ORIGINAL ARTICLE

Identification and Molecular Detection of *ChuA* gene of Pathogenic *Escherichia coli*

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ABSTRACT

During the period of October, 25th 2019 and January, 15th 2020, twenty sampleswere collected from different clinical specimens (stool and urine) from patients attended to the (Rapareen Padiatric and Hawler Teaching hospitals) in Erbil city. The sample was bringing to the laboratory and re-identified by performing morphological, cultural, biochemical tests and confirmed by API 20E systems.The isolates appeared to be varied in their resistance to eleven antibiotics; Imipenim (IMP) was the most effective antibiotic against all isolates of *E. coli*and all isolates were resistant 100%, to Ticarcillin (TIC), Cephalothin(CEP) and Amoxycillin+ Clavulonic acid (AMC). The resistance rate of the isolates for Amikacin (AK) were 10 %, 50% for Nalidixic acid (NA) and Nitrofurantoin (NIT), 60% for Ceftriaxone (CTR), 65% for Cefotaxime (CTX), 85% for Piperacilin(PI), 95% for Ampicillin(AMP), respectively. All*E. coli*isolates were screened for the presence of (*ChuA*) gene on genomic DNA using polymerase chain reaction (PCR) assay. Results showed that all isolates were positive for the existence of *ChuA* virulence gene.

Keywords: *E. coli*, antibiotic susceptibility**,**PCR.

INTRODUCTION

Escherichia coli are the predominant facultative organism in the human gastrointestinal tract. Pathogenic forms of *E. coli* can cause a variety of diarrheal diseases in hosts due to the presence of specific and virulence factors and pathogenicity associated genes which are generally not present in other *E. coli*. However, a small percentage of *E. coli* is capable of causing extra intestinal infections (Darvishi*et al*., 2020). *E. coli* it is a frequent cause of outpatient urinary tract infections in women worldwide, septicemia, diarrhea and meningitis (Faraji Hormozi*et al.,* 2018).

Although most strains exist as harmless symbiosis, there are many pathogenic *E. coli* strains that can cause a variety of diseases in animals and humans (Michael and Thomas, (2001).*E. coli* is the predominant cause of both community and nosocomial urinary tract infection (UTI). UTI is the second most common infectious presentation in community practice. The constant increase in the antibiotic resistance of clinical bacterial strains has become an important clinical problem. The accelerated emergence of antibiotic resistance among the prevalent pathogens is the most serious threat to the management of infectious diseases (Adamus-Bialek*et al*., 2013).

The evolution and spread of various mechanisms of antimicrobial resistance among common human pathogenic members of Enterobacteriaceae is of increasing concern and lead to narrowing of available therapeutic options (Boucher *et al*., 2009).

ChuA a gene required for hem transport in enterohemrrhagic, which encoding the 69 kDa protein was chromosomally encoded (Carlos *et al*., 2010).The aims of this study was isolation and identification of *E. coli* from different clinical sources,study the antimicrobial resistance patterns of the isolated *E. coli* to different antibiotics and reaction (PCR) assay.

MATERIALS AND METHODS

Specimen's collection: Twenty samples were obtained from clinical specimens include; urine and stool within the period between October, 25th 2019 and January, 15th 2020. The samples were taken and cultured on nutrient agar incubated for 24 hours at 37 °C, then single colonies weretransferred to MacConkey and Eosin methylene blue, Simmons citrate agar and incubated for 24 hours for screening of *E. coli*, isolateswere identified according to morphology, cultural and biochemical characteristics, and biochemical tests was performed like; oxidase, indole, and motility tests and confirmed by API 20E system.

Inoculum preparation and susceptibility studies: The antibiotic sensitivity of the twenty isolates of *E. coli*were performed againsteleven antibiotics which include Imipenim (IMP10µg), Nitrofurantoin (NIT300 µg), Nalidixic acid (NA30 µg), Ampicillin(AMP 10 µg), Cephalothin (CEP 30 µg) and Amoxycillin+ Clavulonic acid (AMC 30 µg), Ceftriaxone (CTR 30 µg), Piperacilin(PI 100µg), Cefotaxime (CTX 30 µg), Ticarcillin (TIC 75 µg), and Amikacin (AK30 µg), as shown in Table (1) and determined according to Clinical and Laboratory Standards Institute (CLSI) standards. Muller Hinton agar (MHA) melted and cooled at 45 °C was poured into sterile petri dishes and allowed to solidify completely, the plates were allowed to dry before applying antibiotic disc. Antibiotic resistance patterns of the isolates were determined using the Disc diffusion (Kirby Bauer) method; bacterium inoculate was adjusted to 0.5McFarland standard of Clinical and Laboratory standards institute ® (CLSI, 2012). The test inoculums were spread onto Mullur-Hinton agar using a sterile cotton swab and the disk applied carefully then incubated overnight. The zones of inhibition:

DNA extraction: DNA was extracted from *E. coli* isolates and used for detection of *ChuA* gene by PCR technique. A single bacterial colony was used to inoculate 5 ml Nutrient broth, which was incubated overnight in a shaker incubator at 37^{o} C. A volume of 3 or 5 ml of each isolate in an

eppendorff tube was centrifuged for 30 sec at 13,000 rpm and the supernatant discarded. The bacterial pellet re suspended by vortexing in 650 µl of TE buffer (10 mMTris – 1 mM EDTA, pH 8.0), until no clumps of the cell pellet remain. 30 µl of 10% of SDS (Sodium dodecyl sulfate) and 3 µl of Proteinase K was added and mixed thoroughly by vortexing. The sample incubated at 42 ° C for~ 10 min in water bath. Hundred µl of 5 M NaCl was added to the sample, mixed thoroughly by quick shacking. The sample incubated at 65 ° C for~ 10 min in water bath, until the cells are completely lysed. Added 650 µl of Chloroform to the sample then mix vigorously by hand back & forth until you can see a milky solution, then centrifuged for 3 min. Three layers produced, the upper aqueous phase (contains DNA), the bottom organic phase (contains chloroform) and the middle white phase which contain protein and cell debris. After centrifugation, upper phase of supernatant slowly transferred in to new Eppendorf tube. Six-hundreds of Phenol/ Chloroform (25: 24) was added, and then centrifuged for 2 min at 8,000 rpm. The upper aqueous layer transferred to a new Eppendorf tube; again avoid the middle white phase. Four- hundreds µl of Isopropanol was added to the micro tube and mixing gently by hand back and forth, Then centrifuged for 2 min. Poured the supernatant and washed the pellet by adding of 750 µl of 70% of ethanol, then mixed gently back and forth once or twice. Centrifuge the tube for 1 min. Poured the ethanol and let the tube to dried, then added 20 µl of TE buffer (to dissolve the pellet), then placed it in the 45 °C bath for 30 min, then store in 4 °C overnight then in the freezer.

Protocol of PCR technique: DNA extract was used as a template in the PCR technique. PCR was performed in a 25µl of reaction volume as in Table (2). PCR was used to detect the (*ChuA*)gene in the genomes of the *E. coli* isolates. The *ChuA*primers used were forward 5'- GAC GAA CCA ACG GTC AGG AT -3' and reverse: 5'- TGC CGC CAG TAC CAA AGA CA -3'. The PCR cycles were: denaturation at 94°C for 30 sec., annealing at 56°C for 30 sec., and extension at 72°C for 30 sec., repeated 30 times (Koga *et al*., 2014).

Agarose electrophoresis technique (Sambrook *et al.,* 2001)

Preparation of 1% agarose gel: The gel (1%) was prepared by dissolving 1 gm of agarose powder in 100 ml of 1 X TBE (Tris/borate/EDTA)) buffer, boiled until all agarose was dissolved and left to cool at 50 ºC, the gel was poured into the glass plate that contained appropriate comb, the gel was left to solidify and the comb was removed gently, the gel was soaked in a gel tank containing TBE buffer that should cover the surface of the gel.

Sample loading: To prepare samples for electrophoresis, 3 µl of 6X gel loading dye solution (BPB) for every 7 µl of DNA solution, and the mixture was slowly loaded in to the wells on the gel, also a 100 bp DNA ladder (Gene dire) was used as molecular markers. The amplified products were visualized by ethidium bromide staining after gel electrophoresis; the gel was visualized by UV-trans illuminator, and then photographed.

RESULTS AND DISCUSSION

Identification of bacterial isolates: Twenty isolates of *E. coli* were isolated from clinical specimens include; 13 isolates from urine and 7 isolates from stoolwithin the period between October, 25th 2019 and January, 15th 2020, and all isolates were inoculated in different culture media to study their characteristics, results of biochemical tests and the results of our studies are shown in Table (3). Confirmation of all the isolates of *E. coli*was done by gram staining and various biochemical tests in which indole and motility were positive and negative for citrate and oxidase. On MacConkey agar it produces pink colonies, bright metallic green sheen colonies on Eosin Methylene Blue (EMB) agar. On Kligler 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 no H_2S production. These results werereinforced by API 20 E system(Ibrar*et al.,* 2014 and Zahera *et al.,* 2011).

Antibiotic resistance pattern of *E. coli* isolates: Table (4) indicated that all the isolates of *E. coli* revealed different resistance rate to most antibiotics used and all isolates resistant to Ticarcillin, Cephalothin and Amoxycillin+ Clavulonic acid 100%. The resistance rate of the isolates for Amikacin were 10 %, 50% for Nalidixic acid and Nitrofurantoin, 60% for Ceftriaxone, 65% for Cefotaxime, 85% for Piperacilin, 95% for Ampicillin, and Imipenim was the most effective antibiotic against isolates of *E. coli* and 100% of these isolates were susceptible to IPM.

This result strongly confirmed the result obtained by Nanakali and Abdulrahman, 2012 who stated that all isolates were sensitive to Imipenem and multiple resistance among isolates were common. The results revealed high resistance to Ticarcillin, Cephalothin and Amoxycillin+ Clavulonic acid 100%, Ampicilin 95. %, 85% for Piperacilin, similar results were reported byAghazadeh, *et al*., (2015). The most effective antibiotics against *E. coli* isolates were Amikacin. These results do agree with those reported by (Sharma *et al*., 2016) who found that Amikacin and Nitrofurnatoin showed good activity against uropathogen isolates.

Antibiotic resistance has increased over years, varies from country to country, and is a major clinical problem in treating infections caused by these microorganisms. Maximum isolates (85–90%) showed high resistance to Cefotaxime, Nalidixic acid and Amoxycillin-Clavulanic acid while resistance to Amikacin was found to be 16%. Increased resistance might be due to widespread, inappropriate use of antibiotics and production of extended spectrum beta lactamases in these isolates (Sharma *et al*., 2016).

The high resistance of the bacterial isolates in this study to different antibiotics may be related to the presence and dissemination of plasmids within heterogeneous population of these bacteria which can transfer between genera and species of bacteria lead to the prevalence of resistance by conjugation and transformation (Dionisio*et al*., 2002). In addition to weakling immunity system in some human due to poor nutrition or heredity factors makes bacteria to be more resistant (Sharma *et al*., 2009). Also the main reasons for this increase are patient-to-patient

transmission of resistant organisms and selection of resistant organisms because of antimicrobial exposure. Previous studies have shown that feedback of reliable reports of rates of antimicrobial use and resistance to clinicians can improve the appropriateness of antimicrobial usage (Mendonça*et al*., 2016).

Molecular Analysis: Detection of *ChuA*gene among *E. coli* isolates using PCR technique: After extraction of genomic DNA from *E. coli* isolates, polymerase chain reaction (PCR) and gel electrophoresis were carried out in order to determine the presence of *ChuA* gene in the DNA of *E. coli* isolated. PCR was performed on a pure DNA template (free from RNA contamination) of each bacterial isolate separately for amplification of *ChuA* gene via the use of specified oligonucleotide primers that flanked DNA sequence to be amplified. After amplification of *ChuA* gene in each bacterial samples separately, PCR products of all bacterial samples were visualized the amplified products by ethidium bromide staining after gel electrophoresis to detect the presence of the gene in the sample. Results in figure (1) indicate that *E. coli*isolates which contain *ChuA* gene, exhibit positive PCR products on gel which all of the isolates contained that gene Moreover; the produced band is of 279 bp, which represents the presence of *ChuA* gene.

Table 1: Antibiotics name, abbreviations, and Final concentration

Antibiotics name	abbreviations	Disk content (µg/ml)
Nitrofurantoin	NIT	300
Ticarcillin	TIC	75
Ampicillin	AMP	10
Nalididixic acid	NA	30
Cefotaxime	CTX	30
Amikacin	AK	30
Cephalothin	CEP	30
Amoxycillin+ Clavulonic acid	AMC	30
Ceftriaxone	CTR	30
Piperacilin	PI	100

The gene is part of a larger locus, termed the heme transport locus, which appears to be widely distributed among pathogenic *E.coli* strains. The ability to use heme and/or hemoglobin might be especially advantageous to pathogenic bacteria. These pathogens often secrete cytotoxins, which gain access to the intracellular heme reservoir besides initiating tissue invasion. Cytotoxin production coupled with the capability to utilize heme and/or hemoglobin could serve as an effective iron acquisition strategy during the progression of infection (Nagy *et al*., 2005).

ChuA, the haem receptor of *E. coli*, is thought to contribute to the pathogenicity of *E. coli* strains causing extraintestinal infections. No difference in *chuA* prevalence was found between commensals isolated from the intestine of healthy volunteers and isolates from extra intestinal infections. The results indicate that *ChuA* might be involved in the colonization of human hosts (Hoffmann*et al*., 2001).

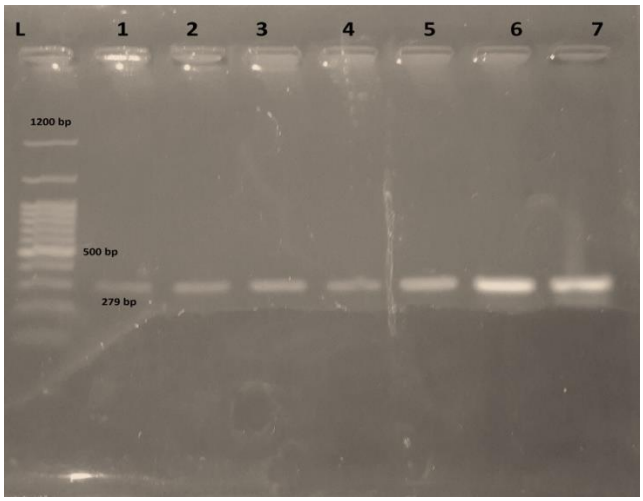
Table 2: Reaction mixture (25µl) for PCR

Component	Volume	Final Concentration
Master Mix	12.5 µl	1X
Forward Primer	1 µl	0.1-1 µl
Reverse Primer	1 µl	0.1-1 µl
Template DNA	1 µl	10 pg-1 µg
Sterile deionized Water	9.5 µl	
Total	25µl	

Table 3: Biochemical characteristics of *E. coli*

Colonies and tests	Results
Gram reaction	Gram negative
Eosin methylene blue agar	Metallic sheen colonies
Indole	+
Citrate utilization	–
Oxidase	–
Motility	+
Kligler Iron Agar	A/A
H_2S Production	No

Figure (1): Products of polymerase chain reaction performed with *ChuA* Primers and the specified *E.coli* DNA



Lane 1: 100 bp DNA ladder
Lane 1-8: Amplified PCR product of *ChuA* gene

Table 4: Sensitivity of *E. coli* isolates to different antibiotics and percentage of resistance

No. of isolates	Antibiotics at final concentration										
	AK	AMP	AMC	CEP	CTR	CTX	IMP	NA	NIT	PI	TIC
1	S	R	R	R	R	R	S	R	S	S	R
2	S	R	R	R	R	S	S	S	S	R	R
3	S	R	R	R	R	R	S	R	S	R	R
4	S	R	R	R	R	S	S	S	S	R	R
5	S	R	R	R	R	S	S	S	S	R	R

6	S	R	R	R	S	R	S	R	R	R	R
7	S	R	R	R	R	R	S	R	R	R	R
8	S	R	R	R	S	S	S	S	R	R	R
9	S	R	R	R	S	R	S	S	S	R	R
10	S	R	R	R	S	R	S	S	R	R	R
11	R	R	R	R	R	S	S	R	S	S	R
12	S	R	R	R	S	R	S	R	S	S	R
13	S	R	R	R	R	R	S	S	S	R	R
14	S	R	R	R	S	R	S	S	S	R	R
15	R	S	R	R	R	S	S	R	S	R	R
16	S	R	R	R	S	R	S	S	S	R	R
17	S	R	R	R	R	S	S	R	S	R	R
18	S	R	R	R	R	R	S	R	S	R	R
19	S	R	R	R	S	R	S	S	S	R	R
20	S	R	R	R	R	R	S	R	S	R	R
No. of resis.ls	2	19	20	20	12	13	0	10	5	17	20

R= Resistant, S = Sensitive

CONCLUSION

In conclusion, in despite of successful findings being reported in this study, the most active antibiotics against *E. coli*is Imipenim so they should be considered as empirical treatment of choice. ChuA gene located on chromosome and as an important virulence factor expressed by all *E.coli* isolates.

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