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ORIGINAL ARTICLE

Detection of Gyrase Enzyme among Clinical Isolates of *E. coli* Resistance to some Quinolone Antibiotics

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KEYWORDS

Bacterial topoisomerases;

Quinolone;

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ABSTRACT: Multiple mutations in the quinolone-determining resistant regions of topoisomerase enzymes are usually associated with high levels of fluoroquinolone resistance in E. coli. Gyr A gene encodes (gyrase II) enzyme that changes topology by introducing transient double-stranded breaks into DNA and passing a second double-stranded DNA segment through the break before resealing it. The current investigation aims to reveal the correlation between functional gyrase enzyme and non-functional gyrase with level MIC of quinolone resistance under study. Patients with various illnesses such as urinary tract infections (UTIs), gastro enteritis (diarrhea) and wound infections were involved in the investigation. A total of 200 clinical samples were obtained. There are 30 samples diagnostic as *E. coli* clinical strain after identification by biochemical test, VITEK-2 compact system, and by molecular method using 16SrDNA marker. Antimicrobial susceptibility testing and minimal inhibition concentration (MIC) for Nalidixic acid, Norfloxacin, ciprofloxacin, levofloxacin, and Gatifloxacin was performed by broth microdilution method. According to manufacturing company (Topogen-USA); cell lysate are produced;then Bacterial gyrase assay (Topogen-USA) has been used to show the activity of gyrase enzyme by observation of negative supercoiling of *E. coli* DNA. Gyr *A* gene is detectable in all tested *E. coli* (100%); but There are only 16/30 isolates have active topoisomerase type2 with percentage 53.33% only; while; (14) isolates with non-functional gyrase enzyme since they not produce negative supercoiling DNA bands after gel electrophoreses with ethidium bromide stain. non-functional gyrase enzyme are associated with high *E. coli* resistance level against quinolone antimicrobial under study. MIC value for two groups are show significantly different concerning Nalidixic acid, Norfloxacin, while no significant difference concerning Levofloxacin and Gatifloxacin antibiotics.As a result, this research representsto be an effective method for determining the primary target of a quinolone drug in *E. coli* clinical isolates, and for the purification and characterization of DNA Gyrase so. *E. coli* are highly isolated from vagina samples 40% and then from urine samples (32%) followed by (24%) from wounds infections and (21%) from stool .There are correlation between the reduced sensitivity of quinolone under study and bacterial topoisomerases activity in *E. coli* isolates.

INTRODUCTION

During a variety of biological activities, including as replication, transcription, and cell division, the type II DNA topoisomerases (Top2) act as nanomachines that regulate DNA topology in the DNA molecule[1].

They are responsible for the transit of a DNA duplex across a double-strand break in order to execute DNA relaxing, decatenation, and unknotting processes. DNA gyrase is a crucial component of the bacterial genome's compacting process, and it is the only type II topoisomerase capable of introducing negative supercoils into DNA, a reaction that is related to ATP hydrolysis, as previously described.[2].The resistance to FQ involves structural alterations in its targets—DNA gyrase (Gyr A) which is essential for all bacterial species. This enzyme provide a balancing DNA uncoiling/coiling function critical for sustaining a sufficient level of chromosomal

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relaxation necessary for replication[3].A single QRDR mutation in gyrA gene provides only an intermediate level of resistance and usually occurs first in position 83S,while the highest minimum inhibitory and bactericidal concentration levels are provided by additional QRDR mutations in both gyrA and parC[4].

Multiple mutations in topoisomerase enzymes' quinolone-determining resistant regions (QRDR) are generally linked to high levels of fluoroquinolone resistance in *E. Coli* strains [5]. Catenated kDNA is one of the DNA markers used in the gyrase Assay. Since it is specialized for type II reaction mechanisms, kDNA is a suitable substrate for topoisomerase II tests. Even in the presence of a substantial excess of topoisomerase I, researchers may test for a type II enzyme. As a result, kDNA is a good way to measure type II activity in crude cell extracts, which are often overloaded with topoisomerase I. Prokaryotic enzymes like DNA gyrase function particularly well with the kDNA substrate. Because of the presence of topo I in partly purified fractions, assays for topo II activity based on relaxing of supercoiled DNA using crude extracts might be complex. Contaminating nuclease activity (due to Mg^{++}) degrades or nicks the supercoiled substrate, causing further difficulties. By utilizing a catenated DNA substrate made from the kinetoplast of the insect trypanosome Crithidiafasciculata, these issues can be circumvented. KDNA is a collection of interconnected DNA mini circles (usually 2.5 kb) that create extraordinarily massive molecular-weight networks. As a result, these networks do not pass through an agarose gel. Mini circular DNAs are efficiently released after incubation with topo II, which engages DNA in a double-stranded breaking and reunion cycle (decatenated). Because of their small size, the decatenated mini circles travel quickly into the gel. With topoisomerase I, this process will not occur[6].

MATERIALS AND METHODS

A total of 200 clinical samples were obtained from individuals with several illnesses, like urinary tract infections, gastro enteritis (diarrhea), vaginitis, and wound infections. The blood samples were collected from the volunteers during (December 2019 to April 2020).There are 30 samples diagnostic as *E. coli* clinical strain after identification by biochemical test ,VITEK-2 compact system, and by molecular method using 16SrDNAmarker. Antimicrobial susceptibility testing and minimal inhibition concentration (MIC) for Nalidixic acid, Norfloxacin, ciprofloxacin, levofloxacin, and Gatifloxacin was done via broth micro dilution method. Due to manufacturing company (Topogen-USA); cell lysate are produced; thenBacterial gyrase assay (Topogen-USA) has been used to show the activity of gyrase enzyme by observation of negative supercoiling of *E. coli* DNA.

Study design

Case-Control study.

Sample processing

The suitable specimens for bacteriological examination are specified in the following sections. Those samples were obtained in the correct manner to avoid contamination [7].

The used quinolone antibiotics used in the current study in order to investigate antibiotics susceptibility against *E. coli* strains are: Nalidixic acid 30 mg μL^{-1}, Ciprofloxacin 5mg μL^{-1}, Norfloxacin 10 mg μL^{-1}, Levofloxacin 5mg μL^{-1}, Gatifloxacin 5 mg μL^{-1}.

Determination of MIC

The E-test was performed to determine the antibiotics' MICs. The E-test utilized a nonporous plastic strip with a prepared antimicrobial exponential gradient running the length of 60 mm[8]. Depending on the substance, the gradient spans a concentration range of 0.002 to 32 mg L^{-1}, 0.016 to 256 mg L^{-1}, or 0.064 to 1024 mg L^{-1}. In a traditional MIC technique, this range equates to 15 twofold dilutions. The results of the analysis were obtained in accordance with[9].

Polymerase chain reaction

PCR reactions were carried out in a final volume of 25 µl, as shown in the Table 1. The thermal cycler was then used to do DNA amplification.

PCR reactions were done in a final volume of 25µl as in Table 1. Then DNA amplification was performed with the thermal cycler.

Table 1. Contents of the Reaction Mixture

No.	Contents of reaction mixture	Volume
1	Gold multiplex PCR (master mix)	5µl
2	Upstream primer	2µl
3	Downstream primer	2µl
4	DNA template	4 µl
5	Nuclease free water	12 µl
Total volume		25 µl

Preparation of Primers

The upstream and downstream primers are produced in accordance with the manufacturer's instructions (Bioneer, Korea), and they are kept at -20°C until needed. Details are tabulated in Table 2.

Table 2. Primers sequences and PCR condition

Primer name	Primer sequence (5´-3´)	Size product	Condition	Reference
Gyr A	F: 5-GTA TAA CGC ATT GCCGC R:5-TGC CAG ATG TCC GAGAT	215	94 ºC 7 min 94 ºC 1 min 46 ºC 1min 35x 72 ºC 30 sec 72 ºC 10 min	[10]
16SrDNA	F:5’-AGAGTTTGATYMTGGCTCAG R: 5’ CTACGGCTACCTTGTTACG	1500	94 ºC 5min 1x 94 ºC 1 min 54 ºC 1min 35x 72 ºC 7 min 95ºC 30sec 51.2ºC 1min 35x 72ºC 2min 72ºC 5min 1x 94ºC 45sec 53ºC 45 sec 32x 72ºC 1min	[11]

Methods

Preparation of bacterial culture extract

The following is the procedure followed by the manufacturing company: (1)10 ml of binding buffer (0.5 M NaCl, 5 mM Imidazole, 20 mM Tris-HCl (pH8), and Protease Inhibitor cocktail) are added into a tube containing cells obtained from single colony grown overnight, diluted, and grown further until the optical density of the cells reaches 0.8 to 1.0, after which cells are harvested and resuspended in the binding buffer (2). Sonication: Amplitude 50, Timer 3 mins, Pulser 2.Then cell lysate are centrifuged with 18k rpm at 4c for 25 minutes and the cell extract are collected

Sample reaction (20 ul, order of addition) of DNA Gyrase Assay Kit User Manual:

Distilled water 14 ul firstly are added to into 1.5 ml eppendorf micro centrifuge tube; then 4 ul of 5x Assay Buffer followed by addition of 1 ul of the marker KDNA , finally 1 ul of *E. Coli* bacterial extract to yield a final reaction volume range are 20-30 ul. Water, buffer, and the substrate kDNA are added to microfuge tubes before the reactions are constructed. Finally, 1/5 volume of the stop buffer was added to the reactions after the test fractions were added. The reactions were then incubated at 37° C for 15-30 minutes (or longer if necessary) before being stopped.

Gyrase gene and topoismerase detection

The sample reaction tube is incubated for 30-60 minutes at 37 degrees Celsius. 1/5 vol of stop buffer/loading dye (5 ul) is then added to the mixture. Chroform: isoamyl alcohol (24:1 mixture) is added to a volume of 20 ul, vortex momentarily, and then removes the blue (aqueous) phase from the mixture. Inject 1 percent agarose

Ethidium Bromide gel onto a 96-well plate (including Ethidium Bromide at 0.5 ug ml^{-1} in Gel and one liter of 1xTAE buffer). The TAE buffer (242 g Tris, 57.1 mL glacial acetic acid, and 100 mL 0.5M EDTA) is 50 times the concentration of the EDTA. Run the electrophoresis until the dye moves approximately 5 cm and then remove it from the gel box. Finally, the destaining process with distilled water is carried out for 10-30 minutes at room temperature before being photographed with an ultraviolet transilluminator [12].

Statistical analysis

Calculation of the comparative data through Software of Statistical Package for the Social Sciences (SPSS), version 26.0, to explain the differences of study parameters between the two groups. t-tests were employed to compare two independent groups as appropriat. It is considered a significant difference when P value <0.05.

RESULTS

In the current investigation, a total of 200 clinical samples were examined, with thirty isolates being identified as *E. coli* in the process. In this study, the internally transcribed 16S–23S rDNA spacer (ITS1) sections of *E. coli* were examined, following which the PCR product was submitted to gradient analysis, which enabled the identification of the bacterium. This study's findings indicated that all isolates (30) samples of *E. coli* isolated from diverse types of sources produced positive results (100 percent) for the presence of 16S-rDNA genes sequence, as seen in Figures 1 and 2.

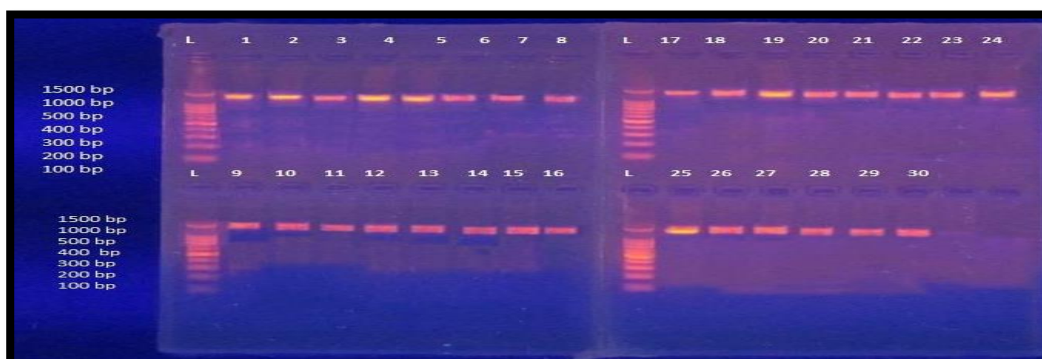

Figure1. Gel electrophoresis of PCR product (1500bp), for Escherichia coli 2% agarose gel at 5 volt /cm for 2 hours. Lane 1-30: PCR product positive for 16S rDNA genes.

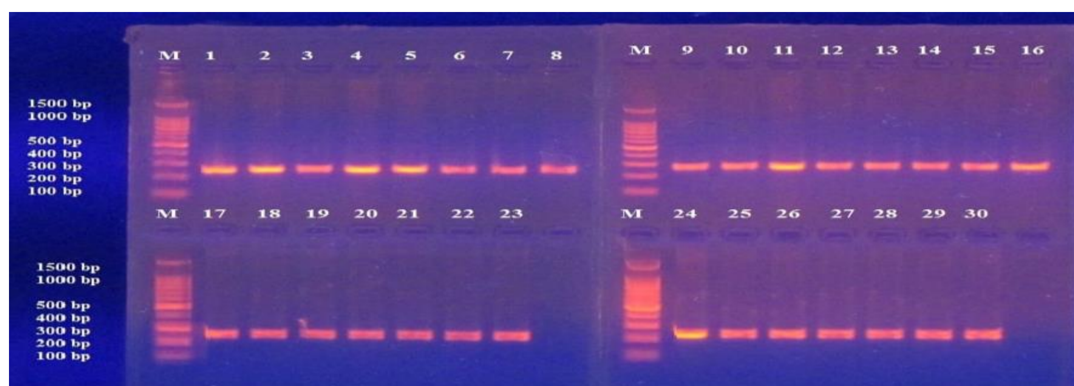

Figure 2. After staining with ethidium bromide, 1 percent Agarose gel electrophoresis at 70 volts for 50 minutes was performed for Gyr A PCR products, which were then seen under ultraviolet illumination at 280 nm. L1: 1500 bp DNA marker; all tested isolates have the gyraseA gene. L2: 1500 bp DNA marker 251bp is the size of the product.

Out of (30) isolates have been recovered, where [13] (32%) obtained from urine samples,4 (21%) from stool samples, 8 (40%) from women vagina and 5 (24%) from wounds Tables 3 and 4.

Table 3. Numbers and Percentages of *E. coli* Isolates from different sources.

Source of the sample	Total No. of samplestested	No. of E .coli	Percentage (%)
Urine	81	isolates	32%
Stool	38	13	21%
Vaginal swabs	40	4	40%
Wound swabs	41	8	24%
Total	200	5	15%

Table 4. Antibiotics resistance of different source *E. coli* isolates**.**

Antibiotics	Rate of resistance (%)	Rate of intermediate	Rate of sensitive
Norfloxacin	30 63.33%/19	5 16.66%	6 20%
Gatifloxacin	4/30 13.33 %	620.00 %	20 66.66%
Ciprofloxacin	20/3066.66%	4 6.66%	6 20%
Nalidixic acid	25/3086.36%	310.00%	26.66%
Levofloxacin	8/3027.27%	10 33.33 %	1240%

E. coli resistance to the studied quinolone antibiotics and its relation with activity of gyrase activity

There are only 16 out of 30 isolates have active topoisomerase type 2 with percentage 53.33% only while the rest;[14] isolates with non-functional gyrase enzyme. Non-functional gyrase enzyme is associated with high *E. coli* resistance level against quinolone antimicrobial under study. (Figure 3 and Table 5)

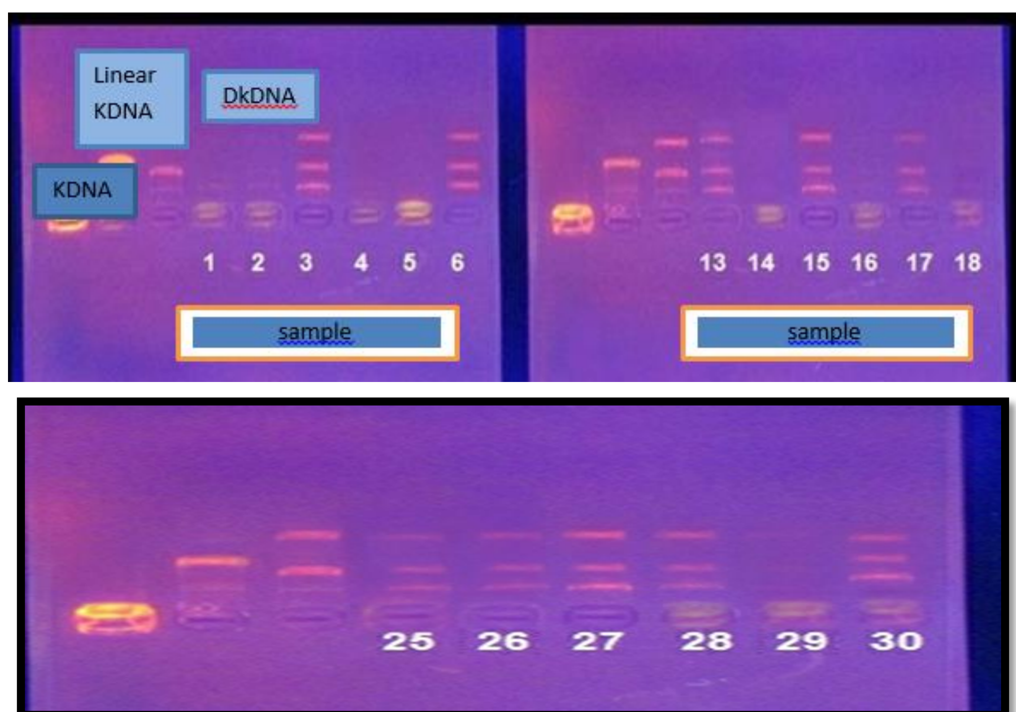

Figure 3. Topoisomerase activity in producing negative DNA supercoiling.
KDNA: is the marker for the open minicircular DNA.
DkDNA: is the marker for the action of bacterial gyrase action by producing negative supercoiling in *E. coli* DNA

Table 5. Fluoroquinolone resistance and active gyrase enzyme

Quinolone antibiotics	Total resistance isolates	Non-Functional gyrase	Functional gyrase
Nalidic acid	25	14 (56%)	11 (44%)
Norfloxacin	19	11 (57.89%)	8 (42.11)
Ciprofloxacin	20	13(65%)	7(35%)
Levofloxacin	8	4 (50%)	4 (50%)
Gatifloxacin	4	2 (50%)	2 (50%)

DISCUSSION

The isolation of *E. coli* from urine is (32%) in the current investigation in comparison to other investigation may correlate with [13] who have found that the prevalence *E. coli* is (36.84%) in urine samples and [14]who have reported that the prevalence *E. coli* is (38%) in urine samples , but the rate is lower than those obtained from some local studies as [15] who found that 7.5% UTIs were caused by *E. coli.*[16]investigated that the UTIs due to *E. coli* are quite common in patients who have undergone instrumentation or catheterization of the urinary tract and resulted in 80% of subjects had *E. Coli* among Gram-negative pathogens as a cause of catheter-associated UTIs. The current study reveals that *E. coli* are isolated from stool samples (21%) which is lower from that conducted by [17] in *E. coli* that isolated from patients with diarrheal stool are represent about 12/46 (26.1%) while lower than obtained by [18] who reveals that the prevalence *E. Coli* is (36%) from children with heavy diarrheal stool in Iraq respectively . This due to different criteria of collected samples .Concern *E. Coli* that isolated from vagina swap samples (40%) are coordinate with study done [19] that *E. Coli* prevalence in *Kirkuk* province as a causative agent of vaginitis are 50% and by [20] in Wasit province who indicated that *E. coli* is (45%) in women with vaginitis infected women . Finally *E. coli* that isolated from wound swap samples (24%) are coordinate with study done by[21] in Iraq who reveals that *E. coli* found in 30% of collected clinical samples isolated from different clinical samples. Also compatible with [22]with prevalence rate (29%). While [23]reported the prevalence rate about 24.2% . The incidence of *E. coli* isolation from urine, vagina, and wounds is influenced by a number of parameters, including the virulence of isolates, the health state of patients, and the effect of environmental circumstances.

According to Antibiotics resistance of different source *E. coli* isolates. It was found that the bacterial isolates showed resistance to first generation (Nalidixic acid 86.36%),second generation (Ciprofloxacin 66.66%) and (Norfloxacin 63.33%), third generation (Levofloxacin 27.27%), and fourth generation (Gatifloxacin 13.33%). These results were consistent with those obtained by[24] as well as those obtained by[25] in Iraq, who found that resistance to ciprofloxacin, levofloxacin, and nalidixic acid ranged from 50 to 70%, and resistance to nalidixic acid was more than 70%. However, a study conducted in Pakistan by[26] found that the rates of resistance to ciprofloxacin, levofloxacin, and norfloxacin remained at 60%, 58%, and 57%, respectively, despite the introduction of new antibiotics. There was a significant difference between the findings of the present investigation and the findings revealedin a previous investigation performed in Nigeria[27], which displayed that the ratio of resistance against ciprofloxacin was higher (27%). It was discovered that plasmid-mediated antibiotic resistance was common among *E. coli* isolates. The raised resistance rate among isolates was primarily due to overuse of antibiotics, disuse of medical prescriptions, and the use of empirical therapy. All of these factors contribute to raise antibiotic pressure and raised likelihood of resistance transfer. When it comes to vaginal samples, *E. coli* is strongly isolated in 40% of cases, followed by urine samples in 32%, wound infections in 25% of cases, and stool samples in 21% of cases.

The current study reveals that all clinical isolates of *E. coli* contain *Gyr* A gene as shown in Figure 1. Gyr *A* gene is detectable in all tested *E. coli* in this study (100%). This result is consistent with the findings of[28], who reported that *gyr*A was identified in 100 percent of *E. Coli* isolates tested. GyrA molecules that have not

been integrated into functioning gyrase might be the cause[29] since there are some of isolates that lose their ability to produce active and functional gyrase enzyme which is the only enzyme that produce negative supercoil . In terms of resistance evolution, mutations that result in the up-regulation of drug efflux mechanisms occur at a higher rate than mutations in the structural genes for topoisomerases, which are responsible for the structure of the enzyme. Because the genetic target in efflux-regulating genes is far greater than the particular amino acid changes necessary for Gyrase-mediated resistance, this is the case[30] and[24].

Topoisomerase activity in tested E. Coliclinical isolates

There are only 16 out of 30 isolates have active topoisomerase type 2 with percentage 53.33% only while the rest; [14]isolates with non-functional gyrase enzyme. non-functional gyrase enzyme are associated with high *E. Coli* resistance level against quinolone antimicrobial under study. Interestingly, this finding is consistent with that of [31], who finds that fluoroquinolone resistance is produced by changes in chromosomal genes encoding quinolone targets, namely DNA gyrase and topoisomerase IV, in susceptible bacteria. As a result, PMQR is not the only mechanism that is resistant to quinolones as well as efflux pump mechanism.

E. coli resistance to the studied quinolone antibiotics and its relation with activity of gyrase activity II

There is a relationship between the number of gyrA in a population and the amount of quinolone resistance in that population. As suggested by the GyrA codon 83 mutation [32]supercoiling DNA changes might be generated in clinical *E. Coli* isolates, which could affect the expression of virulence proteins in the bacteria. The research group of [33] has also proven that changes in supercoiling have an impact on basic biological processes, such as transcription. quinolone resistance is resulted from mutations in the QRDR of the gyrA, which is the main mechanism of resistance[34]. Previous investigations revealed that this mutant enzyme exhibited altered DNA cleavage selectivity even in the absence of a pharmacological treatment regimen[35]. In E. coli, gyrA mutations are most commonly seen at serine-83, where leucine or tryptophan is replaced, resulting in increased resistance to quinolone antibiotics. The substitution of alanine for serine, on the other hand, results in a lesser degree of resistance[36]. Asparagine and/or valine are commonly substituted for aspartate-87 in a number of different mutations[37].

Gyrase in E. coli and its relation with MIC value of quinolone antibiotics

Out of 30 isolates, 16 (53.33 %) were with active gyrase enzyme since the enzyme introduce negative supercoiling. It is not possible to predict the degree of supercoiling in DNA since the structure of DNA is constantly changing in response to environmental stress and cellular activities like transcription, DNA replication, and recombination[38]. Concerning MIC level; strains with non-functional gyrase enzyme appears to have a higher average than those with functional gyrase enzyme as reveals in Figure 4.

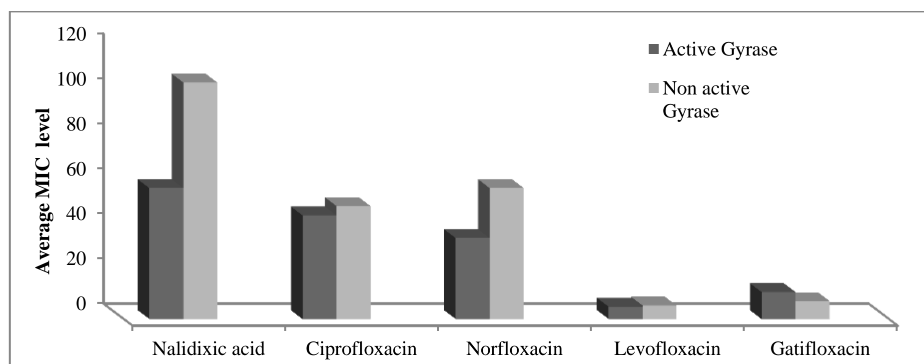

Figure 4. The Distribution of Resistance *E. Coli* isolates According to Their MIC (µg mL^{-1}) average for quinolone antibiotics under study.

Nalidixic acid MIC value in 14 isolates with non-functional isolates was 4 isolates with MIC value 32 µg mL^{-1}, 5 isolates with 64µg mL^{-1}, 2 isolates with MIC value 128 µg/mL and 3 isolates with MIC value 256

µg/mL; while in functional gyrase-isolates; the MIC value 32 µg mL^{-1} are in 6 isolates, 64 µg mL^{-1} in 3 isolates and 2 isolates with 128 µg mL^{-1}. There are a significant difference between both type of strains (p>0.01). The range of MIC value against resistance nalidixic acid was higher than other quinolone antibiotics under study (32-256 µg mL^{-1}) which is similar range to what reports by[39, 40].

Concerning ciprofloxacin MIC value in 13 isolates with non-functional isolates ; there are 5 isolates with MIC value (1 µg mL^{-1}), 2 isolates with MIC (4 µg mL^{-1}), 4 isolates with 64 µg mL^{-1} and only 1 isolate for each concentration (128 µg mL^{-1} and 256 µg mL^{-1}) of MIC. Functional gyrase strains distribution according to MIC concentration; there are 3 isolates with concentration (1 µg/mL); 3 isolates with MIC value (64 µg mL^{-1} and finally 1 isolates with concentration 128 µg mL^{-1}. Form results of study; there is no significant difference in MIC average value of strains with functional gyrase (46.1%) and strains with non-functional gyrase (50.2%). In addition to the existence of additional processes implicated in fluoroquinolone resistance as reduced membrane permeability and overexpression of efflux pumps, this might be owing to the presence of other factors.

This result has also been documented by[40-43] or due to the strains under study, which has displayed that a point mutation in the gyrA protein can only slightly reduce the susceptibility of *E. coli* to ciprofloxacin , but that high-level resistance is associated only with double mutations in the GyrA protein, as reported by several research's in the past few years [38, 40, 44]. According to other research, changes in the parC gene may be the primary source of ciprofloxacin resistance[45].

Concerning Norfloxacin MIC average value; *E. coli* strains with non-functional gyrase (58.2 µg mL^{-1}) have been significantly (P>0.01) higher than *E. coli* strains with functional gyrase (36 µg mL^{-1}). MIC value in 11 isolates with non-functional isolates; there are 4 isolates with MIC value (32µg mL^{-1}), 6 isolates with MIC (64 µg mL^{-1}) and 1 isolates with 128 µg mL^{-1}. Functional gyrase strains distribution according to MIC concentration; there are 4 isolates with concentration (16 µg mL^{-1}); 3 isolates with MIC value (64 µg mL^{-1} and finally 1 isolates with concentration 32 µg mL^{-1}. The results are compatible with [46-48].

Concerning levofloxacin MIC average value; *E. coli* strains with non-functional gyrase (6 µg mL^{-1}) have no significantly (P>0.01) difference and *E. coli* strains with functional gyrase (5.5 µg mL^{-1}). MIC value distribution in 4 isolates with non-functional isolates was 2 isolates with MIC value (4 µg mL^{-1}) and 2 isolates with concentration (8µg mL^{-1}). Functional gyrase strains distribution according to MIC concentration; there are 1 isolates with concentration (2 µg mL^{-1}) and 1 isolates with MIC value (4 µg mL^{-1}) and 2 isolates with MIC value (8µg mL^{-1}).The results are compatible with [49-51] in that there was not a clear correlation between frequency of distribution of each alteration type and levofloxacin MIC values, which ranged from 2 to 64 µg mL^{-1}.

Finally; Gatifloxacin MIC average value has been indicated; that *E. coli* strains with functional gyrase (12 µg mL^{-1}) have higher average of MIC value than *E. coli* strains with non-functional gyrase (8µg mL^{-1}). This result are compatible with[52]who reports even when the *gyrA* has double mutants, the C-8-methoxy group improved lethal action against most of the *gyrA* mutants , as was the case when inhibition of growth was measured.

CONCLUSIONS

Using this test, it appears to be possible to detect which *E. coli* clinical isolates are the major targets of quinolone drugs, as well as to purify and characterize type II topoisomerase enzymes (DNA Gyrase) that have the capacity to supercoil or do not have the ability to supercoil. There appears to be a relationship between the lowered sensitivity (lower MIC value) of the quinolone under investigation and the activity of bacterial topoisomerases in *E. coli* isolates.

ETHICAL CONSUDERATION

Volunteers were asked permission prior to take any blood specimen. In addition, the investigation concept was accepted by the Research Ethical Committee at the College of Medicine / University of Babylon.

Conflict of interest

The authors declare that there is no conflict of interest.

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