ORIGINAL ARTICLE

Phenotypic Detection of Some Beta-lactamases and Enterobacterial Repetitive Intergenic Consensus Genotyping (ERIC) of Natively Isolated Uropathogenic *Escherichia coli*

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ABSTRACT

Background: *β-lactamases comprise wide number of enzymes sharing a common goal which is the hydrolysis of β-lactam anibiotics. Enterobacterial Repetitive Intergenic Consensus –Polymerase Chain Reaction is considered as one of the most reliable fingerprinting technique that provides sharp and fast results with relatively low cost.* ***Objective***: *The current study aimed to isolate Uropathogenic E. coli and explore the possession of some β-lactamases phenotypically and to genetically typing the β-lactam resistant isolates.* ***Methodology***: *During the study period between November 2023 and January 2024, (80) urine samples were collected from (66)female and (14)male patients , who visited hospitals in Al- Najaf Al-Ashraf province in order to isolate uropathogenic Escherichia coli. Identification was done using cultural and biochemical tests supported by utilizing Vitek-2 system. Antibiotic sensitivity test was used to determine pattern of resistance, Screening tests of β-lactamases comprised (DDST) and (CST) on those showed resistance to ceftriaxone. Genetic study included the use of Enterobacterial Repetitive Intergenic Consensus –Polymerase Chain Reaction (ERIC-PCR) technique to determine genetic variation of those isolates.* ***Results***: *In this study (26) isolates were obtained. AST results showed that total number of isolates were resistant to tetracycline, while (84.6%) were resistant to trimethoprim, (53.8%) were resistant to ceftriaxone. While resistant and sensitive isolates were equal regarding ciprofloxacin.. Results showed that out of 14 cefitriaxone resistant isolates three (21.4%) of isolates were resistant to imipenem. Screening method of (MBLs) showed that threes isolates (EC3, EC8, and EC14) gave positive results (MBL producers) for both tests. Regarding the screening the Extended spectrum beta lactamases, results were negative where the isolates were either susceptible to β-lacatam antibiotics involved in the test, or resistant to all antibiotics used. Furthermore, genetic analysis of (ERIC-PCR) revealed at similarity ≥ 70% the presence of 9 different clusters, eight of them with single isolate, other three clusters with two identical pattern each. Identical pattern was shown in two of MBL producers (EC3, EC14).* ***Conclusion***: *We conclude that native Escherichia coli isolates had the ability to produce metallo beta-lactamases, they produced various ERIC patterns, which reflects the high genetic variation these bacteria possess, though they caused the same infection (urinary tract infection), and presence of convergence may reflect phenotypic similarity of resistance pattern.*

INTRODUCTION

Escherichia coli capable of infecting urinary tract, the so called Uropathogenic (UPEC) is one of the most important leading causative agents causing urinary tract infections proceeding other gram negative bacteria since it can produce this infection in hospitalized as well as community patients. Several scenarios are suggested by which *E.coli* initiate urinary tract infection, some by ascending way starting by attaching to uroepithelium then migrate to bladder resulting in cystitis, then to the urethra and kidney causing pyelonephritis, others include the descending route by which organisms

entering blood stream can reside in the kidney tissue causing pyelonephritis.[1]

Dissemination of antibiotic resistance is a worldwide health issue.[2] Multiple endogenous and exogenous means fastening this phenomenon especially in Gram negative organisms.[3] Utilizing low cost and fast outcomes procedures are available and provide an easy way to predict resistance pattern and accordingly aiding in suggestion of effective antibiotic treatment.[4]

Various biological methods have been introduced to provide applicable techniques to type microorganisms in order to evaluate their evolutionary relatedness, ecological distribution and determining the most

prevalent strains causing certain infections. One of those methods is Enterobacterial Repetitive Intergenic Consensus-polymerase chain reaction (ERIC-PCR) technique. [5]

This method depends on the existence of palindromic sequences, which are conserved and repeated spread intergenically in wide range of microorganism, gram positive, and gram negative bacteria even in some fungi species. These sequences are usually found in multiple copies in the chromosomal DNA. The ERIC-PCR technique relies on the fact that these sequences have numerous copies which are randomly distributed within genome. Various patterns could be introduced when amplifying specific primers and examining result by gel electrophoresis.[6]

The current study aimed to detect of beta lactam hydrolyzing enzymes phenotypically and to define the distribution of ERIC patterns in uropathogenic *E. coli* strains.

METHODOLOGY

Isolation and Identification

During the study period from (November 2023 to January 2024), eighty urine samples were collected from patients suffering from symptoms of infection in urinary tract. Identification was primarily done by cultural characteristics and by biochemical tests, furthermore final identification was accomplished by Vitek-2 system.

Antibiotic Susceptibility test

All isolates were submitted to Antibiotic Susceptibility testing (AST) by disk diffusion method.[7] The antibiotics included (Erythromycin, Tetracycline, Ceftriaxone, Chloramphenicol, Trimethoprim, Ciprofloxacin and imipenem). Interpretation of results was according to guidelines. [8]

Double Disc Synergy Test (DDST)

The isolates that showed resistance to ciprofloxacin, (14) isolates were undergone screening tests for both metallo beta lactamases and extended spectrum beta-lactamases. Regarding EDTA DDST, it was performed according to[9], with slight modification. The isolates suspensions were adjusted to a turbidity equivalent to that of a 0.5 McFarland Standard and used to inoculate Mueller–Hinton agar plates. According to the test, a 10 mg Imipenem disc was laid on the lawn, and placing a (6 mm in diameter, Whatman Filter paper no. 2) blank filter paper disc at an edge to edge distance of 10, 15 and 20 mm. A volume of 10 microlitres of a 0.5 M EDTA solution was added to the blank filter paper disc. Plates were incubated for 24 hours; positivity of test was determined by the presence of any synergistic inhibition zone.

Combined Disk Test (CDT)

According to [10] procedure with slight modifications, the combined disk test was performed. Plates inoculated with the tested organisms were used to place two of 10 μg Imipenem discs on, and 10 μL of 0.5 EDTA of as disodium salt dehydrate solution was added to one of the discs. Followed by incubation in air at 35°C for eighteen hours. Results were considered as positive when the difference between the diameter of inhibition zone around imipenem plus EDTA disc and imipenem alone is ≥7 mm.

Extended Spectrum Beta lactamases Detection

This test was done by preparing isolates and media as in classical disc diffusing method and by placing ceftazidime, imipenem, ceftriaxone, cefotaxime, aztreonam and cefepime discs around the central amoxicillin/clavulanic acid disc. Also called DDST. The DDST is considered positive when the inhibition zone of any of the antibiotics is larger towards the clavulanic acid disc. [11]

DNA Extraction (Total DNA):

Genomic DNA was extracted done by boiling method, bacterial suspensions (3-5) isolated colonies of each isolate were suspended in (300) μl of distilled water were boiled at 100°C for fifteen minutes, and immediately cooled onto ice for 30 minutes. Then left to reach room temperature and centrifuged at 8000 rpm for 5min then the supernatant containing DNA was collected in 1.5ml eppendorf tube. [12]

Genotyping by ERIC-PCR

Polymerase chain reaction (PCR) was done using the primers mentioned by [13] table (1). The reaction mixture was prepared as follow: In a 0.2 ml eppendorf tube with a reaction volume of 25μl, PCR reaction materials (12.5μI Go Taq® Green Master Mix, 2μI upstream primer, 2μI downstream primer, 5μI volume DNA template, and 3.5μI water). The PCR cycling program parameters conditions used were: initial denaturation 93/3min, 30 cycles off denaturation 94 C° /5 min followed by annealing 52 C° /2 min, extension at 72 C° /5 min and final extension for five minutes at 72 C°. Detection of the result was done by agarose gel electrophoresis. 5μll from each product was added to a single well, followed by addition of 5μl of ladder. An electric current of 60 volts was administered for 1.5 hours.

Table 1: Oligonucleotide primers sequences

Oligonucleotide primers	Sequence(5′to3′)
ERIC F	ATG TAA GCT CCT GGG GAT TCA C
ERIC R	AAG TAA GTG ACT GGG GTG AGC G

RESULTS

Isolation and identification

Out of the 80 urine samples, 26 *E. coli* isolates were obtained as a result of primary and confirmatory identification methods with a percentage of (32.5%). According to sex, results showed that out of 26 isolates, (23) were obtained from female patients, while other three isolates were from male patients as shown in figure 1.
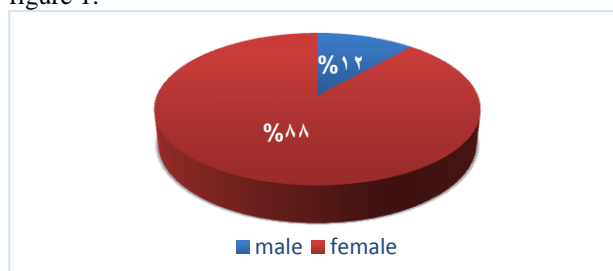

Fig. 1: Distribution of *E coli* isolates according to sex

Antibiogram Profile of uropathogenic *E. coli* Using Disk Diffusion Method:

Tested antibiotics comprised those mostly used in treatment of UTIs. Results showed that all isolates were resistant to tetracycline, while (84.6%) were resistant to trimethoprim. The rate of resistance toward ceftriaxone was (53.8%). While resistant and sensitive isolates were equal regarding ciprofloxacin. Finally, the most effective antibiotic among those used in present study were erythromycin and chloramphenicol where only seven isolates (26.9%) were resistant to each agent (Fig. 2).
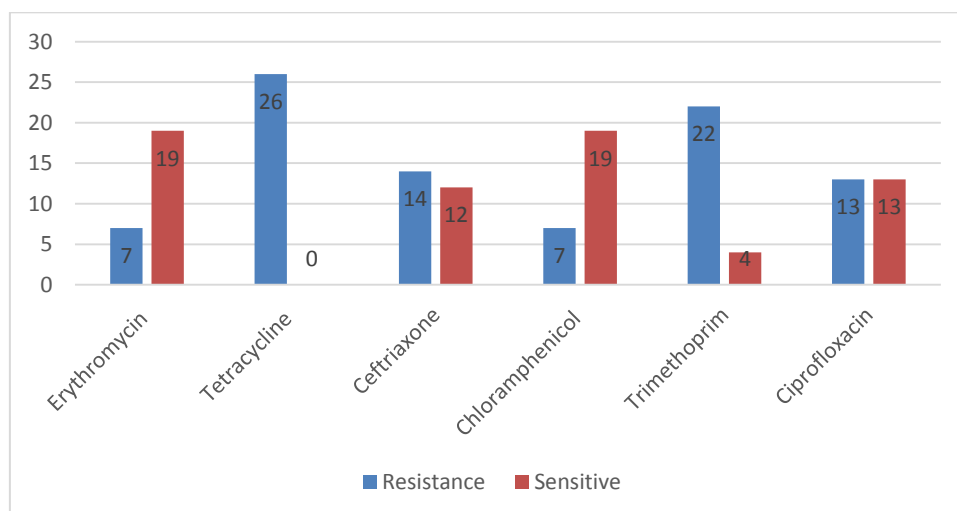

Fig. 2: Resistance rates of antimicrobial agents

Imipenem susceptibility testing

As a member of carbapenems, the substrate of metallo-betalactamases; susceptibility to imipenem was tested. Results showed that only three of fourteen ceftriaxone resistant isolates was resistant (21.4%) and the rest were sensitive (78.6%) the diameter of inhibition zone of sensitive isolate >23, Resistance 19≤ intermediate 20-22 as shown in fig (3)
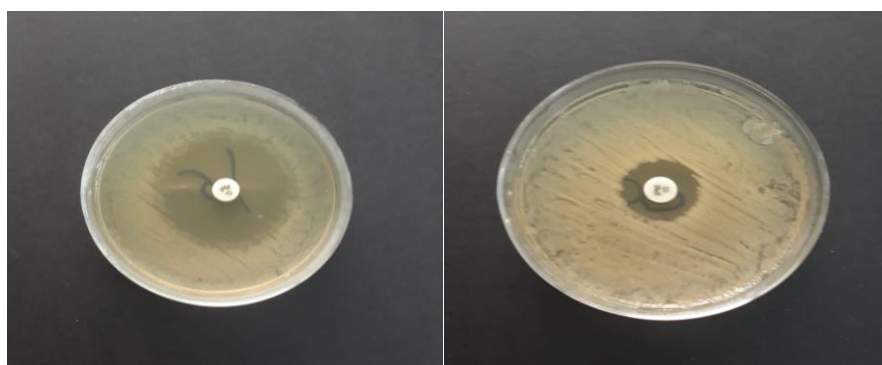
A: **sensitive**   **B: resistant**
Fig. 3: Antibiotic Susceptibility test of Imipenem

Screening the Metallo Beta - lactamases

Results of screening the (MBLs) with the double disk synergy test showed that the isolates (EC3, EC8, EC14) gave positive results where the three distance of EDTA disks showed synergistic zone with the disk of imipenem. As shown in fig (4)

Furthermore results of combined disk synergy of imipenem revealed that the isolates EC3, EC8 and EC14 gave positive result where the differences of between the diameter of inhibition zone of imipenem disk and the inhibition zone of imepenem with EDTA was were(10, 8, and 9) for the three isolates respectively as shown in fig (5).

Fig. 4: Screening MBLS enzymes. Double Disk Synergy Test Positive result. B: Combined Disk Test Positive result

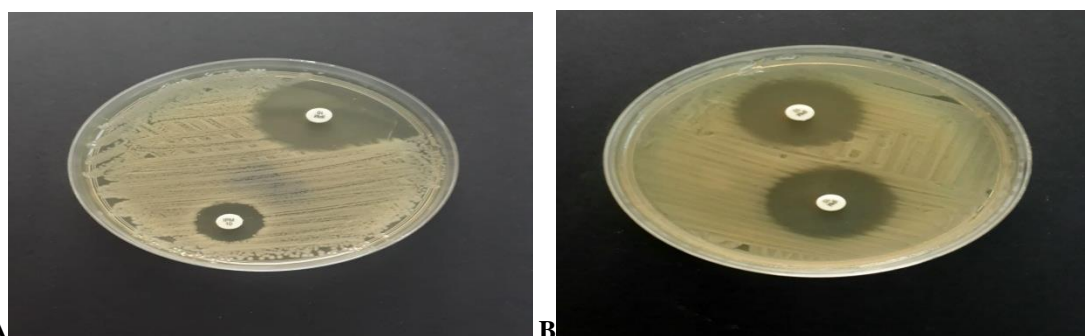**A**   **B**
Fig. 5: Screening MBLS enzymes. Combined Disk Test Positive result A. Negative result (indifference combination) B.

Screening the extended spectrum Beta-lactamases

Results of screening the (ESBLs) exhibited that various pattern of resistance were obtained headed by

isolate EC8, which was resistant to all antibiotics, used in this method, representing negative result for (ESBLs) production as shown in fig (6).
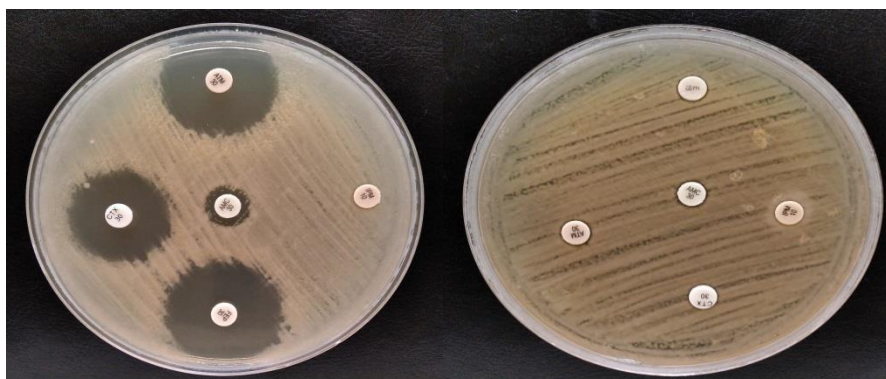
Fig. 6: Screening the ESBLs both images show negative result

Genotyping of isolated *E. coli* by ERIC-PCR

The principle of ERIC-PCR relies on the use of PCR technique to detect the non- coding sequences, which are highly conserved in number of bacterial species, the so- called Enterobacterial Repetitive Intergenic consensus sequences. Isolates that exhibited resistance to ceftriaxone (14 *E coli* isolates) were submitted to this test. Genetic analysis of the ERIC-PCR result was done by Past software version four, which revealed at similarity($\geq$ 70%) the presence of 9 different clusters fig (7), eight of them with single isolate, other three clusters with two identical pattern each table(2). Notably similar pattern was recognized with the isolates EC3, EC8, and EC14 which where MBL producers.

Table 2:Clusters Produced by ERIC-PCR Technique

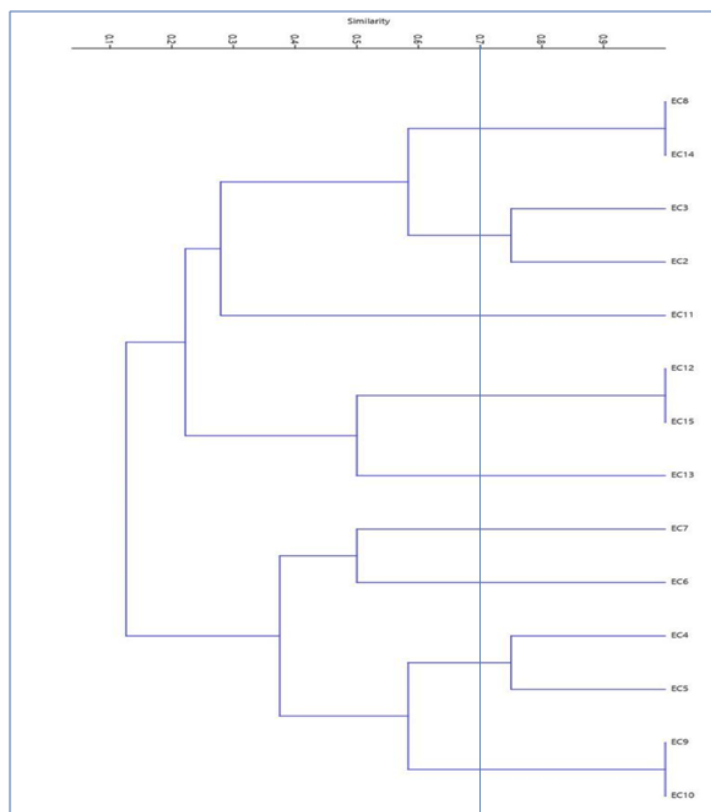| Cluster Number	Isolate
Cluster 1	EC8, EC14
Cluster2	EC3, EC2
Cluster3	EC11
Cluster4	EC12, EC15
Cluster5	EC13
Cluster6	EC7
Cluster7	EC6
Cluster8	EC4, EC5
Cluster 9	EC9, EC10

Fig. 7: Phylogenetic dendrogram of *E. coli* isolates obtained by ERIC-PCR.

DISCUSSION

Gram negative bacteria is well known for their extreme resistance patterns to β-lactams rendering the most effective ones useless. These bacteria have the capacity to cause various infections. Urinary tract infection is one of most common human disease, caused by wide range of bacterial agents headed by *E. coli*.[14]

Examining the frequency of infection with urinary tract between males and females, our study findings revealed that the rate of *E. coli* urinary tract infection in female patients was (88%). This may be due to several physiological and anatomical differences between males and females [15].

Antibiotic resistance is considered as one of the life-threating problem since there are several bacterial strains capable of overcome wide range of clinically available antibacterial agents.[16] Our study showed various patterns of antimicrobial agents' resistance. Where (100%) of isolates were resistant to tetracycline, followed by (84.6%) of them showed resistance to trimethoprim, while the rates of sensitive and resistant isolates toward ciprofloxacin were equal. The latter result is relatively greater than that of *Jadoon et al* [17] who concluded that Ciprofloxacin resistance frequency of *E.coli* were high; approximately 40% . Whereas regarding tetracycline another study showed that the most prevalent type of drug resistance was against tetracycline, where (95.0%) of isolates were resistant .[18] While other studies on erythromycin showed that resistance rate was (89%) in *E. coli,* which is higher than ours.[19] Our findings exhibited that (53.8%) of isolates were resistant to ceftriaxone, which came in accordance with that of a study in the United States, which showed that the rate of ceftriaxone-resistant *E coli* still rising reaching an estimate of 53%. [20]. Trimethoprim, a preferred treatment for UTIs, is becoming neglected due to the wide spread of resistant *E. coli*, as mentioned in a study by Bahosle *et al* [21] which is in agreement with ours.

Production of enzymes that hydrolyze the agents of β- lactams is the leading means of resistance. There are wide range of these enzymes, which are different in their molecular structure as well as their function, in particular, the spectrum of substrates they can inactivate. One of most significant beta-lactamases are the metallo beta lactamases .(MBLs), which can inactivate carbapenems.[10] Additionally it has been well documented that the activity of MBLs is reliant on zinc or cadmium, hence several screening methods incorporating the use of metal chelating agents, such as ethylenediaminetetraacetic acid (EDTA) had been suggested by *Khosravi et al*[22]. One of them is the imipenem (IPM)-EDTA double-disk synergy test (DDST) which can discriminate MBL-producing from MBL-nonproducing Gram-negative bacilli.[9]

The study outcomes showed three of tested isolates gave positive results regarding the screening methods of (MBLs) possession. In these methods, we obviously note the significance of EDTA as chelating factor of zinc, the pivotal factor for activity of MBLs. Our result is somehow similar to that by *Chika et al* [23] who revealed that out of 40 isolates 11 isolates gave positive result regarding combined disk test. While other study showed that (6%) of isolates were MBLS producer phenotypically. [24] Differently, *Panchal et al* [25] showed that (25%) of *E.coli* isolates were MBL producer.

Occurrence of spontaneous mutations may result in novel β-lactamases that can turn extended-spectrum cephalosporins, penicillins, and aztreonam inactivate. These β-lactamases are so called extended-spectrum β-lactamases (ESBLs). The core principle of screening methods regarding these enzymes is the reduced order in sensitivity for detection, cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, and aztreonam. Where results showed that the isolates were either resistant to all agents used in the test (isolate EC8), or variably resistant, that represent an overall negative result to the screening method. Where no synergistic zone towards the clavulanic acid- containing disc were observed, which (ESBLs) are susceptible to. The simultaneous production of a carbapenem-hydrolyzing metalloenzyme and an aztreonam-hydrolyzing OXA enzyme can readily lead to resistance to all β-lactam antibiotics [26], which can be noticed in the isolate number (8) of the current study. However, in the gram-negative bacteria, resistance is frequently returned to a combined action of both acquired β-lactamases, and the natural overexpression of gene products increasing impermeability and efflux features [19].

Multiple studies have implemented ERIC-PCR and other PCR-based typing methods to discriminate between closely related bacterial strains in order to explore their epidemic potency in initiating outbreaks that disseminate throughout the population. Results showed that nine different patterns were generated using ERIC-PCR. Comparing current findings with other studies revealed that this is relatively different from that of *Ardakani and Ranjbar* [6] who showed that the detected strains were classified into six clusters (E1-E6). Different ERIC patterns could be produced while in a study by Movahedi *et al* [15] results showed that 27 different patterns were obtained by using this technique on *E.coli* isolated from urinary tract infection. This technique provides an easy tool to find out the relatedness among various isolates, which reflect the origin of dissemination of certain infection or determine the similarity and dissimilarity of certain organism isolated from various hosts or sources.

Furthermore, in a study by *Dala-Costa et al* [27] on diarrhoeagenic *E. coli* results showed ability of obtaining 25 ERIC type from 122 isolates. In India a

study to compare between RAPD and ERIC-PCR patterns on EPEC strains the study concluded the similarity between the two methods and the similarity among the isolates regarding possession of virulence factors. [28] While in a study in Nigeria based on 13 isolates to compare between using 16S rRNA and ERIC-PCR, researchers concluded the benefit of using this method (ERIC-PCR) to genetically identification of closely related organisms. [29] Natively in a study by *Alttai et al* [30] who aimed to explore the genetic variation of 12 *E. coli* isolates, seven different genotypes were obtained. In a study by *Leung et al* [31] their finding showed the ability of this method to discriminate between host source where they isolated *E. coli* from three different sources.

Presence of the identical clusters may be due to that the two isolates share common features as example the antimicrobial agents' response or they may belong to same strain. The current study showed the relatedness between three pairs of isolates, which may reflect their ancestry relatedness, which means that they could be originated from the same source. By following the similarity among the closely related isolates we can conclude their environmental or host source consequently, efforts could be made in order to eliminate those pathogens.

CONCLUSION

Production of metallo beta- lactamases detected in the current study among *Escherichia coli* isolates by MBLs screening methods. ERIC-PCR method showed both time and cost saving features and discriminating power in typing *E.coli* isolates. Where it had the capacity to yield various ERIC patterns, which reflect the high genetic variation these bacteria possess though they caused the same infection.

Ethical Approval Declaration

The procedures followed in this study were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki). In addition, each participant provided written consent following a concise overview of the project.

Declarations:
Consent for publication: Not applicable
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Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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