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Molecular Characterazation and *Gyrase* Production In *Eschrichia coli* Resistance To Fluoroquinolones Antibiotics Isolated From Different Clinical Samples

A Thesis

Submitted to the Council of College of Medicine, University of Babylon in Partial Fulfillment of The Requirement for the Degree of Doctorate of Philosophy In Medical Microbiology/ Molecular Bacteriology

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بسم الله الرحمن الرحيم

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

صدق الله العلي العظيم

[المجادلة: الآية 11]

Dedication

To my Father, who have ordained delineate me,

To my mother, who have given me color

To my heart dicky –bird …… My sweet heart the clearest Suns

To my darlings my husband, sister and brothers

To my friends of high and beautiful

Iman

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Iman

Summary

Escherichia coli showed increasingly high antimicrobial resistance against Fluoroquinolone antibiotics due to its ability to produce varies virulence factors. Quinolone resistance most often develop in *E.coli* as a result of spontaneous chromosomal mutations in the quinolone resistance determining region (QRDR) of the genes encoding DNA *gyrase* and via plasmid-mediated transfer through acquisition of plasmid mediated quinolone resistance (PMQR). Additionally, PMQR determinants have been shown to coexist with resistance genes causing resistance toward other antimicrobials, which enables co-selection. The aims of this work is to study molecular characterization of quinolone resistance genes *qnrA, qnrB, qnrS, gyrA* , *marRAB* and sox*RS* genes as well as to analysis the role of *gyrase* enzyme in eliciting high quinolone resistance in *E.coli* clinical samples.

A total of 200 clinical samples collected from patients suffering from different infections such as UTI, gastro enteritis(diarrhea), vaginitis and wound infections; who attended to two main hospitals in Holy Karbala governorate : Al-Husseini General Teaching Hospital, Karbala Hospital for Maternity and Pediatric during a period ranging from (February to June 2020). Samples were taken from patients with the range of age from 5 to 66 years of male and female.

Only 30 out of 200 samples gave positive for *E. coli* from both sex and different ages after identification by phenotypic characterization, biochemical tests ,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.

Summary

All strains were screened for PMQR genes namely *(qnr*A, *qnr* B, *qnr*S) as well as efflux pump operons(*mar*RAB, *rob*, *sox*RS) and *gyrase* A encoding gene ; by PCR method after DNA extraction from clinical isolates of *E.coli.* In addition to *gyrase* enzyme activity.

The results showed that *E. coli* are highly isolated from vagina samples (40%) followed by urine samples (32%), then from wounds (24%) and (21%) from stool .The large occurrence of *E. coli* isolates was at the age group (31-45) years at a rate (33.33%), while the lower occurrence was at age group (> 60) year old (10%). Women have a notable increase frequency of *E . coli* versus males with ratio (female: male 87%:13%).

It was found that the bacterial isolates showed high resistance to first generation (Nalidixic acid 86.36%), and second generation (Ciprofloxacin 66.66%);(Norfloxacin 63.33%), while to the third generation (Levofloxacin 27.27%) and fourth generation (Gatifloxacin 13.33%).

Molecular investigation showed the total *E. coli* isolates that harboring at least one of *qnr* genes are 21 isolates (70%); However, Only 12 isolates gave positive for *qnr* A gene (40%), and 13 isolates gave positive results for *qnr*B gene (43.3%), where is 16 isolates gave positive results for *qnr*S gene (53.33%).

The multiple antibiotic resistance (*mar*) operon of *Escherichia coli* is found in 90% (27/30) of isolates as well as for right origin binding protein (*rob*) regulon have been found in 27 isolates with prevalence (90%);while 22 isolates have superoxide response regulon (*sox*RS) gene at a rate(73.33 %).

However , it was shown that all *E.coli* isolates have at least two of multidrug resistance genes; where 16 isolates (53.3%) have all the genes under this study(*mar*RAB, rob, and *sox*Rs).

Gyrase gene type A (*gyrA*) gene was detected in all tested of *E.coli* obtained in this study .

Summary

Functional *gyrase* was calculated according to the presence of three bands; which was catenated DNA, linear DNA, and decatenated DNA while nonfunctional *gyrase* the presence of one bands; which was catenated DNA .

It was found that only 16 isolates gave positive results for *gyrase* activity (53.33%) or so named functional *gyrase*. Non-functional *gyrase* was found only in 14 isolates.

However functional *gyrase* against Nalidixic acid resistance isolates was positive at a rate (56%) and for Ciprofloxacin (35%) with functional *gyrase* enzyme. Norfloxacin- resistance *E.coli* strains (42.11%). Levofloxacin and Gatifloxacin resistance *E.coli* strains were the same in both of non-functional and functional *gyrase* activity isolates with a rate (50)%.

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List of Abbreviations

Abbreviation	Key
CFU	colony forming units
CFX	Ciprofloxacin
CLSI	Clinical Laboratories Standard Institute
DDT	Disk Diffusion Test
dkDNA	Decatenated DNA
Dps	DNA-binding protein from starved cells
Fis	factor for inversion stimulation
FQ	Fluoroquinolone
Gyr	Gyrase enzyme
HGT	horizontal gene transfer
H-NS	histone-like nucleoid structuring protein
IHF	integration host factor
KDNA	Catenated DNA
Lrp	leucine-responsive regulatory protein
mar	Multiantibiotics resistance
NAPs	nucleoid-associated proteins
PCR	Polymerase chain reaction
PEC	pathogenic *E. coli*

PMQR	Plasmid Mediated Quinolone Resistance
qepA	Quinolone efflux pump
qnr gene	quinolone resistant gene
QRDRs	quinolone resistance-determining regions
RND	resistance-nodulation-division family
rob	right origin binding protein
soxRS	superoxide response regulon

1.1. Introduction

Escherichia coli belongs to the family Enterobacteriaceae and it is a Gram negative, facultative anaerobic bacterium. It is genetically heterogeneous and usually non pathogens that are predominant microflora of the alimentary tract of healthy individuals (Jang *et al* ., 2017).However, it may be a causative agents of many diseases in human in urinary tract due to its ability to produce varies virulence factors.

E.coli showed antimicrobial resistance which is increasingly high (Allocati *et al* ,2013) due to increasing the use of antibiotics which has been recognized as a major factor driving *E. coli* resistance over the last decades (Bell *et al* ,2014).

Fluoroquinolones (FQs) are broad spectrum antibiotics widely used in the treatment of infections caused by *E.coli* with high tissue penetration, and ease of use (Hassanshahi *et al*,2020) . Indeed, acquisition of virulence features, chiefly by mobile molecules like transposons, insertion sequences, bacteriophages and plasmids (Henry *et al* ., 2017).

Quinolone resistance most often develop in bacteria as a result of spontaneous chromosomal mutations in the quinolone resistance determining region (QRDR) of the genes encoding DNA gyrase or topoisomerase IV (Gosling *et al*., 2012; Hooper and Jacoby, 2015) or by point mutations in the DNA *gyrase* and via plasmid-mediated transfer (Holmes *et al* , 2016) through acquisition of plasmid mediated quinolone resistance (PMQR) determinants, including *qnrS, qnrA, qnrB* (Gosling et al.,2012;Machuca *et al*.,(2014),(2016); Yamaski *et al* ,2015).Additionally, PMQR determinants have been shown to coexist with resistance genes causing resistance toward other antimicrobials, which enables co-selection (Huang *et al.*, 2012; Slettemeås *et al*., 2019).

Quinolone resistance frequently develop in a stepwise fashion, where a single mutation in *gyrA* is often the initial step (Huseby *et al.*, 2017).

DNA *gyrase* and *topoisomerase* IV (type II topoisomerases) are the prime targets of quinolones. DNA *gyrase*, a tetramer of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes respectively, is responsible for uncoiling the intertwined circles of double-stranded bacterial DNA that arise after each round of replication, hence relieving topological stress. Quinolones obstruct the normal process of DNA synthesis by establishing a stable ternary complex with *gyrase* and DNA (Sharma *et al* ,2009). The quinolone-*gyrase*-DNA complex prevents the broken strands of DNA from resealing thus leading to accumulation of double stranded breaks, disruption of cell growth and finally apoptosis (Varughese *et al* ,2018).

DNA *gyrase* is the main target in gram-negative organisms (for example, *Escherichia coli*) while quinolones target *topoisomerase* IV in gram-positive organisms (for example, *Streptococcus pneumoniae*) (Hooper and Jacoby, 2016).

Further mutations in either the same gene or in other potential quinolone resistance genes, e.g., *marR*, can confer increased resistance toward quinolones, but may also be associated with a fitness cost (Marcusson *et al.,* 2009). However, some mutation combinations have been shown to increase both relative fitness and resistance levels, suggesting that resistant mutants may have an advantage whether quinolones are present or not (Marcusson *et al.,* 2009; Huseby *et al.,* 2017). It is observed that *mar*A, *sox*S and *rob* are activator of membrane fusion protein AcrA and the outer membrane protein TolC which belongs to the resistance-nodulation-division (RND) family of efflux pumps.Overexpression of these regulators have resulted in multidrug resistant phenotype (Huseby *et al.,* 2017).

The aims of this work to detect:

Molecular characterazation and *Gyrase* production in *Eschrichia coli* resistance to fluoroquinolones antibiotics isolated from different clinical samples by using the following objective:

1- investigate the phenotypic and genotypic genomic-mediated resistance mechanisms in *E. coli* strains isolated from different clinical samples of patients in Karbala province - Iraq.

2-Study molecular characterization of quinolone resistance genes *qnrA*, *qnr*B, *qnr*S, *gyr*A , *mar*RAB and soxRS genes by PCR technique .

3- Analysis the role of *gyr*A in eliciting high quinolone resistance in *E.coli* isolates.

1.2. Literatures Review:

1.2.1. *Escherichia coli*

Escherichia coli represents the most frequent sources of blood stream and urinary tract infections worldwide. A continual increase in *E. coli* antibiotic resistance burdens medical facilities throughout the world by causing difficult to treat infections among patients (Hassan *et al*,2020) by limiting the choices of antimicrobials that can be used in the treatment of bacterial infections (Nadda *et al* ,2016).The common eubacteria *E. coli*, has single circular chromosome with genomic size of ~ 4:64 Mbps.

Escherichia coli, chromosome is organized into four structural macrodomains (Ori, Ter, Left, and Right chromosomal arms) and the two unstructured regions, each of which consists of small (average~10 kb) topologically independent microdomains (Hołówka and Zakrzewska-Czerwińska, 2020).This hierarchical structure ensures the accessibility of particular chromosomal regions for DNA-dependent processes, such as replication, transcription, DNA repair, and recombination (Jeon *et al*, 2017; Joyeux, 2019).

Although the contour length is ~ 1:5mm, it is packed compactly into a nucleus like structure called as nucleoid, inside a1:5-2:5μm long cell. (Remus *et al* ,2019). Nucleoid in condensed form, having rosette like structure and arranged into topologically independent loops and sustained with the help of proteins and RNA molecules.The nucleoid composition suggest that the whole nucleoid is composed of ~ 80% DNA, ~ 10% RNA polymerase and ~ 10% protein (Gupta *et al*, 2020).

During transcription, the moving RNA polymerase creates a region of positive supercoiling (overwinding) in front of itself and negative supercoiling (underwinding) behind. In *Escherichia coli*, these negative supercoils are relaxed by the action of Topoisomerase I and the positive

supercoils are relaxed by ATP-dependent DNA gyrase, so that the balance between the activities of the two enzymes determines the overall level of supercoiling(Lal *et al* , 2016).This is referred to as unconstrained supercoiling. Supercoiling can also be constrained by a group of DNA binding proteins referred to as nucleoid-associated proteins (NAPs) that are functionally analogous to histones in a broad sense and play a crucial roles in the ability of a bacterium to adapt to unfavorable conditions, particularly stress (Talukder and Ishihama, 2015).

NAPs are highly abundant and constitute a significant proportion of the protein component of the nucleoid (Shahul and Hameed *et al.*, 2019).

There are at least 12 NAPs identified in *E. coli* include HU (heat-unstable protein), IHF (integration host factor), H-NS (histone-like nucleoid structuring protein), Lrp (leucine-responsive regulatory protein), Fis (factor for inversion stimulation), and Dps (DNA-binding protein from starved cells) (Hołówka and Zakrzewska-Czerwińska, 2020).

The HU is present in ~30,000 dimers per cell and is the most conserved NAP across bacterial species. It binds across the bacterial chromosome and has been shown to constrain negative supercoils on DNA *in vitro* (Dillon and Dorman, 2016) . In *Escherichia coli*, HU was shown to play important mechanistic roles in very basic cellular functions, such as initiation of replication, chromosome partitioning and organization (Berger *et al* ,2016) .

The FIS (Factor for Inversion Stimulation) is another important nucleoid associated protein. It binds to DNA in both, specific and non-specific manner, and plays a role in gene regulation as well as nucleoid organization(Gupta *et al* ,2020).

1.2.2. Pathogenicity of *E. coli*:

The most important biological properties contributing to the pathogenesity of this bacteria is adhesion to the epithelial cells, then ability to invade and multiply within these cells, and their capacity to produce the enzymes besides resist killing by phagocytosis process (Alan and Douglas, 2010). Pathogenicity has been correlated with the presence of different genes encoding virulence factors organized on large chromosomal region, called pathogenicity islands (Franzin and Sircili, 2015) . It has been shown, further, that this pathogenicity islands can transfer horizontally between distinct *E.coli* isolates when are located on plasmids, bacteriophages, or even in bacterial chromosome (Croxen and finaly, 2010). *E.coli* isolates are responsible for urinary tract, intra-abdominal and soft-tissue infections, meningitis and pneumonia (Dale and Woodford, 2015), often leading to bacteremia **(**Matsuura *et al.*, 2018).

The most important biological properties contributing to the pathogenesis of these bacteria are their interference with the complementary immune system(or alternative pathway) and their ability to resist the killing by the phagocytosis (Alan and Douglas, 2010). *Escherichia coli* have many virulence factors such as: Biofilm formation and toxins such as Shiga like toxins (*Stx1* and *Stx2*) and hemolysin *(hlyA*), Cytotoxic necrotizing factors (*CNF*). Adhesions (type I fimbriae),(p-fimbriae), Attaching and effacing (A/E),Adherence (intimin) ,capsule, iron-scavenging systems (siderophores), iron transporting system (*feoB*), IbeA invasins(invasion of brain endothelial cells) (Yun *et al*., 2014).

Uropathogenic *E.coli* raised from non-pathogenic strains by acquiring new virulence factors (including toxins and siderophores) by DNA

horizontal transfer which often organized into pathogenicity islands located on chromosomal or mega plasmid (Tourret *et al.*, 2016).

According to state of patients , the pathogenicity of the bacteria and the infection ranged from asymptomatic bacteriuria to chronic Pylonephrities **(**Kudinha , 2017).

1.2.3. Epidemiology of *E.coli*:

Escherichia coli is present in the digestive tract of postpartum children directly within the intestinal flora, but some *E.coli* strains have the potential to become opportunistic and cause various diseases, especially when they are transmitted from the intestines to other parts of the body,especially urinary infections or any member of the cavity when appropriate conditions are available for infection such as weak immunity to the body, as well as found in the intestines of animals. *E. coli* is a source of hospital infections, especially in children with low immunity and under nutrition, leading to the taking of antibiotics in abundance, and this is why the strains of bacteria are resistant to antibiotics (Brooks *et al.*, 2010 ; Korzeniewska *et al.*, 2013).

The failure of treatment due to antibacterial resistance to antibiotics, especially the first-choice antibiotics in the treatment of infections such as the third-generation of cephalosporin, leads to recurrence of infection and thus increases the mortality rate. Bacterial resistance to antibiotics is the acquisition of bacterial strains of resistance determinants and expansion in the acquisition of resistance already present in *E. coli* bacteria that is most important (Barber *et al.*, 2019; Costa *et al.*, 2019).

Finally *E. coli* is the most common causes of morbidity of children with diarrhea around the world. It is also one of the most common bacterial

species responsible for UTIs, especially in pregnant women, with a prevalence of 90% (Alizade,2018).

1.2.4. Quinolone antibiotics:

The quinolone antibiotics (Figure 1- 1) are the most successful class of topoisomerase inhibitors to date. They are synthetic antimicrobials (Bisacchi,2015) used to treat different bacterial infections caused by both Gram-positive and Gram-negative bacteria, including, but not limited to, urinary tract infections (UTIs), pyelonephritis, gastroenteritis, sexually-transmitted diseases, such as Gonorrhoea, tuberculosis, prostatitis, community-acquired pneumonia and skin and soft-tissue infections (Gillespie, 2016).

A major reason for the comparative success of FQs is that they target the bacterial type II topoisomerases, DNA *gyrase* (gyrase) and DNA topoisomerase IV (topo IV) .DNA topoisomerases are enzymes that catalyse the interconversion of different topological forms of DNA (e.g., relaxed-supercoiled and catenated-decatenated) and are crucial for several DNA-associated processes, such as replication and transcription. (Bush, 2015).

All topoisomerases can relax DNA, but only *gyrase* can introduce negative supercoiling .*Gyrase* is present and essential in all bacteria but absent from higher eukaryotes (e.g., humans), making it an ideal target for antibacterials; however, gyrase does occur in plants and plasmodial parasites(Bush,2020).

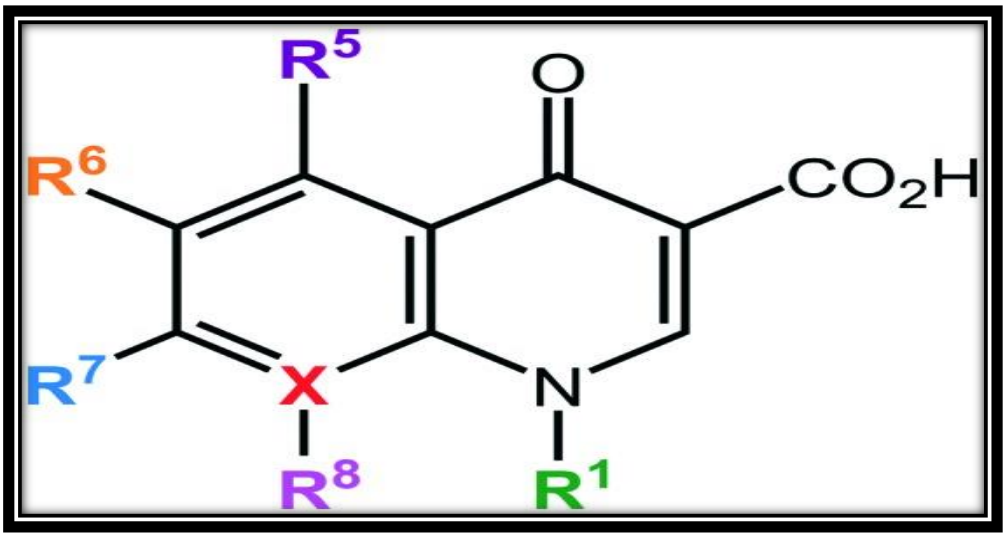


Figure (1-1):Core structure of quinolone antibiotics.There are 6 important positions for modifications to improve the activity of the drug: R_1,R_5,R_6,R_7,R_8, and X, X= C defines quinolones, X=N defines naphthyridones. (Pham *et al* ,2019).

1.2.5. Quinolones classes:

The first quinolone antibiotic is generally considered to be nalidixic acid, which was the prototypical quinolone, nalidixic acid (technically a naphthyridone), was discovered in the 1960s as a by-product during the synthesis of anti-malarial quinine compounds 7-chloro-1-ethyl-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid. It was as part of a series of 1-alkyl-1,8-naphthyridines (Correia,*et al* ,2017) acting by inhibiting the activity of bacterial topoisomerase type II enzymes, inhibiting the bacterial replication.(Pham *et al* ,2019).

In 1967, nalidixic acid was approved for clinical treatment for urinary tract infections (UTIs) caused by Gram negative bacteria. However, its use was limited because of the narrow spectrum of activity, low serum concentrations achieved, high inhibitory concentration required, and several adverse effects (Bisacchi, 2015). The cellular pathway targeted by nalidixic acid was revealed already in 1964. By measuring the incorporation of C14-labeled thymine in DNA, it was shown that it

inhibited the DNA synthesis. Five years later, in 1969,Hane and Wood genetically mapped mutations in two distinct genes, *nalA* and *nalB*, that conferred different levels of nalidixic acid resistance. *nalA* was subsequently identified as *gyrA*, encoding the subunit of the DNA gyrase responsible for nicking and re-ligation of the DNA (Nicolas-Chanoine *et al*,2014). Quinolones with fluorine in their structure are called FQs, (Zhang, *et al* ,2018).

Numerous of FQ derivatives have been synthesized by modifying the chemical structure of the 1,8 naphthyridine core in order to increase their antibacterial efficiency and improve their pharmacokinetics (Suaifan and Mohammed, 2019). These modifications have led to the synthesis of second-generation compounds; norfloxacin and ciprofloxacin, two antibiotics with increased activity against Gram-negative bacteria, which are still marketed (Recacha *el al* ,2017) .

After almost three decades in clinical use, ciprofloxacin remains one of the most commonly prescribed antimicrobial drugs, being listed by the World Health Organization (WHO) as an essential medicine and a critically important antibiotic (Collignon *et al*., 2016).

The major differences from the first to the second-generation compounds were the addition of a fluorine at position C6 and a piperazine or methyl-piperazine substituent at C7(WHO, 2015). These two additions to the quinolone core, improved both the bacterial spectrum, but also the pharmacokinetic and pharmacodynamics significantly (López Cerero,2014).

Since then both third and fourth generation fluoroquinolones has made its way into the clinic. The third generation fluoroquinolones like, levofloxacin, sparfloxacin and grepafloxacin expanded the bacterial spectrum to include streptococci and had prolonged half-lives. The fourth generation fluoroquinolones, was the first generation with activity against

anaerobes like, *bacteroides fragilis*, in addition to an enhanced activity against Gram positives (Opperman and Nguyen,2015). Furthermore, the 8-methoxy group possessed by two of the fourth generation drugs, gatifloxacin and moxifloxacin, eliminated the phototoxicity observed for earlier generations (Opperman, and Nguyen,2015).

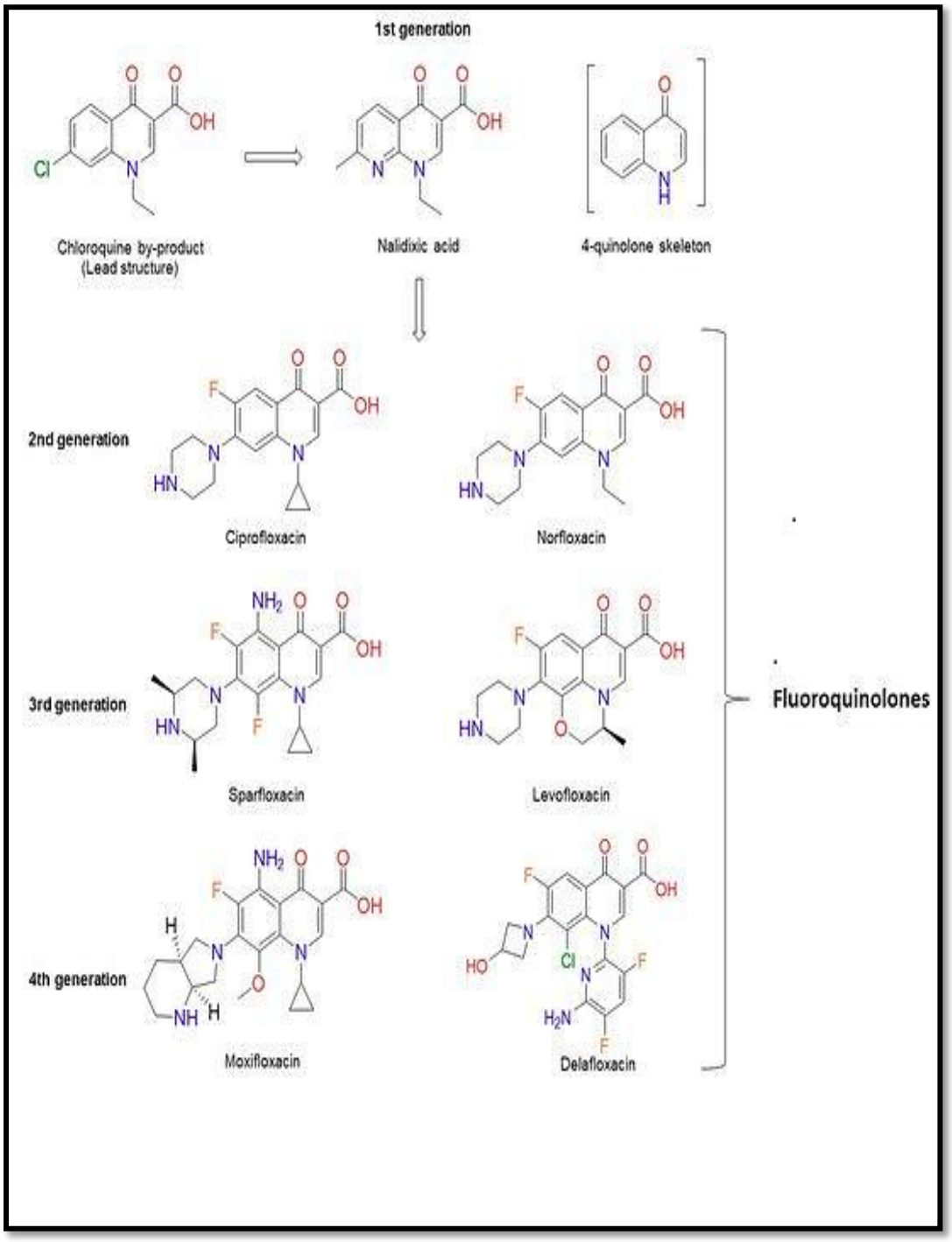
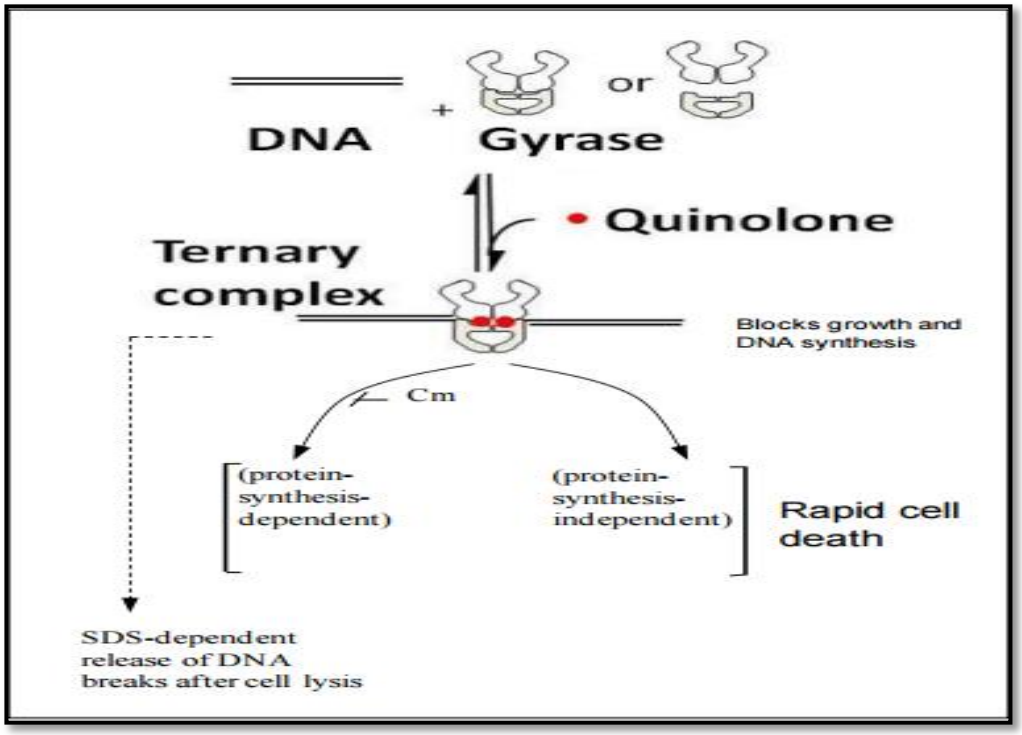


Figure (1-2):Generation of Quinolone antibiotics (Bush *et al* , 2020)

1.2.6: Fluoroquinolone mechanism of action:

Fluoroquinolones form a ternary complex with the DNA and DNA *gyrase*/*Topo* IV, which then becomes trapped, disrupts replication, and triggers various cell death mechanisms (Drlica, *et al* 2009) as showed in figure (1-3).



Figure(1-3):Mechanism of Fluoroquinolone Action(Drlica *et al*, 2009)

The quinolone binds the DNA topoisomerase complex to form a ternary complex, which may lead to cell death by multiple pathways. Rapid cell death can occur via two different pathways: one requiring protein synthesis and one that does not. Cm = chloramphenicol, a known protein synthesis inhibitor(Drlica *et al*.,2008).

Topoisomerase binds and nicks each DNA strand in the replication process, with four DNA base pairs in between the nicked sites. The fluoroquinolones then binds the topoisomerase nicked DNA complex in each of the nicked sites. However, it is not certain as to what the exact order of binding is. Once the ternary complex is formed, different

mechanisms of cell death are observed by different fluoroquinolones (Drlica *et al*,2009) .With older generation fluoroquinolones such as nalidixic acid and ciprofloxacin, slow cell death is observed. In vitro work with purified enzyme shows that ternary complex formation is fully reversible after the older generation drugs are removed because DNA religation is observed. With these agents in the in vitro purified enzyme assays, all of the DNA is not religated after the fluoroquinolone is removed.

In cells, this effect is observed as chromosomal fragmentation. Rapid cell death also appears to be able to occur via two different mechanisms: one requiring protein synthesis and the other in the absence of protein synthesis . Additionally, fluoro- quinolone interactions with DNA may contribute to which mechanism of cell death are observed as showed in figure (1-3) (Malik, 2006).

1.2.7. Resistance to quinolones:

Antibiotic resistance is generally acquired by 3 major mechanisms:

(1) horizontal transfer of novel resistance genes on mobile elements,

(2) overexpression of resistance-associated genes, and 3) structural alteration of essential bacterial proteins that are targeted by antibiotics (Munita and Arias , 2016) .The latter mechanism usually involves acquisition of point mutations in core chromosomal genes and often requires multiple mutations to co -occur either in different positions of the same gene or in separate genes (Hughes and Andersson, 2017) .

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 relaxation necessary for replication (Tchesnokova,*et al*,2019).The FQ resistance mechanisms are similar in *E.*

coli and many different pathogenic species, and involve acquisition of mutations in the so-called quinolone resistance-determining regions (QRDRs) of the proteins. Although many QRDR mutations were found both in nature and in laboratory experiments, the most common are amino acid changes in positions S83 and D87 of *Gyr*A and in positions 80S (Saksena *et al.*,2018) .

A single QRDR mutation in *gyr*A 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 *gyr*A and *par*C (Johnson *et al* ,2015).

Multiple mutations in the quinolone-determining resistant regions (QRDR)of topoisomerase enzymes are usually associated with high-level of fluoroquinolone resistance in *E. coli* strains (Shams *et al*,2015).

Resistance to quinolones is also by the alteration of the efflux pumps and the *qnr* protein, produced from quinolone resistance genes located on plasmid (Ranjbar and Farahani, 2017). About 100 *qnr* genes variant have been described mainly from *Enterobacteriaceae*, and grouped into 5 distinct families: *qnrA, qnrB, qnrC, qnrD* and *qnrS*.(Salah *et al* ,2019).

The *Qnr* genes are known to confer a low-level resistance to fluoroquinolone in *Enterobacteriaceae*. They are often found on the same resistance plasmids as extended spectrum β-lactamase (ESBL) and constitute the most common antibiotic resistance mechanism; but in *E. Coli* strains resistant to ciprofloxacin and nalidixic acid, and some others quinolone class can suggest the presence of another mechanism of resistance to quinolones such as mutations in the gyrase and topo-isomerase IV genes (Redgrave *et al* ,2014). So far, three families of plasmid-mediated mechanisms associated with quinolone resistance have

been identified: i/*Qnr* proteins protecting target enzymes DNA gyrase and topoisomerase IV from quinolone inhibition ii/ aminoglycoside acetyltransferase *Aac(6')-Ib-cr* acetylating several fluoroquinolones such as ciprofloxacin and norfloxacin and iii/ efflux pumps *QepA* and *OqxAB* removing antibiotics from bacterial cells. (Röderova *et al* ,2017).

1.2.8. Quinolone resistance genes

1.2.9.*Qnr* genes:

The first identified plasmid-mediated quinolone resistance (PMQR) gene was discovered in 1998 and termed *qnrA1* encoding an 218 amino acid long protein (Röderova *et al*,2017) and was identified in a clinical strain of *Klebsiella pneumoniae* isolated in Alabama (Andres *et al*,2013).

The introduction of a letter to define the exact *qnr* gene was first introduced in 2005, when a new *qnr* exhibiting only 59% amino acid identity with the only known *qnr* at that time (currently *qnr*A1) was detected in *Shigella flexneri* in Japan; this new *qnr* was named "*qnr* S" because of the microorganisms from which it was recovered (Hata *et al* ,2005). Thereafter, the *qnr* gene, which was first isolated in 1998, was rapidly reported as *qnrA* to avoid confusion. The subsequent names *qnr*B, *qnr* C, and *qnr* D were adopted in alphabetical order for gene name assignation. Meanwhile, *qnr*VC maintains the first name proposed, related to its presence in *Vibrio cholerae* (Fonseca *et al* ,2008).

The pentapeptide repeat family *qnr* proteins includes mainly (*qnr*A, *qnr*B, *qnr*S, *qnr*C, and *qnr*D). These proteins confer quinolone resistance by physically protecting DNA gyrase and topoisomerase IV from quinolone acts.(Correia *et al* ,2017).This condition may provide a selective advantage for the development of quinolone resistance which could result in therapeutic failure(Malekzadegan *et al* ,2019).

*qnr*B and *qnr*S share have only about 40% and 60% amino acid identity with *qnr* A, respectively, and were also usually found in multidrug resistance plasmid among *Enterobacteriaceae* especially *E. coli* and species of *Klebsiella* (Jacoby *et al* ,2014).

The presence of *qnr* genes may increase the selection of mutations with high-level quinolone resistance (Rath and Padhy,2015).In 2005, Hata *et al*. described another *qnr* gene from a *Shigella flexneri* isolated during an outbreak of food poisoning in Japan. This strain contained a plasmid, designated pAH0376, containing a gene with high similarity to *qnr*, which was designated *qnr*S.

The *qnr*S protein was also a 218-aminoacid protein with 59% similarity to *qnr*A that conferred low level resistance to fluoroquinolones (Hopkins *et al.*, 2007). In 2006, Jacoby and colleagues described a third gene encoding quinolone resistance, *qnrB*. This gene was first found in a *Klebsiella pneumoniae* isolate from India and encoded a 214-amino-acid protein of the pentapeptide repeat family, which had 41% amino acid identity with *qnr*A and 39% amino acid identity with *qnr*S (Jacoby *et al.*, 2006). To date, a total of 6 *qnr*A, 4 *qnr*S, and 20 *qnr*B variants (Jacoby *et al.*, 2008).

Wang and colleagues described another *qnr* gene, *qnrC*, which was found in *Proteus mirabilis*; however, its sequence is not yet publicly available, but it was found that *qnrC* encodes a 221-amino-acid protein with different amino acid identities from *qnrD*, which indicates that the gene is different from *qnrD* (Tamura *et al.*, 2007).PMQR provide only a low level of quinolone resistance, not reaching the clinical breakpoints defined by the Clinical and Laboratory Standard Institute criteria (CLSI, 2015). However, PMQR genes may facilitate the selection of higher-level

resistance in the presence of quinolones and lead to treatment failure (Ranjbar and Farhani, 2017; Jacoby *et al*., 2014).

The PMQR mechanisms thought to enable the occurrence of chromosomal mutation which then lead to clinically relevant resistance levels (Ranjbar and Farhani, 2017; Marchisio *et al*., 2015; Wiedemann, *et al*., 2014).The prevalence of *qnr* genes in clinical isolates varies depending on geographical location, patient characteristics (e.g. higher prevalence in patients than outpatients (Longhi *et al*., 2011) or associated susceptibility patterns (Pasom *et al*., 2013; Briales *et al*., 2012).

1.2.10 Mechanisms of *qnr* action:

The exact manner as to how *qnr* interacts with DNA, type II topoisomerases, and/or quinolones to confer quinolone protection remains controversial, and further studies are needed to establish the definitive mode of action of *qnr*.

Initial studies to determine the mode of action of *qnr* showed that *qnr* minimizes the action of quinolones by interacting with type II topoisomerases (Tran *et al* ,2005). Furthermore, it was observed that this interaction did not need either DNA or quinolones, suggesting the presence of different possible mechanistic explanations (Tran *et al* ,2005).

Thus, an alteration of the quinolone-binding pocket conformation was considered, which hinders further quinolone-target interactions and leads to a reduction of the presence of the cleavage complex among quinolones, DNA, and DNA gyrase/topoisomerase IV (Tran *et al* ,2005). Other options have also been considered, such as the destabilization of these cleavage complexes, which subsequently allows DNA replication, or the reduction of the interactions between DNA and DNA gyrase, leading to a lower number of replication forks (Tran *et al* ,2005).

Xiong *et al*. (2011) analyzed the structure of *qnr in silico*, and its interactions with DNA gyrase led to the proposal of an interaction model in which *qnr* is located within the DNA *gyrase* structure interacting by electrostatic charges through faces 1 and 2. In this model, loops A and B interact with the topoisomerase-primase (TOPRIM) domain of *Gyr*B and the *Gyr*A "tower," respectively, although a specific orientation of the GyrA and GyrB subunits is needed (Xiong *et al* , 2011). The authors propose that this specific *Gyr*A/*Gyr*B orientation is favored when quinolones interact with topoisomerase and subsequently allows *qnr* to interact with and disrupt the complex between quinolones and topoisomerase , and the latter recovers its catalytic activity.

At around the same time, working with a crystallographic *qnr*B1 model, Vetting *et al*. (2011) proposed a model in which *qnr* acts in a posterior step, when the DNA-poison-*topoisomerase* is cleaved, destabilizing the cleavage complex and restoring DNA replication.

Subsequent studies analyzing the effect of point mutations of either loop A or loop B found no correlation between the qnr-topoisomerase interaction and final protection levels, proposing that the loops are not involved in direct interactions with topoisomerase but rather are involved in proper *qnr* positioning in gyrase, blocking the access of quinolones. Thus, when the *qnr*-gyrase complex interacts with DNA, *qnr* is removed, and the enzyme becomes catalytic (Kim *et al* ,2015).

1.2.3. *mar*RAB gene:

The *Escherichia coli* multiple antibiotic resistance (*mar*) locus was identified during studies of mechanisms leading to increased resistance to antibiotic tetracycline in *E. coli*, and is used as a template for understanding the mechanisms of intrinsic resistance in enteric bacteria as a determinant for cross-resistance to quinolones.(Sharma *et al*,2017).

The *mar* phenotype results from induction of an operon designated *marRAB* (El-Meouche *et al* ,2016). The *mar* system consists of two divergently positioned transcriptional units: *marC*, which encodes for a putative integral membrane protein with no known function in antibiotic resistance; and the *marRAB* operon, which encodes genes for three proteins (*marR*, *marA*, and *marB*) (Prajapat *et al* ,2015). While *marA* and *marR* are transcription factors, the role of marB was not characterized until 2013, when it was reported to repress expression from the *marRAB* operon by reducing the rate of *marA* transcription via an unknown mechanism (Vinué *et al* ,2013).

1.2.4. *soxRs* gene:

In bacteria, the defense system deployed to counter oxidative stress is orchestrated by three transcriptional factors–*sox*S, *sox*R, and *oxy*R (Dixon and Stockwell ,2014). In the *sox*RS system, the activation of a target gene occurs *via* a two-step process wherein *sox*R acts as a sensory protein recognizing elevated levels of ROS (Reactive oxygen species). Under normal conditions (non-stressed), the binuclear iron sulfur clusters [2Fe-2S] in the *sox*R protein remain reduced. In the presence of enhanced levels of superoxides, the [2Fe-2S] clusters are oxidized. Oxidization of the *sox*R protein enhances the open complex formation of *sox*R with RNA polymerase, thereby activating the transcription of *sox*S (Krapp *et al* , 2011).

The soxs protein is a transcriptional activator belonging to the XylS/AraC family. soxR dependent induction of *sox*S, in turn, activates the transcription of many other genes (denoted collectively as the *sox*RS regulon).In turn, oxidized *sox*R up-regulates the AcrAB efflux pump that likely expels fluoroquinolone in to out of bacterial cells (Gerstel *et al* ,

2020).Efflux pumps can have a range of substrate specificities. For example, FQ efflux systems tend to be broad-ranged and able to transport many drugs and toxic compounds. This means that, often,mutations in these efflux pumps can cause resistance to FQs (Bush *et al* , 2020).

The corresponding pumps are multidrug transporters, though they do display some specificity towards the structure of FQ that they bind. NorA only transports the more hydrophilic FQs (such as norfloxacin and ciprofloxacin), whilst NorB and NorC transport norfloxacin, ciprofloxacin and the less hydrophilic compounds (such as levofloxacin) (Costa *et al*,2013). In *E. coli*, the overexpression of efflux pumps is often linked to mutations in *mar*RA, *sox*RS and *rob* regulons, which are involved in the regulation of these efflux pumps, as well as many other pathways in the cell (Li *et al* ,2015).

1.2.5 *16SrDNA* sequence:

The identification of pathogenic bacteria was traditionally performed by isolating the organism and studying it phenotypically by means of Gram staining and culture and biochemical methods, which has been the gold standard of bacterial identification (Griffin *et al* ,2012). With the invention of polymerase chain reaction (PCR) and automated DNA sequencing, the genome of some bacteria has been sequenced completely .A comparison of the genomic sequences of bacterial species showed that the *16S rDNA* gene is highly conserved within a species and among species of the same genus and, hence, can be used as the new gold standard for the specification of bacteria (Garrity, 2016).

The use of *16S rDNA* sequencing in clinical microbiology laboratories for bacterial identification, the discovery of novel bacterial genera and species, the detection of uncultivable bacteria and the diagnosis of

culture-negative infections are reviewed by some studies (Barghouthi, 2011).

To study bacterial phylogeny and taxonomy, the *16S rDNA* gene sequences are very useful. With the gene presence in almost all bacteria, often existing as a multigene family, or operons, the function of the *16SrDNA* gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the *16SrDNA* gene (1500 bp) is large enough for informatics purposes (Clifford *et al* ,2012).

Using *16SrDNA* sequences, numerous bacterial genera and species have been reclassified and renamed; classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species have been facilitated(Fattahi *et al*, 2013) .This method has been successful in identifying Enterobacteriaceae species from a bone marrow transplant recipient, and the use of this method to identify or discover novel bacteria in clinical microbiology laboratories has successfully been reported also by (Zemb *et al* , 2020).A potential limitation of quantitative *16SrDNA* detection, however, is that the copy number of *16S rDNA* genes per genome differ between bacterial species, whereas most species-specific genes occur in only one copy (Vetrovsky and Baldrian, 2013) .

The *16SrDNA* copy number per genome for the three most prevalent pathogens in community-onset, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *E. coli* (Laupland *et al* ,2014) has been estimated at five to six, four, and seven, respectively (Kembel *et al* ,2012).

1.2.6 *.rob* gene:

rob, (right origin binding protein), was first discovered to bind to a site close to DnaA binding site to the DNA, However, its precise role in controlling DNA replication is not known (Jain and Saini ,2017). Crystal structure of *rob* further revealed structural homology with transcriptional regulators *MarA* and Sox and has been classified as AraC/XylS type transcriptional regulator family(Jain and Saini ,2016) .However, unlike *MarA* and *SoxS*, transcription from the *rob* promoter is constitutive (the protein in its uninduced state is present in the cell as agglomerate) and the protein amounts are regulated post translationally, in presence of cognate inducer bile slats(Chubiz *et al* ,2012) .

Rob binds with higher affinity than *mar*A or *sox*S to the marbox, however, most of the Rob proteins are inactive due to post-transcriptional sequestration. This prevents activation and in turn prevents activation of other promoters regulated by *rob* (Griffith *et al* ,2009). Sequestrated Rob clumps are not formed when compounds such as 2,2′-dipyridyl activate Rob. When activated, *rob* regulates many promoters by binding to the marbox in response to antibiotics and organic solvents (Weston *et al* ,2017).

Not all *rob* is in the inactive form. Likely, only a fraction aggregates within clusters, leaving some of it in the free and active form even in the absence of its cognate inducer. (Chubiz *et al* ,2012).

1.2.7. Bacterial *gyrase* gene (*Gyr*A) :

The type II DNA topoisomerases (*GyrA*) are nanomachines that control DNA topology during multiple cellular processes such as replication, transcription and cell division (Bush *et al* ,2015) .

These enzymes catalyze the transport of a DNA duplex through a double strand break to perform DNA relaxation, decatenation, and unknotting. DNA *gyrase* plays a vital role in the compaction of the bacterial genome and is the sole type II topoisomerase able to introduce negative supercoils into DNA, a reaction coupled to ATP hydrolysis.(Broeck *et al* , 2019).

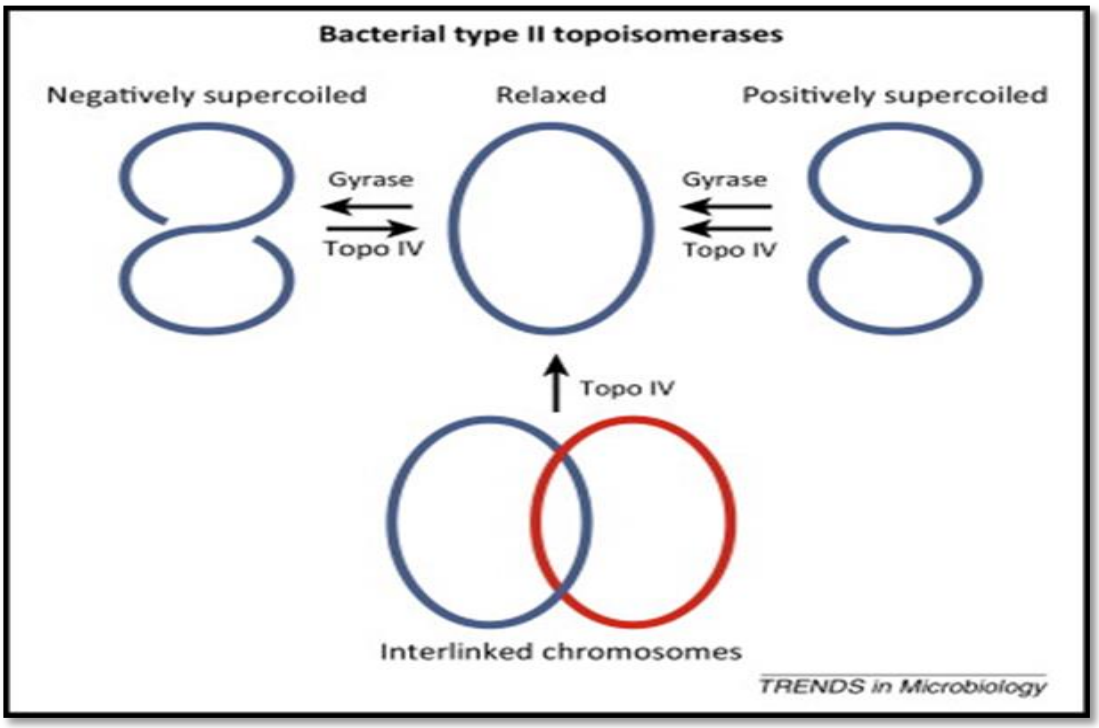


Figure (1-4):Action of type II *topoisomerases* on DNA.(Broeck et al , 2019)

The *gyrase* of *E. coli* is a 374-kDa heterotetramer formed from two *GyrA* (97 kDa) and two *GyrB* (90 kDa) subunits. *Gyr*A subunit consists of two domains: an amino-terminal domain of 59 kDa (*GyrA*59) and a carboxy-terminal domain of 38 kDa (*Gyr*A-CTD). *Gyr*A59 contains the active-site tyrosines responsible for the cleavage and re-ligation of the DNA (the G segment).(Collin *et al*, 2011).

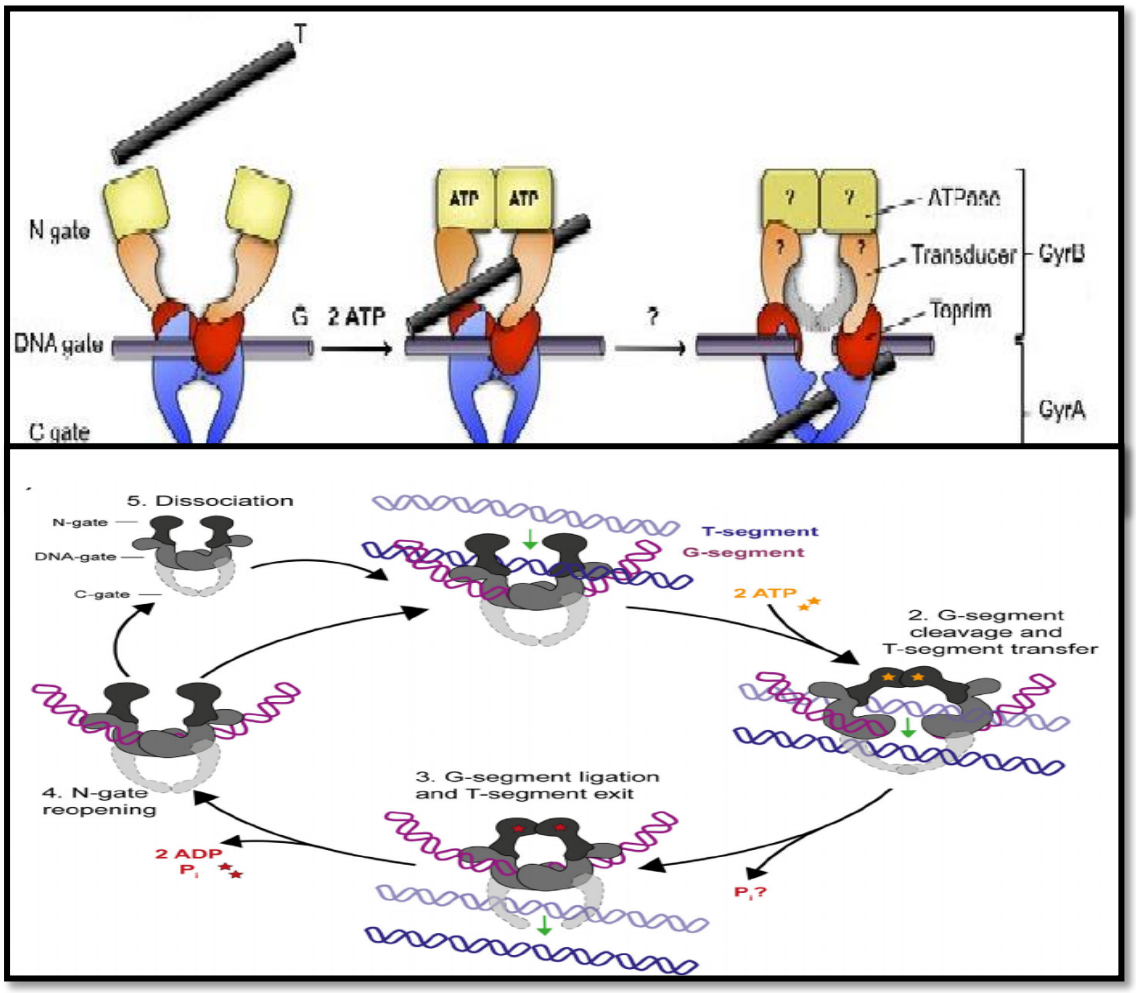
The *GyrB* subunit is comprised of three domains: the N terminal ATPase domain (GHKL family), the transducer domain, and the C-

terminal TOPRIM domain. (Stanger *et al* ,2014).*GyrB* (*Gyr*B43) that comprises the ATPase domain and the central transducer domain (Collin *et al*, 2011). The structure showed a tight dimer with contacts mainly mediated by the ATPase domains (N-gate). As *gyrase* is essential for bacterial viability, and absent in humans, it has had significant and ongoing clinical success as an antibacterial target (Collin *et al*, 2011).

The enzyme cleaves the G segment in both strands of the DNA, leaving a four base stagger, involving amino acid residues in both subunits and entailing the formation of covalent bonds between the 50 ends of the broken DNA and the active site tyrosines in *GyrA*. This allows passage of the T segment through the G-segment break (strand passage), enabling the alterations in DNA topology, such as relaxation or decatenation. In the case of gyrase, the T and G segments are located close together on the same piece of DNA, enabling vectorial strand passage and, thus, the introduction of negative supercoils (Cheng *et al* ,2013) .

The mechanism requires the hydrolysis of two ATP molecules. Type II topo strand passage mechanism includes:

(1) G-segment is bound at the DNA-gate(G- segment) and the T-segment(Transported segment) is captured. (2) ATP binding stimulates dimerisation of the N-gate, the G-segment is cleaved and the T-segment is passed through the break. (3) The G-segment is re-ligated and T-segment exits through the C-gate. For type IIB topos, there is no C-gate so once the T-segment passes through the G-segment, it is released from the enzyme. (4) Dissociation of ADP and Pi allows N-gate opening, a scenario where the enzyme either remains bound to the G-segment, ready to capture a consecutive T-segment, or (5) dissociates from the G-segment (McKie *et al* , 2020).



Figure(1-5):Mechanisms of *gyrase* action in *E.coli* (Stanger *et al* , 2014)

1.2.8 Bacterial *gyrase* II assay (Topogen- U.S.A):

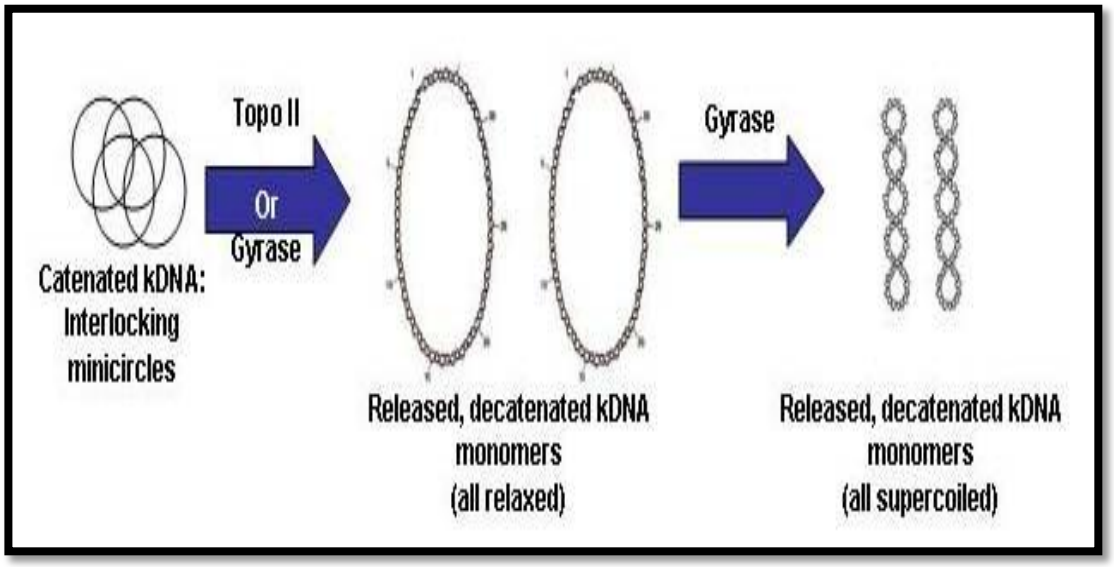
According to manufacture company;The *gyrase* II assay is designed to allow quick and specific detection of DNA *gyrase* and facilitates the purification and characterization of type II topoisomerase enzymes (DNA *Gyrase*) that either have or do not have the ability to supercoil.

The *Gyrase* Assay is based upon a two-step Process:

1.Decatentation of kinetoplast DNA (kDNA) .

2. Supercoiling of the resulting decatenated monomer kDNA species

Reaction products are resolved using a novel gel system that allows extremely rapid and unambiguous detection of *gyrase* activity.



Figure(1-6):Steps of *gyrase* reactions in the Bacterial *gyrase* II assays under study (Phillips *et al* , 2011).

1.2.9. DNA markers included in the *gyrase* assay

1.2.9.1. Catenated kDNA :

kDNA is the ideal substrate for *topoisomerase II* assays because it is specific for type II reaction mechanisms. Researchers can even assay for a type II enzyme in the presence of large excess of topoisomerase I. Thus, kDNA works well to quantify type II activity in crude cell extracts.

1.2.9.2. Decatenated kDNA:

The kDNA substrate works exceptionally well prokaryotic enzymes, such as DNA *gyrase*. Assays employing crude extracts for topo II activity based upon relaxation of supercoiled DNA can be complicated due to the presence of topo I in partially purified fractions. Additional complications arise with contaminating nuclease activity (due to Mg++) which degrade or nick the supercoiled substrate.

These problems can be avoided by using a catenated DNA substrate prepared from the kinetoplast of the insect trypanosome Crithidia fasciculata. KDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb) that form extremely large networks of high molecular weight. As a result, these networks fail to enter an agarose gel. Upon incubation with topo II, which engages DNA in a double stranded breaking and reunion cycle, minicircular DNAs are effectively released (decatenated).

The decatenated minicircles move rapidly into the gel owing to their small size. This reaction will not occur with topoisomerase I.

2.1. Materials and Methods:

2.1.1. Specimens :

This study includes 200 samples which were collected from patients suffering from urinary tract infections, vaginitis, diarrhea and wounds from both sexes and different ages, who attended to two main hospitals in Holy Karbala governorate :Al-Husseini General Teaching Hospital, Karbala Hospital for Maternity and Pediatric during a period ranging from February to June 2020. Samples were taken from patients with the range of age from 5 to 66 years of male and female.

2.1.2. Ethical approval:

1- The study was done and the samples were collected after getting the agreement of the patients (verbal acceptance).

2- Approval of Babylon medical college ethical committee. 3-Before study, permission were taken from Babylon health direct orate.

2.1.3. Study design:

Cross sectional study. The schematic figure of current study reveals in figure (2-1):

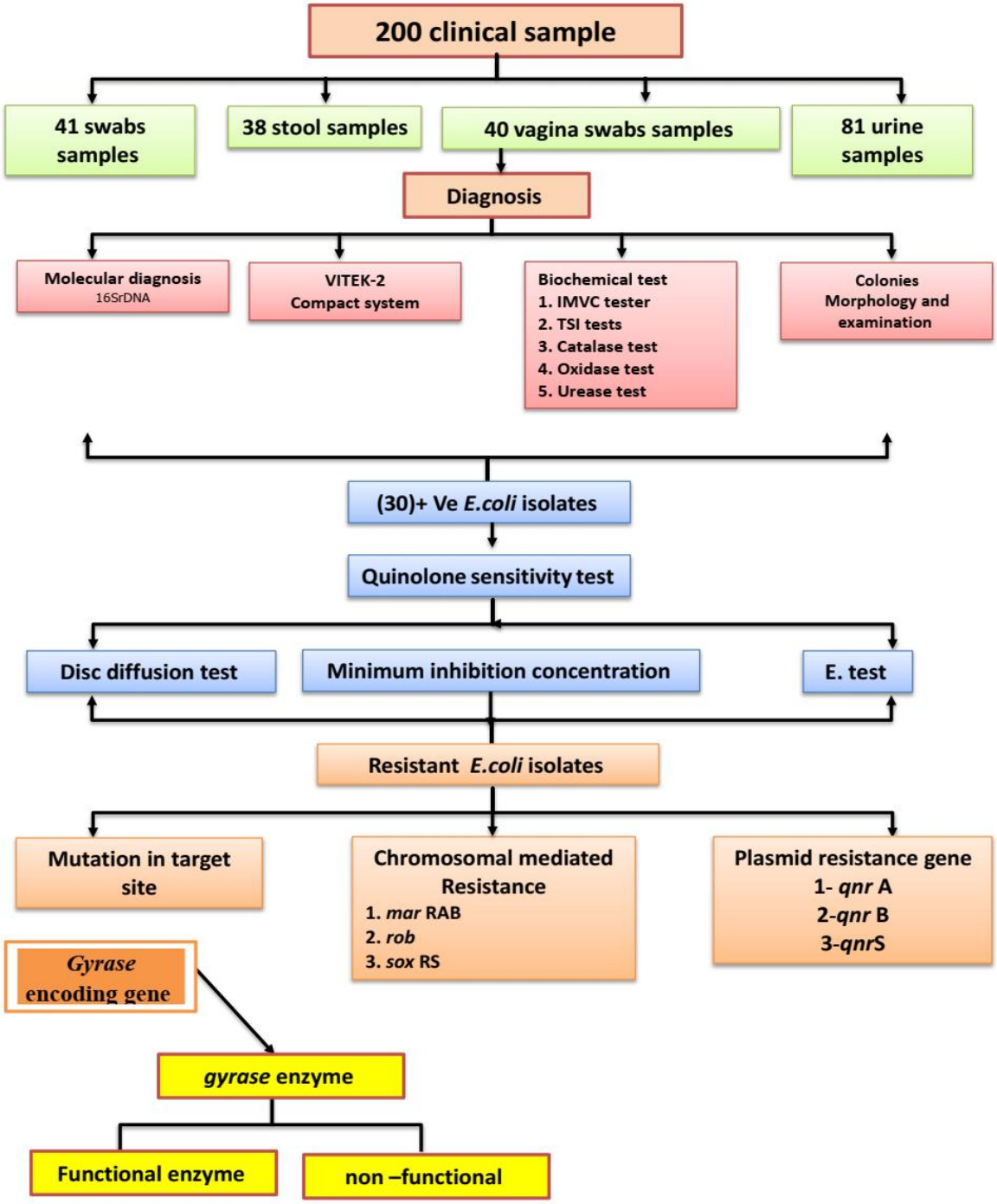


Figure (2-1): The schematic figure of current study

2.1.4. Laboratory apparatuses and instruments:

The main instruments with disposable materials respectively that employed during the present study were listed down in table (2-1).

Table (2-1) Laboratory apparatus and instruments

Instruments	Company	Country
Autoclave	Stermite Olympus A & D	Japan
Benson burner	Labogene	Germany
Distillator	GFL	
DNA extraction tubes.	Eppendorf	
Digital camera	Samsung	Japan
Gel electrophoresis	Clever	USA
Glass slides, flasks and beakers	Hirschmann	Germany
gloves, facial mask, head cap, filter paper	Unimed	K.S.A
Hood	Labogene	Danemark
Incubator, Water bath, Oven	Memmert	Germany
Light microscope	Stermite Olympus A & D	Japan
Micro centrifuge		
Micropipettes 5-50 µl ,100-1000 µl, 0.5 – 10 µl		
	Eppendorf	Germany
Nano drop	Avans biotechnology corp	Taiwan
Medical gloves	Broche	PRC
PCR tube	Clever	U.S.A
Platinum wire loop	Himedia	India
Petri dishes	Dolphin	Syria
Refrigerator	Concord	Italy.
Screw capped bottles 30 ml	Hirschmann	Germany
Sensitive electron balance	Stermite Olympus A & D	Japan
Sterile swab for streaking	Lab. Service	S.P.A.
UV-transilluminator	Clever	U.S.A
Vortex	Germmy	Twain
Vitek 2 Compact Autoanalyzer	BioMerieux	France
ViteK 2 system cards(ID)	BioMerieux	France

2.1.5 Chemical and biological materials:

2.1.5.1 Chemical materials:

Table (2-2) Chemical Supplies

Chemical	*Company/nation*
95% and 70% alcohol (Ethanol), Urea solution	Flukachemika/ Switzerland
Ethidium bromide (10mg\ml), Loading dye (bromophenole blue), Agarose, nuclease free water.	(Promega, U.S.A)
Gram Stain kit	Crescent/KSA
H_2O_2, Glycerol ($C_3H_8O_3$), Kovac's reagent.	Flukachemika/Switzerland
HCl, KOH, NaCl	Merk Darmstadt/Germany
Methyl red reagent, Alpha-nephthol reagent(A), KOH reagent(B), McFarland tube standard (0.5), Tetramethyl Para-Phenylene diamine dihydrochloride (oxidase reagent).	Searle/England
Normal saline specific for Vitek(PH=5.0-7.0)	Mexico /U.S. A
TRIS -EDTA(TE)buffer PH=8	BioBasic/ Canada
Tris-Borate-EDTA (TBE) buffer	BioBasic/ Canada

2.1.5.2 Biological materials

Table (2-3) The Culture Media

Medium	Company/nation
Eosin Methylene blue (EMB) agar, Blood agar medium, MacConkey agar, Nutrient agar, the Brain heart infusion agar, Brain heart infusion broth. Peptone water broth, Muller Hinton agar, MR-VP broth, Luria broth	Himedia – Accumax, India
Urea agar base, Triple sugar iron agar, Simmon's citrate agar, Lysine Decarboxylase broth , ornithine Decarboxylase broth.	Diffco – Michigan

2.1.6. Bacterial isolates:

The main bacterial isolates which used as throughout present study were planned in table (2-4)

Table (2-4): Control strain and local isolates used in the study

Strain	Genotype	Origin
Escherichia coli HB101	Wild type	Institute of genetic engineering /Baghdad University / Department of Microbiology
Escherichia coli	Local isolates of current study	Al-Husseini General Teaching Hospital, Karbala Hospital for Maternity and Pediatric

2.1.7. Antibiotics disks:

Table (2-5): Antibiotics disks. (Bioanalyze / Turkey)

Group	*Antibiotics*	*Potent(µg per disk)*
Fluroquinolons	Nalidixic acid	30
	Ciprofloxacin	5
	Norfloxacin	10
	Levofloxacin	5
	Gatifloxacin	5

2.1.8 Molecular commercial kits:

Table (2-6)Commercial kits used in study

Type of kits	Company/country
DNA extraction kit	Geneaid / UK
Gold multiplex PCR PreMix kit (master mix)	Bioneer- korea
DNA ladder 100- 1500bp ,100-2000bp	Bioneer-Korea
Bacterial *gyrase* assay kit	Topogen-U.S.A

Geneaid (DNA extraction) kit

Materials:
GT Buffer, GB Buffer, GD Column,
2 ml Coll
ection Tube, W1 Buffer,
Wash Buffer (ethanol added), Elution Buffer , Proteinase K.

Gold multiplex PCR Kit PreMix

1- DNA polymerase enzyme (Taq).
2- Pyrophosphatase and pyrophosphate .
3- dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm dTTP).
4- Reaction buffer (pH 8.3) with $MgCl_2$ (2mM).
5- Stabilizer and tracking dye.

DNA ladder

1- Ladder consist of 12 double-stranded DNA with size 100-1500bp, include 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 bp .
2- Loading Dye which has a composition of (15% Ficoll, 0.03%bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

2- Bacterial *gyrase* assay kit

1. kDNA (20 ug total) substrate at the concentration specified on the tube provided.
2. Decatenated kDNA marker (25 ul) in gel loading buffer
3. Linearized kDNA marker (25 ul) in gel loading buffer.
4. 5x Gyrase Assay Buffer (600 ul). 1x buffer contains 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl2, 1 mM ATP, 2 mM Dithiothreitol, 1.8 mM Spermidine, 6.5% glycerol, 100 ug/ml BSA
5. 5x Stop Buffer/gel loading dye (600 ul): 5x buffer is 5% Sarkosyl, 0.125% bromophenol blue, 25% glycerol

Samples reaction (20 ul, order of addition):

Distilled water 14 ul (Vary as needed to bring final volume up to 20 ul)
5x Assay Buffer 4 ul
KDNA 1ul (vary as needed to give 100-200 ng)
Test Extract of protein 1 ul

2.1. 9. Primer sets: Primers sequences of bacterial used during this study were scheduled in the table (2-7):

Table (2-7):- Primers sequences and PCR condition

Primer name	Primer sequence (5´-3´)	Size product bp	Condition			Reference
			Temp	Time	No of cycles	
marRAB	F: GTA CAC CGA GGC CAT CAA CGA CT R: GAC CGG GCT GTA TAT CAA TGT A	1,543	95 ºC	15 min		Karczmarczy *et al,*2011
			94ºC 52 72 72	15sec 30sec 5 min 5 min	35x 1x	
soxRS	F: TTA AAA ACG ATC GCT GAA GG R: TTG ACG TCG GGG GAA AC	1,132	95 ºC	15 min		
			94ºC 52 72 72	15sec 30sec 5 min 5 min	35x 1x	
rob	F: ACT GGG ATG CCT GGT GA R: CAA GCC CTA AAA CAT ACT CTA CTA	1,172	95 ºC	15 min		Karczmarczy *et al,*2011
			94ºC 52 72 72	15sec 30sec 5 min 5 min	35x 1x	
gyr A	F: GTA TAA CGC ATT GCCGC R: TGC CAG ATG TCC GAGAT	215	94 ºC	7 min		Ramírez-Cas - tillo *et al,* 2018
			94 ºC 46 ºC 72 ºC	1 min 1min 30 sec	35x	
			72 ºC	10 min		
16s rDNA	F: AGAGTTTGATYMTGGCTCAG R: CTACGGCTACCTTGTTACG	1500	94 ºC	5min	1x	Varughese *et al* ,2018
			94 ºC 54 ºC 72 ºC 72	1 min 1min 7 min 5 min	35x 1x	
qnrA	F: ATTTCTCACGCCAGGATTTG-3 R: GATCGGCAAAGGTTAGGTCA-3	516	95ºC	3min	1x	Shams *et al.*2015
			95ºC 51.2ºC 72ºC	30sec 1min 2min	35x	
			72ºC	5min	1x	
qnrB	F : GATCGTGAAAGCCAGAAAGG-3 R: ACGATGCCTGGTAGTTGTCC-3	469	94ºC	5min	1x	
			94ºC 53ºC 72ºC	45sec 45 sec 1min	32x	
			72ºC	5min	1x	
qnrS	F: ACGACATTCGTCAACTGCAA-3 R: TAAATTGGCACCCTGTAGGC-3	417	94ºC	5min	1x	
			94ºC 53ºC 72ºC	45sec 45sec 1min	32x	
			72ºC	5min	1x	

2.2. Methods :

2.2.1. Collection of specimens :

The proper specimens collected for bacteriological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination (Forbes *et al*., 2007).

1- Urine samples:-

The specimens were generally collected from patients suffering from UTIs. Mid-stream urine samples were collected in sterilized screw-cap containers.

2- Vagina swabs:-

The specimens were generally collected from women (pregnant and non-pregnant) suffering from vaginitis. The swabs were taken by gynecologists. Swab for culture should be placed in tubes containing normal saline to maintain the swab moist until taken to laboratory.

3- Stool samples:

The specimens was collected in feces container. Then, the swab was inserted into feces and inoculated on culture media.

4-Wound samples:

Collect most wound specimens with either a sterile swab or a syringe. Always perform hand hygiene and wear clean gloves when obtaining a wound sample. If the wound is bandaged, remove the dressing and clean the wound with sterile water or normal saline solution prior to collection. Collection of specimens were from the center of the wound, not from the wound's edges, as they might be contaminated with outside skin flora. Then use a sterile swab or syringe to obtain a sample of the drainage in the wound. The swabs was plased into a sterile test tubes for transport.

2.2.2. Preparation of reagents and solutions:

2.2.3. Reagents:

2.2.3.1. Oxidase reagent:

It was prepared freshly in a dark bottle by dissolving 1gm Tetramethyl Para-Phenylene diamine dihydrochloride in 100 ml D.W(Forbes *etal*., 2007).

2.2.3.2. Catalase reagent:

This reagent was used at a concentration (3%) using H_2O_2 in D.W. (v/v) and stored in a dark container. It was used for identification of catalase producing bacteria (Forbes *et al*., 2007).

2.2.3.3. Kovacs reagent:

It was prepared by dissolving 5 gms of P-Dimethyl amino Benz aldehyde (DMAB) in 75ml amyl alcohol. Afterwards, 25 ml of concentrated HCl was gradually added to this mixture. It was used to detect the indole production (MacFaddin, 2000).

2.2.3.4. Methyl red reagent:

Methyl red reagent was prepared by dissolving 0.1 gm of methyl red in 300 ml of (95%) ethanol. The volume was then completed to 500 ml by adding distilled water. It was used to detect complete glucose hydrolysis (MacFaddin, 2000).

2.2.3.5. Vogues –Proskauer reagent (Barrett's reagent) :

1-Solution A (α-naphthol) : prepared by dissolving 5 gm of α-naphthol in 100 ml of ethanol.

2-Solution B (40 % KOH) : prepared by dissolving 40 gm of KOH in 100 ml D.W. Two drops of 40% KOH solution and 6 drops of 5% solution of α-naphthol were added to each test tube (MacFaddin, 2000).

2.2.4 Solutions:

2.2.4.1 Normal saline solution:

It was prepared by dissolving 8.5 gm of NaCl in a small volume of

distilled water, then completed to 1000 ml, pH fixed at 7.2 pH were adjusted using 1M NaOH or 1M HCl, and sterilized in autoclave at 121°C for 15 minutes, then kept at 4°C (MacFaddin, 2000).

2.2.4.2. McFarland (0.5) turbidity standard:

The solution of tube No.0.5 was prepared mixing 0.05ml of barium chloride with 9.95ml of concentrated sulfunic acid in which result in turbidity approximately equal to bacterial cells density of 1.5×10^8 cell/ml. The McFarland standard tube were sealed with parafilm to prevent evaporation and stored for up to 6 months in the dark at room temperature. Accuracy of the density of a prepared 0.5 McFarland standard was checked by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be between 0.08 and 0.1(CLSI, 2020).

2.2.4.3 Agarose gel:

It was prepared by dissolving 1.5 gm agarose powder in 100 ml (1X TBE) buffer.

After boiling, leave the solution left to cool until 50 C before adding florescent dye. Just 0.5-1 µl of ethidium bromide stock solution were added to 100ml of melting agarose gel (Sambrook and Russel, 2001).

2.2.5. Preparation of culture media (pH7):

A group of culture media were prepared according to the instructions of the company and serialized by autoclaving at 121°C for 15 minutes.

2.2.5.1. Eosin Methylene Blue(EMB)medium:

Eosin methylene blue (EMB) agar medium was prepared according to the method recommended by the manufacturing company (36 gm /L).

Lactose fermenting colonies were either dark or possess dark centers with transparent colorless peripheries. While organisms that did not ferment lactose remain uncolored. The purple color was due to the absorption of the

eosin methylene blue complex formed in the presence of acid. Certain members of *E. coli* exhibit a greenish metallic sheen in reflected light (Murray *et al.*, 2003).

2.2.5.2. MacConkey agar medium:

MacConkey agar medium was prepared according to the method recommended by the manufacturing company (52 gm /L) and it was used for the primary isolation of most Gram negative bacteria and differentiation of lactose fermentative from the non-lactose fermentative (Winn *et al*., 2006) .

2.2.5.3.Peptone water medium:

This medium was prepared by dissolving (8 gm/L) peptone of in distilled water, then distributed into test tubes, and autoclaved. It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, 2000).

2.2.5.4. Nutrient agar medium:

Nutrient agar medium was prepared according to the manufacturing company (28 gm/1L). It was used for general experiments, cultivation and activation of bacterial isolates when it is necessary (MacFaddin, 2000).

2.2.5.5 Brain Heart Infusion Broth:

This medium was prepared by dissolving 37gm of medium in 100 ml distilled water, then sterilized by autoclave at 121^{o}C for 15 minutes (MacFadden, 2000).

2.2.5.6.Brain Heart Infusion(BHI) Broth–Glycerol Medium:

This medium was prepared by mixing 5 ml of glycerol with 95 ml of BHI broth (sterilized by autoclave). It was used for preservation of bacterial isolates as stock for a long time (Forbes *et al*., 2007).

2.2.5.7. Brain Heart Infusion Agar:

Brain Heart Infusion agar was prepared according to the manufacturing company (52 gm/1L) (MacFadden, 2000).

2.2.5.8. Methyl red Vogues – Proskauer medium:-

The medium was prepared according to the manufacturing company (17 gm/1L), then sterilized by autoclave at 121°C for 15 minutes (MacFadden, 2000).

2.2.5.9. Blood agar medium :

Blood agar medium was prepared by dissolving 40gm blood agar base in one liter of distilled water. This media was autoclaved at 121°C for 15 minute, and then cooled to 50°C. Then, (5%) of fresh human blood was added. This medium was used as enrichment medium for cultivation of bacterial isolates and to determine their ability of blood hemolysis (Forbes *et al*., 2007).

2.2.5.10. Muller-Hinton agar medium:

Muller-Hinton agar was prepared according to the manufacturing company (38mg/IL). It was used in anti-bacterial susceptibility testing (MacFaddin, 2000).

2.2.5.11. Triple sugar Iron agar Medium:

It was prepared according to the manufacturing company (64.4gm/IL).It used for determining glucose , lactose and sucrose fermentation and possible hydrogen sulfide H2S production as a first step in the identification of Gram- negative bacilli (MacFaddin, 2000).

2.2.5.12. Simmon's Citrate agar:

This medium was prepared according to the method suggested by the manufacturing company (24gm/L). It was used for the demonstration of the

bacterial ability to use citrate as sole source of energy. The positive result was conversion of color from green to blue (Forbes *et al*., 2007).

2.2.6. Laboratory diagnosis:

2.2.6.1 Bacterial identification assays:-

According to the diagnostic procedures recommended by Forbes *et al*,(2007); Benson, the isolation and identification of *E. coli* associated with patients under study were performed as follows:

2.2.6.2.Colonial morphology and microscopic examination:

A single colony was taken from each primary positive culture. Its identification depended on the morphology properties (colony size, shape, color, translucency, edge, and elevation of texture). The colonies were then investigated by gram stain to observe bacterial cells. Specific biochemical tests were done to reach the final identification.

E .coli appeared with circular shape , entire margin, raised, punctiform, small size, smooth texture, green shiny appearance, on culture media (EMB).

2.2.6.3. Staining :

Gram stain was used to differentiate Gram negative from Gram positive bacteria and to identify their shape and arrangement in steps declared by (Winn *et al*., 2006).

2.2.7. Biochemical tests:

2.2.7.1. Catalase test:

Catalase is an enzyme that catalyses the release of oxygen from hydrogen peroxide. Nutrient agar medium was streaked with the selected bacterial colonies and incubated at 37ºC for 24 hrs, then the growth was transferred by the wooden stick and it was put on the surface of a clean slide,

a drop of 3% H_2O_2 was added. Formation of gas bubbles indicates a positive result (Forbes *et al*., 2007).

2.2.7.2. Oxidase test:

The test depends on the presence of certain bacterial oxidases that would catalyze the transport of electrons between electron donors in the bacteria and a redox dye (tetramethyl-ρ-phenylene-diaminedihydrochloride) , the dye was reduced to a deep purple color.

A strip of filter paper was soaked with a little freshly made reagent, and the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep purple color which appeared within 5-10sec. (Forbes *et al*., 2007).

2.2.7.3. Indole test:

This test was performed by inoculating peptone water medium with bacterial growth by the loop, and it was inoculated for 24 hour at 37°C. Indole test was done by adding 6-8 drops of Kovac's reagent (p-dimethyl amino Benzaldehyde in amyl alcohol). The positive reaction was characterized by the formation of red color ring at the top of the broth (MacFaddin, 2000).

2.2.7.4. Methyl –red test:

The tubes of the MR-VP broth were inoculated with selective bacterial colonies and were incubated at 37°C for 24 hour. Five drops of methyl red reagent were then added to it. The appearance and observation of red colour means a positive result and a complete hydrolysis of glucose (MacFaddin, 2000).

2.2.7.5. Vogues –Proskauer test (VP):

The tubes of the MR-VP broth were inoculated with selected bacterial colonies and were incubated at 37°C for 24 hours. The result was then read

by adding 5-12 drops of alpha nephthol (reagent A) and 4 drops of 40% KOH solution (reagent B) the appearance of red color after 15 minutes -1 hrs means a positive result due to the partial hydrolysis of glucose, which produced acetoin or Acetyl - methyl - carbinol (MacFaddin, 2000).

2.2.7.6 Citrate utilization test:

After the sterilization of Simmons Citrate slants by autoclave, the bacterial colonies were inoculated and incubated for 24 hours at 37^{o}C. The change of color of media from green to blue indicated that the organisms were able to utilize citrate as sole carbon source (Benson , 2001).

2.2.7.7. Triple sugar Iron agar medium:

If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in butt and in the slant. If lactose is not fermented but only amount of glucose is fermented, the slant remain red while the butt is yellow with acid . If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids , if H_2S is produced, the black color of Ferrous Sulfide is seen(Brooks *et al*., 2010).

2.2.8. Rapid Identification System by VITEK-2 Compact System:-

VITEK-2 compact system include a strip consist of 47 biochemical tests. Bacteria was suspended in 3 ml normal saline test tube then inserted into dense check machine for standardization of colony to MacFarland standard solution (1.5 x 10^8 cell/ ml). A test tube containing the microorganism suspension was placed into a special rack (cassette) and the identification

card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube.

The filled cassette was placed manually into a vacuum chamber station. After the vacuum was applied and air was re-introduced into the station, the organism suspension was forced through the transfer tube into micro-channels that fill all the test wells.

A transmittance spectrophotometer is used to read of test reactions using different wavelengths. During incubation, each test reaction is read every 15 minutes to measure either turbidity of bacterial mass growth or color products of substrate metabolism.

All reagents and equipments needed for processing supplied by manufacturer company. All isolates introduced to the computer before processing and inoculated cards were processed in the instrument within 30 min of inoculation.

The card thus connected to the sample ID number , then the cassette is placed in the filler module, when the card are filled , transferred the cassette to the reader incubator module. All following steps handled by the instruments, the instruments controls the incubation temperature, the optical reading of the cards and continually monitors and transfers test data to the computer for analysis (Biomerieux, France).

2.2.9. Antibiotic Susceptibility Testing:

2.2.10. Disc diffusion test:

The Kirby-Bauer method is a standardized system for this test that takes all variables into consideration (Jorgensen and Ferraro,2009) It was performed by using a pure culture of previously identified bacterial organism(10^7 CFU). The inoculums to be used in this test was prepared by adding growth from 5 isolated colonies grown on blood agar plates to 5 ml of nutrient broth, this culture was then incubated for 2 hrs. to produce a

bacterial suspension of moderate turbidity that compared with turbidity of ready-made (0.5) McFarland tube standard. A sterile swap was used to obtain an inoculums from the standardized culture, this inoculums was then swabbed on Mueller–Hinton plate.

1. The antibiotic discs were placed on the surface of the medium at evenly spared intervals with flamed forceps, then incubated at 37ºC for a full 18 hrs (Weigelt, 2007).
2. Antibiotics inhibition zones were measured using a transparent ruler. Zone size was compared to standard zones (CLSI, 2020) to determine the susceptibility of organism to each antibiotics.

2.3. Determination of Minimum Inhibitory Concentrations (MICs) of *E. coli* isolates:

The E-test was used for determination of MICs of antibiotics. E-test used a nonporous plastic strip covered with preformed exponential gradient of an antimicrobial along the 60 mm of length (Andrews *et al*., 1993). The gradient of agent covers a concentration range of 0.002 to 32 mg/L, 0.016 to 256 mg/L, or 0.064 to 1024 mg/L, depending on the agent. This range corresponds to 15 twofold dilutions in a conventional MIC method.

On the other side of the strip, calibrated MIC values covering 15 twofold dilutions are marked according to the antimicrobial gradient coated. When the strips applied on the surface of agar plate inoculated with the test organism, the continuous drug gradient is formed on the agar by diffusion. The areas with inhibitory concentration of the antimicrobial to the test organism show no bacteria growth while the confluent lawn growth covers the rest of the area on the plate. As the result of the response of the bacteria to the test drug in different concentrations, an elliptic growth inhibitory zone was formed around the strip.

The point on the E-strip at which the inhibition zone intersects is determined as the MIC. Muller Hinton agar plate was inoculated with PEC bacterial suspension

and the inoculum prepared from colonies grown after 24- or 48-hr is standardized to the density of a McFarland standard recommended by the manufacturer for the particular organism–antimicrobial combination tested.

The E-strips are stored at −20◦C or −70◦C to prevent loss of drug activity. They should be removed from the freezer and equilibrated to the room temperature for 30 min before use. After overnight incubation, the MIC is read at the point of intersection of the elliptical zone with the strip.

2.4. Genotyping assays:

2.4.1. DNA extraction from *E.coli* isolates:

DNA was extracted from clinical isolates. One colony of each isolate cultured and inoculated into 5 ml of BHI (Brain Heart Infusion) and grown overnight at 37ºC. from these isolate cultures, DNA was purified from bacterial cells using genomic DNA kit supplemented by the manufacturing company. DNA obtained were used as templates for all PCR experiments. The DNA concentration is measure by Nanodropes machines at 260/280nm give 1.8 purify DNA.

The PCR reactions were carried out in a Thermal Cycler. before PCR assay, DNA profile were performed by using bacterial DNA and loading buffer without thermal cycling conditions, and according to the following steps :

First- Sample preparation of *E.coli* isolates:

1. Overnight bacterial cells (up to 1 x 10^9) was transfered to a 1.5 ml microcentrifuge tube.
2. Tubes were centrifuged at 14.000 rpm for 1 min to pellet the cells and the supernatant was removed.
3. 180µl of GT Buffer was added then re-suspend the cell pellet by vortex or pipette.

4. 20µl of *Proteinase* K was added after ddH2O was added and incubate at 60ºC for at least 10 minutes. During incubation, the tubes were inverted every 3 minutes.

Second - Cell Lysis:

5. 200µl of GB Buffer to each samples were added and mix by vortex for 10 seconds.; then tubes were incubated at 70ºC for at least 10 minutes to ensure the sample lysate is clear. During incubation, the tubes every were invert 3 minutes. At this time, pre-heat of elution buffer (200 µl per sample) to 70ºC .

Third- DNA Binding:

6. 200µl of absolute ethanol was added to the clear lysate and immediately mixed by shaking vigorously for 10 minutes.
7. GD Column were placed in a 2ml collection tubes.
8. All of the mixture was transferred (including any precipitate) to the GD column and centrifuged at 14,000 rpm for 2 minutes.
9. The collection tubes were discarded containing the flow through and the GD columns were placed in a new collection tubes.

Forth – Wash step:

10 - 400 µl of W1 buffer was added to the GD Columns ;then Centrifuged at 14,000 rpm for 30– 60 seconds.

11 - The flow-through was discarded and the GD column placed back in the 2ml collection tubes.

12 - 600 µl of wash buffer (Ethanol) was added to the GD column, and centrifuged at 14,000 rpm for 30 seconds.

13 - The flow-through was discarded and the GD column placed back in the collection tubes and then centrifuged again for 3 minutes at 14,000 rpm to dry the column matrix.

Fifth - Elution step:

14-The dried GD columns were transferred to a 1.5 ml centrifuge tubes.

15- 100 µl of pre-heated elution buffer or TE was added to the center of the matrix, and stand for at least 3 minutes to ensure the elution buffer is absorbed by the matrix and centrifuged at 14,000rpm for 30 seconds to elute the purified DNA.

16- The DNA were stored at -20ºC to avoid degradation.

2.4.2 .Measuring DNA purity:

The extracted DNA was checked by using Nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (280 nm) as following steps:

1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2- A dry paper-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH_2O onto the surface of the lower measurement pedestals for blank the system.
3- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then pedestals was cleaning off the and 1µl of extracted DNA carefully pipet onto the surface of the lower measurement pedestals, then the concentration and purity of extracted DNA was checked. (Sambrook and Russell, 2001).

2.4.3 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction were performed in a final volume of 25µl as in table (2-8). Then DNA amplification was carried out with the thermal cycler.

Table (2-8) 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

2.4.4 Preparation of primers:

The upstream and downstream primers are prepared according to the manufacturing company instructions (Bioneer,Korea) and stored at-20ºC.

2.4.5 Detection of amplified products by agarose gel electrophoresis:

Successful PCR amplification was confirmed by agarose gel electrophoresis (Lodish *et al.*,2004). Agarose gel was prepared by dissolving 1.5 gm of agarose powder in 100ml of TBE buffer (pH:8) then volume completed to 100 ml deionized water, in boiling water bath, allowed to cool to 50°C and Ethidium bromide at the concentration of 2-3µl/ml was added.

The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was powered gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covering the surface of the gel, 4µl of DNA sample was transferred into the signed wells in agarose gel, and in one well we put the 5µl DNA ladder .The electric current was allowed at 70 volt for 45 min. UV transilluminater was used for the observation of DNA bands, and gel was photographed using a digital camera.

2.5. Preparation of bacterial culture Extract:

According to manufacturing company procedure(Topogen-U.S.A.);cell lysate are done as following :

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 tube containing the cells that obtain from single colony grown overnight, diluted, and grown further until the optical density reaches 0.8 to 1.0, after which cells are harvested then resuspend cell pellet.

2. Sonication at Amplitude 50, Timer 3 mins, Pulser 2 was done.

3. cell lysate were centrifuged with 18000 rpm at 4c for 25 minutes.

4. The cell extract were collected

2.6. DNA *gyrase* assay kit

2.6.1 Assay protocol

2.6.2. Sample reaction (20 ul, order of addition):

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 was 20-30 ul. Reactions was assembled in microfuge tubes with water, buffer and substrate kDNA.

The test fractions was added last and the reactions incubated at 37° C for 15-30 min; then terminated with 1/5 volume of the stop buffer.

2.6.3. DNA *gyrase* assay kit user manual procedure

1. Sample reaction tubes were incubated for 30-60 min at 37° C.
2. A 1/5 volumes of stop buffer/loading dye (5 ul) were added

3. The Chloroform plus isoamyl alcohol were mixed with a ratio (24:1) then 20 ul of the mixture was added to each tubes, then vortex briefly, and withdraw blue (aqueous) phase were done

4.1% agarose Ethidium Bromide gel was loaded (include EB at 0.5 ug/ml in Geland 1xTBE buffer. (50x TBE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA).

5.Electrophorese was run until dye travels about 5 cm, then removed from gel box .Finally destain step with distilled water were done for 10-30 min at room temperature and then picture are given using UV transilluminator

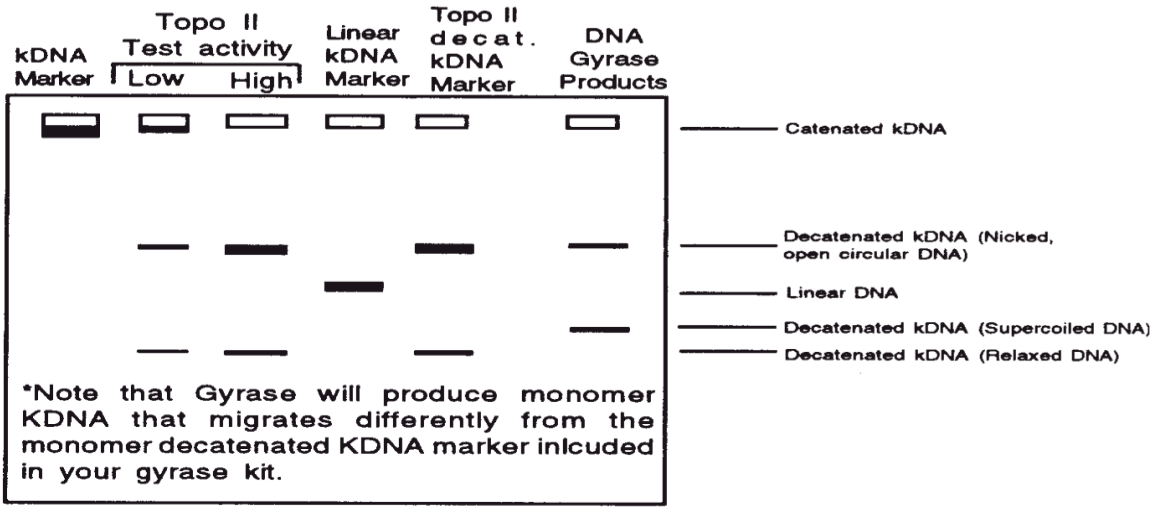


Figure (2-2) :Schematic of *gyrase* assay kit results (Topogen,2010).

Both *gyrase* and eukaryotic *topo II* have the ability to decatenate kDNA. Topo II cannot supercoil the monomeric kDNA forms;thus, the covalently closed circular monomers will migrate as a relaxed kDNA minicircle.*Gyrase* can supercoil these circular decatenation products and yield a slower migrating monomeric kDNA product. The nicked monomeric kDNA cannot be supercoiled since it cannot retain *gyrase* induced supercoils (due to the rotation around the nick site).

3.1.Isolation and identification of *Escherichia coli* isolates:

In the present study, a total of 200 clinical samples were collected from patients suffering from different infections such as UTI, gastro enteritis(diarrhea), vaginitis and wound infections. Three techniques are used for identification of *E.coli* , one of these is by conventional technique through using cultural and biochemical features. However 30 isolates have been recovered, where 13 isolates (32%) obtained from urine samples,4 isolates (21%) from stool samples, 8 isolates (40%) from women vagina and 5 isolates (24%) from wounds as shown in table (3-1)

Table (3-1):Numbers and percentages of *E.coli* isolates from different sources

Source of the sample	Total No. of tested samples	No. of *E .coli* isolates	Percentage(%)
Urine	81	13	32
Stool	38	4	21
Vaginal swabs	40	8	40
Wound swabs	41	5	24
Total	200	30	15

This table has conducted that *E. coli* are highly isolated from vagina samples 40% and from urine samples (32%) followed by (24%) from wounds infections and (21%) from stool . The results are close to those obtained by (Alsaadi ,2018) who found that the prevalence of *E.coli* is approximately (46%) in vagina, (33%) in urine ,(30 %) from wounds as well as Wonyong *et al* .,(2013) who found that the prevalence of *E.coli* is approximately (42%) in vagina, (35%) in urine ,(29%) from wounds.

The isolation of *E.coli* from urine was 32% in the present study when compared to other studies may correlate with (Hamza ,2019) who have found that the prevalence *E.coli* was 36.84% in urine samples and Saenz *et al*., (2015) who have reported that the prevalence *E.coli* was 38% in urine samples , but the rate is lower than those obtained from some studies as in Brusch *et al* .,(2016) who found that 7.5% of UTIs were caused by *E. coli.*

Kohinoor *et al* ., (2013) investigated that the UTIs due to *E.coli* were 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 Otaiwi *et al*, (2019) in *E. coli* that isolated from patients' stool are represent about 12/46 (26.1%); while lower than obtained by Aljanaby *et al* ,(2017) who reveals that the prevalence *E.coli* was 36% from childrens' stool in Iraq respectively ,This may be due to the uses of antibiotics in different disease which influence on the rate of isolates of this bacteria.

Concern *E. coli* that isolated from vaginal swab samples 40% were coordinate with study done by Hussien *et al*,(2020) that *E.coli* prevalence in *Kirkuk* province as a causative agent of vaginitis are 50% and by Al-Mayahie (2013) in Wasit province who indicated that *E.coli* was 45% in women with vaginitis infected women .

Finally *E. coli* that isolated from wound swab samples 24% are coordinate with study done by Ali (2018) in Iraq who reveals that *E.coli* found in 30% of wound collected clinical samples.

Also compatible with Abdulqader and Saadi, (2019) with prevalence rate 29%. While (Adhikari *et al* ,2020) reported the prevalence rate about 24.2% .

The isolation rate of *E.coli* from urine ,vagina and wounds depends on several factors like virulence of isolates, health status of patients and effect of environmental conditions. Moreover, The study made by Yonas *et al* ., (2017) investigated that the UTI caused by *E. coli* affecting both sexes and all ages groups across the life span but women are more susceptible than men, due to short urethra, pregnancy ,easy contamination of the urinary tract with fecal flora and people with anatomical malformations of the urinary tract.

Although this bacteria lives in human intestine as natural habitat for it, but not all stool samples obtained in this study have this bacteria(negative isolates), this means that this bacteria is may be not a causative agents for diarrheal stool or this bacteria is absent due to drug abused. Sarowska, *et al* ., (2019) shown that the bacterial pathogens have evolved numerous strategies to exploit their host's cellular processes so that they can survive and persist.

E.coli includes in variety of different disease, ranging from highly specialized pathogenic strains causing worldwide outbreaks of severe diseases to opportunistic pathogens which have the potential to cause disease if the human host defenses are compromised and to a virulent isolates which are part of the normal intestinal microbiota, or which are well-characterized and safe laboratory strains (Chu *et al* ,2018).

However ; mutations gene duplications and horizontal gene transfers (HGT) are frequent in *E. coli*, which make its genome highly flexible and dynamic (Chu *et al* ,2018). These evolutionary mechanisms may result in

changes in the physiology or lifestyle of the bacterium for instance, a strain may gain pathogenic capacity, the ability to use a carbon source, the ability to take upon a particular ecological niche or the ability to resist antimicrobial agents (Peterson and Kaur , 2018).

3. 2 .The Characterization of *Escherichia coli*

3. 2.1. Confirmation of the isolates

3.2.2. Culturing of bacterial *E. coli* isolates

The identification of *E.coli* depends mainly on the cultural and biochemical characteristics and also microscopic patterns. During the present investigation of samples which was processed to isolate *E.coli* morphologically typical colonies were verified by Gram staining, to determine the size, shape and arrangement of bacteria. The organisms revealed gram negative, pink colored with rod shaped appearance and arranged in single or in pair were suspected as *E. coli.*

A series of biochemical tests were performed which included catalase are positive, oxidase test are negative, TSI test was performed by incubation at 37ºC for 24 hours, the tubes showing acid butt (yellow), acid slant (yellow), with gas production and no H_2S production were interpreted to be positive for *E. coli*, this give rise to be consider the *E. coli* is an acid resistant food borne pathogen that survives in the acidic environment of stomach and colonize the gastrointestinal tract (Xu,*et al* ,2020). Furthermore, it also increases the survival of *E. coli* for extended periods, especially at refrigeration temperature (Paswan and Park ,2020).

E.coli can be differentiated from other members of the *Enterobacteriaceae* on the basis of a number of sugar fermentation and

other biochemical tests. Classically an important group of tests used for this purpose are known by the acronym IMVIC.

Most of isolates of *E. coli* showed fermented, glucose, lactose and sucrose with the production of both acid and gas.

The diagnostic features of bacteria are summarized in table (3-2). However, some other characteristic of *E.coli* should be considered to confirm the identification of this bacteria through using specific markers via PCR techniques.

Table (3-2): The most important traditional tests used in identification of *E.coli* isolates

Tests	Results
Gram stain	Gram negative short rods
Growth on the EMB agar	Green metallic sheen
Catalase	Positive
Oxidase	Negative
Indole (I)	Positive
methyl red(MR)	Positive
Voges prauskar(VP)	Negative
Citrate	Negative
Carbohydrate fermentation (glucose, lactose, sucrose)	Positive
H_2S	Negative
Urease	Negative

3.2.3.Identification of *E coli* by Vitek2 System:

After identification of bacterial colonies on the conventional biochemical test as were summarized in table (3-2). The isolates were

identified with Vitek2 compact auto analyzer system. The results produced by the machine were analyzed using compact software.

In this study the identification of *E. coli* isolates were confirmed by using Vitek2 system compact, which is the standard biochemical identification system for bacteria.The analytical profile index of this system has showed the probability rate of identification scored more than 97 % with excellent confidence.The vitek2 system may provide on accurate microbial identification, as phenotypic confirmatory tool, and it is the most automated platform available, with rapid results, and high degree of specificities, with improved confidence, and minimal training time than with manual microbial identification techniques.

The results revealed that 30(100%) samples of clinical were confirmed as *E. coli* .The results in the present study are similar to the results of Modest *et al*., (2015) who reported the rate of *E. coli* within clinical isolates was (98%).

3.2.3.Molecular identification of *E. coli* with 16srDNA:

The one aim of this study was to develop a rapid molecular diagnostic test for identity and purity of bacterial isolates based on the internally transcribed 16SrDNA profiling, comparison with of traditional criteria and that they may be good candidates to be used as genotypic markers for confirmatory identification of *E. coli* isolates from different sources. The clinical isolates had been classified previously as presumptive *E. coli* by routine phenotypically testing and it was found that all isolates of were positive at rate 100% as shown in table (3-2).The internally 16SrDNA gene of *E.coli* were analysed with respect of 30 specimens of *E.coli* were amplified by PCR and the PCR product was subjected to gradient analysis, which allowed the identification of the

bacteria.According to the results; it was revealed that all isolates (30) samples of *E. coli* isolated from different types of sources gave positive result (100%) containing sequence as shown in figure (3-1)

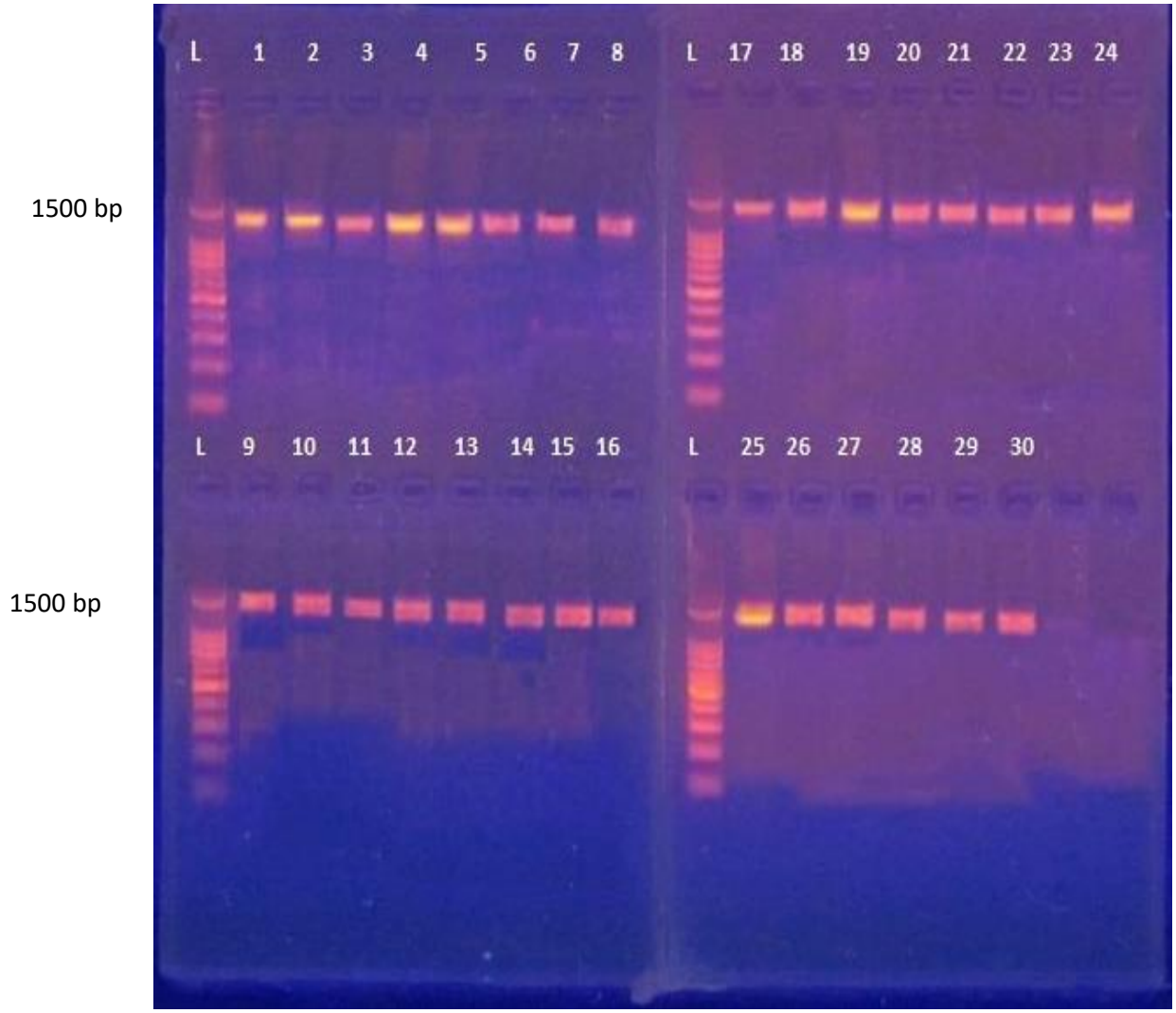


Figure (3-1): Gel electrophoresis of PCR product (1500bp), for *Escherichia coli* 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min . Lane 1-30: PCR product positive for *16S rDNA* gene, marker for DNA ladder (100-1500)bp .

3.2.4: The frequency of *E.coli* isolates according to the age groups:

Table 3-3; showed that the large occurrence of *E. coli* isolates was in the age group (31-45) years at a rate 33.33%, while the lower occurrence was at age group (>60) years old was 10% in female ;Also the highest incidence of *E. coli* in male patients with age group of (31-45) years old at a rate 6.67% while the lowest incidence in the age group (1-15) years at a rate 0%. The high occurrence of *E.coli* in both male and female in age (16-30) and (31-40) years old and may be attributed to sexual activity in both sex in this age, pregnancy in female also a prior UTI, vaginal infection, diabetes, obesity and genetic susceptibility (Hannan *et al*.,2012).

Table(3-3):The frequency of *E.coli* isolates in different age groups

No.	Age groups (Years)	No. of isolates (%)		Patients	
		Female	Male	No.	%
1.	1-15	3 (10.00 %)	0(0.00%)	4	13.33
2.	16-30	6(20.00%)	2(6.67%)	8	26.66
3.	31-45	8(26.67%)	2(6.67%)	10	33.33
4.	46-60	4(13.13%)	1(3.33%)	5	16.66
5.	> 60	2(6.67%)	1(3.33%)	3	10
Total		25 (83.33%)	5 (16.67)	30	100%

The results were differed from the results of Dawood, (2020) whose revealed that the highest prevalence of *E.coli* was observed in the age

group of (>70) years at a rate 46% and the lowest was seen in the age group of (14- 40) years at a rate 8% among male patients.

Females have a notable increase frequency of *E . coli* versus males, this difference in frequency could be due to several clinical factors, including anatomic differences, hormonal effects, and behavioral patterns Tabasi *et al*., (2015).This result agreed with (Prakapait *et al* ,2019) at a rate (female, male 87%:13%).

3.2.5:Effect of some quinolones antibiotics on *E.coli* isolates

Five types of quinolone antibiotics are used to show their effects on *E.coli* isolates. It was found that the bacterial isolates showed resistance to first generation Nalidixic acid with resistance percentage 86.36%, second generation Ciprofloxacin; with resistance percentage 66.66% and Norfloxacin with resistance percentage 63.33%, third generation ; Levofloxacin with resistance percentage 27.27%, and to fourth generation Gatifloxacin with resistance percentage 13.33%.

Table (3-4): Quinolone antibiotics resistance of (30) *E. coli* isolates

Quinolone Antibiotics	Rate of resistance No.	(%)	Rate of intermediate No.	(%)	Rate of sensitive No.	(%)
Norfloxacin	19	63.33%	5	16.66%	6	20%
Gatifloxacin	4	13.33 %	6	20.00 %	20	66.66%
Ciprofloxacin	20	66.66%	4	6.66%	6	20%
Nalidixic acid	25	86.36%	3	10.00%	2	6.66%
Levofloxacin	8	27.27%	10	33.33 %	12	40%

Alssadi (2018) as well as the results recorded by Naji *et al*.,(2017) in Iraq who were showed, resistance range (50 to 70%) to ciprofloxacin, norfloxacin, and above 70% to nalidixic acid. However in Pakistan by Ali *et al.*, (2016) showed that the rate of resistance against ciprofloxacin, levofloxacin and norfloxacin were remained 60%, 58% and 57%, respectively. The findings of the present study were found to be more than the results recorded in Nigeria by Ekwealor *et al.*, (2016) who reported the rate of resistance against ciprofloxacin was (27%). Previous studies have found that ciprofloxacin showed a higher number of resistant strains than levofloxacin among the clinical isolates of Enterobacteriaceae (Brink *et al*,2012; Fu ,2013).

The increased resistance rate among isolates was mostly due to over use, disuse of medical prescription with empirical therapy that increase antibiotic pressure and increase probability of resistance transfer such as plasmid-mediated antibiotic resistance that found to be common in *E. coli* isolates. This variation in resistance among quinolones can be explained by the intrinsic structural drug differences between older and newer quinolone classes. Nalidixic is devoid of any cyclic derivatives whereas cip and levo have substituents at positions C-6, C-7 and C-8, that offer greater spectrum/potency of activity (Bhatnagar and Wong ,2019).Thus, the modified quinolone substituents likely reduce resistance levels by increasing the affinity for *gyr*A, and by stabilizing the quinolone-DNA complex.

3.3. Molecular characterization of quinolone resistance genes *(qnr* genes) by PCR

Current study out of 30 clinical isolates ; 12 isolates were positive for *qnrA* gene (40%) (figure 3-2) , and 13 isolates were positive for *qnrB*

gene (43.33%) while there are 16 bacterial isolates have *qnr*S positive (53.33%). The *qnrS* was the most prevalent, followed by *qnrB* and finally *qnr A.* Present findings concurred with a previous European survey that *qnrS* was more frequently detected than other *qnr* genes in clinical *Enterobacteriaceae* isolates (Alm'amoori *et al* ,2020).

The total percentage of *E. coli* isolates that harboring at least one of studied *qnr* genes 21/30 (70%); this composed from 14/30 isolates harboring *qnr* in combined or mixed form (46.66%) and 7/30 (23.33%) isolates harboring *qnr* in single form (3 isolates harboring *qnr*A gene alone, 1 isolates harboring *qnr* B alone, 3 isolates harboring qnr S alone).It seems there is a high frequency of transconjugation or transformation mechanisms of the *qnr* genes.

Other study revealed that 59.88% of the *E.coli* isolates from clinical sample in south of Iran harbored the *qnr* genes. (FarajzadehSheikh *et al*,2019); while Nsofor *et al* ,(2021) reports that electrophoresis analysis revealed that 28 out of the 30 (93.3%) *E.coli* isolates from different source were harbored at least one fluoroquinolone resistance gene. Mokhtari-Farsani(2016) mentioned 80.34% of clinical *E.coli* samples are resistant to at least one quinolone.

These data show that the distribution of *qnr* genes in *E. coli* varies from one region to the other. The regionally diverse and wide distribution of *qnr* genes in *E. coli* suggests that resistance evolved independently on many occasions. However, the rational and controlled use of fluoroquinolone antibiotics is an effective way to control the development of higher levels of resistance.

The results of this study regarding PMQR were differed from the results conducted by Al-Hillali, (2015) who showed that only one (1%)

isolate carried *qnr*A gene, (4.8%) isolates carried *qnr*B gene, and (6.7%) isolates carried *qnr*S gene, while, no amplified product for *qnr*C gene.

Although the results of this study were found to be lower to these obtained by Mokhtari-Farsani *et al*., (2016) who pointed that (19.15%) of isolates were carrying to *qnr*A, Whereas *qnr*B, *qnr*S were detected in (88.30%) and (78.72%) respectively.

However data obtained in the present study were higher than those of Al-Hasnawy *et al* (2018) in Iraq which revealed the percentage of *qnr* genes was (31.8, 56.5 and 28.9%) for *qnr*A and *qnr*B, *qnr*S respectively.

This difference may be due to the selected *E. coli* population and change in environmental conditions and geographical areas. *E. coli* tested from different clinical samples were found harboring at least one of the *qnr* genes consisting of 47.74% *qnrB*,47.10 % *qnrS* and 12.58% *qnrA* (Salah, *et al* ,2019).

The prevalence of plasmid-mediated quinolone resistance (PMQR) determinant was investigated in many countries . Tarchouna *et al*, (2015) found that (32%) of *E. coli* strains isolated from different clinical samples in several Arab country like in a Tunisian hospitals were positive for the presence of *qnr* genes, and only (12.5%) of them were carried *qnrB* gene .

Another study conducted in Egypt by EL-Mahdy (2015) who revealed that (53%) of *E. coli* were positive for the *qnr* genes; while *qnrA* and *qnrB* genes were not found among the clinical isolates from UTI in Iran- Tehran . Differences in distribution of the *qnr* genes may be attributed to difference in geographical area , or may be due to difference in selection criteria (EL-Mahdy , 2015).

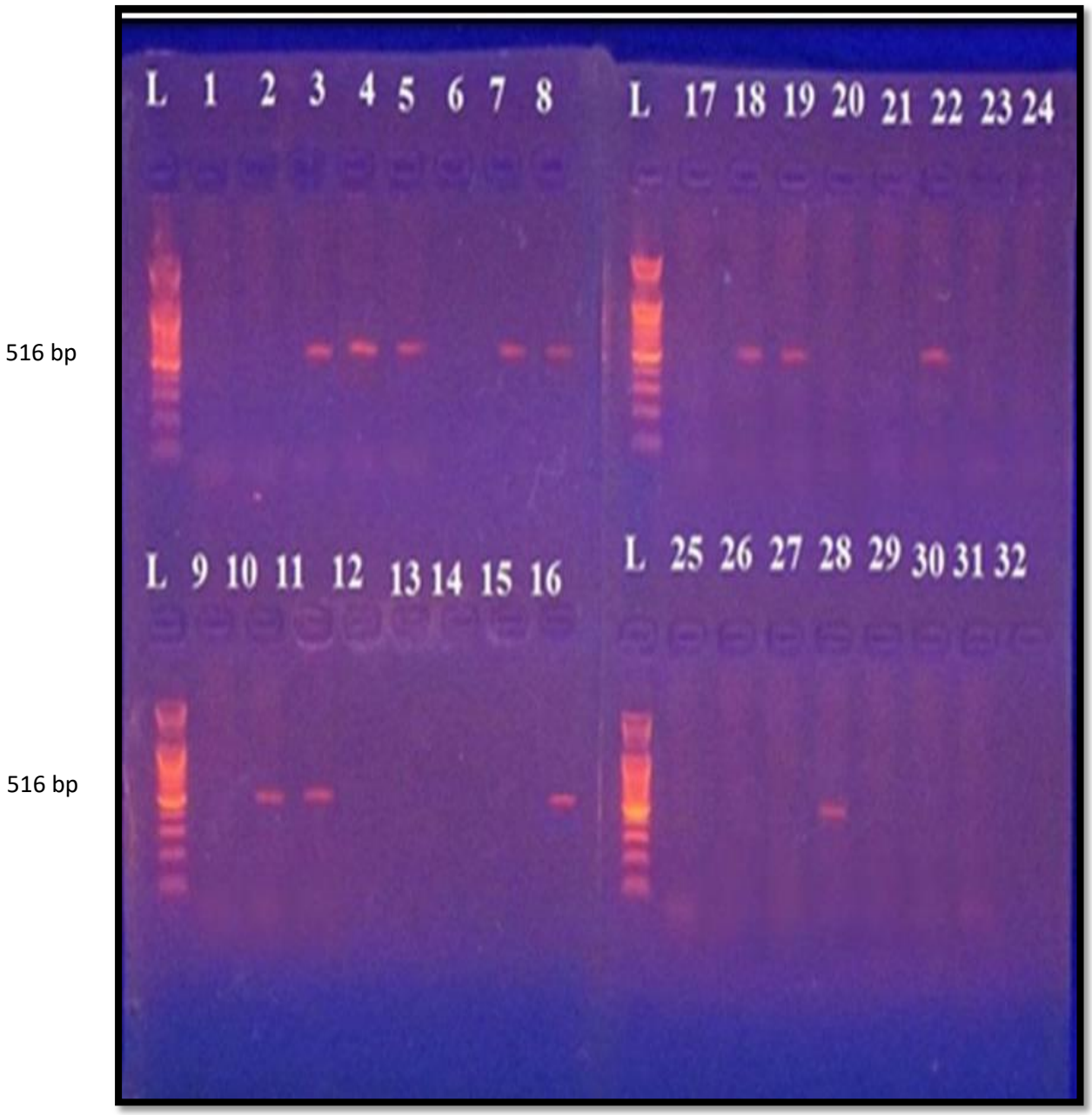


Figure (3-2):Gel electrophoresis of PCR product size (516 bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (3,4,5,7,8,10,11,16,18,19,22,28) PCR product were positive for *qnr* A gene, marker for DNA ladder (100-1500)bp .

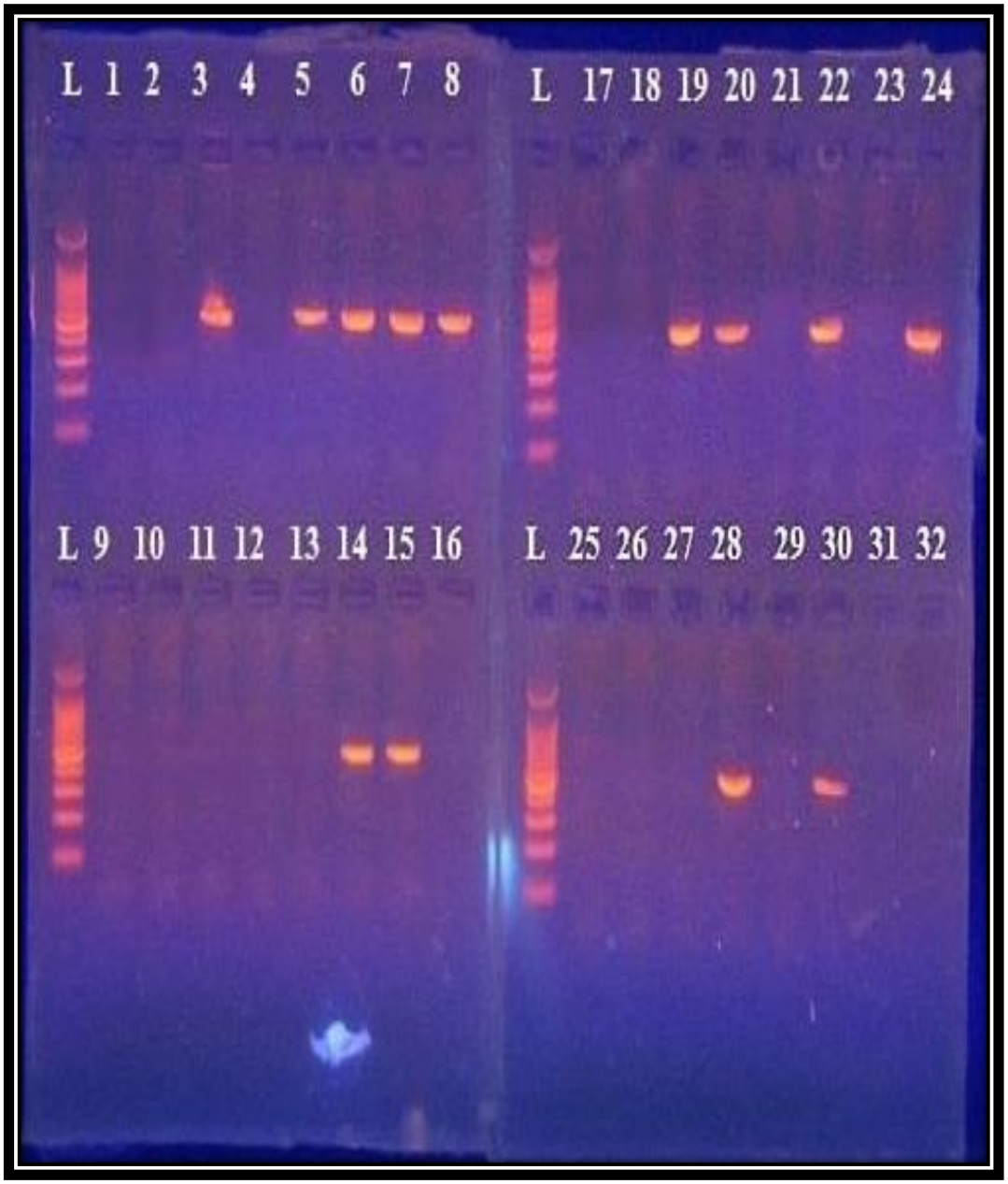


Figure (3-3): Gel electrophoresis of PCR product size (469 bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (3,5,6,7,8,14,15,19,20,22,24,28,30) PCR product were positive for *qnr* B gene, marker for DNA ladder (100-1500) bp .

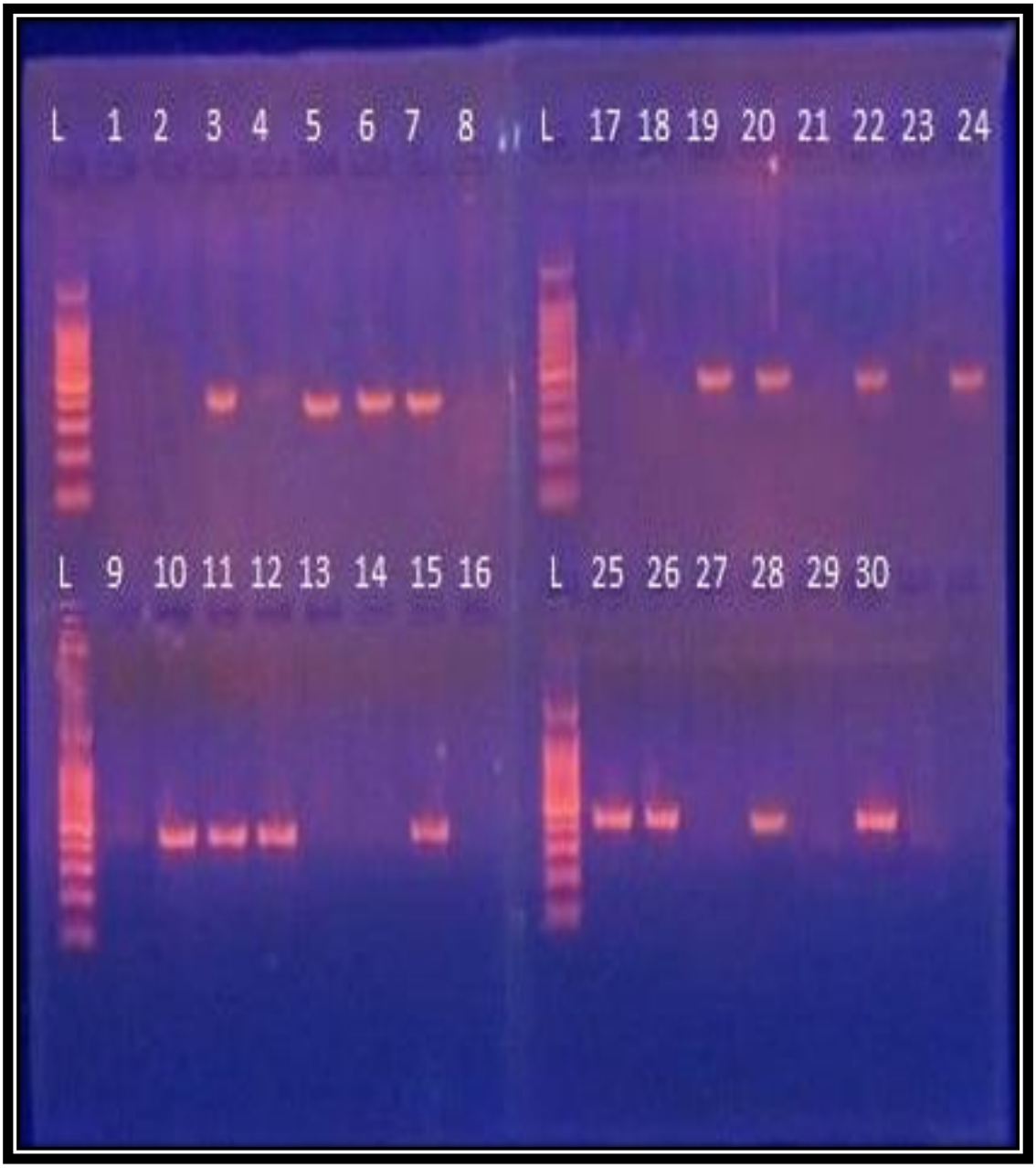


Figure (3-4): Gel electrophoresis of PCR product size (417 bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (3,5,6,7, 10 ,11, 12, 19, 20, 22, 24,25,26,28,30) PCR product were positive for *qnr* S gene, marker for DNA ladder (100-1500)bp .

3.3.1. *Qnr*-genes harboring *E.coli* and its relation with MIC value:

In case of *qnr* A gene , there are three samples (4,16,18) out of twelve samples with single *qnr* A gene with percentage 3/12(25%); while in case of *qnr* B , there is only one sample(sample number 14) out of thirteen samples with single *qnr* B gene alone with percentage 1/13 (7.69%).

Finally in case *qnr* S gene , there are three samples (12,25,26) out of sixteen samples with single *qnr* S gene with percentage 3/16 (18.75%).

All isolates with single *qnr* only have low-level resistance concerning MIC value. This result are compatible with Wang *et al* ,(2017) who refers that MIC value was below the CLSI resistance breakpoint or may provide low level of resistance .

The plasmid-mediated mechanisms provide only low-level resistance that by itself does not exceed the clinical breakpoint for susceptibility but nonetheless facilitates selection of higher-level resistance and makes infection by pathogens containing PMQR harder to treat. (Jacoby *et al* ,2014).Thus, some strains have coexistence of two or three tested *qnr* genes on a multiple resistance plasmid.

The current study demonstrated that the coexistence of three or two *qnr* genes can cause an increase in MIC more than the presence one of *qnr* genes for all quinolone antibiotics under study (figures: 3-5,3-6,3-7 , 3-8, 3-9). The coexistence of *qnr* A,B,S genes are observed in six samples out of fourteen tested clinical isolates of *E.coli* (42.86%) (samples number 3,5,7,19,22,28) while there are eight sample out of fourteen (57.14%) harboring two *qnr* genes (sample number 6,8,10, 11,

15,20,24,30). The distribution of coexistence of three PMQR genes on a multiple resistance plasmid reveals in table (3-5).

Table(3-5):Coexistence of three PMQR genes on a multiple resistance plasmid

Coexistence of different genes	No. of isolates	Percentage
QnrA+QnrB+QnrS	6	42.86
QnrA+QnrB	1	7.14
QnrA+QnrS	2	14.29
QnrB+QnrS	5	35.71
total	14	100

This results are agreement with Machuca *et al* ,(2014) (Machuca, *et al* ,2016) who reported that the presence of any combined in plasmid-mediated *qnr* genes increased the MIC levels in all *E. coli* genotypes.

This may be back to the role of *qnr* genes plays in providing additional protection to the resistant *E.coli* isolates such as added structural features (loops, N-terminal extension) of *qnr* proteins allow interactions with regions of *gyrase* besides the DNA binding groove (Xiong , 2011) and could allow more specific binding to and destabilization of the topoisomerase-DNA-quinolone cleavage complex.

Other study reported that *qnr* genes is involved in the response to DNA damage and nucleic acid secondary structure.(Gil-Marqués *et al* ,2021). *Qnr* plasmid-mediated resistances highly facilitate the spread and increase their frequency (Salah,*et al* ,2019).

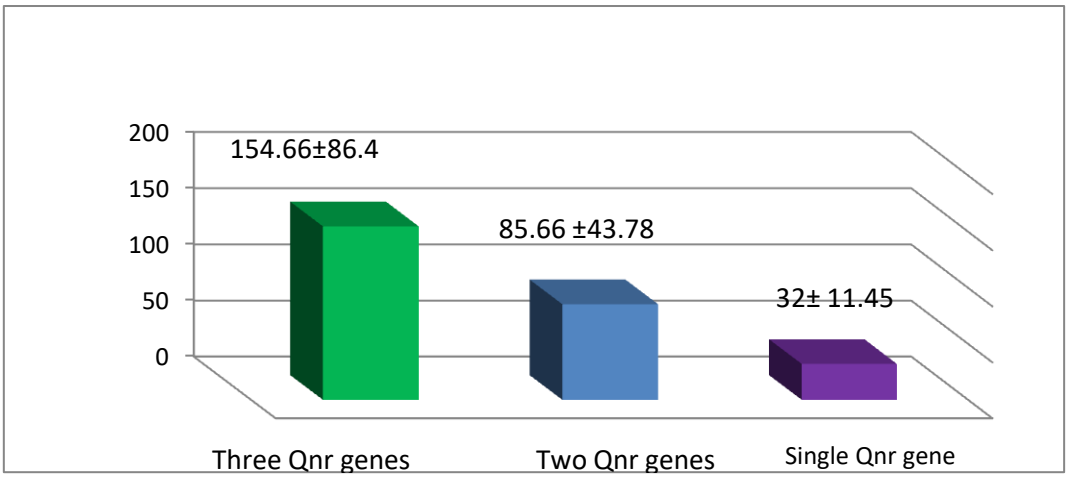


Figure (3-5): Nalidixic acid antibiotics MIC value

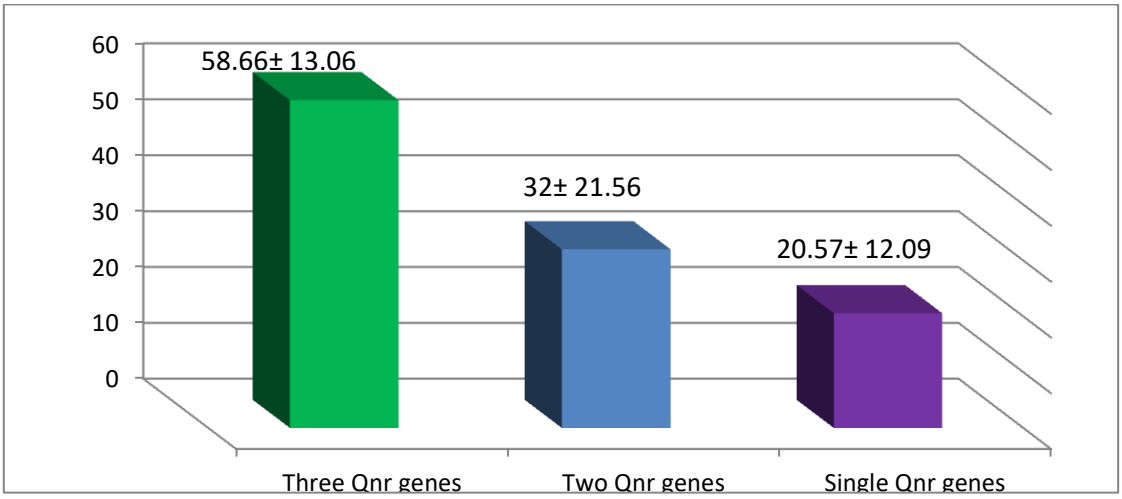


Figure (3-6) :Norfloxacin antibiotic MIC Value

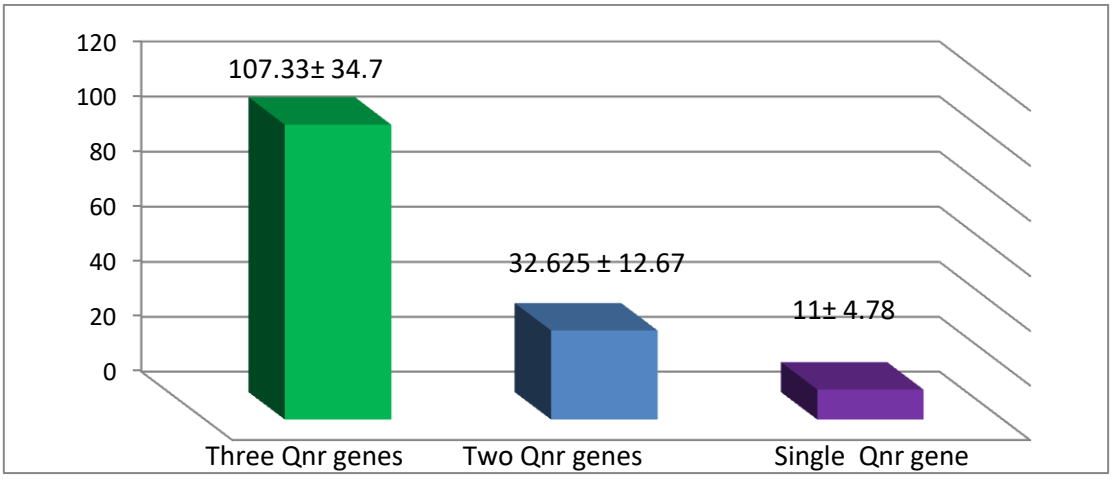


Figure (3-7) :Ciprofloxacin antibiotic MIC Value

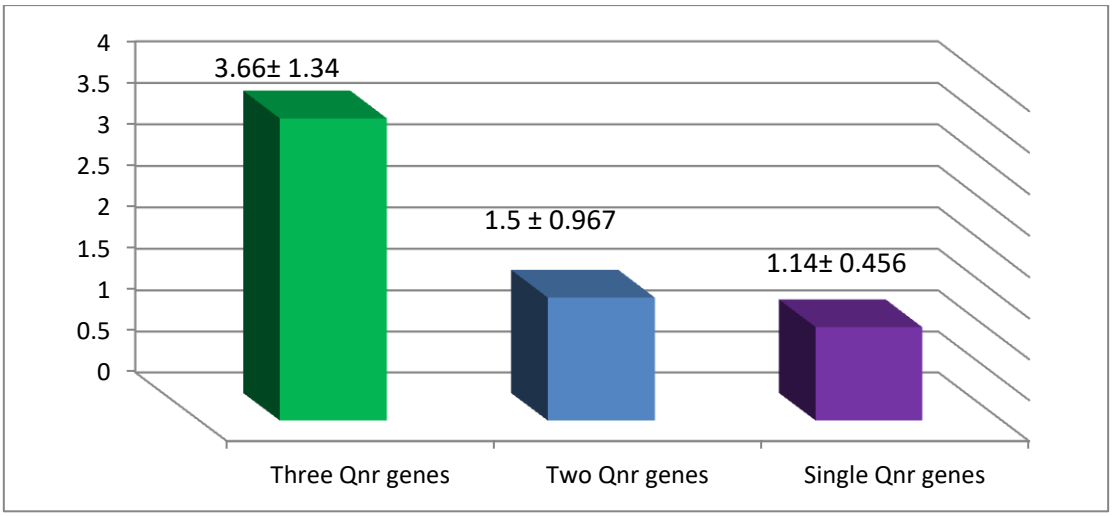


Figure (3-8) :Levofloxacin antibiotic MIC Value

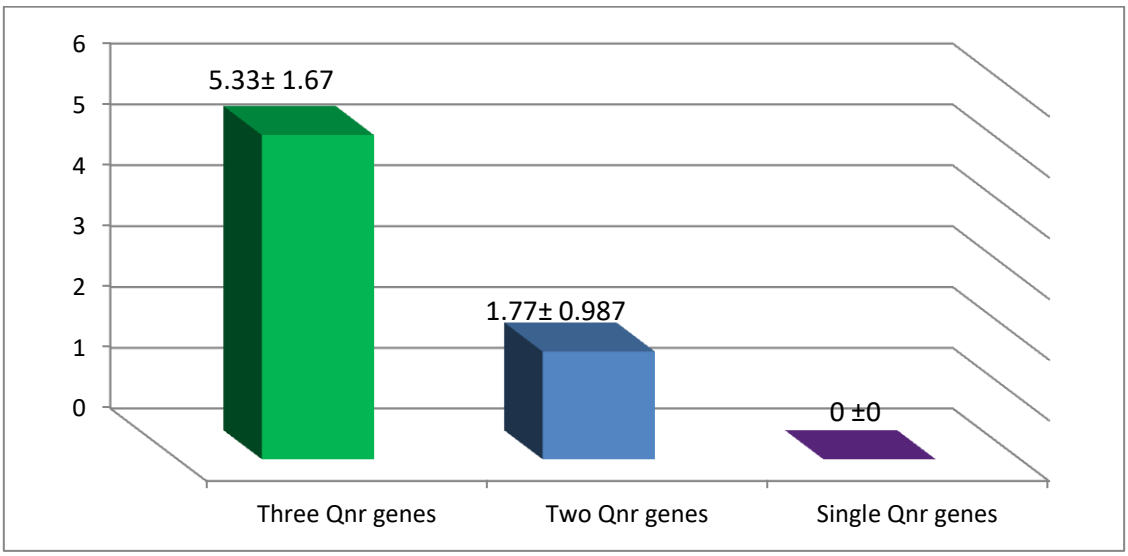


Figure (3-9) :Gatifloxacin antibiotic MIC Value

3.3.2 *E.coli* isolates resistance to quinolone antibiotics and its relation with *qnr* genes:

E.coli resistance to the quinolone antibiotics and its relation with *qnr* gene prevalence are revealed in figure (3-10).

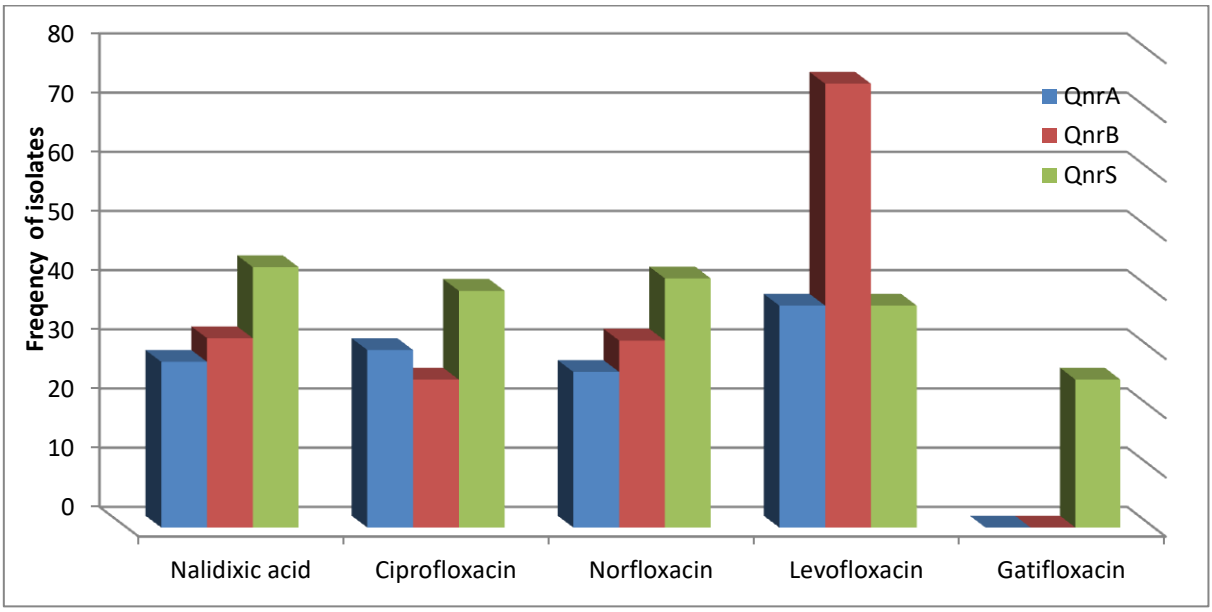


Figure (3-10):Distribution of *qnr* genes prevalence in clinical *E.coli* isolates

Among Nalidix acid resistance *E.coli* isolates (25 clinical isolates) encoding studied *qnr* genes (A, B and S) in our study, 28%,32%,44% respectively were resistant to Nalidixic acid. The prevalence of plasmid-mediated quinolone resistance (PMQR) determinant in NA-resistance isolates was investigated by Taha *et al* (2019) in Iraq who report that the frequency of *qnr* A, in NA-resistant *E.coli* was (24.5%) and Al-Hasnawy (2018) who found that the frequency of *qnr* B in NA-resistant *E.coli* was 14.% while; Malekzadegan *et al* ,(2019) was found that the frequency of *qnr* S in NA-resistant *E.coli* was 34.7%.

Ciprofloxacin resistance isolates that are carry *qnrA* , *qnr* B and *qnr* S genes was 30.0%,25.0%, 40.0% respectively. The isolates exhibited high-level ciprofloxacin resistance. (Doma *et al* ,(2020) reports that *qnrA* gene was observed only in 12.0% of isolates of CFX–resistance-*E.coli* obtained from human subject. Sedighi *et al* (2015) reported that CFX-resistance isolates that carry *qnr B* prevalence was 6.7% ; while Firoozeh *et al*.(2014) reported that 14% of CFX–resistance isolates are harboring *qnr* B.

This result are compatible with (FarajzadehSheikh *et al* ,2019) and Hamed,*et al* (2018) who reported that *qnr* genes were harbored by 32.5% in ciprofloxacin resistance *E.coli* isolates from blood and stool samples.

Prevalence rate of *qnr* S gene was higher than other tested *qnr* type (*qnr* A, *qnr* B). This means *qnr* S gene seems to play a more significant role than *qnr*A and *qnr*B with regard to quinolones resistance since 62.5% (10/16) of the isolates harboring the *qnr*S gene were resistant to all quinolones . The combination of *qnr* S and B detected in five strains have increased the level of resistance to quinolone-containing antibiotics nearly to 100% (4 of 5), suggesting that the synergistic effects resulting from the combination of *qnr*S with *qnr*B in a strain may increase their defensive positions. However, some other factors such as the overexpression of efflux pump or declined intracellular concentration and mutations (in *topoisomerase* IV and DNA *gyrase* enzymes) could also be equally effective in this regard.

Norfloxacin–resistant *E.coli* isolates percentage that harbor *qnr* A,B and S in the present study are 26.3 %,31.6%,42.1% respectively .All of isolates shows higher level of MIC value than ciprofloxacin.

Other studies report that resistant of norfloxacin and ciprofloxacin in clinical *E.coli* are interrupted with presence of *qnr* genes (Saeed ,2014; Alm'amoori *et al* ,2019) in Iraq as well as in study done by Hooper and Jacoby,(2015) whom mentioned that *qnr* genes not partially protects *E. coli gyrase* against ciprofloxacin and norfloxacin inhibition but also inhibits ATP-dependent supercoiling activity of gyrase. However horizontal transferable resistance genes is often compensated by the regulation of transcription factors encoded by other genes harbored onto the same plasmid.(Dionisio *et al* ,2019)

Levofloxacin resistance isolates that are carry *qnr*A , *qnr* B and *qnr* S genes was 37.5%,75.0%,37.5% respectively. Results of this study indicate that levofloxacin presents the highest frequencies of *qnr* A,B,S genes more than other tested FQ antibiotics and was compatible with (Malekzadegan *et al* ,2019) who mention that levofloxacin presents the highest frequencies of *qnr* in their study of quinolone-resistant pathogenic *Escherichia coli* in a tertiary care hospitals in Iran.

Also; current study compatible to several studies concerning MIC value (Nordmann and Poirel ,2005; Martinez-Martinez *et al* ;2003) that reported *qnr* A,B,S positive clinical strains of *E.coli* determinant raised MICs of levofloxacin to 32-fold compare to wild type ;reaching MIC values of up to 8 mg/L. Finally ; lowest prevalence of *qnr*A,B,S -positive *E.coli* strains was against Gatifloxacin antibiotic since ; the prevalence rate of *qnr* A,B,S (0% ,0% , 25%).This may due to gatifloxacin is the newest and last reported generation of flouroquinolone antibiotics.

3.3.3 Prevalence of resistance *E.coli* clinical isolates with negative *qnr* genes

In the current study ;there are resistance *E.coli* clinical isolates with negative *qnr* genes which are reveal in table (3-6) . The total prevalence of these isolates are 11 % , 10.2%, 9.0% for *qnr* A,B,S respectively.

Table(3-6):Distribution of resistance *E.coli* isolates with negative *qnr* genes

Quinolone-antibiotics	Total No. of resistance isolates	-ve *qnr* A		-ve *qnr* B		-ve *qnr* S	
		No	%	No	%	No	%
Nalidixic acid	25	18	72.0	17	68.0	14	56.0
Ciprofloxacin	20	14	70.0	15	75.0	12	60.0
Norfloxacin	19	14	73.7	13	68.4	11	57.9
Levofloxacin	8	5	62.5	2	25.0	5	62.5
Gatifloxacin	4	4	100	4	100	3	75.0
Total (%)		55	11.0	51	10.2	45	9.0

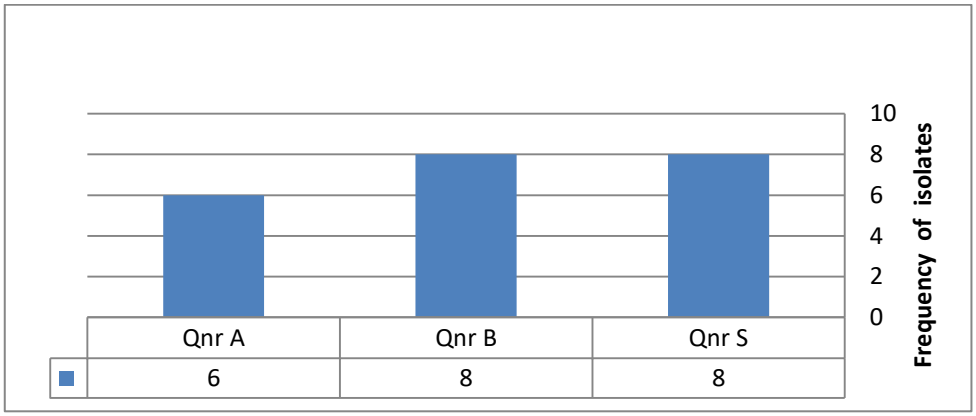
These findings form the result above showed that *qnrA*, *qnrB*, and *qnr* S-negative isolates were also resistant to NA and fluoroquinolones with percentage (12.0%,13.0% and 10.5%) respectively; which signifies that other *qnr* genes like *qnr* C and *qnr* D or resistance mechanisms,such as mutations in the target enzyme (e.g., DNA *Gyr A* and *topoisomerase* IV) and/or activation of efflux pumps, may be involved. However that ; *qnr* gene is involved in the response to DNA damage and nucleic

acid secondary structure (Gil-Marqués *et al* ,2021). In *vitro* results of studies and detailed analysis of several fluoroquinolone-resistant and *qnr*-negative isolates against Nalidixic acid showed that chromosome and plasmid-mediated quinolone resistance determinants have additional effects. (Mammeri, *et al* ,2005). Indeed, mutations in *gyrA, gyrB,* and in efflux pump and porin genes may increase plasmid-mediated quinolone resistance (NCCLS , 2004). This observation explains the higher level of resistance to quinolones in clinical isolates compared with those observed for wild type in several studies including our study.

The CFX- resistance in these isolates but can be back to contribution of both mutated chromosomal *gyrA* gene and plasmidic *qnrA* resistance genes in most of the high ciprofloxacin resistant bacterial strains, besides the overuse of antibiotics, which can increase the emergence of resistant strains (Zahedi *et al*,2018). Several studies refer that ciprofloxacin and norfloxacin are only fluoroquinolones which possess the piperazynil amide side chain, which is acetylated by the enzyme encoded by *aac*(6′)*Ib-cr* (Robicsek *et al*, 2006; Luzzaro,2008; Cavaco and Aarestrup, 2009) which may contributed to their high MIC value in resistance *E.coli* isolates. This mechanisms also reported by Oviaño *et al*,(2017) that *AAC(6')-Ib-cr* acetyltransferase plasmid-associated gene that encodes a variant of a common aminoglycoside acetyltransferase that is capable of acetylating and reducing the activity of certain FQs, mainly ciprofloxacin and norfloxacin, but not levofloxacin. Gatifloxacin, which differ only in presence of an 8-methoxy group, respectively, this moiety at the 8 position appears to exert its greatest influence in reducing the effect of common resistance mutations on drug activity (Ince and Hooper,2020).

3.3.4. Prevalence of unexpressed *qnr* genes in non-resistance fluoroquinolone isolates:

The presence of some unexpressed PMQR genes in fluoroquinolone sensitive isolates was also observed. The study also demonstrated that extra-intestinal *E. coli* isolates might carry silent antibiotic resistance genes, since a eight fluoroquinolone sensitive isolates (26.6%) of *E. coli* harboured *qnr* A,B and *qnr*S. Perhaps this gene did not express in these isolates as phenotypic resistance. The silencing phenomenon was due to the chromosomal effects of the host. Later, it was also found that the silencing was reversed at a low frequency of 10^{-6}-10^{-10} in the original host (Enne *et al* , 2006).Deekshit *et al* , (2012) suggested that the deletion of promoter region was the main reason for unexpressive nature of acetyltransferase (*catA*) gene in *Salmonella weltevreden*.Unexpression of gene related to mutation in upstream region or mutation encoding region as well as there is no secretion system or there is no gene function. These reports show the future risk associated with the global emergence of plasmid-borne resistance pattern of clinical pathogens and these PMQRs can also contribute to the elevated levels of quinolone minimum inhibitory concentrations (MICs) in clinical isolates.



Figure(3-11):Prevalence of unexpressed *qnr* genes in fluoroquinolone sensitive isolates

3.3.5. Prevalence of genes *mar* RAB, *rob* and *sox*RS by PCR:

In current study; high prevalence of genes *mar* RAB, *rob* and *sox*RS are showed in figure(3-12)(3-13)(3-14).Twenty seven out of thirty of *E.coli* isolates have *mar*RAB regulon with prevalence rate (90%) . The *rob* regulon have been found in also twenty seven isolates with prevalence (90%) ; while twenty two of thirty tested *E.coli* isolates have *sox* RS gene with the percentage (73.33 %) .These result are compatible with Gupta, *et al*.(2019) who reported that multiple antibiotic resistance (*mar*) operon and *rob* is one of the main regulators of drug resistance in *Escherichia coli*.

The result are in coordinate with Kirti and Saini (2017) who reported that *rob* has been observed to control similar downstream targets as *marA* and *soxS* genes while Sharma *et al* ,(2017) found that *rob* usually binds marboxes with a higher affinity than either *mar*A or *sox*S. Iraqi study done by Obeed and Dhahi (2020) in Bagdad shows (87.5%) of *E.coli* isolates were harboring *mar*RAB regulon, while these results were expected, as all three regulators bind to the same sites (Chubiz *et al* ,2012).

Similar antibiotic resistance was conferred by expression of a *rob* fragment containing only the N-terminal 123 residues that constitute the *sox*S-*mar*A homology are reported by Singh (2012).The multiple antibiotic resistance (*mar*) operon of *Escherichia coli* is a paradigm for chromosomally encoded antibiotic resistance in enteric bacteria.(Sharma *et al*,2017).The locus is recognized for its ability to modulate efflux pump and porin expression via two encoded transcription factors, *mar*R and *mar*A (Sharma *et al*,2017). The *mar*RAB, *sox*RS and *rob* regulons,

are involved in the regulation of AcrAB-TolC multidrug efflux pumps.(Bush *et al* ,2020).

It is observed that *mar*A, *sox*S and *rob* are activator of AcrAB-TolC tripartite efflux pump systems. Over expression of these regulators has resulted in multidrug resistant phenotype (Chetri, *et al* ,2020). In *E. coli*, the overexpression of efflux pumps is often linked to mutations in *mar*RA regulon (Correia *et al*.,2017). The *rob* and *sox*RS regulons, which are involved in the regulation of these efflux pumps, as well as many other pathways in the cell (Correia *et al*.,2017).

In current study; high prevalence of genes *mar* RAB, *rob* and *sox*RS are showed in figure(3-12)(3-13)(3-14).Twenty seven out of thirty of *E.coli* isolates have *mar*RAB regulon with prevalence rate (90%) . The *rob* regulon have been found in also twenty seven isolates with prevalence (90%) ; while twenty two of thirty tested *E.coli* isolates have *sox* RS gene with the percentage (73.33 %) .These result are compatible with Gupta, *et al*.(2019) who reported that multiple antibiotic resistance (*mar*) operon and *rob* is one of the main regulators of drug resistance in *Escherichia coli*.

The result are in coordinate with Kirti and Saini (2017) who reported that *rob* has been observed to control similar downstream targets as *marA* and *soxS* genes while Sharma *et al* ,(2017) found that *rob* usually binds marboxes with a higher affinity than either *mar*A or *sox*S. Iraqi study done by Obeed and Dhahi (2020) in Bagdad shows (87.5%) of *E.coli* isolates were harboring *mar*RAB regulon, while these results were expected, as all three regulators bind to the same sites (Chubiz *et al* ,2012). Similar antibiotic resistance was conferred by expression of a *rob* fragment containing only the N-terminal 123 residues that constitute the

*sox*S-*mar*A homology are reported by Singh (2012). Efflux has been shown to be crucial for the development of high-level fluoroquinolone resistance, because the inactivation of major efflux systems prevents the selection of fluoroquinolone-resistant mutants and strains carrying specific target site mutations are no longer clinically resistant if efflux pumps are inactivated (Mohammad *et al* ,2018).

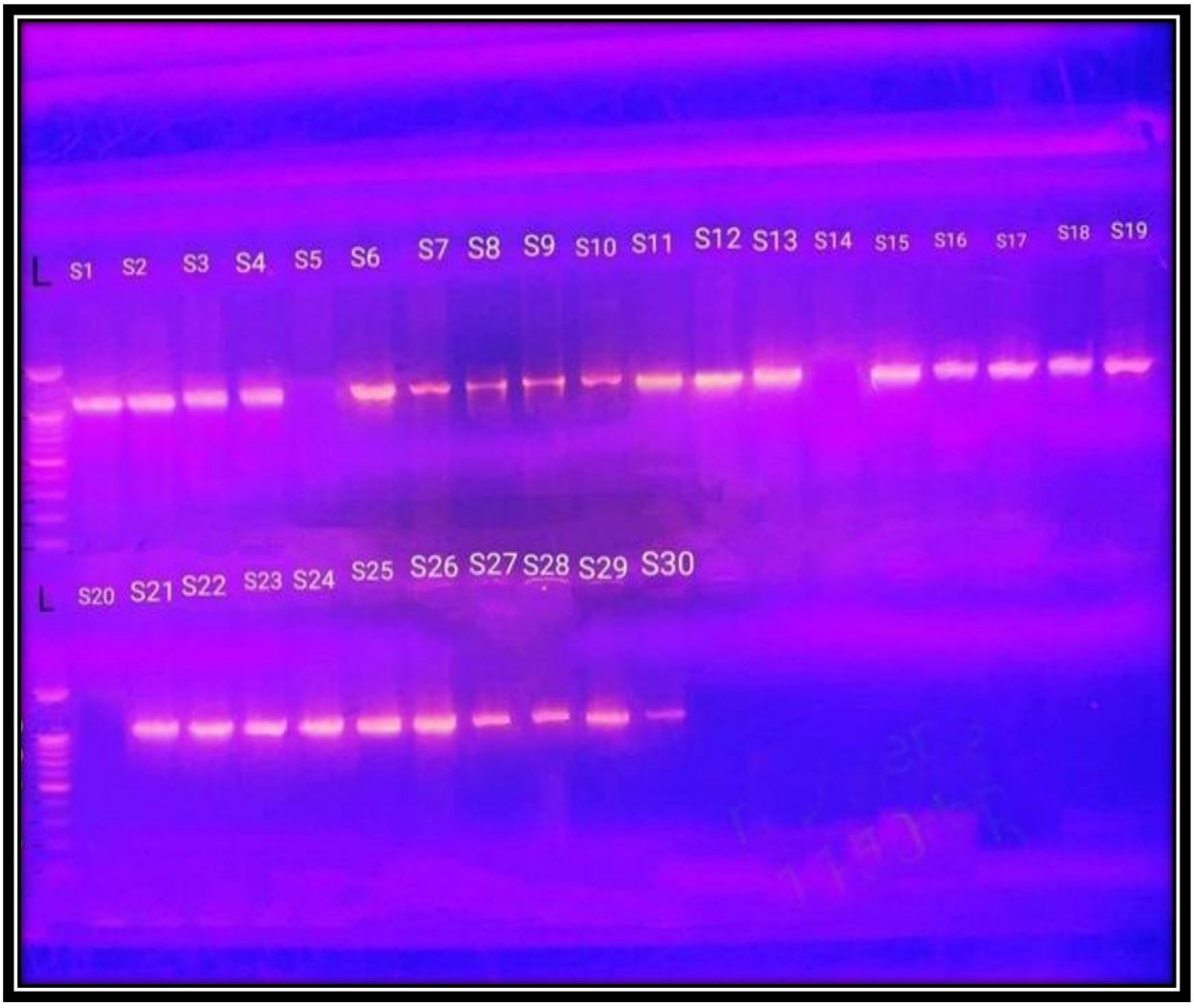


Figure (3-12): Gel electrophoresis of PCR product size (1543bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (1 ,2 ,3 ,5 ,6 ,7 ,8 ,9 ,10 ,11 ,12 ,13 ,15 ,16 , 17, 18,19 ,21 ,22 ,23,24 ,25, 26 ,27 ,28 , 29 ,30) PCR product were positive for *mar* RAB gene, marker for DNA ladder (200-2000)bp .

The results also compatible with Hooper and Jacopy ,(2015) who reported that less often mutations in efflux pump structural genes have been associated with changes in pump substrate profiles that include quinolones.

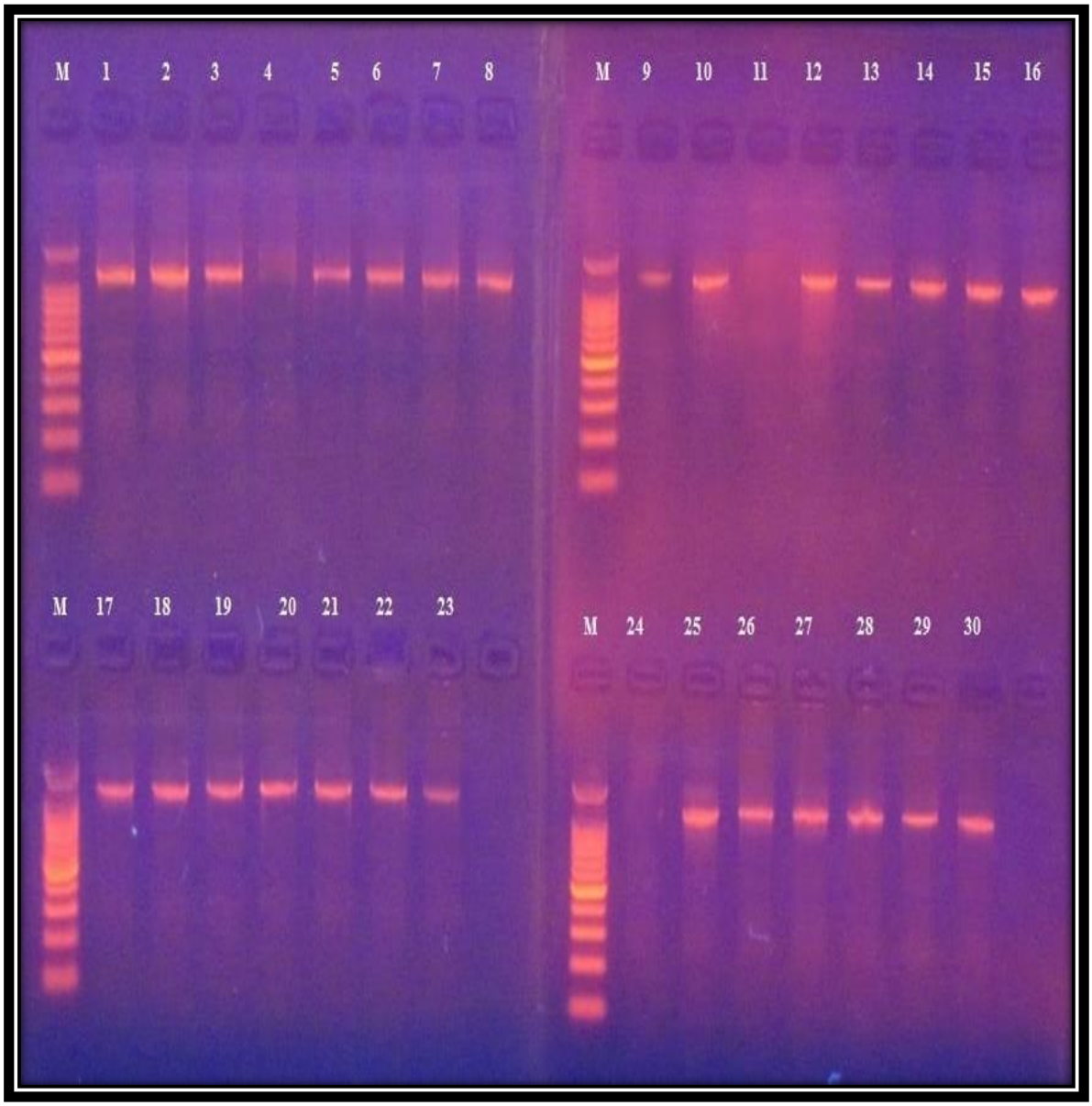


Figure (3-13): Gel electrophoresis of PCR product size (1172 bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (1 ,2 ,3 ,5 ,6 ,7 ,8 ,9 ,10 ,12 ,13 ,15 ,16 , 17, 18,19 ,21 ,22 ,23, 25, 26 ,27 ,28 , 29 ,30) PCR product were positive for *rob* gene, marker for DNA ladder (100-1500)bp .

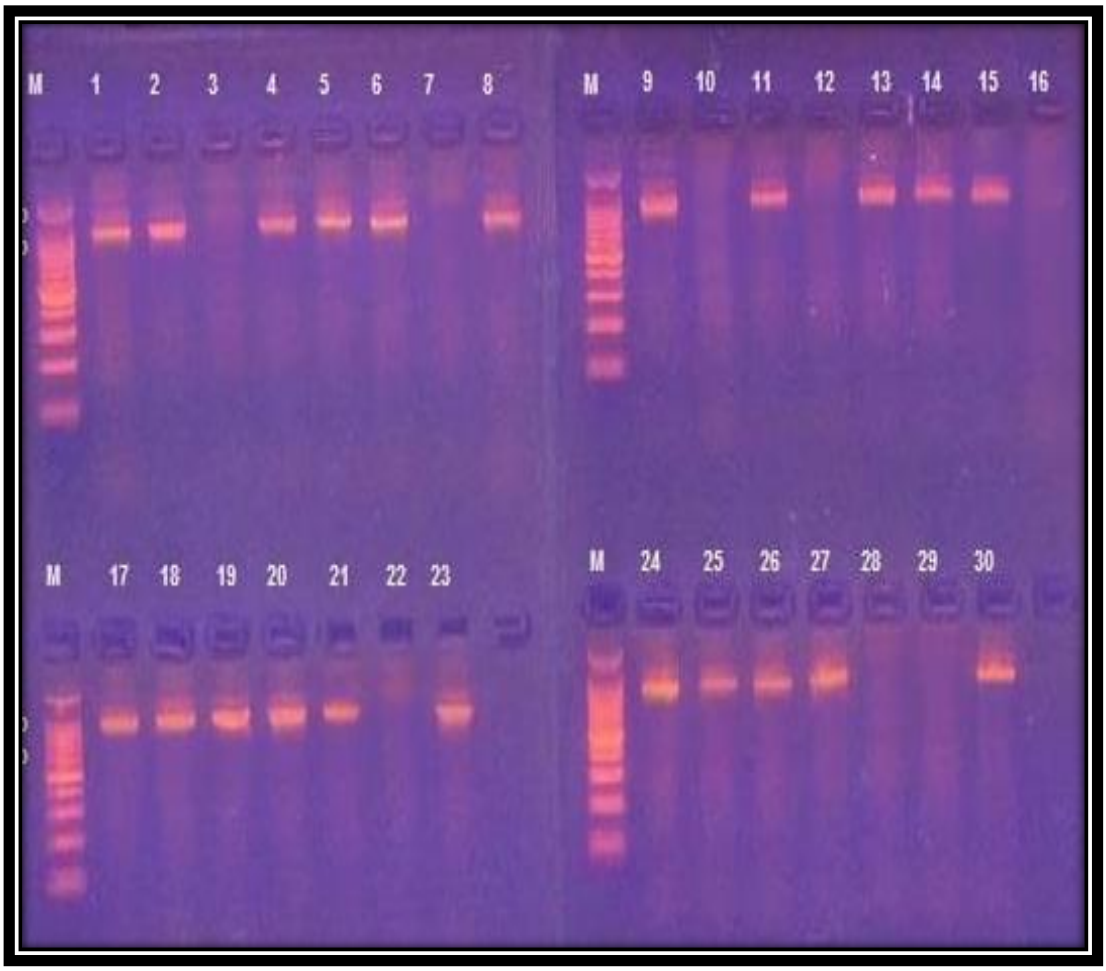


Figure (3-14): Gel electrophoresis of PCR product size (1132bp), for *Escherichia coli* in 1.2% agarose gel at 100 volt /cm for 10 min and 80 volt for 40 min. Lane (1 ,2 ,4 ,5 ,6 ,8 ,9 , 11 ,13 ,15, 17, 18,19 ,21 ,23,24 ,25, 26 ,27,30) PCR product were positive for *sox*RS gene, marker for DNA ladder (100-1500)bp .

3.3.6 *E.coli* resistance to quinolone antibiotics and its relation with chromosomally encoded antibiotic resistance (*mar*RAB, *sox*RS and *rob*)

The results of this study showed that there is relation between *mar* and *rob* systems which considered as regulators; since there is 8 of 16 clinical isolates (53.16%) where harboring for both of *mar*RAB and *rob* regulone and were with high level of resistance in vitro. This compatible

with results reports by (Jain and Saini, 2016). However results indicate that there exists a complex control of gene regulation in the *mar–sox–rob* regulons; Mechanistic details of this control are likely quite complex, and may involve additional regulators.

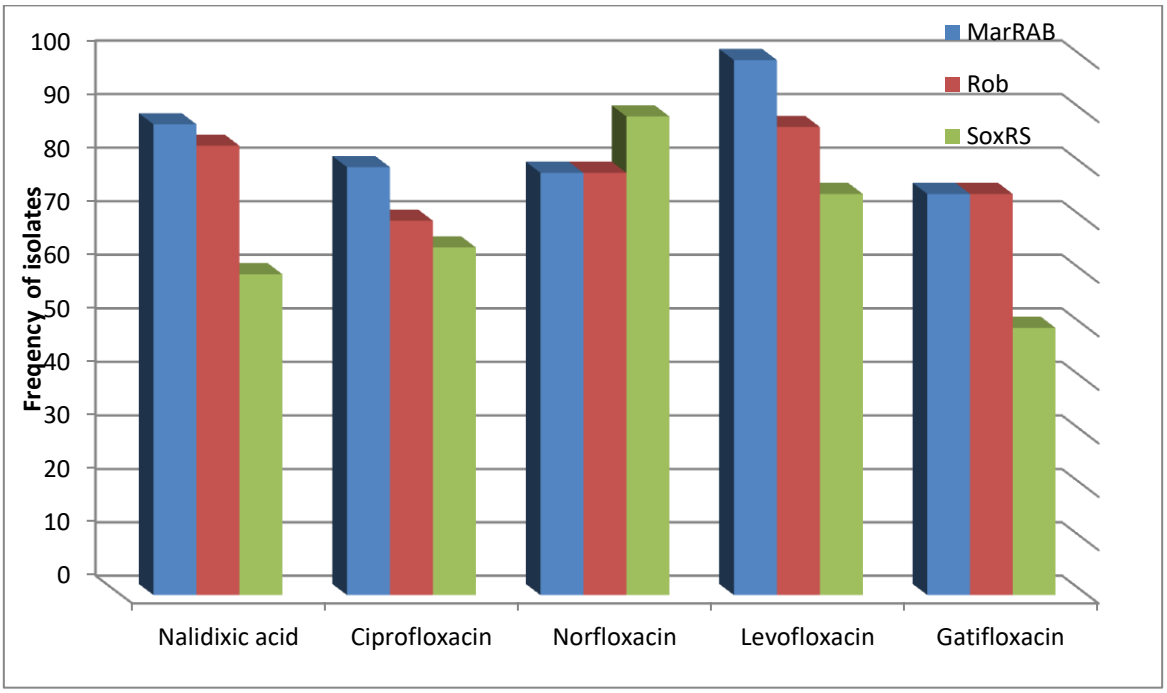


Figure (3-15):Distribution of *mar*RAB, *rob* and *sox*RS genes prevalence in clinical *E.coli* isolates.

A study carries by Atac *et al* (2018) indicates that *mar* - RAB regulatory genes of AcrAB–TolC efflux pump system has a significant impact on quinolone resistance and progression to MDR profile in *E.coli* clinical isolates . Tyrosin-Histidin mutation in *mar*R may have a role in increased expression of *mar*A, *acr*A *and acr*B, and may be result in the initiation of multidrug resistance phenotype in *E.coli* clinical isolates (Obeed and Dhahi , 2020) .Thus overexpression of AcrAB have been documented in the fluoroquinolone resistance of clinical *E. coli* (Alav *et al* ,2021).

The three isolates that are negative for *mar*RAB, may back to the acting of *mar*R since the transcription of *mar*RAB will only occur when repression of *mar* R is disrupted (Weston *et al* ,2017).Transcription of *mar* RAB can be due to the presence of certain ligands (e.g. phenolic compounds such as sodium salicylate), antibiotics, oxidative stress or mutation of *mar*R, *mar*O or the *mar*R binding site (McDermott *et al*,2008). The *sox*S failed to activate the *mar*RAB promoter when *mar*R is still repressing it. (Chubiz *et al* ,2012). High percentage of *rob* gene in the present study (90%) may back to the fact that not all *rob* gene is in the inactive form. Likely, only a fraction aggregates within clusters, leaving some of it in the free and active form even in the absence of its cognate inducer (Chubiz *et al* ,2012).

Our results suggest that the *mar*RAB and *rob* operons function together in a conditional manner and that the two systems should not be viewed as autonomous systems but rather as an integrated network in their own right.

The strong overexpression of *mar*RAB was also found in most levofloxacin-resistant clinical strains from current study with prevalence (87.5%) from total resistance isolates against levofloxacin antibiotic .This result also documented by (Li *et al* ,2015 and Yasufuku *et al* ,2011). High percentage of *sox*RS system in the present study (73.33%) may back to the fact constitutive expression of *sox*S due to an in-frame internal deletion of *sox*R amino acids 136 to 144 (affecting the last 19 amino acids) has been described (Fàbrega *et al*,2010).

A second mutation within *sox*R, Gly74Arg, also been found in several *sox*S-overexpressing *E.coli* clinical isolates. Oxidative stress, however, inactivates *sox*R enabling *sox*S expression (Fàbrega *et al*,2010).

Both *mar*A and *sox*S are further regulated post-translationally by protein degradation from the ATP-dependent on protease such that mutations confers increased AcrAB efflux activity and MDR (Weston *et al*,2018).

The network architecture between the *mar-sox-rob* loci is dependent on the inducer present in the environment since the set of relevant regulatory interactions between the three systems is dependent on the precise chemical environment (inducer) of the cells (Vinue *et al*,2013). In the present study ;Concerning to coexisting of the three genes in the same isolates , out of 30 clinical 30 isolates (100%) have been harboring at least two of the studied multidrug resistance genes of the efflux pumps (*mar* RAB, *rob* and *sox*RS) .

Table (3-7) : Prevalence of *mar* RAB, *rob*, and *sox*RS combination in *E.coli* isolates

Coexistence of different efflux pump genes	No. of Isolates	Rate of resistance (%)
mar RAB, *rob, and sox*RS	16	53.33
mar RAB *and rob*	8	26.67
mar RAB *and sox*RS	3	10
*rob and sox*RS	3	10

Efflux has been shown to be crucial for the development of high-level fluoroquinolone resistance, because the inactivation of major efflux systems prevents the selection of fluoroquinolone-resistant mutants and strains carrying specific target-site mutations are no longer clinically resistant if efflux pumps are inactivated (Redgrave *et al*, 2014).In

general, there was a direct correlation between the number of resistance phenotype in a strain carried of three studied efflux pump genes and the MIC value. This result is also reports by Machuca *etal*,(2014). Novel mutation in *soxR* appear to have a role concerning increasing MIC level of norfloxacin ;since 7 out of of 8 isolates that have deletion in soxR have increase in MIC level raise to MIC>64 μg/mL. This observation is also documented by (Fàbrega *et al* ,2020).

Many studies have evaluated the *gyrA* and *parC* mutations that accompany *qnr* in clinical isolates (Briales *et al*,2012),(Silva-Sánchez *et al*,2013) and (Xue *et al* ,2017) and a few investigations have examined expression of efflux genes in quinolone-resistant strains (Vinué *et al*,2018) , but this study is the first to evaluate both topoisomerase mutations and pump expression in *qnr*-containing clinical isolates.

3.4 *Gyrase* II encoding gene :

The current study reveals that all clinical isolates of *E.coli* contain *Gyr* A gene as shown in figure (3-16). *Gyr* A gene encodes essential enzyme (gyrase II) that change topology by introducing transient double-stranded breaks into DNA and pass a second double-stranded DNA segment through the break before resealing it .(Bush *et al* ,2015).

This result are compatible with Zahedi *et al* .(2018) who reported that *gyr*A is present in 100% of *E.coli* isolates. Possibly *GyrA* molecules not incorporated into functional *gyrase*.(Stracy,*et al* ,2019) 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 up-regulation of drug efflux mechanisms arise at higher rates than mutations in the structural genes for topoisomerases. This is due to the fact that the genetic target in efflux-regulating genes is much larger than the specific amino acid substitutions required for *Gyrase*-mediated resistance (Marcusson *et al*,2009 ; Alsaadi ,2015).

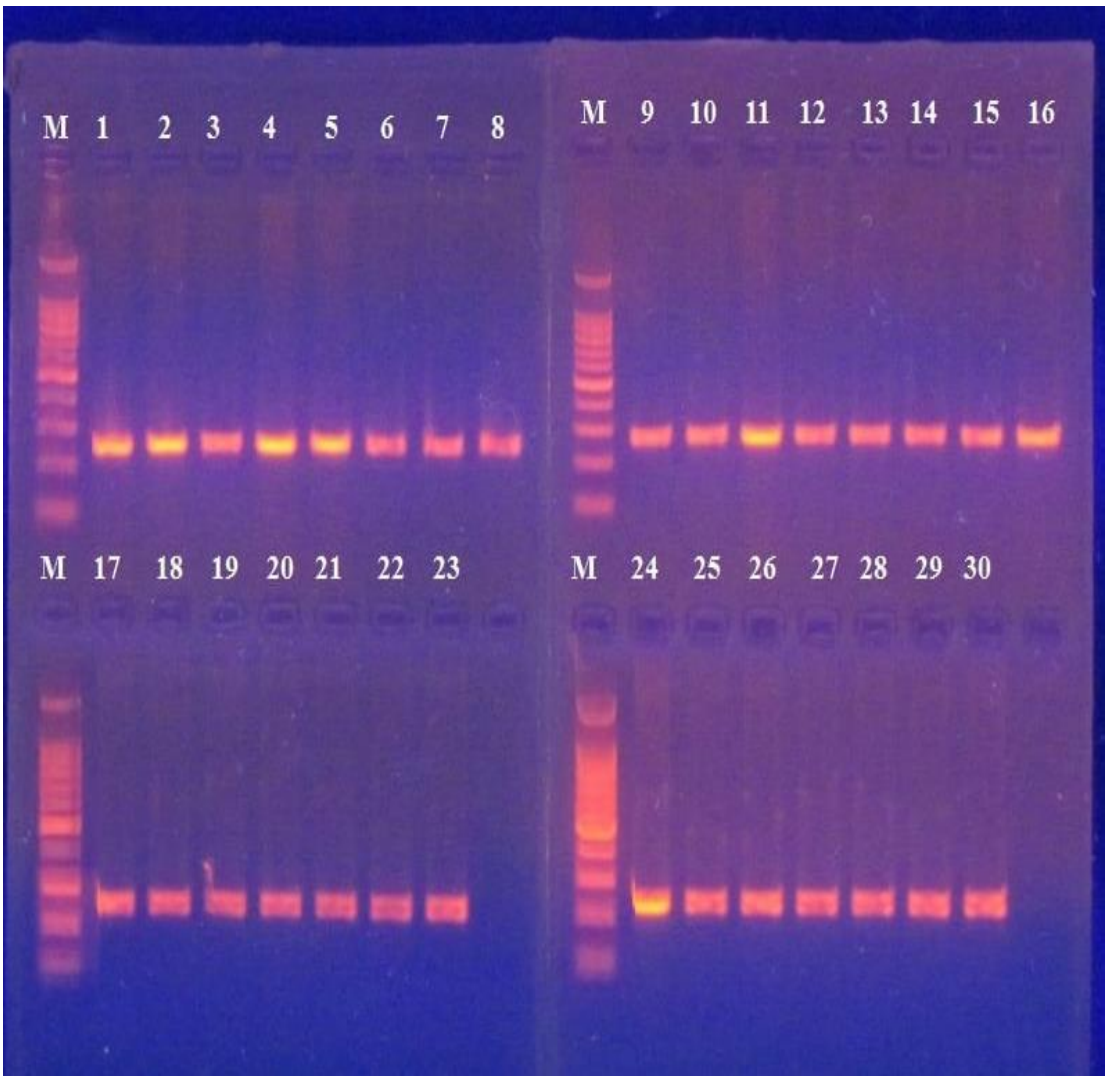


Figure (3-16):1% Agarose gel electrophoresis at 70 volt for 50 min for *Gyr A* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L1: 1500 bp DNA marker ;all tested isolates have *gyrase* A gene .Size of product 251bp.

3.4.1 Detection of *Gyrase* activity in *E.coli* clinical isolates:

There are only 16 out of 30 isolates have active topoisomerase type II 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.

The results were compatible with Reza *et al*.(2018) who reports that fluoroquinolone resistance is caused by mutations in chromosomal genes encoding the quinolone targets, mainly DNA *gyrase* and t*opoisomerase* IV. So PMQR is not the only resistant mechanism to quinolone as well as efflux pumps mechanisms.

Figure (3-17):*Gyrase* activity in producing negative DNA supercoiling; Samples (1-12)

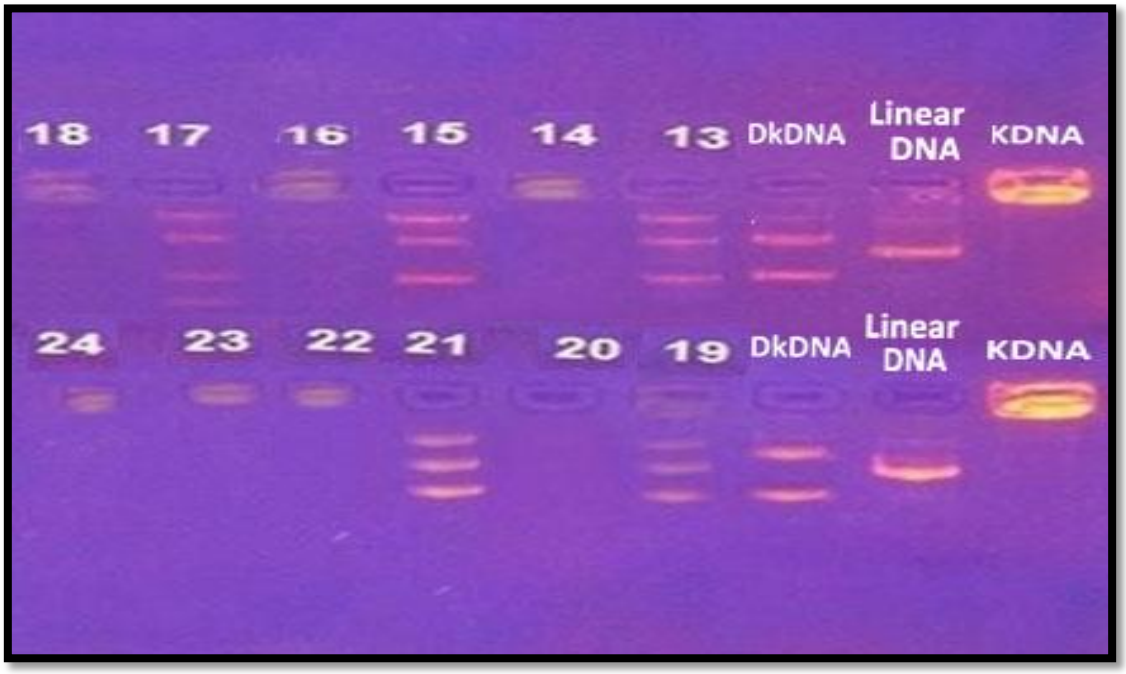


Figure (3-18):*Gyrase* activity in producing negative DNA supercoiling ;samples (13-24)

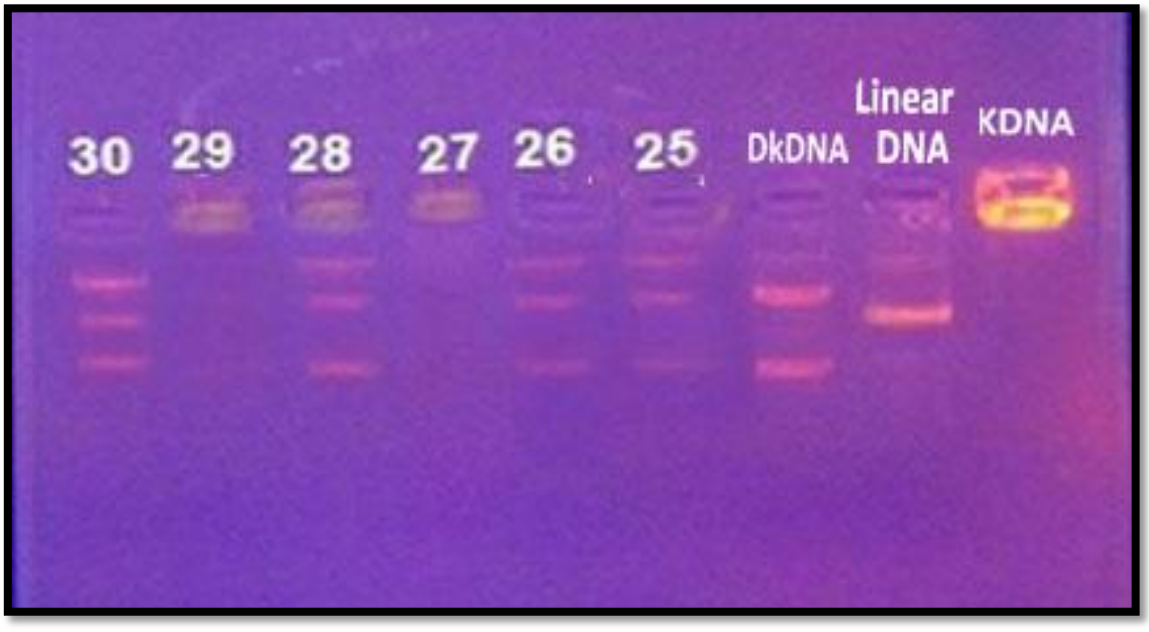


Figure (3-19):*Gyrase* activity in producing negative DNA supercoiling; Samples (25-30)

1-KDNA: : is the marker for the open minicircular DNA. 2-DkDNA: is the marker for the action of bacterial gyrase action by producing negative

supercoiling in *E.coli* DNA .There is no linear DNA alone in this study ; because there is no contamination in the DNA samples ; and also ;the DNA samples are free from nuclease enzyme (Topogen,2010)

3.4.2 *E.coli* resistance to the quinolone antibiotics and its relation to the activity of *gyrase* activity:

Fluoroquinolone resistance and *gyrase* activity was analyzed as showing in table (3-8) ;However it was found that the *gyrase* was produced and active against nalidixic acid at a rate 44% and to norfloxacin 42% , and to ciprofloxacin 35% ; where as to levofloxacin and gatifloxacin at a rate 50% for each. There is a positive correlation between the number of non-functional *gyrA* isolates and the level of quinolone resistance. Silva and Mendonça (2012) suggested that the *gyr*A codon 83 mutation generates supercoiling DNA alterations that could modify the expression of virulence factors of clinical *E.coli* isolates.

Table (3-8): Quinolone antibiotics and its relation with activity of *gyrase* activity

Quinolone antibiotics	Total resistance isolates	Non-Functional *gyrase*	Functional *gyrase*
Nalidixc 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%)

In addition, Weber *et al.* (2013) demonstrated that alterations in supercoiling affect fundamental cellular processes, including transcription. Because the primary mechanism of quinolone resistance involves mutations in the QRDR of the *gyrA* (Gomig *et al* ,2015). Previous studies showed that this mutant enzyme had alterations in DNA cleavage specificity even in the absence of drug (Gruger *et al*,2004).

In *E. coli*, *gyrA* mutations usually occur at serine-83, which is substituted by leucine or tryptophan and causes high resistance to quinolones. However, the replacement of serine by alanine causes a lower resistance level (Lorestani *et al* ,2018). Another common mutation is the substitution of aspartate-87 by asparagine and/or valine (Krishnan *et al*, 2012).

3.4.3.Relation between *gyrase* activity in *E.coli* and MIC value of quinolone antibiotics:

Supercoiling of DNA degree is not fixed and there is continuous remodeling of DNA topology in response to environmental stress and cellular processes such as transcription, DNA replication, and recombination.(Redgrave *et al*,2014). In this study ; two distinct parameters are considered, functional *gyrase* and non-functional *gyrase*.

In the first parameters, functional *gyrase* is the active enzyme where it can cut the DNA at both strands, whereas non-functional means the *gyrase* is inactive ;but it is present and cannot cut the DNA strands.

Concerning MIC level ; *E.coli* with non-functional *gyrase* enzyme appears to have a higher average than those with functional *gyrase* enzyme as reveals in figure (3-20):

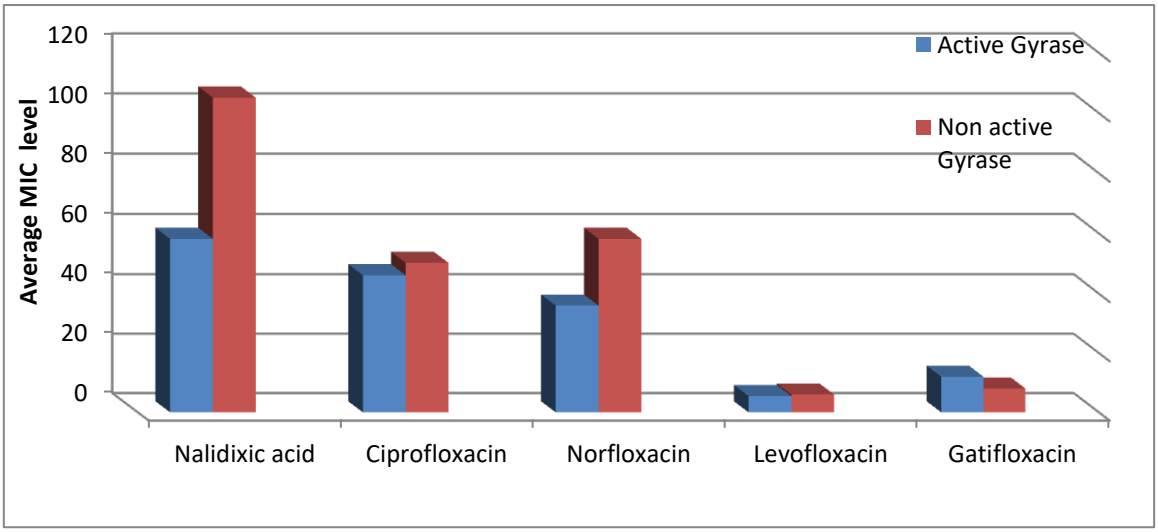


Figure (3-20):The distribution of resistance *E.coli* isolates according to their MIC (µg/mL) average for quinolone antibiotics under study.

Nalidixic acid MIC value in 14 isolates with non-functional isolates was 4 isolates with MIC value 32 µg/mL,5 isolates with 64 µg/mL,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 are in 6 isolates ,64 µg/mL in 3 isolates and 2 isolates with 128 µg/mL.

There are a significant difference between both type of strains ($p>0.05$). The range of MIC value against resistance nalidixic acid was higher than other quinolone antibiotics under study (32-256 µg/mL) which is similar range to what reports by Shenagari, *et al* (2018); Haggag *et al* (2020) .

Concerning ciprofloxacin MIC value in 13 isolates with non-functional-*gyrase* isolates ; there are 5 isolates with MIC value (1 µg/mL), 2 isolates with MIC (4 µg/mL), 4 isolates with 64 µg/mL and only 1 isolate for each concentration (128 µg/mL and 256 µg/mL) 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 and finally 1 isolates with concentration 128 µg/mL. 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%).This may be due to presence of other mechanisms involved in fluoroquinolone resistance such as decreased membrane permeability and overexpression of efflux pumps.

This results were also documents also by Bin *et al* (2009);Cavaco and Aarestrup (2009) ;Oviaño *et al* (2017); and Haggag *et al* (2020) or due to strains under study has shown that a point mutation in the *gyrA* which can only slightly reduce the susceptibility of *E. coli* to ciprofloxacin, but high-level resistance is associated only with double mutations in the *Gyr*A protein as reported by several studies (Hopkins *et al*,2005; Ogbolu *et al* ,2012; Lorestani, *et al*,2018).Other studies reveal that mutations in *parC* may the main cause of ciprofloxacin resistance (Abbasi and Ranjbar ,2018).

Regarding , Norfloxacin MIC average value; *E.coli* strains with non-functional gyrase (58.2 µg/mL) have been significantly (P>0.01) higher than *E.coli* strains with functional *gyrase* (36 µg/mL). MIC value in 11 isolates with non-functional isolates ; there are 4 isolates with MIC value (32µg/mL), 6 isolates with MIC (64 µg/mL) and 1 isolates with 128 µg/mL. Functional *gyrase* strains distribution according to MIC concentration; there are 4 isolates with concentration (16 µg/mL); 3 isolates with MIC value (64 µg/mL and finally 1 isolates with concentration 32 µg/mL. The results are compatible with (Pfeiffer and Hiasa ,2007; Cesaro *et al*;2008 ;Yousefi *et al* 2018).

Concerning levofloxacin MIC average value; *E.coli* strains with non-functional gyrase (6 µg/mL) have no significantly (P>0.01) difference and *E.coli* strains with functional *gyrase* (5.5 µg/mL). MIC value distribution in 4 isolates with non-functional isolates was 2 isolates with MIC value (4 µg/mL) and 2 isolates with concentration (8µg/mL) .

Functional *gyrase* strains distribution according to MIC concentration; there are 1 isolates with concentration (2 µg/mL) and 1 isolates with MIC value (4 µg/mL) and 2 isolates with MIC value (8µg/mL).The results are compatible with (Drago *et al* ,2010, Minarini and Darini,2012; Bhatnagar and Wong 2019) 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.

Finally; Gatifloxacin MIC average value has been indicated ; that *E.coli* strains with functional *gyrase* (12 µg/mL) have higher average of MIC value than *E.coli* strains with non-functional *gyrase* (8µg/mL); since two isolates with non-functional *gyrase* are with MIC value (8 µg/mL) while in functional-*gyrase* ; there is one isolates for each concentration(16 µg/mL and 8 µg/mL) of MIC value. This results are compatible with Lu *et al* (2020) who have reported the C-8-methoxy group improved lethal action against most of the *gyrA* mutants, even when *gyrA* has double mutants, as was the case when inhibition of growth was measured.

Conclusions:

1. The highest prevalence of *E.coli* was found in vaginal samples, and then from urine samples followed by wounds infections and stool.
2. Gatifloxacin and levofloxacin (quinolones), are still the drugs of choice for treatment of *E.coli* infection with probability of emerging resistance to ciprofloxacin in the future
3. *E.coli* isolates showed highest resistance rate to Nalidixic Ciprofloxacin; Norfloxacin, whereas lowest is against Levofloxacin Gatifloxacin.
4. The study shows that the frequency of quinolone resistance qnr genes among *E.coli* isolates is high and enhance the increasing resistance rate of *E.coli* against quinolone antibiotic under study as detailed by MIC results.
5. There are correlation between the reduced sensitivity (MIC value) of quinolone and bacterial topoisomerases II expression in *E.coli* isolates.
6. Non-functional *gyrase* enzyme are associated with high *E.coli* resistance level against quinolone antimicrobial under study.
7. *Gyrase* enzyme are produced at high levels when Nalidixic acid, Ciprofloxacin are used.

Recommendations:

1.Further studies with other techniques like, pulsed-field gel electrophoresis, multilocus sequence typing is needed to determine the dissemination source and studying the epidemiology of PMQR producing isolates by studying of dominant mutation in *E.coli* genome that related to quinolone resistance antibiotics.

2.Studies should be done to search new generation of quinolone and show their effect on bacteria .

3.Determination of the resistance rate to quinolones and to other type of antibiotics by new methods in vivo and in vitro.

4.The isolates have evolved resistance through multiple mechanisms including plasmid mediated resistance interrupted with chromosomal mediated resistance mechanisms so ;containment strategies to limit the spread of quinolone-resistant *E.coli* need to be deployed to conserve quinolone effectiveness and promote alternatives to their use.

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MIC for Nalidixic acid

No of isolates	R≥16	I=4	S≤32
1	256 mg/µl	0	0
2	32 mg/µl	0	0
3	0	0	4 mg/µl
4	256 mg/µl	0	0
5	32 mg/µl	0	0
6	32 mg/µl	0	0
7	0	0	2mg/µl
8	64 mg/µl	0	0
9	0	0	4 mg/µl
10	64 mg/µl	0	0
11	32 mg/µl	0	0
12	0	0	2 mg/µl
13	64 mg/µl	0	2 mg/µl
14	64 mg/µl	0	0
15	0	0	16 mg/µl
16	256 mg/µl	0	0
17	32 mg/µl	0	4
18	32 mg/µl	0	0
19	128 mg/µl	0	0
20	64 mg/µl	0	0
21	32 mg/µl	0	0
22	128 mg/µl	0	0
23	32 mg/µl	0	0
24	64 mg/µl	0	0
25	32 mg/µl	0	0
26	64 mg/µl	0	0
27	128mg/µl	0	0
28	128 mg/µl	0	0
29	64 mg/µl	0	0
30	32mg/µl	0	0
	N=25	N= 0	N=5

MIC for Norfloxacin

No of isolates	R≥16	I= 8	S≤ 4
1	32mg/µl	0	0
2	64 mg/µl	0	0
3	64 mg/µl	0	0
4	0	8 mg/µl	0
5	32mg/µl	0	0
6	16 mg/µl	0	0
7	0	0	2 mg/µl
8	0	0	2 mg/µl
9	0	0	1mg/µl
10	128 mg/µl	8	0
11	64mg/µl	0	0
12	0	8mg/µl	0
13	0	8mg/µl	0
14	32mg/µl	0	0
15	0	0	1mg/µl
16	64 mg/µ	0	0
17	0	0	2mg/µl
18	0	8mg/µl	0
19	64mg/µl	0	0
20	32mg/µl	0	0
21	16 mg/µl	0	0
22	64 mg/µl	0	0
23	0	8mg/µl	0
24	64 mg/µl	0	0
25	32mg/µl	0	0
26	16mg/µl	0	0
27	64 mg/µl	0	0
28	16mg/µl	0	0
29	64 mg/µl	0	0
30	0	0	1mg/µl
	N=19	N= 5	N= 6

MIC for Ciprofloxacin

No of isolates	R≥1	I= 0.5	S≤0.25
1	0	0	0.25 mg/µl
2	1 mg/µl	0	0
3	0	0	0.125 mg/µl
4	64 mg/µl	0	0
5	4 mg/µl	0	0
6	0	0.5 mg/µl	0
7	128 mg/µl	0	0
8	0	0	0.125 mg/µl
9	0	0	0.25 mg/µl
10	4 mg/µl	0	0
11	0	0	0.25 mg/µl
12	0	0	0.125 mg/µl
13	1 mg/µl	0	0
14	1 mg/µl	0	0
15	0	0	0.125 mg/µl
16	64mg/µl	0	0
17	1 mg/µl	0	0
18	1 mg/µl	0	0
19	0	0.5 mg/µl	0
20	256 mg/µl	0	0
21	0	0	0.25 mg/µl
22	128 mg/µl	0	0
23	1 mg/µl	0	0
24	64 mg/µl	0	0
25	64 mg/µl	0	0
26	1 mg/µl	0	0
27	64 mg/µl	0	0
28	64 mg/µl	0	0
29	1 mg/µl	0	0
30	64 mg/µl	0	0
	N= 20	N= 2	N=8

MIC for Levofloxacin

No of isolates	R ≥2	I= 1	S≤ 0.25
1	0	0	0.15 mg/µl
2	0	0	0.25 mg/µl
3	2 mg/µl	0	0
4	0	0	0.15 mg/µl
5	8 mg/µl	0	0
6	0	1 mg/µl	0
7	0	1 mg/µl	0
8	4 mg/µl	0	0
9	0	0	0.25 mg/µl
10	4 mg/µl	0	0
11	0	0	0.25 mg/µl
12	0	1 mg/µl	0
13	0	0	0.15 mg/µl
14	0	1 mg/µl	0
15	0	1mg/µl	0
16	4 mg/µl	0	0
17	0	1 mg/µl	0
18	0	0	0.15 mg/µl
19	8 mg/µl	0	0
20	8 mg/µl	0	0
21	0	1 mg/µl	0
22	0	1 mg/µl	0
23	0	0	0.25 mg/µl
24	0	0	0.15 mg/µl
25	8 mg/µl	0	0
26	0	0	0.15 mg/µl
27	0	1 mg/µl	0
28	0	0	0.25 mg/µl
29	0	0	0.25 mg/µl
30	0	1 mg/µl	0
	N=8	N= 10	N=12

MIC for Gatifloxacin

No of isolates	R≥8	I= 4	S≤ 2
1	0	0	2 mg/µl
2	0	0	1mg/µl
3	0	4 mg/µl	0
4	0	0	1mg/µl
5	0	1 mg/µl	0
6	0	0	2 mg/µl
7	0	0	1mg/µl
8	0	0	1 mg/µl
9	0	0	1 mg/µl
10	0	2mg/µl	0
11	0	0	1 mg/µl
12	0	0	0.5 mg/µl
13	0	4mg/µl	0
14	0	0	1 mg/µl
15	0	0	0.5 mg/µl
16	0	0	1 mg/µl
17	0	0	0.5 mg/µl
18	0	0	1 mg/µl
19	16 mg/µl	0	0
20	0	0	1 mg/µl
21	0	0	2 mg/µl
22	0	4mg/µl	0
23	8mg/µl	0	0
24	0	0	0.5 mg/µl
25	0	0	0.5 mg/µl
26	0	4mg/µl	0
27	8 mg/µl	0	0
28	8 mg/µl	0	0
29	0	0	1 mg/µl
30	0	0	2 mg/µl
	N=4	N= 6	N= 20

الخلاصة

تم جمع 200 عينة سريرية من مرضى يعانون من عدوى مختلفة مثل التهاب المسالك البولية والتهاب المعدة والأمعاء (الإسهال) والتهاب المهبل والتهابات الجروح ؛ الذين حضروا إلى مستشفيين رئيسيين في محافظة كربلاء المقدسة: مستشفى الحسيني التعليمي العام ، ومستشفى كربلاء للولادة والأطفال خلال فترة (شباط - حزيران 2020). تم أخذ عينات من المرضى الذين تتراوح أعمارهم من 5 إلى 66 سنة من الذكور والإناث. اظهرت النتائج ان هناك 30 عينة تم تشخيصها على أنها سلالات سريرية للإشريكية القولونية من كلا الجنسين وأعمار مختلفة بعد تحديدها عن طريق اجراء الاختبارات الكيمياء الحيوية ، ونظام VITEK-2 ، وبالطريقة الجزيئية باستخدام ماركر 16SrDNA. تم إجراء اختبار الحساسية لمضادات الميكروبات وتركيز التثبيط الأدنى (MIC) لحمض النااليديكسيك ، نورفلوكساسين ، سيبروفلوكساسين ، ليفوفلوكساسين ، وجاتيفلوكساسين بطريقة التخفيف الدقيق للمرق. وكذلك تم فحص جميع السلالات لجينات PMQR وهي (qnrA، qnr B، qnrS) وكذلك عوامل مضخة التدفق (Mar RAB، rob، SoxRS) وجين الترميز *Gyrase* A ؛ بواسطة طريقة تفاعل البوليميراز المتسلسل بعد استخلاص الحمض النووي من العزلات السريرية المختبرة للإشريكية القولونية. بالإضافة إلى تقدير نشاط إنزيم *Gyrase* في العينات قيد الدراسة.

أظهرت نتائج عزل بكتيريا الإشريكية القولونية ;تواجدها بدرجة عالية في عينات المهبل وبنسبة (40٪) ثم من عينات البول (32٪) تليها (24٪) من التهابات الجروح و (21٪) من البراز. وكانت اعلى نسبة (33.33٪) في المجموعة العمرية (31-45) سنة بينما كان أقل حدوث في المجموعة العمرية (60) وبنسبة (10٪) عند الإناث. كذلك فان الإناث كانت لديها زيادة ملحوظة في وتيرة E. coli. مقابل الذكور بنسبة (إناث: ذكور 87٪: 13٪).

في هذه الدراسه ;خضعت العزلات لاختبار الحساسيه (DDT) وفقا لدليل CLSI 2020,ووجد أن العزلات البكتيرية أظهرت مقاومة عالية للجيل الأول (حمض النااليديكسيك 86.36٪) ، والجيل الثاني (سيبروفلوكساسين 66.66٪) ، (نورفلوكساسين 63.33٪) ، بينما في الجيل الثالث (ليفوفلوكساسين 27.27٪) ، وأخيراً الجيل الرابع (سيبروفلوكساسين). جاتيفلوكساسين 13.33٪).

تم استخدام بادئات نوعيه (Primers) في تقنية تفاعل البلمرة المستلسل (PCR) للتحري عن الجينات الخاصة بمقاومة مضادات الكيونيلون (qnr) وأظهر الفحص الجزيئي ان النسبة الإجمالية

لعزلات الإشريكية القولونية التي تحتوي على جينات *qnr* كانت (70) (21/30٪). وكانت 30/14 عزلة تؤوي جينات qnr في صورة مختلطة أو مجمعة بنسبة (46.66٪) و 30/7 (23.33٪) عزلات تؤوي qnr في شكل منفرد .كان معدل انتشار *qnr A* و qnr B و qnrS بنسبة 40, ٪ 43.33 ٪ و 53.33٪ على التوالي . اظهرت عزلات *E.coli* المقاومة لمضاد Nalidix احتواءها على جينات qnr A و B و S) في دراستنا بنسبة 24٪ ، 12٪ ، 36٪ على التوالي في حين كانت العزلات المقاومة للسيبروفلوكساسين والتي تحمل جينات qnrA و qnr B وS qnr 15.8٪ و 5.3٪ و 26.3٪ على التوالي. ظهرت عزلات *E.coli* المقاومة الليفوفلوكساسين التي تحمل جينات qnrA و qnr B وqnrS بنسبة 37.5٪ و 75.0٪ و 37.5٪ على التوالي. أخيرا؛ كان أدنى معدل انتشار لجينات qnrA و B و S ضد المضاد الحيوي Gatifloxacin حيث كانت ؛ بنسبة (,0%,0% 25)٪ على التوالي.

تم العثور على أوبرون مقاومة المضادات الحيوية المتعددة للإشريكية القولونية (mar RAB) في 90٪ (30/27) من العزلات وكذلك بلنسبة للجين المشفر للارتباط البروتيني الصحيح(rob) gene في 27 عزلة مع نسبة انتشار (90٪) ؛ في حين أن اثنين وعشرين عزلة من ثلاثين عزلة من بكتيريا الإشريكية القولونية المختبرة تحتوي على جين soxRS بنسبة (73.33٪). (100٪) ؛ بينما لا يوجد سوى 16 عزلة من أصل 30 عزلة تواجد فيها انزيم الالتفاف الفائق نشط وبنسبة(53.33٪) فقط بينما البقية ؛ (14) عزلة كانت حاوية على إنزيم الالتفاف الفائق (gyrase enzyme) بشكل غير وظيفي وبنسبة (46.67%).

اظهرت الدراسة ارتباط وجود انزيم الالتفاف الفائق بمستويات عالية من المقاومة لمضادات الكيونولون قيد الدراسة ; حيث ظهرت العزلات المقاومة للمضاد الناليديكسيك مع انزيم فائق الالتفاف غير فعال بنسبة (44٪) من مجموع العينات السريرية المقاومة NA.اما مضاد في العزلات المقاومة لسايبروفلوكسساسين ، فقد كان الانزيم فعالا بنسبة 35% من مجموع العزلات الكلي المقاومة لمضاد السايبروفلوكساسين .كما وجد ان الانزيم كان فعالا بنسبة (42.11%) وغير فعال بنسبة (57.89%) من المجموع الكلي من العزلات المقاومة لمضاد النورفلوكساسين . واخيرا فان سلالات الإشريكيا القولونية والمقاومة ليفوفلوكساسين مع انزيم فعال كانت بنسبة (٪ 50) مع عزلات وكذلك الحال في السلالات المقاومة للمضاد الحيوي جاتيفلوكساسين بنسبه (50%).

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دراسة الخصائص الجزيئية وانتاج انزيم *Gyrase* في بكتريا الاشريكية القولونية *Eschrichia coli* المقاومة للمضادات الحيوية الفلوروكيونيلون Fluoroquionlons والمعزولة من عينات سريرية مختلفة

أطروحة مقدمة الى

مجلس كلية الطب/ جامعة بابل

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة علوم / الأحياء المجهرية الطبية

من قبل

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بكالوريوس علوم حياة 2010
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بإشراف

الأستاذ الدكتور

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