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Original Research Paper

DETECTION OF MUTATIONS IN GYRA GENE AMONG CIPROFLOXACIN RESISTANT *ESCHERICHIA COLI* ISOLATED FROM CLINICAL SAMPLES IN BAGHDAD CITY

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Abstract: Quinolone resistant Escherichia coli (QREC) isolation rate was elevated due to the prolonged use of this drug in clinical diseases. Various mechanisms play role in developing the quinolone resistance, mutations in DNA gyrase and topoisomerase II are the most common among them. The aim of this study was to identify the mutations in *gyrA* that correlated with ciprofloxacin resistance among Iraqi *E. coli* isolates. Out of 600 clinical specimens (Urine, Blood and infected wounds), 70 (11.67%) isolates were identified as *E. coli* during 2015 -2016. The higher isolation was recorded from infected wound swabs (23.33%), while the prevalence rate of *E. coli* isolates from urine and blood samples was (12.27% and 4.49%, respectively). The resistance rate of ciprofloxacin for *E. coli* was recorded (52.86%) with minimum inhibitory concentration (MIC) ≥ 4µg/ml. Non-synonymous mutations, Ser-83 were detected in quinolone resistant determining region (QRDR) of *gyrA* in all sequenced QREC isolates (Eco U3, Eco U16 and Eco U58) and other mutations (Val-68-Ile, Ser-72-Pro, Arg-73-Cys and Lys-96-Glu) were also detected. Mutation outside this region was also displayed in Eco U16 isolate, at position (Ala-119) near to the active site of *gyrA* (Tyr-122).

Keywords: Ciprofloxacin Resistance, *Escherichia coli*, Baghdad City, Iraq.

1.Introduction

Escherichia coli is facultative anaerobe, Gram-negative bacilli, belong to Enterobacteriaceae. The lower intestine of human is the natural habitat of this bacterium, which resident in commensalism manner. However pathogenic *E. coli* strains play a critical role in severe infections to human being [1]. *E. coli* gives rise to maximal recurrent infections occur in human such as urinary tract infections, intestinal diseases, neonatal meningitis and septicemia [2]. In third world countries, hospital acquired infections and epidemics that related to urinary and gastrointestinal tracts mainly caused by *E. coli*, especially quinolone-resistant strains [3].

Quinolones are vast antibiotics utilized to cure infections that resulted from Gram-negative and Gram-positive bacteria. These antibiotics had become predominant in the treatment of urinary, urogenital, gastrointestinal, respiratory, intra-abdominal, and skin infections that caused by *E. coli*, since 1960 [4]. QREC strains were markedly developed since 1990 due to the excessive use of these drugs [5]. The different incidence of resistance

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for ciprofloxacin has been documented in various regions of the world. These differences in level of resistance can be referred to many agents such as the average of the drug utilization [6]. Mechanism of action for quinolones depends on changing the replication enzymes (DNA gyrase and topoisomerase IV) into harmful enzymes that dissociate the bacterial chromosome [5].

Quinolone resistance correlated with certain chromosomal mutations in *gyrA* and *gyrB* of DNA gyrase (topoisomerase II) and/or in *parC* and *parE* of topoisomerase IV. In *E. coli*, the mutations in ser83 in the GyrA subunit of DNA gyrase are the most common mechanism developing resistance to quinolones. The second most common mutations in *gyrA* of *E. coli* take place in glutamic acid at position 87 [7]. In addition, other resistance mechanisms include modulation in protein interactions, drug metabolism, and uptake and/or efflux pumps [8]. The present study aimed to determine the chromosomal mutations in *gyrA* gene that related with ciprofloxacin resistance among *E. coli* isolates in Baghdad city.

2.Methodology

Samples collection

A total of (600) clinical samples (481 Urine, 89 Blood and 30 infected wounds) were collected from inpatients admitted at Baghdad Medical City/ Baghdad Teaching Hospital and Child Welfare Teaching Hospital and outpatients visited Baghdad Medical City/ Teaching Laboratories during the period from October/ 2015 to January/ 2016.

Identification of Isolates

All collected samples were inoculated on blood agar, MacConkey agar and Eosin Methylene Blue media for primary identification. They were incubated aerobically at 37˚C for overnight. All E. coli isolates were identified to genus and species level based on the standard biochemical and microbiological methods [13]. API-20 E system was used to confirm the identification.

Antimicrobial Susceptibility Test

All E. coli isolates were tested as initial screening for ciprofloxacin resistance by using the disk diffusion method (Kirby-Bauer Method) as recommended by the Clinical Laboratory Standards Institute (CLSI). The results were interpreted in based on the CLSI, 2014 [14]. The minimum inhibitory concentration (MIC) for 20 representative ciprofloxacin resistant E. coli was determined using of VITEK-2 Compact / AST-GN69 card.

DNA Extraction

Genomic DNA Extraction was carried out for 20 ciprofloxacin resistant *E. coli* isolates that confirmed their MIC by VITEK-2 and 3 ciprofloxacin sensitive E. coli isolates (as a control) by using ZR Fungal/Bacterial DNA MiniPrepTM kit.

Amplification of gyrA and gyrB genes

The quinolone resistant determining region (QRDR) in gyrA gene was amplified from extracted genomic DNA (23 E. coli isolate). A 952-bps fragment of gyrA was amplified using the primer forward (5'-GCT CCT ATC TGG ATT ATG CGA TGT-3') and reverse (5'-GCC ACC ATG TTG ATA CCG AAA GA-3') [15]. The PCR reaction mixture was composed of 25µl of green master mix, 6µl of template DNA, 4µl of each forward and reverse primer (10µM), then the volume completed to 50 µl by free nuclease water. Amplification was carried out with the following thermal cycling profile: 5 min. at 94˚C and 35 cycles consisting of 10 Sec at 98˚C, 30Sec at 60˚C and 1 min at 72˚C and 5 min at 72˚C for the final extension. Amplified DNA products were resolved by electrophoresis on 1% agarose gels containing RedSafeTM Nucleic acid staining.

Sequencing for gyrA and gyrB

25µl from PCR reaction products for 3 representative QREC isolates (Eco U3, Eco U16 and Eco U58) and 3 QSEC isolates (Eco U22, Eco U28 and Eco U30) with 50µl of forward for gyrA gene were send to (Microgene) company (Korea) to determine the DNA sequencing for QRDR in this gene.

Sequencing Analysis

Sequence analysis was done by using software BioEdit/ ClustalW was used to perform the multiple sequence alignment. The sequences compare the result with NCBI control strain (E. coli K-12 substrains MG1655). DNA

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pairwise alignment, single nucleotide polymorphism and protein of gene were also done with the same software.

3. Results and discussion

E. coli is one of the most important causes of both community-acquired and nosocomial infections. It is therefore considered of clinical importance and can be isolated from different clinical samples [16]. Out of 600 specimens of the present study, 70 E. coli isolates (11.67%) were recovered. This result was matched with that of another study, E. coli isolated from (14.2%) of clinical samples [17].

Although Gram-negative bacteria do not typically reside in the dry environment of normal skin [18], the wounds infection remains major global problem and leading to many complications and increased both morbidity and mortality [19]. The reported findings of this study demonstrated that the prevalence rate of E. coli was (23.33%) in infected wounds, as indicated in Table (1).

Urinary system was the most common location to be infected. E. coli is argued to be the major pathogens in UTI cases [20]. In this study, the prevalence of E. coli isolates was 12.27% from urine samples. This result was near to that reported by Hassan (2014) in Thi-Qar governorate, who found the rate of isolation of E. coli from urine was (18.5%) [21]. Other local studies have reported higher prevalence rates. In Baghdad City, (Ahmed, 2016) documented that the prevalence of E. coli isolates isolated from urine was 65.45% [22]. Fayroz-Ali, (2012) recorded that E. coli with a rate of (55.7%) was the most frequent organism isolated in urine samples suspected of UTI in Najaf Province [23]. Yaseen (2014) in Kirkuk city also found that E. coli rate in UTI was (49.36%) [24].

In despite E. coli is a major etiologic agent of Gram-negative bacteremia worldwide [25], the results obtained in this study revealed low rates of E. coli isolation from blood sample (4.49%), which convergent to the result that was reported by El Duah (2013), who found that E. coli isolation rate from blood was (9%) [26]. In another study done in Iraq by Al-Saadi et al., (2011) in Karbala Province, found *E. coli* account for about (22.2%) that is higher than what was reported in current study [27].

More than half isolated E. coli isolates 37 (52.86%) showed resistance to ciprofloxacin (MIC ≥4 μg/ml). This result agreed with result of study in Erbil city conducted by Alsamarai et al., (2016) who found that the resistance level of *E. coli* against ciprofloxacin antibiotic was (57.6%) [28]. In another study in Baghdad city (2012), ciprofloxacin resistance rate in *E. coli* was lower (37%) [29]. In Erbil city, Kirecci et al., (2015) [30] found that the antimicrobial susceptibility test shows that ciprofloxacin was the most effective antibiotic in-vitro testing by the effectiveness against (81.43%) of *E. coli* isolates. This variation in the prevalence of resistance rate is depending on several factors, include; the patient population, geographic location, patient ward/unit, patient's prior antibiotic use, hospital and other factors [31].

The preceding decade has witnessed a very high usage of fluoroquinolones [32]. This extensive usage of fluoroquinolones has led to the emergence of Enterobacteriaceae isolates with reduced susceptibility to them [33]. Major mechanism for fluoroquinolone resistance in E. coli is mainly by chromosomal mutations in the quinolone resistance-determining region (QRDR) of gyrA and gyrB, which encode DNA gyrase subunits A and B respectively, parC and parE which encode topoisomerase IV subunit A and B respectively [34]. In E. coli and related Gram-negative bacteria, DNA gyrase is the first target for fluoroquinolones. If gyrA has resistance-conferring mutations, the primary target of fluoroquinolone switches from DNA gyrase to topoisomerase IV [35]. In present study, chromosomal quinolone related gyrA gene among 23 of E. coli isolates were conducted using specific primers for gyrA. Fig. 1. shows the result of PCR amplification. PCR products corresponding to gyrA (952 bp) were appeared in all isolates (n=23).

Table 1: Distribution of *E. coli* isolates among clinical samples.

Source of clinical samples	**Total No.**	***E. coli* isolates**	
		No.	**%**
Urine sample	481	59	12.27%
Blood sample	89	4	4.49%
Infected wound swab	30	7	23.33%
Total	600	70	11.67%

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Table 2: Mutations in quinolone resistance determining region (QRDR) for *gyrA* gene determined after a ClustalW alignment against reference sequences and control strains.

Bacterial Strain	Mutations Nucleotide Substitutions	Mutations (Amino acid Alteration)
***Eco* U3 Strain**	C153T (CGG → TGG)	Arg-52 →Trp
	G201A (GTA → ATA)	Val-68 →Ile
	T213C (TCG → CCG)	Ser-72 →Pro
	C216T (CGC → TGC)	Arg-73 →Cys
	T246A (TCG → ACG)	Ser-83 →Thr
	A285G (AAA → GAA)	Lys-96 →Glu
	C417T & G419T (CGG → TGT)	Arg-140 →Cys
***Eco* U16 Strain**	C192T (CGG → TGG)	Arg-65 →Trp
	G201A (GTA → ATA)	Val-68 →Ile
	T213C (TCG → CCG)	Ser-72 →Pro
	C216T (CGC → TGC)	Arg-73 →Cys
	T246A (TCG → ACG)	Ser-83 →Thr
	A285G (AAA → GAA)	Lys-96 →Glu
	G294T (GCG → TCG)	Ala-99 →Ser
	G306A (GAA → AAA)	Glu-103 →Lys
	G309A (GAT → AAT)	Asp-104 →Asn
	C355T (GCT → GTT)	Ala-119 →Val
***Eco* U58 Strain**	C97T (CGG → TGG)	Arg-52 →Trp
	G201A (GTA → ATA)	Val-68 →Ile
	T213C (TCG → CCG)	Ser-72 →Pro
	C216T (CGC → TGC)	Arg-73 →Cys
	T246A (TCG → ACG)	Ser-83 →Thr
	A285G (AAA → GAA)	Lys-96 →Glu
	C417T (CGG → TGG)	Arg-140 →Trp

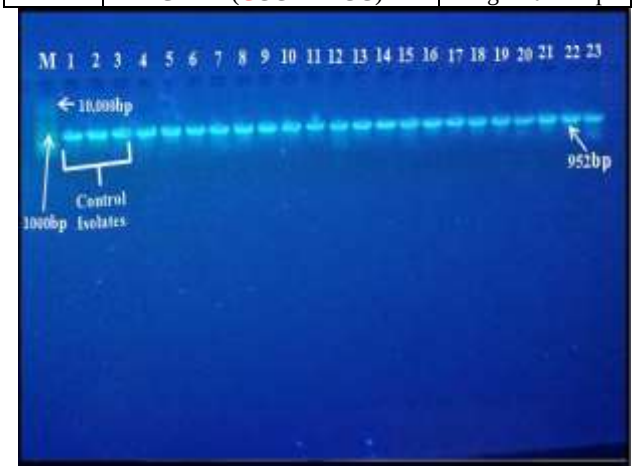

Fig.1. Analysis of *gyrA* gene of *E. coli* isolates. Lane (M):1kb bp DNA Marker. Lane(1-3): Quinolone Sensitive *E. coli* (control isolates from the present study). Lane (4-23): Quinolone Resistant *E. coli*. All isolates are positive for *gyrA* gene and they are generated (952bp) PCR product.

The result of sequence comparison revealed that ciprofloxacin resistance property was associated with alterations in nucleotide sequence of gyrA gene. Table 2. shows the quinolone-resistant isolates had several non-synonymous substitutions in the QRDR of gyrA that yield to amino acid changes. Among the studied 952 nucleotide bases compromising for gyrA gene (8, 10 and 7) sites were variable in Eco U3, Eco U16 and Eco U58 respectively.

DNA sequence analysis has shown that most of the mutations have been located in the first half of gyrA gene in the region called quinolone resistance determining region (QRDR). QRDR is a small region from amino acid 67 to amino acid 106 in GyrA (A subunit) [36]. Amplified region located between nucleotides 56-1007 [15]. This region is in close relation with the active site of gyrA (Tyr-122), which interacts with DNA and quinolone [37]. In this study, the mutation, encoding Serine at position 83 was detected in QRDR of gyrA in all sequenced QREC isolates. This result was in agreement with those in previous studies, which reported that alteration at S83 in gyrA are common and lead to high level of quinolone resistance [15,38,39].

In Eco U3 and Eco U58 isolates, five mutations were located within QRDR, and two mutations were located outside this region, while Eco U16 isolate had 8 mutations located in QRDR, and two mutations were located outside this region. Mutations outside QRDR were also reported in other study [36].

In Eco U16 isolate, one mutation located at position 119 (from Ala to Val) near to the active site of gyrA (Tyr-122) [37]. This result was identical to other study carried by Jaktaji and Mohiti (2010) in Iran, who found the mutation at Ala-119 among Iranian E. coli strains [36].

In addition to substituted mutation (Ser-83-Thr), four mutations (valine to isoleucine at position 68, serine to proline at position 72, arginine to cysteine at position 73 and lysine to glutamine at position 96) were most frequented mutations which present in all sequenced QREC (Eco U3, Eco U16 and Eco U58).This result suggested the role of these mutations in resistance to quinolone among Iraqi E. coli isolates.

Conclusion

This study underscores the critical clinical effect of quinolone-resistant E. coli (QREC) within different hospitals in Baghdad city. A highly alarming result is that more than half of the isolated E. coli strains appeared high resistance level to ciprofloxacin,

reinforcing the global concern regarding the excessive and prolonged utilization of quinolones in clinical settings.

Molecularly, genetic sequencing confirmed that ciprofloxacin resistance is associated with specific chromosomal mutations within the QRDR of the gyrA gene. The Ser-83 →Thr substitution was recorded in all sequenced resistant isolates, establishing its important role as a major driver of quinolone resistance among Iraqi E. coli strains. In addition, other mutations within QRDR (such as, Val-68 →Ile, Ser-72 →Pro, Arg-73 →Cys, and Lys-96 →Glu) were recorded as further characterizes the genetic landscape of these resistant local isolates.

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Author's Contributions

All authors made a remarkable contribution to this study in the study design, sample collection, diagnosis, analysis, and interpretation. All authors participated in writing, revising, and reviewing this article; gave terminal acceptance of the article to be published; and agreed to be responsible for all aspects of this work.

Ethics

This study was conducted under approval by the medical ethics committee at the Middle Technical University (2017). Verbal and written consent was provided by parents and agreement for publication was obtained from both participants and researchers.

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