA'3. SHV-1 (Forward) 5'TACCGGATTGTCATACCGT'3, SHV1(Reverse) 5'AATTCTTCCCTCAGTAGAAG'3 (CinaGencompany).

I. Temperature cycling program

DNA amplification was carried out for 35 cycles in thermal cycler for *bla* CTX-M as following:Initial denaturation at 94 °C for 5 minute (1 of cycle).The total number of cycles was 35 of the following steps:Denaturation at 94°C for 50 sec Annealing at 50°C for 40 sec,Extension at 72°C for 1 min ***[18]***. DNA amplification was carried out for 30 cycle in thermal cycler for *bla* TEM as following:Initial denaturation at 95°C for 4 minute (1of cycle).The total number of cycles were 30 of the following steps:Denaturation at 95°C for 1 minute.Annealing at 48°C for 1 minute.Extension at 72°C for 1 min .Final extension at 72 °C for 10 min. DNA amplification was carried out for 30 cycle in thermal cycler for *bla* SHVas following:Initial denaturation at 95°C for 4 minute (1of cycle).The total number of cycles were 30 of the following steps:Denaturation at 95°C for 1 minute.Annealing at 60°C for 1 minute.Extension at 72°C for 1 min .Final extension at 72 °C for 10 min***[19]***.About 5 µl of PCR product were analyzed on 1.2% Agarose gel containing ethidium bromide, the DNA was then visualized on UV transilluminator and photographed.The amplicon (PCR product) generated from*E. coli O157:H7* gene bythis PCR method was DNA fragment of (369bp for *bla* CTX-M, 410 bp for *bla* TEM and 545 bp for *bla* SHV-1) length. The molecular size of the band was verified by comparing its migration to that of a DNA molecular size marker (100 bp DNA ladder) run on the same gel.

Results and discussion

A. Identification of E. coli O157isolates

The identification of *E. coli O157* isolates performed according to the following steps:

B. Morphological identification

They produce bright metallic green sheen colonies on Eosin Methylene Blue (EMB) agar and on Sorbitol MacConkey agar colonies of*E. coli* O157:H7 appear as colorless colony due to non-sorbitol fermenting which consider as a selective media ***[13]***.

C. Smear preparation

Bacterial cells from smear preparation are gram negative short rods, motile, non-spore forming and presumptively are *Escherichia coliO157:H7* which in accordance with previous observations ***[20]***.

D. Biochemical identification

The biochemical tests for all bacterial isolates were negative for citrate, oxidase, and urease production test, but they were positive for catalase and Indole test. On Kligler's Iron Agar (KIA) medium, all isolates of*E.coli* understudy produce a yellow slant and a yellow butt A/A reaction due to the fermentation of lactose and glucose and negative for H2S production ***[19]***.

E. Serological test

Samples serotyped by application of solid agglutination test, using anti *Escherichia coli* O157:H7. Once the agglutination was detected, the reaction was considered positive according to the materials used.Three drops of physiological normal saline were added on a clean slide, and then with the aid of the loop, a small piece of fresh bacterial growth was mixed with the 3 drops in order to obtain a homogenous mixture. After that a drop of polyvalent antisera was added to the two drops (mixture) while the third drop

was considered as control test. The appearance of clear agglutination within one minute indicates a positive result.

F. Antibiotic resistance pattern of E. coli O157 isolates

Antibiotic test for 25 isolates of *E. coli O157* was done against 14 antibiotics (AK, AMP, C, CIP, CTX, KF, CN, IPM, FEP, CRO, MET, TE, TMP and SXT).

Table: 1 indicated that all the isolates of *E. coli O157* revealed different resistance rate to most antibiotics used and resistance percent was 100 % for AMP, 96% for MET, 76% for CTX, 72% for TE and KF, 68% for TMP, 56% for FEP, 52% for C and SXT, 48% for CRO, 28% for AK, 20% for CIP, while the lowest percent 16% was for CN, and all isolates were sensitive for IPM.

Table- 1: Percentages of resistance of bacterial isolates to different antibiotics

Antibiotics	***Abbreviation***	***No. of resistant isolates***	***Percentage of resistance %***
Amikacin	*AK*	*7*	*28*
Ampicillin	*AMP*	*25*	*100*
Chloramphenicol	*C*	*13*	*52*
Cephalothin	*KF*	*18*	*72*
Ciprofloxacin	*CIP*	*5*	*20*
Cefepime	*FEP*	*14*	*56*
Cefotaxime	*CTX*	*19*	*76*
Ceftriaxone	*CRO*	*12*	*48*
Gentamicin	*CN*	*4*	*16*
Imipenem	*IMP*	*0*	*0*
Metronidazole	*MET*	*24*	*96*
Tetracycline	*TE*	*18*	*72*
Trimethoprim	*TMP*	*17*	*68*
Trimethoprim + Sulphamethoxazole	*SXT*	*13*	*52*

IPM was the most effective antibiotic against isolates of *E. coli O157* and 100% of these isolates were susceptible to IPM, this resultwas agree with ***[21]***. They reported that all *E. coli* isolated from different clinical source were sensitive for IPM. The high efficiency of these antibiotics may be the usage rarely in studied area and it is expensive drug.In the present work, 68% of the isolates were resistant to TMP, this result was in agreement with ***[22]*** who stated that 52 % of *E. coli* isolates were resistant for TMP, also ***[23,24]*** found that 59.03 % and 72.2% of *E. coli* isolates were resistant to TMP.This may be result from mutational changes that lead to over productionof the bacterial dihydrofolate reductase***[25]***. High resistance pattern for AMP reported with a percentage of 100%, similar result obtained by ***[21]*** who found the resistance to AMP was 100%. This was explained by the overuse of antibiotics especially AMP in Erbil Hospitals, and also to the missuses of these antibiotic as they are prescribed without sensitivity test ***[26]***. The resistance to CIP was 20 %, this result in agreement with ***[21,27]*** recorded 25% and 17.9% respectively. This is due to that the ciprofloxacin is newly used in treatment in comparison with other antibiotics. Moreover,***[28]*** showed that increasing uses of antibiotics was associated with development of resistance against them. The clinical use of fluoroquinolones in children should be restricted because of potential cartilage damage that occurred in research with immature animals, the safety and efficacy of oral ciprofloxacin in children is understudy ***[29]***. ***[30]***Showed that the resistance in bacterial population can be spread either by transfer of bacteria between people or transfer of resistance genes between bacteria (usually

on plasmids) and by transfer of resistance genes between genetic elements within bacteria, on transposons or may chromosomally located. Susceptibility to antibiotics is changing in general and increase in antibiotic resistance has been shown worldwide. The main reason for this trend is theincrease in antibiotic consumption, the abuse of board spectrum antibiotics or self-medication. ***[31]***Showed that multi-resistance is usually related to production of ESBL that lead to inappropriate use of antibiotics and treatment failure. These variation of antibiotic resistance in *E.coli* return to the genes that located on conjugant plasmids mostly and transferred by conjugation, transformation or transduction processes, to susceptible recipient *E.coli,* or the resistance genes may be located on bacterial chromosome and jumped to resistant plasmids by transposition process, many composite transposons contain genes for antibiotic resistance, and some bear more than one resistance genes, this is found in both G-ve and G+ve bacteria ***[25]***.

G. Detection of ESBLs production in E.coli O157

All isolates of *E.coli O157* were tested for extended- spectrum β-lactamase (ESBLs) production, they were determined that 32% showed ESBLs positive as detected by double disc synergic test. The main results are illustrated in (*Table: 2*).

The results of present study demonstrated that ESBL production was 32 % this is in agreement with ***[32]*** who reported 30 % of*E.coli* isolated from clinical sources was ESBLs positive. In Egypt, ***[33]***confirmed that ESBLs production was 42 % among *E. coli* isolates using combined disc diffusion method.

Table- 2: Number and percentage of ESBL producing in *E.coli O157:H7* isolates

Production of ESBLs	***E.coli O157 isolates***	
	Number	***Percentage %***
Positive	*8*	*32*
Negative	*17*	*68*
Total	*25*	*100*

[34]Reporting the first isolation of an ESBL-producing *E.coli 0157* strain from cattle in Nigeria. ***[35]***in India reported that production of ESBL was noted in *E. coli* at a rate of 66.78 %. Similar finding have been reported by [36] who found that 71.5 % of *E.coli* isolated from clinical sources were ESBL producers.While less than 10 % of *E. coli* isolates produce ESBL in the Scandinavian countries ***[37]***. ***[38]*** found that among 194 isolates of *E. coli* 9.27% were ESBL producers.***[39]*** Mentionedthat production of ESBL was noted in *E. coli* at a rate of 58.5%. The spread of ESBL producing bacteria has become strikingly rapid worldwide, indicating that continuous monitoring systems and effective infection control measures are absolutely required ***[40]***. An ESBL resistance mechanism was mediated by the production of metallo β-lactamase, lack of antibiotic penetration due to mutations in the porins or due to the loss of certain outer membrane proteins and the efflux pumps ***[41]***.The random and over use of β-lactam antibiotics might stimulate the bacterial population to develop different defense mechanisms as production of ESBL enzymes ***[42]***.Many clinical laboratories are not fully aware of the importance of ESBL and how to detect those, laboratories may also lack the resources to control the spread of these resistance mechanisms. This lack of understanding or resources is responsible for a continuing failure to respond appropriately to prevent the rapid worldwide dissemination of pathogens possessing beta lactamases ***[43]***.

H. Plasmid profile of E.coli O157:H7

The results in (*Figure: 1*) revealed that most isolates have one band with molecular weight more than 10 kb while (E11, E13 and E21) have two bands with molecular weight more than 10 Kbp, E16, E20 and E24 have three bands but E15 which is resistant for 13 antibiotics have 5 bands with molecular weight ranging between (2kbp- to more than 10kbp) bp in size.Analysis of plasmid DNA content by Agarose gel

electrophoresis showed variation in their size and more than one DNA bands appeared in many isolates. These results revealed that among 25 *E.coli O157*isolates, the size of the bands ranged from 2Kbp to more than 10Kbp. The presence of more than one DNA band in the Agarose gel is a good indication for existence of more than one plasmid DNA species.

Plasmid isolation procedure is based on the fact that plasmids usually occur in the covalently closed circular (supercoiled) configuration within the host cells. After gentle cell lysis all intracellular macromolecules have to be eliminated whereas plasmid DNA is enriched and purified ***[44]***. The variation in the molecular weights of plasmids might be a result of these plasmids carrying different gene cassettes for resistance against different classes of antibiotics ***[45].*** The reported results indicate the dissemination of plasmids among *E. coliO157:H7* isolates which may be carrying resistant genes against wide spectrum of clinically used antibiotics, which may explain the reason of evolution antibiotic resistant patterns in studied bacterial cultures.Similarresults were obtained by***[46]*** who detected various plasmid bands with different molecularweight among*E.coli* isolates. Various investigators reported that most *E. coli* isolates contain at least one large plasmid in addition to other smaller plasmids, which encode for several virulence factors such as hemolysin, toxins, siderophores Productin (aerobactin), in addition to antibiotic resistant markers ***[47]***. R-plasmids have been detected in many members of the Enterobacteriaceae. The antibiotic resistance conferred by plasmids broadly reflect the agents commonly used against their bacterial hosts, whereas resistance to tetracycline and to certain penicillins and aminoglycosides occurs on plasmids from bacteria of either Gram type. Plasmids may also confer resistance to other antimicrobial agents. These include heavy metal ions such as mercury***[48]***.

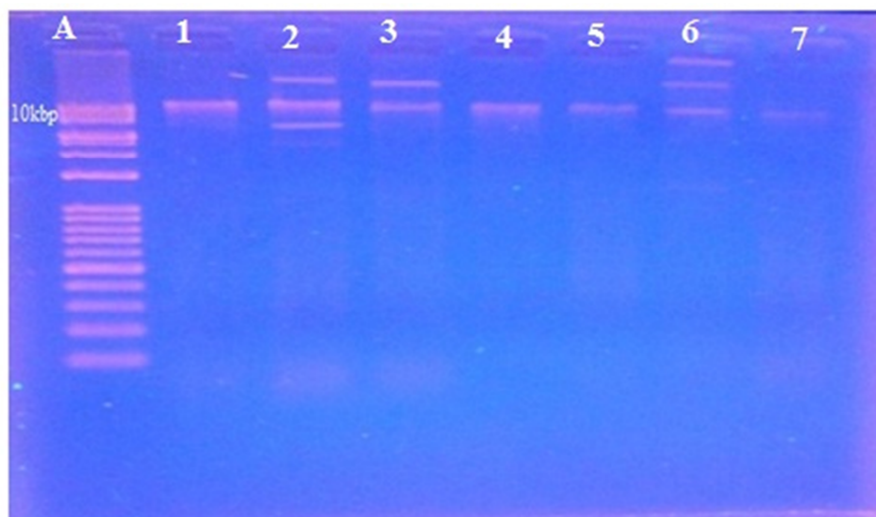

Figure- 1: Plasmid profile of some *E. coli O157:H7* isolates
A: Ladder 1-7: plasmid bands

I. Genetic site determination of the antibiotic resistance genes in E. coli O157:H7 by genetic transformation

The process of genetic transformation is depended on the ability of laboratory *E. coli* DH5α strain, which have known genotype to be transformed from sensitive strain to resistant one for some antibiotics under study after treating with $CaCl_2$ and exposing to the prepared plasmid DNA from chosen bacterial isolates using heat shock according to the method of ***[17]***. Extracted plasmid DNA from the two isolates (E1 andE15) that represents the most resistant isolates to antibiotics under study which shown in (*Table: 3*) was plated on LB agar to ensure either it is contaminated or not, after incubation period, there was no growth which means that the extracted plasmid DNA is not contaminated during the extraction process. *Table: 4* shows *E. coli* DH5α had the ability to receive purified plasmid DNA from *E. coli O157:H7* isolates and

transformed successfully. Ten colonies of *E. coli* DH5α transformants were chosen to be purified on LB agar media and then these colonies were subjected to antibiotic resistance test using Kirby-Bauer method and the main result were recorded in (*Table: 4*). From (*Table: 4*), it's clear that extracted plasmid DNA from E1 and E15 transferred successfully to *E. coli* DH5α strain, in which the transformant colonies of *E. coli* DH5α showed resistance for KF, AMP, FEP, MET and TMP for E1, for KF, FEP,CTX , CRO,MET and TMP for E15.

Table- 3: Antibiotic resistance pattern of tested isolates before transformation

Bacterial Isolates and Laboratory strain	*Antibiotic resistance pattern*											
	*AK**	*C*	*KF*	*CIP*	*FEP*	*CTX*	*CRO*	*CN*	*MET*	*TE*	*TMP*	*SXT*
DH5-α	S	S	S	S	S	S	S	S	I	S	S	S
E1	R	R	R	R	R	R	R	R	R	R	R	R
E15	R	R	R	R	R	R	R	R	R	R	R	R

Table- 4: Antibiotic resistance pattern after transformation of *E. coli* DH5α with purified plasmid from *E. coli O157:H7*

Bacterial Isolates and Laboratory strain	*Antibiotic resistance pattern*											
	*AK**	*C*	*KF*	*CIP*	*FEP*	*CTX*	*CRO*	*CN*	*MET*	*TE*	*TMP*	*SXT*
DH5-α	S	S	S	S	S	S	S	S	I	S	S	S
E1	S	S	R	S	R	S	S	S	R	S	R	S
E15	S	S	R	S	R	R	R	S	R	S	R	S

Figure: 2 showed the plasmid profile of transformant cells of *E. coli* DH5α strain, after transformation with purified plasmid from E1 and E15 isolates and the results revealed that one plasmid in E1 and two plasmids in E15 had been transformed successfully with molecular weight of more than 10 Kbp. This may due to that some plasmids may degrade especially those with low molecular weight during storage and cannot entered to the host cell or unable to replicate inside host cell and this may explain why only plasmid with high molecular weight were obtained in gel electrophoresis.In the present study, variation in resistance of *E. coli* DH5α transformant colonies to different antibiotics observed, this might be indicated that the antibiotic resistant genes located on different plasmid fragments which were known as r-determinant. However, in general R-plasmids consist of two major fragments, the RTF-Tc which was carrying several numbers of genes specialized for replication process, copy number of plasmid and bear the genes which were responsible for the resistance to tetracycline only, and the r-determinant which contains all genes that were responsible for the resistance antibiotics ***[49]***.***[50]***Found that *E. coli* DH5α had the ability to receive purified O157:H7 plasmid DNA isolates and transformed successfully. The transformant colonies became resistance to Amoxicillin, Amoxiclave, and Nalidixic acid, Nitrofurantoin, Rifampicin, Ampicillin, Cephalexin, Cefixime, Cefotaxime, Doxycycline, Gentamycin, Streptomycin and Tetracycline.

[51]Reported that multidrug resistance plasmids may carry genes encoding resistance to other antibiotics such as aminoglycosides. ***[52]***Have reported that horizontal gene transfer is a factor in the occurrence of antibiotic resistance in clinical isolates and suggested that the high prevalence of resistance to a particular antibiotic does not always reflect antibiotic consumption as previously suggested by others ***[53]***.[54]Reported that the frequency of transformation for *E.coli* isolates was 10^{-7} and they concluded that urinary tract isolates being exposed to antibiotics are developing high level of antibiotic resistance, which

involves the intrageneric transfer of the corresponding genes ***[55]***. ***[56]*** reported different transforming colonies for *E. coli* isolates and he concluded that all genes responsible for confirming resistance to Ampicillin, Erythromycin, Chloramphenicol, Lincomycin, Nalidixic acid, Tetracycline and Gentamicin were located on plasmid DNA, while those confeing resistances to Streptomycin and Trimethoprim might be chromosomally encoded.

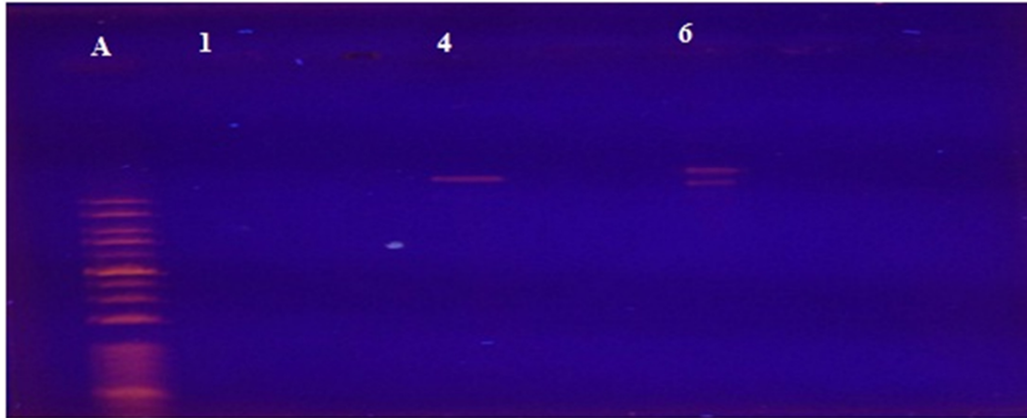

Figure- 2: Plasmid profile of transformant colonies

Lane A 1000 bp DNA ladder Lane 1 *E. coli* DH5α laboratory strain(Plasmidless)
Lane 4 E1 transformant colonies Lane 6 E15 transformant colonies

J. Detection of ESBL genes (blaCTX-M - blaSHV-and blaTEM) among isolates using PCR assay
Detection of blaCTX-M gene

Results in (*Figure: 3*) indicate that *E. coli O157:H7* isolates which screened for*bla*CTX-M gene, exhibit positive PCR products on the gel in which 88% of the isolates contained that gene except (E1,E5 and E6). Moreover, the produced band is of 369 bp, which represents the presence of *bla*CTX-M gene. This result was nearly similar to previous reports by ***[57]*** who performed PCR to confirm the presence of *bla*CTX-M gene in ninety-three *E. coli*. Their results revealed that all isolates contain *bla*CTX-M.

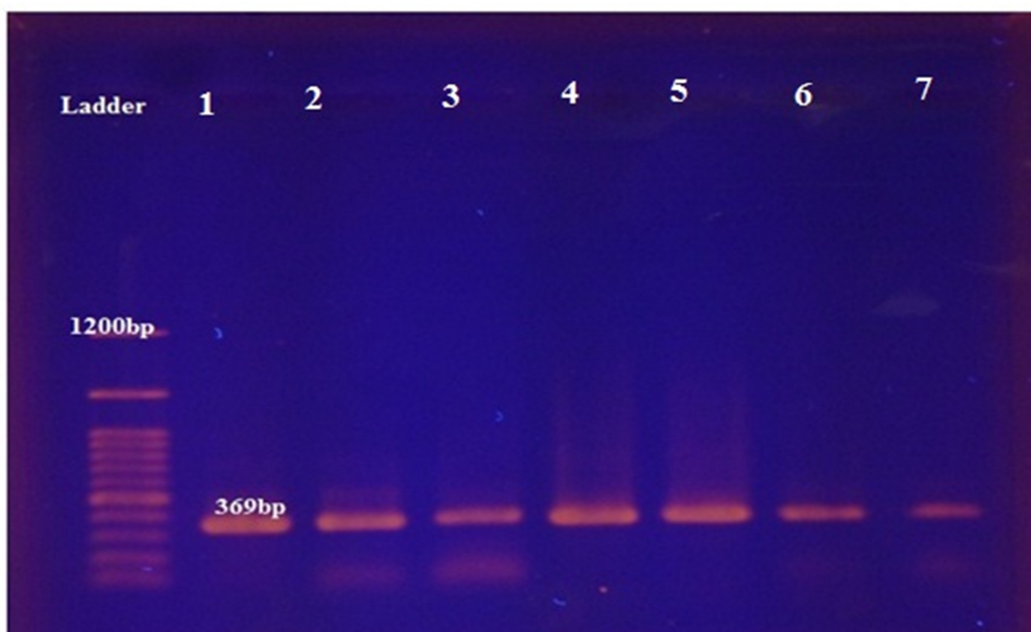

Figure- 3: Products of polymerase chain reaction performed with *bla*CTX-MPrimers

These results also are in agreement with other investigations. (58,59) demonstrated that most *E. coli* isolated from different clinical source produced high amount of *bla*CTX-M gene, while our result are in disagreement with ***[34]*** in Nigeria who found that one isolate among *E. coli O157:H7* express *bla*CTX-M. ESBLs can easily hydrolyze this group of β -lactam antibiotics, such as Cefpodoxime, ceftriaxone, Cefotaxime, and Ceftazidime***[60]***. *bla*CTX-M genes are usually present on large plasmids that also carry additional resistance genes, but have been found on plasmids ranging in size from 7 to 430 kb. The *bla*CTX-M gene is mainly associated with incompatibility group FII plasmids, which are narrow host-range plasmids and are characterized by low copy numbers. These plasmids, which have the propensity to readily acquire and disseminate resistance genes in Enterobacteriaceae members, are termed as 'epidemic resistance plasmids'. It is believed that IncFII plasmids were widely distributed in Enterobacteriaceae and well adapted to the hosts even before the introduction of antibiotics. Broad host-range plasmids such as IncN, IncI1 and IncL/M have also been involved in the dissemination of CTX-M genes. These plasmids are also known to harbor additional genes conferring resistances to aminoglycosides and fluoroquinolones. IncL/M plasmids have been involved in the spread of *bla*CTX-M-3 in Poland and several other Eastern European countries. IncHI2 seems to have preference for *bla*CTX-M-9 whereas IncK has preference for *bla*CTX-M-14. The spread of *bla*CTX-M-14 in Southern Europe has been mainly on IncK plasmids whereas its spread in Northern Europe has been on IncI1 and IncFII plasmids ***[61]***. Many of the CTX-M ESBL producing isolates are often resistant to fluoroquinolones and aminoglycosides. The IncFII plasmids that bear *bla*CTX-M genes also bear *aac(6')-Ib-cr*gene, which encodes an aminoglycoside- and fluoroquinolones-modifying enzyme. Genes encoding resistance to macrolides, Tetracyclines, sulphonamides, trimethoprim, and chloramphenicol have all been associated with *bla*CTX-M containing plasmids ***[62]***.

K. Detection of (blaSHV-and blaTEM) genes

In the current study all the isolates of *E. coli O157:H7* had both genes of *bla*SHV-1 and *bla*TEM-1 and the produced band are 545 bp and 410 bp respectively as shown in (*Figure: 4*) and (*Figure: 5*). ***[63]***Mentioned that the percentage of isolates for genes *bla*TEM were (54.9%) and *bla*SHV (32.9%) either alone or in combination. Rate of *bla*TEM (16%)and *bla*SHV (19.5%)was reported by ***[64]***, *bla*TEM (27.7%) and *bla*SHV (13.6%) respectively reported by ***[65]*** that disagree with our results. Our result agreed with [66] in Kingdom of Saudi Arabia who found in their study that among 8 strains of *E.coli O157:H7*, all isolates showed amplification for *bla*TEM and *bla*SHVgene.The TEM β-lactamases spread worldwide and it is known to be found in many Enterobacteriaceae. However, *E. coli* shows reduced susceptibility to first and second generation cephalosporins by the production of plasmid-mediated, TEM β-lactamase. Since 1980s, the emergence of resistance to third generation cephalosporins has been reported in strains of *E. coli* ***[67]***. In recent local study, ***[24]*** found that 18 of the 22 enteropathogenic *E. coli* yielded amplification products with TEM-PCR specific primers. Most *E. coli* isolates have chromosomally or plasmid-mediated SHV-1 β-lactamase, which is a narrow-spectrum β- lactamase with activity against penicillins. More than 50 variants of SHV which are important worldwide and currently recognized on the basis of unique combination of amino acid replacement. SHV-2 and SHV-5 (plasmid-mediated β-lactamases) enzymes have been recorded in at least five countries, with the latter type widespread in Greece. In another study, in Thailand it was reported that the frequency of *bla*SHV gene was 8% of the confirmed ESBL producing *E. coli* isolates ***[68]***.

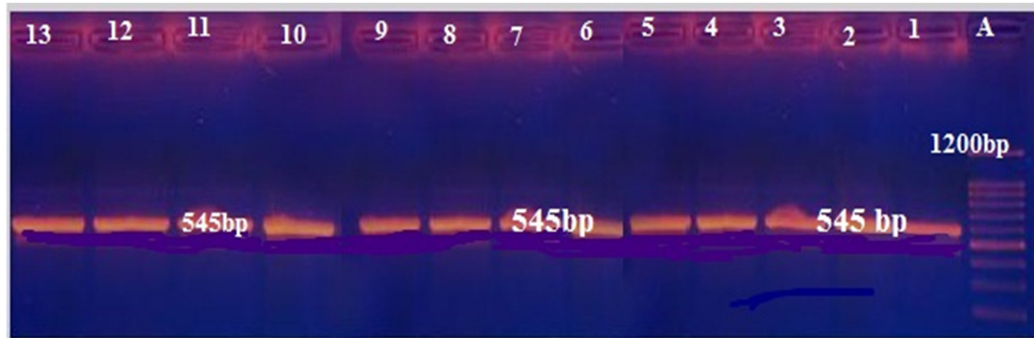

Figure- 4: Products of polymerase chain reaction performed with *bla*SHVprimers

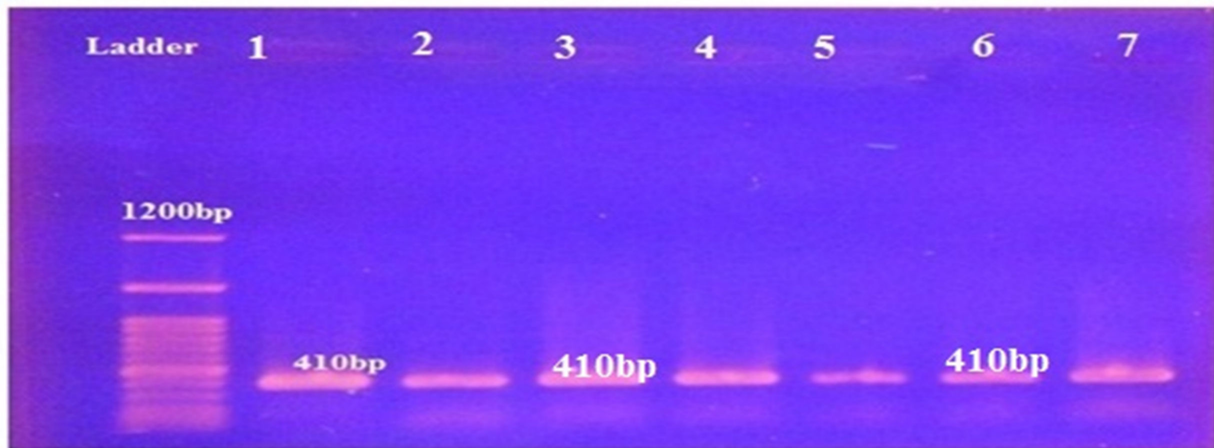

Figure-5: Products of polymerase chain reaction performed with *bla*TEMPrimers

Most *E. coli* isolates have chromosomally or plasmid-mediated SHV-1 β-lactamase, which is a narrow-spectrum β-lactamase with activity against penicillins. More than 50 variants of SHV which are important worldwide and currently recognized on the basis of unique combination of amino acid replacement. SHV-2 and SHV-5 (plasmid-mediated β-lactamases) enzymes have been recorded in at least five countries, with the latter type widespread in Greece. In another study, in Thailand it was reported that the frequency of *bla*SHV gene was 8% of the confirmed ESBL producing *E. coli* isolates ***[68]***. Such differences in prevalence of ESBL (*bla*CTX-M - *bla*SHV-and*bla*TEM)might be due to divergences in geographical variation or differences in association with host characteristics and even weather climate of each regions. The prevalence of ESBL-producing bacteria was increasing every year especially in tertiary hospitals. The major risk factors are long term exposure to antibiotics, prolonged ICU stay, nursing home residency, severe illness, instrumentation or catheterization, length of hospitalization (more than 48 hours of hospitalization), etc. ***[68]***.The genotypic methods help us to confirm the genes responsible for ESBL production. The majority of our strains harbored two or more ESBL genes and the most common phenotypes were TEM, SHV and CTX-M.The correct identification of the genes involved in ESBL-mediated resistance is necessary for the surveillance and epidemiological studies of their transmission in hospitals. Appropriate antibiotic policy and infection control measures in hospital settings are crucial to overcome the problems associated with infections by ESBL-producing strains. *bla*genes are usually carried by large and transferable plasmids. Thus the plasmid localization of the genetic determinants facilitates their horizontal spread in bacterial populations, particularly by means of conjugation, accumulation of resistance genes results in strains that contain multiresistant plasmids ***[68]***.

Conclusion

The high prevalence of ESBLs genes among diarrheagenic *E. coli* O157:H7 explain why such bacteria are resistance to a broad spectrum of β-lactamantibiotics.

Competing interests

The authors declare that they have no competing interests.

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