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Molecular Characterization of Antibacterial Resistant Genes in *Escherichia coli* Isolated from Clinical Samples

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بسم الله الرحمن الرحيم

وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا ﴿١١٣﴾

صدق الله العلي العظيم

سورة النساء

الآية (113)

Dedication

This work is dedicated

To

Al-Imam Ali (Aleh Alsalam)
To

My dear country (Iraq)

And to

The spirit of my father

Alaa 2016

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In the name of God the most merciful compassionate, the peace and the mercy upen our Messenger prophet Mohammed and his sanctified household. All thanks to God for granting me with will, strength, and patience with which this research had been accomplished.

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Alaa 2016

Supervisors Certification

We certify that this thesis entitled (**Molecular characterization of antibacterial resistant genes in *E. coli* isolated from clinical samples**) was prepared under our supervision at the College of Medicine /Al-Qadisiyah University as a partial requirement of the degree of Master of Science in Medical Microbiology

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Summary:

This study was designed to identify the distribution of some genes responsible for antibacterial resistance in *Escherichia coli* isolated from different clinical samples by using phenotypic methods and genotypic technique (by monoplex PCR). A total of 400 samples were collected from urine (n=100), stool (n=75), and blood (n=45) from patients admitted to the Al-Hamza Hospital in Al-Diwaniya City and Al-Sadder medical City in Al-Najaf Governorate-Iraq.

The results demonstrated that there were 220 specimens (55%) were diagnosed as gram negative bacteria. From total of 220 specimens there were 49 isolates (22.27%) identified as *E. coli* by cultural, biochemical characteristics and Vitek-2 system.

The susceptibility of *E. coli* isolates towards 14 type of antibiotics were tested using disk diffusion method. The results showed that all isolates of *E. coli* (100%) were resistant to cefotaxime, 39 (79.59%) isolates were multidrug resistant, 9 isolates (18.36%) were extensive drug resistant. This study proved that 47 isolates (95.91%) were resistant to ampicillin and tobramycin, 41isolates (83.67%) were resistant to amoxicillin+clavulanic acid. The results of the present study demonstrated that 39 isolates (79.59%) and 38 isolates (77.55%) of *E.coli* were resistant to ceftriaxone and ceftazidime respectively. The current study showed that 34 isolates of *E. coli* (69.38%) were resistant to azteronam, 26 isolates (53.06%) were resistant to nalidixic acid, 21 isolates (42.85%) were resistant to ciprofloxacin & gentamycin, 17 isolates (34.69%) were resistant to cefoxitin, 8 isolates (16.32%) were resistant to chloramphenicol, 6 isolates (12.24%) and 2 isolates (4.08%) were resistant to nitrofurantoin & imipenem respectively.

On the other hand, all of *E. coli* isolates (49) were detected as the potential ESBL-producers by using confirmatory method (double disk

synergy test), the results showed that out of 49 isolates of *E. coli* examined in this study, ESBL were detected in only one isolates (2.04%).

Polymerase Chain Reaction has been used to detect of some genes encoding for antimicrobial resistance in *E. coli* isolates. Regarding genes that responsible for ESBL enzymes ($bla_{\text{CTX-M}}$, bla_{OXA} and bla_{TEM}), the current results proved that bla_{TEM} genes have highest rate (100%) followed by $bla_{\text{CTX-M}}$ and bla_{OXA} (91.83 %) for each.

Finally, present study showed some plasmid mediated quinolones resistance genes (*qnrA*, *qnrB* and *qnrS*). Results demonstrated that these genes have the same percentage (100%).

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List of abbreviation

Abbreviation	Key
AmpC	Molecular class C beta lactamases
β	Beta
bla gene	β-lactamase gene
CTX	β-lactamase active on cefotaxime
CTX-M	β-lactamase active on cefotaxime, first isolated at Munich
DDS	Double disk synergy
EDTA	Ethylene diamine tetra acetic acid
ESBLs	Extended-spectrum β-lactamases
PMQR	Plasmid-mediated quinolone resistance
qnr	Quinolone resistance protien
F	Forward of primer
R	Reveres of primer
OXA	lactamase active on oxacillin-β ,Oxacillinases
PCR	Polymerase chain reaction
PDR	Pan-drug resistance
MDR	Multi-drug resistance
XDR	Extensive drug resistance
SDS	Sodium Dodecyl Sulfate
TBE	Tris borate-EDTA buffer
TEM	β-lactamase Named After Patient (Temoneira) providing the first sample
Tris	Tris 2-Amino-2-Hydroxymethylpropane-1,3-Diol

TSI	**Triple sugar iron test**
MBL	**Metallo-β-lactamase**
MHA	**Mueller-Hinton agar**
µm	**Micro mole**
bp	**Base pair**
Ċ	**Degree Celsius**
I U	**International Unit**
Chrome agar	**Orientation chromogenic agar**
ATCC	**American type culture collection**
CLSI	**Clinical and Laboratory Standards Institute**
E. coli	***Escherichia coli***
ICUs	**Intensive care units**
IMP	**β-Lactamase active on imipenem**
MR-VP	**Methyle-red- voges-Proskauer**
NCCLS	**National Committee for Clinical Laboratory Standards**
PBPs	**Pencillin binding protien**
UTIs	**Urinary tract infection**
PFGE	**Pulse field gel electrophoresis**
RFLP	**Restriction fragment lenghth polymorphism**

Chapter One

Introduction & Literature Review

1. Introduction

Antimicrobial resistance (AMR) is when a microbe evolves to become more or fully resistant to antimicrobials which previously could treat it (Retrieved, 2015a; Retrieved, 2016). This broader term also covers antibiotic resistance, which applies to bacteria and antibiotics (Retrieved, 2015b). When a bacterial strain resistant to three or more different antimicrobial classes defined as Multi-drug resistance bacteria (Magiorakos *et al*., 2012). *Escherichia coli* often carry multidrug resistant plasmids and under stress readily transfer this plasmids to other species. Thus, *E. coli* and the other member of Enterobacteriaceae are important reservoirs of transferable antibiotic resistance (Franiczek *et al*. 2006).

Multidrug resistance bacteria afford numerous challenges and problems for healthcare providers, including increases in hospital-acquired infections,reduced treatment options, higher morbidity, and mortality rates, and healthcare cost increases due to longer hospital stays (Partridge *et al*., 2009).

At a molecular level, MDR may develop through clustering of resistence genes on mobile genetic elements. The physical linkage of plasmid-borne resistance genes in integrons and transposons has been shown to assist co-transfer of resistance genes (Rijavec *et al*., 2006).

The development of multi-drug resistance in *E. coli* is of major concern worldwide. Although prevalence rates for resistant *E. coli* strains are significantly distinct for various populations and environments and the impact of resistance to antimicrobial drugs is obiquitous (Von Baum and Marre, 2005). during the last decade, several studies have highlighted the environmental resistance as a source of resistance genes of clinical interest (Wright, 2010). Avariety of genetic elements are strongly associated with the exchange and transfer of antimicrobial resistance genes among grame-negative bacteria.Transposons can transfer resistance

genes intracellularly.On the other hand, integrons are genetic elements that can acquire several resistance genes in the form genes cassettes. These genetic elements sometimes co-exist and work co-operatively. For example, integrons, which are not mobile by themselves, can acquire an ability to transfer when they are incorporated into transposons (Harada, 2012).

Escherichia coli may use various biochemical pathways to escape the lethal action of drugs: (i) decreased intracellular accumulation of the antibiotic by an alteration of outer membrane permeability, diminished transport across the inner membrane, or active efflux; (ii) alteration of the target by mutation or enzymatic modification; (iii) enzymatic detoxification of the drug; and (iv) by passing of the drug target. The coexistence of several of these mechanisms in the same host can lead to multidrug resistance (MDR) (Florence *et al*., 2007).

Resistance to β-lactam antimicrobial agents in *E.coli* is principally mediated by β-lactamase, which hydrolyze the β-lactam ring and thus inactivate the antibiotic (Brinas *et al*., 2005). *E.coli* is intrinsically resistant to therapeutic levels of penicillin G, the first β-lactam introduced into clinical practice, because of its outer membrane barrier. *E. coli* is also resistant to several different classes of antibiotics with distinct mechanisms of action (Johnson *et al*., 2012).

Resistance to quinolones has been a problem ever since nalidixic was introduced into clinical medicine more than 40 years ago. Three mechanisms of resistance to quinolones are now recognized: mutations that modify the drug targets, mutations that reduce drug accumulation, and plasmids that protect cells from the lethal effects of quinolones. The targets of quinolone action are the vital bacterial enzymes DNA gyrase (Jacoby, 2005). Quinolone resistance in *E. coli* frequently the result of chromosomal mutations, leading to alterations in target enzymes or drug

accumulation. In recent times, plasmid-mediated quinolone resistance has been reporting by the acquisition of the *qnr*, *qepA*, and *aac(6')-Ib-cr* genes (Carattoli, 2009).

There are several mechanisms that can cause bacterial resistance to aminoglycoside antibiotics (Zarubica *et al*., 2011; Lindemann *et al*., 2012). In the last few years, alteration of 16S rRNA site by methyltransferase enzymes has emerged as a serious threat to this antimicrobial class. Of particular concern is 16S rRNA methyltransferase *armA* gene that confers pandrug-resistance to aminoglycosides and which is often accompanied by the carbapenemase genes on the same mobile genetic element (Livermore, 2011).

1.1. Aim of study

The aim of this study was to detect the ability of *E. coli* to produce certain resistant genes encoding antibacterial resistance through the following objectives:

1- Isolation and identification of *E. coli* from different clinical sources(urine, stool, blood).

2- Phenotypic and genotypic detection (by technique of monoplex PCR) of some genes that responsible for ESBLs enzymes (bla_{TEM}, bla_{CTX-M}, and bla_{OXA}) in plasmid mediated quinolones resistance-positive isolates.

3- Genotypic detection by technique of monoplex PCR) of some genes(*qnrA*,*qnrB*, and *qnrS*) that responsible for plasmid mediated quinolones resistance -among isolaes.

1.2: Literature Review

1.2.1: Taxonomy and Description of *E. coli*

Escherichia coli is one of the family Enterobacteriaceae, Domain - Eubacteria, Phylum - Probacteria, Class - Proteobacteria, Order - Enterobacteriales, Genus - *Escherichia*, Species - *coli* (Steadman and Topley, 1998). it is a Gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999).

Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (vogt and Dippold, 2005; Retrieved, 2012). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al*., 2001; Reid, 2001). *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards (Russell and Jarvis, 2001).

Escherichia coli and other facultative anaerobes constitute about 0.1% of gut flora, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination (Thompson and Andrea, 2007).

The bacterium can be grown and cultured easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years. *E. coli* is a chemoheterotroph whose chemically defined medium must include a source of carbon and energy. Organic growth factors included in chemically defined medium used to grow *E. coli* includes

glucose, ammonium phosphate, mono basic, sodium chloride, magnesium sulfate, potassium phosphate, dibasic, and water. The exact chemical composition is known for media that is considered chemically defined medium (Tortora and Gerard, 2010).

Escherichia coli can live on a wide variety of substrates and uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide. Since many pathways in mixed-acid fermentation produce hydrogen gas, these pathways require the levels of hydrogen to be low, as is the case when *E. coli* lives together with hydrogen-consuming organisms, such as methanogens or sulphate-reducing bacteria (Madigan and Martinko, 2006).

1.2.2: Growth culture

Optimum growth of *E. coli* occurs at 37 °C, but some laboratory strains can multiply at temperatures of up to 49 °C (Fotadar *et al*., 2005). Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide (Ingledew and Poole, 1984). *E. coli* is classified as a facultative anaerobe. It uses oxygen when it is present and available. It can however, continue to grow in the absence of oxygen using fermentation or anaerobic respiration (Tortora and Gerard, 2010).

1.2.3: Pathogenicity

Escherichia coli may cause several different gastrointestinal infection syndromes, based on definitive virulence factors, clinical manifestation, epidemiology, and different O and H serotypes. There are six distinct groups have been defined within gastrointestinal pathogenic

E. coli commonly associated with intestinal disease: [enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC) and the diffusely adherent *E. coli* (DAEC)] (Gillespie and Hawkey, 2006; Mahon *et al*., 2007).

1.2.3.1: Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is an important cause of childhood diarrhea with fatal consequences for children under 5 years of age (Croxen and Finlay, 2010). It is also common type of diarrhea in the developing world and is the main cause of diarrhoea in travellers (Kaper *et al*., 2004). ETEC-mediated diarrhea has been described by the secretion of characteristic toxins: the heat-stable enterotoxins (STs), the heat-labile enterotoxin (LT) or a combination of these (Croxen and Finlay, 2010). LT is large oligomeric toxin that is closely related in structure and function to the cholera toxin expressed by *Vibrio cholera.* In contrast to LT, the ST is small, monomeric toxin that contains multiple cystein residues, whose disulphide bounds account for the heat stability of this toxin (Eisinghorst and Weitz, 1994). Other ETEC strains produce heat-stable enterotoxins (STs) in addition to or instead of LT. They are classified into two distinct groups (STa and STb) or STI, and genes for ST are found on plasmids, although some have been found in transposons (Gillespie and Hawkey, 2006). STs have a similar but distinct mode of action to that of LT. STa activates guanylate cyclase activity, causing an increase in cyclic guanosine monophosphate, which results in increased fluid secretion (Mims *et al*., 2008).

1.2.3.2: Enteropathogenic *E. coli* (EPEC)

During the 1920s in Germany, Adam showed by serological typing that strains of "dyspepsiekdi" could be implicated in outbreaks of pediatric diarrhea. Many investigators before 1945 had suggested that certain strains of *E.coli* might produce enteritis. However, studies in that year described the associated of serological homogeneous *E.coli* in children with summer diarrhea that the concept of *E.coli* as a cause of human diarrhea was generally accepted (Keskimaki, 2001).

Enteropathogenic *E. coli* is an important category of diarrheagenic *E. coli* which has been linked to infant diarrhea in the developing world. As with other diarrheagenic *E. coli* strains, transmission of EPEC is faecal-oral, with contaminated hands, contaminated foods, or contaminated fomites serving as vehicles. EPEC adhere to the mucosal cells of the small bowel. The result of EPEC infection is watery diarrhea, which is usually self-limited, but can be chronic (Dedeićljubović *et al*., 2009).

The principal factor responsible for the localized adherence phenotype is a surface appendage known as the bundle-forming pilus (BFP), a member of the type IV fimbria family that is encoded on the EAF plasmid (Donnenberg, 1999). EPEC cells cluster because of the ability of BFP to reversibly aggregate into ropelike bundles. If any of the genes required for the formation of BFP are inactivated by mutation, the bacteria fail to form aggregates and do not display localized adherence (Anantha *et al*., 2000). EPEC possessing both the bundle-forming pilus gene (*bfpA*) and intimin gene (*eae*) for *E. coli*-attaching and effacing is a well-recognized pathogen in developing countries as class I EPEC or typical EPEC. However, atypical EPEC organisms possessing *eae* alone have been reported to be more prevalent in both developing and developed countries, and animals can be reservoirs of atypical EPEC, in

contrast to typical EPEC, in which humans are the sole reservoir (Fujihara *et al*., 2009).

1.2.3.3: Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) are characterized as very similar in biochemical, genetic and pathogeni aspects to Shigella spp (Kaper *et al*., 2004). Different studies have reported that *Shigella* and *E. coli* sharing many features taxonomically belong to same specie (Wei *et al*., 2003). They have the same mechanisms of pathogenicity (Croxen and finlay, 2010). Presence of the invasive plasmid with the capability to invade host tissues is one of most important events that seem to have caused the coevolution of both *Shigella* and EIEC (Jafari, 2012). Enteroinvasive *E. coli* (EIEC) attach specifically to the mucosa of the large intestine. They invade the cell by endocytosis by using plasmid associated genes. Inside the cell, they lyse the endocytic vacuole, multiply and spread to adjacent cells, causing tissue destruction, inflammation, necrosis and ulceration, resulting in blood and mucus stool (Mims *et al*., 2008).

The invasive capacity of both EIEC and *Shigella* is dependent on the presence of large (~140 MDa) plasmid coding for the production of several outer membrane proteins involved in invasiveness, the protein are antigenically closely related in EIEC and in *Shigella.* EIEC often resemble *Shigella* in being nonmotile and unable to ferment lactose. Furthermore, EIEC and *Shigella* O antigens show many cross-reactions (Keskimaki, 2001).

1.2.3.4: Eenterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E .coli (*EHEC) strain may produce one or more types of cytotoxins which are collectively referred as shiga-like toxins (SLTs) since they are antigenically and functionally similar to

shiga toxin produced by shigella dysenterica. SLTs were previously known as verotoxin. The toxins provoke cell secretion and kill colonic epithelial cells (Jay, 2000).

Enterohemorrhagic *E. coli* are characterized by presence of SLTs genes, locus for enterocyte effacement (LEE) and higher molecular-weight plasmid that encodes for a hemolysin. These three virulence factors are present in most *E. coli* associated with bloody diarrhea and hemolytic uremic crisis in humans (Radostits *et al*., 2007). The most virulent factor of *E. coli* O157: H7 is the production of cytotoxic SLT. *E. coli* O157: H7 likely gained ability to produce the SLT1 and SLT2 as a result of ingestion with a bacteriophage carrying SLT1 and SLT2 genes (Robinson *et al*., 2000). The SLTs of *E. coli* O157: H7 are cytotoxic to human colon and ileum cells. In animals, toxin has been shown to cause localized fluid accumulation and colonic lesion characterized by sloughing of surface and crypt epithelial cell (Jay, 2000).

1.2.3.5: Enteroaggregative *E. coli* (EAggEC)

Enteroaggregative *E. coli* (EAEC) are increasingly recognized as a cause of persistent diarrhea in both children and adults, and is also commonly recognized as a cause of endemic and epidemic diarrhoea worldwide (Croxen and Finalay, 2010). The EAEC first cause colonization of the colon, followed by secretion of enterotoxins and cytotoxins, resulting in significant mucosal damage (Hicks *et al.*, 1996). In development countries, very little is known about the epidemiology of EAEC.

However, it has been reported that in Germany 2% of children with diarrhea have EAEC in their stools (Huppertz *et al*., 1997). In the United States, EAEC have been linked with diarrhea in human immunodeficiency virus-infected patients (Durrer *et al*., 2000). Toxins

that have also been associated with strains of EAggEC include an *E. coli* heat-stable-like enterotoxin termed enteroaggregative heat-stable toxin-1 (EAST-1) and a heat-labile toxin termed plasmid-encoded toxin (Pet) (Gillespie and Hawkey, 2006).

1.2.3.6: Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) are defined by their diffuse adherence pattern on HeLa and HEp-2 cells and has been associated with the watery diarrhea in both developing and developed countries, and is also responsible for recurring urinary tract infections (Servin, 2005). DAEC have been widely reported as a cause of diarrhoea particularly in children older than one year of age (Nataro and Kaper, 1998). DAEC produce an alpha hemolysin and cytotoxic necrotizing factor 1. They are also known as diffuse-adherent or cell-detaching *E. coli*. Their role in diarrheal disease, especially in young children, is incompletely understood and somewhat controversial, with some studies reporting no association (Mims *et al*., 2008). Few epidemiological or clinical studies permit adequate description of the clinical syndrome associated with DAEC infection. In one study, the majority of patients infected with DAEC had watery diarrhea without blood or fecal leukocytes (Poitrineau *et al*., 1995).

1.2.4: Antibiotics resistance

Antibiotic resistance is a growing problem. Some of this is due to overuse of antibiotics in humans, but some of it is probably due to the use of antibiotics as growth promoters in animal feeds (Johnson *et al*., 2006) . A study published in the journal Science in August 2007 found the rate of adaptative mutations in *E. coli* is "on the order of 10−5 per genome per generation, which is 1,000 times as high as previous estimates," a finding

which may have significance for the study and management of bacterial antibiotic resistance (Perfeito *et al*., 2007**)**.

Antibiotic-resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*, through a process called horizontal gene transfer. *E. coli* bacteria often carry multiple drug-resistance plasmids, and under stress, readily transfer those plasmids to other species. Indeed, *E. coli* is a frequent member of biofilms, where many species of bacteria exist in close proximity to each other. This mixing of species allows *E. coli* strains that are piliated to accept and transfer plasmids from and to other bacteria. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance (Salyers *et al*., 2004).

Many factors may have contributed to the high rates of resistance, including; presumptive treatment without antimicrobial susceptibility testing, poor drug quality, unhygienic conditions, misuse of antimicrobials in both human and veterinary medicine has resulted in the emergence of strains of bacteria that no longer respond to antimicrobial therapy (McDermott *et al*., 2003; Toukam *et al*., 2010).

Bacteria may be intrinsically resistant to one class or more of antimicrobial agents, or resistance can be acquired by de novo mutation or by the acquisition of resistance genes from other organisms (Tenover, 2006). The intrinsic resistance can be described as a natural phenomenon when it is displayed by all members of a species and is a function of the physiological or biochemical structure of that species. For example, *enterococci* are intrinsically resistant to cephalosporins due to a decreased binding affinity to the penicillin-binding proteins(Harbottle *et al*., 2006).

On the other hand, acquired resistance can result from the acquisition of a mutation in the regulatory or structural genes and/or the acquisition of a foreign resistance gene (Harbottle *et al*., 2006). It appears

that the expression of bacterial resistance to antibiotics is frequently regulated, this indicates that modulation of gene expression probably reflects a good compromise between energy saving and adjustment to a rapidly evolving environment. The modulation of gene expression can occur at the transcriptional or translational level, following mutations or the movement of mobile genetic elements, and may involve induction by the antibiotic (Florence *et al*., 2007). Acquired resistance is not present in the entire species but within only a certain lineage of bacteria derived from a susceptible parent (Karah, 2008).

However, plasmid-encoded beta-lactamase encoding gene of the TEM family from an ampicillin-resistant *E. coli* was transferable to a susceptible strain in the gut of a children treated with ampicillin for UTIs (Karami et al., 2007).

Resistance to beta-lactam antibiotics has become a particular problem in recent decades, as strains of bacteria that produce extended-spectrum beta-lactamases have become more common. These beta-lactamase enzymes make many, if not all, of the penicillins and cephalosporins ineffective as therapy. Extended-spectrum beta-lactamase–producing *E. coli* (ESBL *E. coli*) are highly resistant to an array of antibiotics, and infections by these strains are difficult to treat. In many instances, only two oral antibiotics and a very limited group of intravenous antibiotics remain effective. In 2009, a gene called New Delhi metallo-beta-lactamase (shortened NDM-1) that even gives resistance to intravenous antibiotic carbapenem, were discovered in India and Pakistan on *E. coli* bacteria (Paterson and Bonomo, 2005).

1.2.4.1: Genetics of bacterial antibiotics resistance

1.2.4.1.1: Antibiotic resistance via mutations

There is a substantial number of biochemical mechanisms of antibiotic resistance that are based on mutational events, like the mutations of the sequences of genes encoding the target of certain antibiotics (e.g. resistance to rifampicin and fluoroquinolones is caused by mutations in the genes encoding the targets of these molecules, RpoB and DNA-topoisomerases, respectively) (Ruiz, 2003). The variation in the expression of antibiotic uptake or of the efflux systems may also be modified by mutation (e.g. the reduced expression or absence of the OprD porin of Pseudomonas aeruginosa reduces the permeability of the cell wall to carbapenems) (Wolter *et al*., 2004).

1.2.4.1.2: Antibiotic resistance via horizontal gene transfer

A principal mechanism for the spread of antibiotics resistance is by horizontal transfer of genetic material. Antibiotic resistance genes may be transferred by different mechanisms of conjugation, transformation or transduction. Over the last 15 years, β-lactamase enzymes that have an extended spectrum of activity (ESBL) against the majority of β-lactams, including cephalosporins but not carbapenemases, have evolved. One of these, CTX-M-15, initially found in *E. coli* but now found in other members ofEnterobacteriaceae and frequently associated with a specific lineage, uropathogenic clone ST131 (Bush and Fisher, 2011; Woodford *et al*., 2011), has spread worldwide. It is often located on highly mobile IncFII plasmids and associated with mobile genetic element IS26. The risk of infection is particularly high in individuals in association with prolonged hospitalization, catheterization, nursing home residency, previous antibiotic treatment, underlying renal or liver pathology, and travel to high-risk areas (Nordmann *et al*., 2011).

1.2.5: β-lactam antibiotics

β-lactams are a large group of antibiotics, all containing the β-lactam ring. There are four major groups, penicillins, cephalosporins, carbapenems and monobactems, which differ from one another in the nature of the additional ring attached to the β-lactam ring. In penicillins there is a five-membered thiazolidine ring, in cephalosporins a six-membered cephem ring, a double ring in carbapenems whereas in monobactams only the β-lactam ring is present. The various types of β-lactams within each group differ in the side chains attached to the core rings (Samaha-Kfoury and Araj, 2003). The history of β-lactam antibiotics began in 1929 when Alexander fleming description of antibacterial activity of substances produced by pencicillum mould and give it the same pencillin. However, the phenomenon of antibiotic was already well known at this time (Albert and Sussman, 1998).

The production of β-lactamase is the most frequently encounterd mechanism of the bacterial resistance to β-lactam antibiotics. Some variants are able to inactivate newer cephalosporins, are called extended spectrum β-lactamases (ESBLs) and are predominantly plasmid mediated Queenan and Bush, 2007).

1.2.5.1: Mechanism of β-lactams action

β-lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems target transpeptidase enzymes that synthesize the bacterial cell wall and act cytostatically on bacteria by inactivating peptidoglycan transpeptidases irreversibly. The desirable attributes of this class of antibiotic arise from the facts that these enzymes are localized to the outer leaflet of the bacterial cytoplasmic membrane (i.e. are relatively accessible) and that they are specific to bacteria (with

no functional or structural counterpart in the human host) (Walther-Rasmussen and Hoiby, 2006**)**.

The β-lactam antibiotics inhibit the final transpeptidation by forming covalent bond with penicillin- binding proteins that have transpeptidase and carboxypeptidase activities thus preventing formation of cross links. the final bactericidal action is the inactivation of an inhibitor of autoulytic enzymes in the cell wall, which leads to lysis of the bacteria. Some tolerant organisms have defective autolytic enzymes and are inhibited but not lysed in the presence of drug (Rang *et al*., 2006).

1.2.6: β-lactamase inhibitors

These agents structurally resemble β-lactam antibiotics but do not possess any significant antimicrobial action. They bind irreversibly to the catalytic site of susceptible β-lactamases (particularly penicillinase) to prevent hydrolysis of penicillins. These are generally effective against plasmid mediated β-lactamases which are responsible for transferred drug resistance such as those produced by methicillin-sensitive *Staphylococcus aureus, H. influenza, H. ducreyi, E. coli, Klebsiella pneumonia, Proteus mirabilis, Neisseria gonorrhoeae, salmonella species and Shigella species*. These are generally ineffective against chromosomally mediated β-lactamases found in the *Enterobacter, Pseudomonas aeuginosa, Citrobacter and Serratia* and organisms producing inducible extended spectrum β-lactamases. Currently three β-lactamases inhibitor are available; Clavulinic acid, Sulbactam and Tazobactam. Clavulinic acid is combind amoxicillin, sulbactam with ampicillin, and tazobactam with piperacillin and are available as fixed dose combinations (Sharma and Sharma, 2007).

1.2.7: β- Lactamase

1.2.7.1: Classification of β-lactamases

In molecular classification β-lactamases can be classified into four different molecular groups, A, B, C and D, according to amino acid sequence identities (Huovinen *et al*., 1998). Class A, C (AmpC) and D β-lactamases use a catalytically active serine residue for inactivation of the β-lactam drug (Lamotte-Brasseur *et al*., 1994). The enzymes assigned to the molecular class B are metallo-enzymes requiring zinc for their catalytic activity, and they operate through a completely different mechanism (Majiduddin *et al*., 2002). In addition to the Ambler classification, β-lactamases can be classified on their substrate specificity. Penicillinases, ESBLs, carbapenemases and so on are examples of classification of β-lactamases by they preferred substrate specificity. This classification does not follow the phylogeny of these enzymes. For example, ESBLs exist among several different phylogenetically defind β-lactamase groups. For this work, ESBLs are the most important group of β-lactamases (Livermore, 2008).

1.2.7.2: Mechanism of action of β-lactamases

β-lactamases enzymes are able to hydrolyze the β-lactam ring using two types of nucleophile agents (Fisher *et al*., 2005), these are either a serine residue (as in class A, C and D β-lactamases) or a Zn(II)-bound water/hydroxide group (class B or metallo-β-lactamases, MBL's). the serine β-lactamase classes A, C, and D share similarity at the protein structure level, whiches proves that they descended from a common ancestor (Hall and Barlow, 2004). It reaction are in three steps. At first, formation of the non covalent Michaelis-Menten complex by associating non –covalently the enzyme with the drug (binding steps). The lactam

bond of β-lactam drugs nucleophilic is attacked by the three hydroxyl on the side chains of a serine residue at the active site of enzyme, yielding a covalent acyle ester (acetylation step) (Pratt, 2002).

Metallo-β-lactamases, a less commonly encountered group of β-lactamases in which a divalent transition metal ion, most often zinc, linked to a histidine or cysteine residue react with the carbonyl group of the amide bond of most penicillins, cephalosporins, and carbapenems, but not monobactams (Walsh *et al*., 2005; Bebrone, 2007).

1.2.7.3: Type of β-Lactamase

1.2.7.3.1: Extended spectrum β-lactamases (ESBLs)

Extended spectrum β-lactamases-producing strains can increase morbidity and mortality rates, in part as a result of linked resistance to other antibiotic families, which restrict therapeutic options and raises healthcare costs (Inwezerua *et al*., 2014).

ESBL, hydrolyse the oxyimino β-lactams like ceftazidime, cefotaxime, ceftriaxone and monobactams, but have no outcome on the cephamycins, carbapenem and related compounds have emerged as an important mechanism of resistance amongst pathogens (MuKherjee *et al*., 2013). ESBL are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Pitout, 2012). The ESBLs are classified into two subgroups. The first group (type I) consists of variants of the penicillinases TEM-1, TEM-2, and SHV-1 differing by one to four point mutations leading to either an extended hydrolytic capacity to third generation cephalosporins (Tomanicek *et al*., 2010), and do not hydrolyze oxyimino-cephalosporins or aztreonam (Kiratisin *et al*., 2008). The origins of the TEM types remain uncertain, but SHV-1 was later realized to be derived from the chromosomal β-lactamase of *K. pneumoniae*. The spread of plasmid-mediated TEM and SHV enzymes provided a major

impetus for the development of 'β-lactamase-stable β- lactams' from the mid-1970s onwards (Livermore, 2008).

The second and the most widespread group of ESBLs (type II) include the CTX-M-type enzymes that are distantly related to TEM-1 or SHV-1. These enzymes share less than 40% sequence identity with other type I ESBLs, whereas they share a 70% or higher identity within the subgroup (Tomanicek *et al*., 2010). The CTX-M-type β-lactamases, along with a host of smaller groups such as the VEB, PER, GES and some OXA enzymes to form a collection of enzymes referred to as the ESBLs (Evans *et al*., 2007). These enzymes are mostly plasmid-coded and thus more easily horizontally transmissible. They hydrolyze β-lactam antibiotics resulting in resistance to penicillins, cephalosporins, and aztreonam (Reinthaler *et al.*, 2010). CTX-M-Producing E. coli are important causes of community-onset UTIs, bacteremia and intra-abdominal infections (Can-ton and Coque, 2006).

Currently, the most wide spread and prevalent type of CTX-M enzyme among human clinical isolates of *E. coli* is CTX-M-15.EXPEC producing this enzyme often belong to international uropathogenic sequence type named ST131 and to a lesser extened ST38, ST405, and ST648 (Peirano and Pitout, 2010).

ESBL are created by substituting one or more amino acids in the TEM, SHV and OXA lactamases molecules. So far over 200 new ESBL group enzymes have been discovered, which are responsible for the resistance to all cephalosporins including 3rd generation cephalosporins, as well as to all penicillins and aztreonam (Gupta, 2007).

1.2.7.3.2: Carbapenemases

Carbapenemases are ESBL enzymes that hydrolyze or partially hydrolyze imipenem and/or meropenem. Because they often confer only partial resistance and are hard to detect, their presence may be underestod (Siegel, 2008).

These enzymes have been classified into the class A group (clavulanic acid-inhibited carbapenemases) which is a class of serine β-lactamases (most of these β-lactamases have a serine residue at the active center of the enzymes), the class B (metallo-β-lactamase) requiring zinc ion as cofactor for the maximal activity, and the class D (oxacillinases) (Jamklang, 2004).

A few classes of A enzyme (clavulanic acid inhibited carbapenemases) which is class of serine β-Lactamases, remarkably the plasmid-mediated KPC enzymes, are effective carbapenemases as well. As well as, some of class D, OXA-type β-lactamases have carbapenemases activity, augmented in clinical isolates (Pitout, 2012).

1.2.7.3.3: AmpC β-lactamases

AmpC β-lactamases are active-site serine enzymes that are primarily cephalosporinases, they confer resistance to cephalosporins in the oxyimino group, 7-α-methoxy cephalosporins and are not affected by available β- lactamase inhibitors (Thomson, 2001). Organisms expressing these enzymes are not resistant to third-generation cephalosporins unless the AmpC β-lactamase is expressed at high levels (Navarro, 2006).

AmpC β-lactamases confer resistance to a wide variety of β-lactam drugs except for cefepime and carbapenems. They are known to be responsible for nosocomial outbreaks, therapeutic failure, and multidrug resistance (Oteo *et al*., 2010).

This AmpC phenotype may result from overexpression of the chromosomally encoded AmpC enzyme or from the acquisition of a transferable plasmid encoded AmpC gene (Naseer *et al*., 2009).

In many species, inducible AmpC β-lactamases are normally produced at very low levels but are typically induced to several hundred fold higher by the presence of β-lactams (e.g. cefoxitin, cefotaxime, etc.) and certain β-lactam inhibitors (e.g. clavulanic acid) (Arora and Bal, 2005).

Plasmid-mediated *AmpC* genes are derived from the chromosomal *ampC* genes of several members of the family Enterobacteriaceae (Pitout, 2012). AmpC β-lactamases make available resistance to cephamycins as well as to oxyimino-β-lactams and are resistant to inhibition by clavulanic acid. CMY-2 is the most commonly encountered plasmid mediated AmpC β-lactamase, often found in *E. coli* (Doi *et al*., 2009).

1.2.8: Molecular Mechanisms of Action of Quinolones

The DNA topoisomerases control the topological state of the chromosomal DNA to facilitate replication, recombination, and expression through the breaking and rejoining of DNA strands (Hawkey, 2003). Type I topoisomerases cleave one strand of DNA whereas type II topoisomerases cleave both strands in a reaction coupled to ATP binding and hydrolysis (Hawkey, 2003).

The inhibitory function of quinolones is initiated via binding to complexes that form between DNA and gyrase or topoisomerase IV, resulting in conformational change in the enzyme and subsequent cleavage of the bacterial DNA. Importantly, quinolones not only induce nicks in the bacterial genomic DNA but also prevent such nicks from re-ligating, thus perpetuating the inhibitory action (Hawkey, 2003). Inhibition occurs more quickly for DNA gyrase than topoisomerase IV,

because the former is located at the replication fork and the latter is located behind the replication fork (Khodursky and Cozzarelli, 1998). The primary target for quinolones is DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. Although exceptions to this pattern have been seen with some molecules (Andriole, 2005).

1.2.9: Plasmid-Mediated Quinolone Resistance

The discovery of PMQR in the late 1990s by Martínez-Martínez and colleagues, that group was studying pMG252, a plasmid from a multi-resistant strain of *K. pneumoniae* that was isolated from a urine specimen from a patient at the University of Alabama at Birmingham in 1994 (Jacob *et al*., 2009). The first PMQR gene to be described was named *qnrA* and encodes a 218 amino acid pentapeptide repeat protein that is capable of protecting the DNA gyrase from the activity of quinolones **(**Tran *et al*., 2005; Robicsek *et al*., 2006d).

Plasmids carrying *qnrA* variants often carry other antibiotic resistance genes, which may or may not be transferable (Nordmann and Poirel, 2005). Interestingly, hybridization studies for *qnrA* in an *E. coli* clinical isolate revealed integration with chromosomal DNA (Cavaco *et al*., 2007). It is known that *qnrA*genes are often embedded in complex sul1-type integrons (Nordmann and Poirel, 2005). These integrons include a single 5′-conserved segment that contains the integrase gene (intl1), and duplicated 3′-conserved segments, each of which contain quaternary ammonium compound-resistance and sulphonamide resistance protein (sul1) genes. The two 3′-conserved segments surround a common region that contains the putative transposase orf 513, which may act as a recombinase for (Cavaco *et al*., 2007).

The *qnrS* genes are also typically located on plasmids but, in contrast to *qnrA*, they are not part of a *sul1*-type integron structure (Robicsek *et al*., 2006).

later on, the *qnrB* and *qnrS* genes were first discovered in 2005, and 2006, which confer quinolone resistance by binding to DNA gyrase and topoisomerase IV and protect them from quinolones by unknown mechanism (Xiang *et al*., 2011). However A plasmid-mediated *AAC(6`)-Ib-cr*, represents a new variant of a common aminoglycoside acetyltransferase, *AAC(6`)-Ib*, that reduces the activity of both the aminoglycosides, kanamycin, tobramycin, and amikacin and the fluoroquinolones, ciprofloxacin and norfloxacin and hence stands for unpredictable new mechanism of quinolone resistance (Melano *et al*., 2003; Robiscek *et al*., 2006c; Park *et al*., 2006; Jacob *et al.,* 2009). The -cr variant of *aac(6')-Ib* has two amino acid changes, Trp102Arg and Asp179Tyr, both of which are necessary for the N-acetylation of the amino nitrogen of a piperazinyl group (Robicsek *et al*., 2006). This enzyme reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substitute. Ciprofloxacin and norfloxacin are the only fluoroquinolones with diminished activity in the presence of *aac(6`)-Ib-cr* since they are the only compounds harboring an un substituted piperazinyl group (Karah, 2008; Jacob *et al.,* 2009).

Chapter Two

Materials and Methods

2: Materials and methods

2.1: Materials

2.1.1: Instruments and Equipments

The instruments and equipments used in the present study with their remarks are listed in table (2-1) bellow:

Table (2-1): Instruments and equipments used with their remarks

Type of equipment	Manufactured company (Origin)
Autoclave	Hiclave- Hirayama (Japan)
Bench centrifuge	Hettich (Germany)
Calipers	China
Centrifuge	Memmert (Germany)
Distilator	GFL (Germany)
Digital camera	Sony (Japan)
Electrophoresis	Labner (Taiwan)
Electric oven	Memmert
Incubator	Memmert
Laminar flow cabiant	Cruma (Spain)
Millipore filter (0.22µm)	Difco (USA)
Micropipette 1-10 µl-10-1000 µl	Eppendorf (Germany)
Micropipette 2-20 µl, 500 µl	Eppendorf
PCR system	GeneAmp (Singapore)
Petridish 9 cm	China
Petridish 15 cm	China
Sensitive balance	Memmert
UV – transilluminator	Taiwan
Vortex mixer	Thermolyne

2.1.2: Biological and Chemical Materials

The chemical and biological materials used in this work with their remarks are listed in table (2-2) bellow:

Table (2-2): Biological and chemical materials used with their remarks

Biological and Chemical type	Manufacturer (Origin)
Agarose	Promega (USA)
Barium chloride dihydrate ($BaCl_2$). $2H_2O$	Fluka (Switzerland)
Ethanol (96%)	BDH
Ethidium bromide	Sigma (USA)
Ethyenediamine tetra-acetic acid (EDTA)	BDH
Glycerol ($C_3H_8O_3$)	Fluka
Hydrochloric acid (HCl)	BDH
Iodine	Mast Diagnostic (UK)
Isoamyl alcohol	BDH
Kovac's reagent	Himedia (India)
Methyl red	BDH
Phenol	Scharlau
Sodium chloride (NaCl)	BDH
Sodium dodecyl sulfate (SDS)	AppliChem (Germany)
Sodium hydroxide (NaOH)	BDH
Sulfuric acid (H_2SO_4)	BDH
Tris- EDTA (TE) buffer molecular grad	Promega
Tris-(hydroxymethyl)methylamine ($NH_2.(CH_2OH)_3$ (Tris-OH)	BDH
Tris-Borate-EDTA Buffer **(**TBE buffer)	Promega
Urea solution	Mast Diagnostic
α-naphthol (C10H8O)	BDH
Isopropanol	Mast Diagnostic
Gram stain	Himedia

2.1.3: Culture Media

The culture media used in this work with their remarks are listed in table (2-3) bellow:

Table (2-3): Culture media used with their remarks

Media	Manufacturer(Origin)
Brain heart infusion agar	Himedia
MacConkey agar	Himedia
Muller-Hinton agar	Himedia
Nutrient broth	Himedia
Nutrient agar	Himedia
MR-VP broth	Oxoid (UK)
Brain heart infusion broth	Himedia (India)
Peptone water	Biolife (Italy)
Simmons citrate agar	Mast Diagnostic (UK)
Urea agar base	Biolife
Tryptic soy broth	Biolife
Tryptic soy agar	Biolife
Triple sugar iron agar	Biolife
Blood base agar	Himedia

2.1.4: Antibiotics

2.1.4.1: Antibiotic discs

The antibiotics discs used in this work with their remarks are listed in table (2-4) bellow:

Table (2-4): Antibiotics discs used in the present study with their remarks

Class	Subclass	Antibiotic name	Symbol	Concentration	Origin
Penicillin		Ampicillin	AMP	µg 10	Himedia
β-lactam /β-lactamase inhibitor combinations		Amoxicillin +Clavulinic acid	AMC	30 µg	Himedia
Monobactams		Aztreonam	AT	30 µg	Himedia
Quinolones	Flouroquinolones	Nalidixic acid	NA	30 µg	Himedia
		Ciprofloxacin	CIP	5 µg	
Penems	Carbapenem	Imipenem	IPM	10 µg	Himedia
Phenicols		Clorampenicols	C	30 µg	Bioanalyse
Cephems (parenteral)	Cephalosporin III	Cefotaxime	CAZ	30 µg	Biomaxima S.A
		Ceftazidime	CAZ	30 µg	Biomaxima S.A
		Ceftriaxone	CRO	10 µg	Bioanalyse
	Cephamycin	Cefoxitin	FOX	30 µg	Biomaxima S.A
Aminoglycosides		Gentamycin	GEN	10 µg	Himedia
		Tobramycin	TOB	10 µg	Bioanalyse
Nitrofurans		Nitrofurantoin	F	300 µg	Bioanalyse

2.1.5: standard Strain Bacterium

Standard strain bacteria used in this work with it remark is listed in table (2-5) bellow:

Table (2-5): Standard strain bacterium with its remark

Strain Name	LaboratoryIdentifier	Key characteristic	Source
E. coli	(ATCC 35218)	Susceptible to Cefoxitin,Nalidixic acid, Imipenem, Ciprofloxacin, and Nitrofurantoin	(BioMerieux® - France)

2.1.6: PCR Materials

2.1.6.1: Master Mix

The PCR PreMix used in this work with it remark is listed in table (2-6) bellow:

Table (2-6): PCR PreMix with its remark

Go Tag Green Master mix	**Source**
Go Tag DNA polymerase is supplied in 2X Green Taq Reaction buffer pH 8.5, 400µmM dATP, 400µM dGTP, 400µmM dCTP, 400µM dTTP, and 3mM $MgCl_2$	Promega (USA)

2.1.6.2: PCR Amplification PMQR Primers

Plasmid mediated quinolone resistance Primers used in this work with it remark are listed in table (2-7) bellow:

Table (2-7): PMQR Primers and their sequences

Primer name	Oligo sequence (3`-5`)		Product size bp	Reference
qnrA	F	TCAGCAAGAGGATTTCTCA	625	Thi *et al*., 2007
	R	GGCAGCACTATTACTCCCA		
qnrB	F	GATCGTGAAAGCCAGAAAGG	468	Thi *et al*., 2007
	R	ACGATGCCTGGTAGTTGTCC		
qnrS	F	ACGACATTCGTCAACTGCAA	417	Thi *et al*., 2007
	R	TAAATTGGCACCCTGTAGGC		

2.1.6.3: PCR Amplification ESBLs Primers

Extended spectrum beta lactamases Primers Primers used in this work with it remark are listed in table (2-8) bellow:

Table (2-8): ESBLs Primers and their sequences

Primer name	Oligo Sequences (3`-5`)		Product size bp	Reference
bla$_{TEM}$	F	AAACGCTGGTGAAAGTA	822	Paterson *et al*., 2003
	R	AGCGATCTGTCTAT		
bla$_{CTXM}$	F	CGCTTTGCGATGTGCAG	550	Bhattacharjee *et a*l., 2007
	R	ACCGCGATATCGTTGGT		
bla$_{OXA}$	F	ATATCTCTACTGTTGCATCTCC	619	Karami and Hannoun, 2008
	R	AAACCCTTCAAACCATCC		

2.2: Methods

2.2.2: Preparation of Buffers and Solutions

The following solutions and reagents were used in the present study. Those, which require sterilization, were autoclaved at 121˚C for15-20 minutes. Millipore filters (0.22 μm) were used for sterilization of heat-sensitive solutions like urea. The pH of the solution was adjusted using 1M NaOH and 1M HCl.

2.2.2.1: McFarland (0.5) Turbidity Standard

The 0.5 McFarland standard was prepared by adding 0.5 ml of a 1.175% (wt/vol) $BaCl_2.2H_2O$ solution to 99.5 ml of 1% (vol/vol) H_2SO_4.. The turbidity standard was liquated into test tubes identical to those used to prepare the inoculums suspension. The McFarland standard tubes were sealed with parafilm to prevent evaporation and stored for up to 6 months in the dark at room temperature (22-25ºC). The accuracy of the density of a prepared 0.5 McFarland standard equivalent $1.5X10^8$ cell/ml was checked by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be 0.08 to 0.1 (CLSI, 2010).

2.2.2.2: Normal Saline solution

Sodium chloride (0.85 gm) was dissolved in 90 ml D.W. and further completed to 100 ml with D.W (Collee *et al*., 1996).

2.2.2.3: Phosphate buffer solution(PBS)

This buffer consisted of two solutions and prepared as follows:

Solution A: 3.12 gm of $NaH_2PO_4.2H_2O$ was dissolved in 90 ml of D.W. and then completed to 100 ml with D.W.

Solution B: 2.839 gm of Na2HPO4 was dissolved in 90 ml of D.W. and the volume was completed to 100 ml. Then 87.7 ml of solution A was added to 12.3 ml of solution B, and the pH was adjusted to 6. The buffer

was used for the detection of β-lactamase production (Collee *et al.*, 1996).

2.2.2.4: Urea solution

Dissolving 40 grams of urea powder in 100 ml of distilled water, and sterilized by filter paper (0.22mm) (Collee et al., 1996).

2.2.2.5: EDTA Solutions for disks preparation

Disks of EDTA were prepared by dissolving 190 mg of EDTA (EDTA powder must completely dissolved) in 1 ml of D.W., and adjusting it to pH 8, the mixture sterilized by autoclaving. EDTA solution was added to a 6-mm Whatmann filter No.1 disks (n=100) and allowed to dry. Each disk contain approximately 1,900 µg of EDTA and used to detection of metallo-β-lactamases producing isolates (Lee *et al*., 2003).

2.2.3: β-lactam Antibiotic Solutions

β-lactam solutions were prepared as stock solutions with concentrationof 10 mg/ml by dissolving 1gm of the following antibiotics: Amoxicillin,Ampicillin, Cloxacillin and Cephalothin in a small volume of sterile PBS(pH 6.0), or by dissolving in sterile D.W for cefotaxime, piperacillin andmeropenem, while ceftazidime was solubilized in Na2HCO3 . Each of these solutions was further diluted with sterile D.W to volume 100 ml, and stored at 4˚C until use (CLSI, 2010).

2.2.4: Solutions used for β-lactamase detection

These solutions were prepared according to Collee *et al.* (1996) as follows:

Pencillin G solution: this was prepared by dissolving 0.569 gm of penicillin G in PBS (3-2-1-3**)**. The solution was sterilized, dispensed in small vials, and stored at -20˚C.

Starch solution: this solution was prepared by dissolving 1gm of soluble starch in 100 ml of D.W. and boiled in water bath for 10 minutes, and stored in a dark bottle at 4˚C.

Iodine solution: iodine (2.03 gm) and KI (5.32 gm) were dissolved in 90 ml of D.W., then the volume was completed to 100 ml with D.W., and stored in a dark bottle at 4˚C.

2.2.5: Solutions Used in DNA Extraction

2.2.5.1: Tris-EDTA Buffer (TE) Buffer

This buffer was prepared by adding 0.05 M Tris-OH and 0.001 M EDTA to 800 ml D.W, the pH was adjusted to 8 and completed to one litter by D.W, then autoclaved at 121˚C for 15 minutes, and stored at 4˚C until use (Pospiech and Neurmann, 1995).

2.2.5.2: Salt-Tris-EDTA (STE) Buffer

This buffer was prepared by dissolving 10 mM Tris-HCl, 1 mM of EDTA and 100 mM NaCl in 400 ml D.W, the pH was adjust8.0 andthe volume completed to 500 ml by D.W, then autoclaved at 121˚C for 15 minutes (Cheng and Jiang, 2006).

2.2.5.3: Salt-EDTA (SE) Buffer

This buffer was prepared by dissolving 0.1 M of EDTA and 0.15 M NaCl in 750 ml D.W, the pH was adjusted to 8.0 and the volume completed to 500 ml by D.W, then autoclaved at 121˚C for 15 minutes (Cheng and Jiang, 2006).

2.2.5.4: Sodium Dodecyl Sulfate (SDS) Solution (25%)

Sodium dodecyl sulfate (25 mg) was dissolved in 100 ml of D.W, then sterilized in autoclave, and stored at 4˚C (Pospiech and Neurmann, 1995).

2.2.5.5: Sodium Chloride (NaCl) Solution (5 M)

Sodium chloride (14.625 g) was dissolved in 50 ml D.W, sterilized in autoclave, and stored at 4°C until used (Pospiech and Neurmann, 1995).

2.2.5.6: Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

The solvent was composed of 25 ml phenol, 24 ml chloroform, and 1 ml isoamyl alcohol (Pospiech and Neurmann, 1995)

2.2.6: Solutions used in gel electrophoresis

These solutions were prepared as described by Sambrook and Rusell (2001).

2.2.6.1: Tris-Borate-EDTA buffer (TBE)

Tris-OH- 0.08 M, Boric acid-0.08 M, EDTA-0.02 M. The pH was adjusted to 8, autoclaved, and stored at 4˚C.

2.2.6.2: DNA loading buffer

Bromophenol blue-25 mg, Xylene Xyanol-25 mg, Sucrose-4 gm, D.W.- 10 ml

2.2.7: Preparation of Reagents

2.2.7.1: Methyl Red Reagent

It was prepared by dissolving 0.1 g of methyl red in 300 ml of 96% ethanol. Then 200 ml of distilled water was added. This reagent was used as indicator in methyl red test (MacFaddin, 2000).

2.2.7.2: Voges-Proskaur Reagents

The reagents were prepared according to MacFaddin (2000) as follow:

Reagent A: 5% α-naphthol in 96% ethanol.

Reagent B: 40% KOH in distilled water.

These reagents were used as indicators in Voges-Proskour test.

2.2.7.3: Oxidase Reagent

This was prepared by dissolving 1 g of tetramethyl-p-phenylenediaminedihydrochloride in 100 ml of distilled water. This reagent was used as indicator in oxidase test (MacFaddin, 2000).

2.2.7.4: Catalase Reagent

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle and used for detection of ability of isolates to produce catalase enzyme (MacFaddin, 2000).

2.2.7.5: Kovacs Reagent

It was prepared by dissolving 5 gm of P-dimethyl-aminobenzlaldehyde in 75 ml of amyl alcohol.25 ml of HCl is added to this mixture. The Reagent was placed in dark bottle until used ((MacFaddin, 2000).

2.2.7.6: Aqueous Ferric Chloride Reagent

This solution was prepared by dissolving 12 gm of FeCl3 in 2.5 ml of concentrated HCl and completed to 100 ml with D.W(MacFaddin,2000).

2.2.8: Preparation of culture and diagnostic media

2.2.8.1: Ready-Manufactured media

Media used in this study were prepared in accordance with the manufacture's instructions fixed on their containers. All the media were sterilized by autoclaving at 121˚C for 15 minutes. After sterilization, blood agar base was supplemented with 7% human blood, and urea agar base was supplemented with 20% sterile urea solution.

2.2.8.2: Laboratory Prepared Media

2.2.8.2.1: Motility medium

Agar-agar (0.5 gm) was dissolved in to 100 ml of brain heart infusion broth, the contents were dispensed in to test tubes and autoclaved at 121˚C for 15 minutes (MacFaddin, 2000).

2.2.8.2.2: Urea agar medium

Urea agar medium was prepared according to Collee *et al*., (1996), As follows: first, urea base agar medium was prepared depending on the procedure of manufacturer's instructions, then pH was adjusted to 6.8-6.9, then autoclaved, urea base agar was cooled to 45-50°C, then 5ml of sterile filtrated 40% urea solution was added to each 95ml of urea base agar, and mixed well then dispended into sterile tubes (poured as a slant). Urea agar medium was used to verification the ability of bacterial isolates to produce urease.

2.2.8.2.3: Blood agar medium

First, blood agar base medium was prepared according to protocol of manufacturer's instructions and sterilized by autoclave, and it was cooled to approximately 40-45°C, then blood was added (5%) to this medium and poured into sterile petri dishes .Blood agar medium is suitable for the isolation and culturing of bacteria and for the detection type of hemolysis (Collee *et al*., 1996).

2.2.9: Biochemical Tests

2.2.9.1: Indole Production Test

Peptone water medium tube was inoculated with a refresh bacterial culture and incubated at 37°C for 24-48 hours. A few drops of Kovàcs reagent were added to each tube. Formation of pink ring indicates a positive test (MacFaddin, 2000).

2.2.9.2: Methyl Red Test

Methyl red-Voges-Proskauer broth was inoculated with a young bacterial culture and incubated at 37˚C for 24 hours. Five drops of methyl red solution were added to each tube contain 2ml and the result was read immediately. Changing the color to red indicates a positive test (MacFaddin, 2000).

2.2.9.3: Voges-Proskauer Test

Methyl red-Voges-Proskauer broth was inoculated with a young bacterial culture and incubated at 37˚C for 48 hours. One ml of 40%KOH solution and 3 ml of 5% solution of □-naphthol were added to each tube. A positive reaction was indicated by the development of a pink color in 15-20 minutes (MacFadden, 2000).

2.2.9.4: Simmons Citrate Test

Simmons citrate slant was inoculated with a fresh bacterial culture and incubated at 37˚C for 48-72 hours. Changing the color from green to blue is indicating a positive test (MacFaddin, 2000).

2.2.9.5: Triple Sugar Iron Test (TSI)

A heavy growth of tested bacteria was streaked over the surface of the slope and stabbed into the bottom, and incubated at 37˚C for 24 hours. Results were configured as follows:((MacFaddin, 2000).

Slant/butt	Color
Alkaline/Acid	Red/Yellow
Acid/ Acid	Yellow/ Yellow
Alkaline/ Alkaline	Red/ Red
H_2S production	Black precipitate
Gas formation	+/-

2.2.9.6: Urease Test

Urea agar slant was streaked with bacterial culture and incubated at 37˚C. The result was read after 6 hours, 24 hours, and every day for 6 days. Urease test is positive if the indicator was changing the color of medium to purple-pink (MacFaddin, 2000).

2.2.9.7: Oxidase Test

A strip of filter paper (Whatman No.1) was soaked with a little freshly made 1% solution of tetramethyl-P-phenylene-diamine dihydrochloride, and then 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 that appeared within 5-10 seconds (MacFaddin, 2000).

3.2.9.8: Motility Test

Motility medium was used for detection of bacterial motility. Tubes were inoculated with bacterial culture by stabbing and incubated at 37˚C for 24-48 hours. Formation of cloudy growth out of the line of stab indicates a positive result (MacFaddin, 2000).

2.2.9.10: Chrome agar medium test

This medium was used as a selective identification for *E. coli* and other members of Enterobacteriaceae family according to the manufacture company protocol (Orientation company – France): as follows:

All suspected bacterial isolates were streaked on chrome agar medium plates, and then incubated at 37^{o}C for one day. Appearance of metallic blue colonies considered as a positive results for (*E. coli*).

2.2.10: Preservation and Maintenance of Bacterial Isolates

The bacterial isolates were preserved on nutrient agar slant at 4˚C. Theisolates were maintained monthly by reculturing on new medium. Nutrient broth supplemented with 15% glycerol was used for long preservation and the isolates were maintained frozen at -70 $_{o}$C (deep freeze) for several months (long term maintenance) (Collee *et al*., 1996).

2.2.11: Identification of Bacterial Isolates

Escherichia coli isolates were identified depending on the traditional morphological and biochemical tests, and other bacterial isolates were detected according to the methods of MacFaddin (2000).

2.2.11.1: Vitek2® System test:

All isolates of *E. coli* were identified by using Vitek2® system (BioMerieux® -France) according to steps of Manufacture Company.

2.2.11.2: Storage of isolates

2.2.11.2.1: Short term storage

The *E. coli* positive cultures were stored on nutrient agar slant at 4°C until further testing (Thomas, 2007).

2.2.11. 2.2: Long term storage

Fresh 24 hours, blood agar cultures of isolates were frozen in 20% glycerol nutrient broth and stored at -70°C until required (Thomas, 2007).

2.2.11. 2.3: Subculture of frozen stocks

Frozen stock cultures stored at -70°C were sub-cultured on fresh blood agar plates, and then incubated in aerobic condition at 37°C for 24 hours (Thomas, 2007).

2.2.12: Antibiotic Susceptibility Test

Antimicrobial susceptibility testing of *E. coli* isolates performed by identification to susceptibility testing by modified disc-diffusion method (Kirby-Bauer) (Bauer *et al.,* 1966). The selection of antibiotic disc was performed according to the guidelines recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010). Inoculum from the pure culture plate was prepared; a loopful of the growth was suspended in a tube of tryptic soy broth and incubated for one hour at 37°C. The density of the suspension was adjusted depending on 0.5 McFarland standards by adding sterile normal saline. Mueller-Hinton

agar plates were inoculated by sterile swab dipped into the inoculums. The excess inoculums were removed by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. The swab was streaked all over the surface of the medium several times; the plate was rotated through an angle of 60° after each application finally, and the swab was pressed around the edge of the agar surface. Four disks were placed on a plate, approximately 15 mm from the edge of the plate. Each disk gently pressed down to ensure even contact with the medium. The plates were placed in an incubator at 37°C. After 18 hours incubation, the diameter of each inhibition zone (including the diameter of the disk) were measured with a pair of calipers, and recorded in mm. The results then interpreted according to CLSI (2010). A laboratory stock culture of generic *E. coli* ATCC 35218 was used as a quality control organism to confirm accuracy of the antibiotic disk.

2.2.13: Beta-lactam resistance test

The medium was poured into sterilized petri dishes. A young and fresh bacterial isolate was streaked in Muller-Hinton agar plates supplement with 10 µg of ampicillin and incubated at 37˚C for overnight. Formation of any bacterial growth considered as a positive result for β-Lactam resistance (NCCLs, 2007).

2.2.14: Detection of extended-spectrum β-lactamase producing *E. coli*

Two methods were performed for detection of ESBLs in significant bacteriuria *E. coli* isolates.

2.2.14.1: a) Initial Screen Test (primary test)

Extended spectrum β-lactamase production by β-lactam resistant isolates was initially screened by using disk diffusion of cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (10 µg), and aztreonam (30 µg). placed on inoculated plates containing Muller-Hinton agar according to

the CLSI recommendations (CLSI, 2009). After 18 hr incubation at 37°C, the diameters of the inhibition zones around the antibiotics were meatured by caliper. The isolates wich showed inhibition zones < 27 mm for cefotaxime, <22mm for ceftazidime, <25 mm for ceftriaxone, and < 27 mm for aztreonam were suspected for ESBL production.

2.2.14.2: b) Disk Approximation Method (confirmatory method)

This method was carried out as modified by Bedenić *et al.* (2010) as follows: Muller-Hinton agar plate was inoculated with an overnight blood agar culture of the tested bacterial isolate. Disks containing 30 µg for cefotaxime, 30 µg for ceftazidime, 10µg for ceftriaxione, and 30 µg for azetreonam were placed 15 mm (edge to edge) from a disk of augmentin (30 µg). Incubation followed for 16-20 hours at 35˚C. Any enhancement of the zone of inhibition between a β-lactam disk and augmentin disk was indicative of presence of an ESBL. *E. coli* ATCC35218 was used as the negative control.

2.2.15: Molecular detection of antimicrobial resistance genes

2.2.15.1: Isolation of total DNA

This method was carried out according to Omar *et al*., (2014); as follows:

Three to five pure and fresh colonies of *E. coli* were inoculated from MacConkey agar plate into 300 µl of distilled water. Then cells was lysed by heating at 100 ^{o}C for 20 minutes (in water bath), and then immediately the cells were placed in ice for 30 minutes, and the other cellular components was removed by centrifugation at 8500 rpm for 10 min. Finally the supernatant was used as the DNA template.

2.2.15.2: Polymerase Chain Reaction Assay

2.2.15.2.1: Preparing the Primers

The Alpha DNA primers were prepared depending on manufacturer instruction by dissolving the lyophilized product with TE

buffer molecular grad after spinning down briefly. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

2.2.15.2.2: PCR Supplies Assembling and Thermocycling Conditions

Escherichia coli DNA templates were subjected to PCR using 12 sets (F and R) of primers targeting two groups of genes: the first group was applied to determine the quinolone resistant genes and the second group was used to determine ESBLs genes. The reaction mixture moreover contain Go Taq® Green Master Mix, X2 which is premixed ready-to-use solution containing bacteriology derived *Taq*DNA polymerase dNTP, $MgCl_2$, and reaction buffers at optimal concentrations and its recommended for any amplification reaction that to visualized by agarose gel electrophoreses and ethidium bromide staining.

2.2.15.2.3: PCR cycling programs applied

Polymerase chain reaction assays were carried out in a 20 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set, first group and second group depending on their reference procedure, as follows:

A) The first group

Table (2-9): PCR condition for plasmid mediated quinolones resistance genes

Gene name	Initial denaturation	Cycles	Denaturation	Primer annealing	Elongation	Final elongation	Reference
qnrA	94ºC / 10 min	35	94ºC / 30 sec	52ºC / 30 sec	72ºC / 1 min	72ºC / 10 min (then 4°C→ ∞	Thi *et al*., 2007
qnrB	94ºC / 10 min	35	94ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 10 min (then 4°C→ ∞	Thi *et al*., 2007
qnrS	94ºC / 4 min	35	95ºC / 30 sec	53ºC / 30 sec	72ºC / 1 min	72ºC / 10 min (then 4°C→ ∞	Thi *et al*., 2007

b) The second group

Table (2-10): PCR condition for extended spectrum beta lactamases genes

Gene name	Initial denaturation	Cycles	Denaturation	Primer annealing	Elongation	Final elongation	Reference
bla$_{TEM}$	94ºC / 30 sec	35	94ºC / 30 sec	45ºC / 1 min	72ºC / 1 min	72ºC / 10 min (then 4°C→ ∞	Paterson *et al*., 2003
bla$_{CTX-M}$	94ºC / 30 sec	35	94ºC / 30 sec	60ºC / 1 min	72ºC / 1 min	72ºC / 10 min (then 4°C→ ∞	Bhattacharjee *et al*., 2007.
bla$_{OXA}$	94ºC / 5 min	30	94ºC / 50 sec	55ºC / 50 sec	72ºC / 1min	72ºC / 10 min (then 4°C→ ∞	Karami and Hannoun, 2008

2.2.15.2.4: Agarose gel electrophoresis

Agarose gel was prepared according to Sambrook and Rusell (2001) by dissolving 1.5 gm of agarose powder in 100 ml of TBE buffer (pH 8) in boiling water bath, allowed to cool to 5˚C, and 5µl ethidium bromide was added. A tape was placed across the end of the gel tray, the comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently in to the tray, and allowed to solidify at room temperature for 30 minutes. Then the comb was removed gently from the tray and the tap was also removed from the ends of the tray. The latter was fixed in electrophoresis chamber which was filled with TBE buffer has covered the surface of the gel. 5µl of each DNA sample was transferred to Eppendorf tube. 5µl of loading buffer was added to the tube and the mixture was loaded into the wells in agarose gel. The electric current was allowed at 70 volt for 2 hr. UV transilluminater was used at 320-336 nm for the observation of DNA bands, and the gel was photographed using digital camera.

3.2.21: Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 5 software, percentages was used for the comparison between samples of the study.The study was cross sectional.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1: Total specimens

This study was carried out at Al-Hamza Hospital in Al-Diwaniya City and Al-Sadder Medical City in Al-Najaf Governorate-Iraq. A total of 400 specimens from different clinical sources (urine, stool, and blood) were collected.

Out of the 400 total specimens there were 220 specimens (55%) were diagnosed as gram negative bacteria, while there were150 specimens (37.5%) were diagnosed as gram positive bacteria and 30 specimens (7.5%) with no any growth (Table 3-1).

Table (3-1): Numbers and percentage of total specimens. (N=400).

Bacterial growth	No.	Percentage %
Gram negative	220	55
Gram positive	150	37.5
No growth	30	7.5

Total	400	100

Table (3-2): Morphological and biochemical tests for *E. coli* isolates.

Test	Result
Gram stain	Gram negative Cocoobacilli
Growth on MacConky agar	Pink and small colony
Lactose fermenter	+
Oxidase	-
Motility	+
Indole production	+
Methyl red	+
Voges-Proskauer	-
Simmons citrate	-
Urease	-
Triple Sugar Iron Agar	Acid / Acid with Gas
Growth on chrome agar	Metallic blue colonies

3.2: Identification of bacterial isolates

3.2.1: Morphological and biochemical test for *E. coli*

The positive results for cultural, microscopically and biochemical characteristics of *E. coli* were 49 isolates only, (Table 3-2). Primary identification of *E. coli* isolates was based on cultural, morphological characteristics, microscopically diagnosis and biochemical tests , while the confirmatory identification test was based on selective Chrome agar medium (Figure 3-1) and according to Vitek®2 system. Bacterial colonies

developed on plate agar, were studied; on MacConkey agar Small, round, entire, opaque, flat, non viscous, rose pink colour, lactose fermenter colonies (lactose fermenter) colonies , on chrome agar the colonies were metallic blue, while on blood agar *E. coli* were rounded, smooth, convex and beta hemolytic colonies, on EMB agar Black, Dark colour colonies with green Metallic sheen. Microscopic examination showed that the isolates were gram negative Coccobacilli.

3.2.2: Identification of *E. coli* isolates according to Vitek®2 system

Identification of all 49 suspected isolates of *E, coli* was confirmed by Vitek®2-automated system. The results demonstrated that there were full similarity between biochemical tests results, chrome agar and Vitek2 system results.

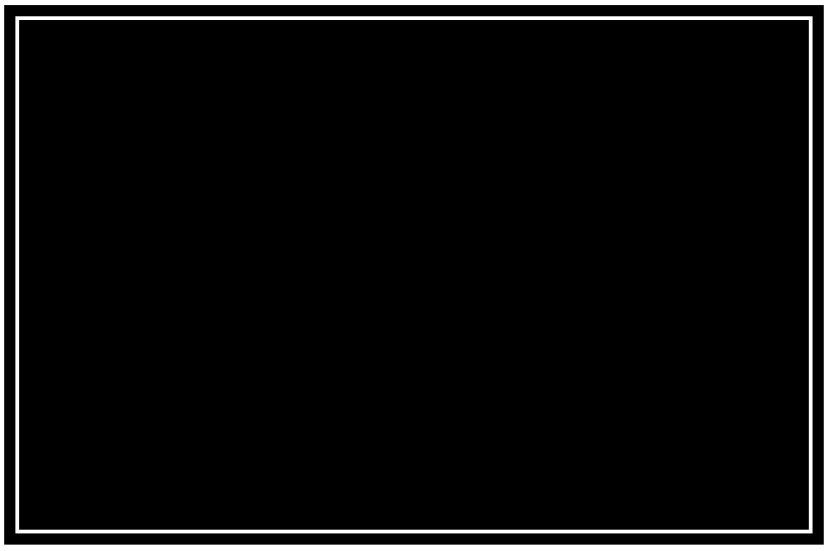

Figure (3-1): *E. coli* showing metallic blue colonies on chrome agar

Table (3-3): Numbers and percentage of *E. coli* isolates according to total gram negative bacterial isolates and sites of infection. (N=220).

Site of infection	Numbers of Gram negative bacteria	*E. coli*	Percentage (100%)
Urine	100	30	30
Stool	75	11	14.66
Blood	45	8	17.77
Total	220	49	22.27

3.3: Numbers and percentage of *E. coli* isolates according to total gram negative bacterial isolates and site of infection

A total of 220 gram negative bacterial isolates were collected from different clinical sources (100 from urine, 75 from stool, 45from blood) there were 30 isolate of *E. coli* (30%) from urine , 11 isolates (14.66%) from stool, 8 isolates (17.77%) from blood (Table 3-3). The results of the present study are in agreement with Akram *et al.,* (2007) who showed that the urinary tract was the most common site of infection by *E. coli* strains. But a disggrement with Ziad and Claude (2011) who reported in their study on urinary tract infections in Lebanese patients that *E. coli* was the most frequent isolate (60.64%) among all isolates throughout the duration of study(ten years). Also differ to those reported by Aiyegoro *et al.,* (2007) who showed that *E. coli* was the most common organism isolated from patients with significant bacteriuria (52.6%).

However, Al-Husseiny (2004) who isolated *E. coli* (40%) from urine of kidney patients and (50%) from stool of colon patients. Hadi (2008) who isolated *E. coli* at percentage (42.6%) from urine samples with significant bacteriuria in Najaf city.

E. coli accounts for more than 90% of the more than 7 million cases of cystitis and 250,000 of pyelonephritis estimated to occur in otherwise healthy individuals every year in the United States (Ryan and Ray, 2004).

Table (3-4): Antibiotics susceptibility test for 49 clinical isolates of *E. coli*

Antibiotics	Concentration (µg)	NO. (%) of Sensitive Isolates	NO. (%) of Intermediate Isolates	No. (%) of Resistant Isolates
Ampicillin	10	0 (0 %)	2 (4.08 %)	47 (95.91)
Amoxicillin +Clavulinic acid	30	4 (8.16)	4 (8.16)	41 (83.67)

Azteronam	30	11 (22.44)	4 (8.16)	34 (69.38)
Nalidixic acid	30	16 (32.65)	7 (14.28)	26 (53.06)
Ciprofloxacin	5	12 (24.48)	16 (32.65)	21 (42.85)
Imipenem	10	42 (85.71)	5 (10.20)	2 (4.08)
Chloramphenicol	30	36 (73.46)	5 (10.20)	8 (16.32)
Cefotaxime	10	0 (0)	0 (0)	49 (100)
Ceftazidime	30	5 (10.20)	6 (12.24)	38 (77.55)
Ceftriaxone	10	3 (6.12)	7 (14.28)	39 (79.59)
Cefoxitin	30	23 (46.93)	9 (18.36)	17 (34.69)
Gentamycin	10	24 (48.97)	4 (8.16)	21 (42.85)
Tobramycin	10	1 (2.04)	1 (2.04)	47 (95.91)
Nitrofurantoin	300	31 (63.26)	12 (24.48)	6 (12.24)

3.4: Antimicrobial susceptibility testing

The results of the present study demonstrated that *E. coli* isolates showed different level of resistance to antibiotics (Table 3-4).They were resistant to cefotaxime with percentage 100%. It was resistant to ampicillin and tobramycin with percentage 95.91 % while amoxicillin/ clavulanic acid and ceftriaxone were 83.67 % and 79.59% respectively. On the other hand *E. coli* isolates were resistant to other antibiotics with different percentage; it was resistant to ceftazidime and aztreonam with percentage 77.55 % and 69.38 % respectively. nalidixic acid 53.06 %, gentamycin 42.85 %, ciprofloxacin 42.85 %, cefoxitin 34.69 %, chloramphenicol 16.32 %, nitrofurantoin 12.24 %, and imipenem 4.08 %. The present result is in agreement with some previous results such as Kargar *et al*., (2009) who noted that *E. coli* was resistance to several

antibiotics like: nalidixic acid (66.67%), gentamicin (44.93%) and chloramphenicol(14.49), Mokracka *et al*., (2012), Oliveira *et al*., (2011), and Rezaee *et al*., (2011) have reported that 0%, 0.5%, and 1.4% of *E. coli* isolates were observed resistance to imipenem . Also our result is similar to other results reported by Allen *et al*., (1999), and Navaneeth *et al*., (2002) have reported increasing resistance in *E. coli* strains to ampicillin other countries, and also to Aiyegor *et al*., (2007) who found that *E. coli* was the principal pathogen isolated from patients with significant bacteriuria, show high resistance to ampicillin. However the current result is similar to other result reported by Al-Hilli (2010) who identified that 75.0% of isolates belonging to *E. coli* isolated from hospital environment were aztreonam resistant.

Morever the current result is close to a study from EsterNepal showed a high prevalence of *E. coli* strains resistance to nalidixic acid, ampicillin, (Srinivasa *et al*., 1999). The high resistance of *E. coli* isolates to ampicillin, may be due to produce β-lactamases enzymes. but its disagreement with Jones *et al*., (2003) reported a resistance to ampicillin 100%, chloramphenicol 84%, gentamicin 79%, and nalidixic acid 68%, Murshed *et al*., (2010) noted resistance rates to, gentamicin, chloramphenicol, , nalidixic acid, and ampicillin were noted as, 54%, 50%, 83%, and 96%, respectively, and with Poovendran *et al*., (2013), who revealed, 95% of *E. coli* isolates resistance to amoxicillin-clavulanic acid. Since clavulanate inhibits the ESBLs, dipping the level of resistance.

However, many studies found the major mechanism of resistance in *E. coli* strains causing clinically significant infection is the expression of β-lactamases of which there are several classes including plasmid

encoded and chromosomally encoded enzymes (Medeiros and Jacoby, 1986; Shlaes *et al*., 1986; Livermore, 1995).

Morewever, the excessive and uncontrolled use of fluoroquinolones in clinical and veterinary medicine led to the increasing resistance of bacteria, which reduces the effectiveness of these drugs for future use (Curtis, 2007).

Research in Split-Dalmatia Country, showed that the rate of resistance in the five year period to ciprofloxacin was increased from 2.48% in 1999 to 7.28% in 2004 (Barišić *et al*., 1999-2004).

According to Al-Hilali (2010), Hadi (2008) and Al-Hilli (2010), demonstrated that all (100%) *E. coli* isolates were susceptible to imipenem. However Al-Hilali (2010) who revealed 4.5% were resistance to gentamycin.

In previous study in Najaf, Hadi, (2008) reported that 42.1%, 36.8% and 55.3% of *E. coli* isolated from patients with significant bacteriuria were resistant to cefotaxime, ceftazidime and ceftriaxone, respectively. However, it might be possible that this high level of resistance to third generation cephalosporins in current study was most probably due to acquisition of β-lactamase, which encode by *bla*-genes possibly during therapy. These antibiotics usually used for treating of urinary tract, respiratory tract and burn wound infections caused by Enterobacteriaceae (Nasser, 2008).

A study accomplished in Najaf by Al-Hilali (2010) who indicated that aztreonam resistance was confirmed in 59.1% of *E. coli*.

The resistance against β-lactamase inhibitors occurs mainly by numerous mechanisms: hyperproduction of β-lactamases, production of β-lactamases resistant to inhibitors, and chromosomal cephalosporinases (Espinasse *et al*., 1997).

Morever Khanal *et al*., (2013) who reported that 66.7% of isolates were resistant to ciprofloxacin. However, Khadgi *et al*., (2013) reported 100% resistance to ciprofloxacin.

Moreover, a high potency of the fluoroquinolones (ciprofloxacin) against uropathogenic *E. coli* was observed by Akram *et al*., (2007) at Aligarh, India.

Bacterial resistance to antibiotics is now widespread and possesses serious clinical threats. The organisms develop resistance to antibiotics by any of the following mechanisms: selection, mutation, phage transduction, and transference. Microbial resistance can be either hereditary in the organism or acquired through the environment (Ibezim, 2005).

The resistance to third generation cephalosporins was caused mainly by mutations in the common group of class A β-lactamases, which consisting of TEM, SHV and CTX-M β-lactamases that has extended hydrolytic spectrum activity on cephalosprins. The high rate resistance to ceftazidime and cefotaxime in this study may be related with production of enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity towards cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby 2004).

Reduced susceptibility to cefoxitin in the *E. coli* may be an indicator of AmpC activity, but cefoxitin resistance may also be mediated by alterations to outer membrane permeability (Tan *et al*., 2009).

nitrofurantoin remains the drug of choice for treatment and management of asymptomatic bacteriuria in pregnant women and symptomatic UTI in general (Onifade *et al*., 2005; Aiyegoro *et al*., 2007). The lack of resistance may be related to the fact that nitrofurantoin has multiple mechanisms of action, requiring organisms to develop more than a single mutation in order to develop resistance. In addition, limited usage of nitrofurantoin for treating uncomplicated cystitis, may also be a contributing factor to the lack of development of widespread resistance to this drug (Ladhani and Gransden, 2003; Gupta, 2003).

Table (3-5): Numbers and percentage of resistance type in 49 clinical isolates of *E. coli* according to site of infection.

3.4.1: Multidrug resistance (MDR), extensive drug resistance (XDR), and pandrug resistance (PDR) of *E. coli* isolates

The results of the current study reported that 39 isolates (79.59%) of *E. coli* were MDR (bacterial isolate can resist to a minimum at least 3 different classes of antibiotics). Also the result shows that out of 49 isolates of *E. coli* there were 9 isolates (isolate No 1, 3 isolated from blood and isolate No 12, 13, 26, 27, 30 isolated from urine and isolate No 19, 22 isolated from stool, with percentage (18.36%) as XDR

Type of resistance	Urine Total Isolates:	Stool Total isolates:	Blood Total isolates:	Total (49)
MDR	25 (51.02%)	8 (16.32%)	6 (12.24%)	39 (79.59%)
XDR	5 (10.20%)	2 (4.08%)	2 (4.08%)	9 (18.36%)
PDR	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ESBL	0 (0%)	1 (2.04%)	0 (0%)	1 (2.04%)

(bacterial isolate remain susceptible to only one or two class of antibiotics).On the other hand the result demonstrated that there was no isolate with percentage (0%) was PDR(bacterial isolate resistance to all sub classes in all classes of antibiotics), (Table 3-5). The present study is an agreement with some local study such as Hadi (2008) also reported that 82.6% of *E. coli* isolates were MDR in Najaf, In Tabriz, northern west of Iran, 84.2% of tested strains were observed as MDR(Rezaee *et al*., 2011). But it differed with that obtained by other local studies such as

Al-Mohana (2004) found that 56.8% of clinical *E. coli* isolates in Najaf were resistant to more than five antimicrobial agents, while Hadi (2008) reported that all 60(100%) of the β-lactam resistant *E. coli* isolates obtained from patients with significant bacteriurea were resistant to at least five antimicrobial agents in Najaf, and MuKherjee *et al*., (2013) revealed, MDR strains was detected in 92.5% of isolates obtained from hospitalized patients in Kolkata, India.

In the another study byAl-Hilli (2010) revealed that all *E. coli* isolate obtained from Merjan Teaching Hospital in Hilla, Iraq, were considered as MDR. The level of MDR amongst the UTI isolates was found to vary from country to country.

According to Ibrahim *et al*., (2012) who founded that a high incidence of MDR *E. coli* in Sudan is (92.2%).

In the another study by D'Agata, (2004) defined multi-drug resistance as resistance to 3 or more antimicrobial classes. He has reported that, multi-drug resistant for all gram negative bacilli increased from 1% to 16% and 0.2% to 4% for *E. coli*.

According to Livermore, (2007) revealed that, the continuous used was observed in Najaf of even a single antibiotic over a period of weeks or months will select bacteria resistant to a different type of antibiotics in addition to the one in use.

Previous studies have indicated lower percentages of MDR compared to this study (Baquero *et al*., 2008; Figueira *et al*., 2011; Ham *et al*., 2012).

However, One study described bacteraemia due to MDR gram-negative bacilli in cancer patients and found the incidence to be 13.7% and out of these 49% episodes were due to MDR *E.coli* (Gudiol *et al*., 2011).

Morever, MDR rates vary from one country to another. Over all Gram-negative isolates from Latin American countries revealed the lowest susceptibility rates to all antibiotics followed by Asian-Pacific isolates and European strains. Strains from Canada exhibit the best global susceptibility testing results (Gales *et al*., 2001). Widespread use of antimicrobial therapy has often been held responsible for co-resistance to four or more unrelated families of antibiotics and the occurrence of multiply resistant *E. coli* strains in hospitals (Maynard *et al*., 2003). A strain of *E. coli* considered as an MDR if it was resistant to at least three classes of antibiotic (Magiorakos *et al.*, 2012).

3.5: Phenotyphc detection of extended spectrum β-lactamases (ESBL)

3.5.1: Confirmatory test for extended spectrum beta lactamase (ESBL)

This test was carried out according to Double Disk Synergy Test (DDST).The results demonstrated that 1 isolate (2.04%) were ESBL producers (Table 3-5).The result of these study is aggrement with some study such as data from the European Antimicrobial Resistance Surveillance System (EARSS), 2.6% of *E. coli* and 1.7% of *K. pneumonia* strains in Sweden were resistant to third-generation cephalosporins in 2010 (EARSS, 2011), In more than 13000 isolates of *E. coli*, the percentages expressing the ESBL phenotype were as follow: in Latin America, 8.5%; in the Western Pacific, 7.9%; in Europe, 5.3%; in the

United States 3.3%, and in Canada, 4.2% (Winokr *et al*., 2001), and In a large study performed in 2001, it was demonstrated that about 5.3% of *E. coli* in the United States harbored ESBLs (Winokur *et al*., 2001). Also, the present result is similar to previous study conducted in 2009 showed that 9% of *E. coli* isolates in Texas were ESBL producers (Bhusal *et al*., 2011). This lower prevalence of ESBL may be due to the selection of only tracheal isolates from ICU(Khanal *et al*., 2013). But a dissageement with some previous studies such as in the United States, ESBL production was detected in 57.69% *E. coli* isolates tested by the Clinical Microbiology Laboratory in Rochester (Robberts *et al*., 2009), Tankhiwalae *et al*, (2004) reported 48.3% and Aggarwal., (2009) reported 40 % ESBL producers *E. coli* isolated from the urine at Rohtak.

In Saudi Arabia, although the exact prevalence of ESBL is currently unknown, the PEARLS study (2001- 2002) reported that the overall ESBL production rate from Enterobacteriaceae was 18.6% (Kader *et al*., 2004).

In the another study, 35.2% of *E. coli* and *K. pneumoniae* isolates obtained from nosocomial blood stream infections were ESBL producer (Superti *et al*., 2009).

However, in a survey of nosocomial isolates of Enterobacteriaceae collected from different wards in Pakistan, 28.57% of *E. coli* isolates were ESBL producers (Ali Shah, 2003).

The prevalence of ESBLs in India has now reached epidemic proportions, ranging from 62% to 100% in *E. coli* as observed in the 10 Indian medical centres sentry study (Mathai *et al*, 2009). Overall the ESBLs rate in the India sentry surveillance was 84%. This higher

prevalence of ESBL may be because of the selection of various types of clinical samples from the ICU patients (Altun *et al*., 2013).

In previous Study from Lucknow, Delhi and Nagpur have reported that there is an increased percentage of ESBL positive isolates (Hawser, 2009).

In Turkey, several studies have shown that ESBL was found in high ratio in clinical isolates in Enterobacteriaceae (Tasli and Bahar, 2005).

However, the prevalence of ESBLs among clinical isolates varies from country to another country and from institution to another (Bradford, 2001).

In study for clinical isolates of ESBL-producing bacteria carried out in Najaf, the prevalence of ESBL-producing *E. coli* isolates was 18.3% (Hadi, 2008).

Other studies reported that a high endemic of ESBL- producing Enterobacteriaceae have been determined in Latin American countries especially in Brazil (Marry *et al*., 2006; Martins *et al*., 2006).

According to Fatemeh *et al*., (2012) found that 26.5% of *E. coli* and 43% of *K. pneumonia* were ESBL positive in their study conducted at the Imam Reza hospital of Mashhad, Iran.

In the another study by Majda *et al*., (2013) reported that 72% of *E. coli* and 65.8% of *K. pneumoniae* were ESBL producers at the Microbiology laboratory of Shalamar Medical College, Lahor.

According to a study conducted at the National Public Health Laboratory (NPHL), Kathmandu, Nepal reported that 31.57% of *E.*

coli were confirmed as Extended Spectrum β-lactamase producers, these isolates further exhibited co-resistance to several antibiotics (Thakur *et al*., 2013).

Although strains that produce ESBL are characteristically resistant to new cephalosporins and/or aztreonam, many strains producing these enzymes appear susceptible or intermediate to some or all of these agentsin vitro, while expressing clinically significant resistance in infected patients (Paterson and Bonomo, 2005).

Table (3-6): Prevalence of extended spectrum beta lactamases genes of *E. coli* according to infections site (n=49)

Genes	Urine Total Isolates (n=30)	Stool Total Isolates (n=11)	Blood Total Isolates (n=8)	Total (49)
bla_{CTX-M}	29 (96.66%)	9 (81.81%)	7 (87.5%)	45 (91.83 %)
bla_{OXA}	29 (96.66%)	9 (81.81%)	7 (87.5%)	45 (91.83 %)
bla_{TEM}	30 (100%)	11 (100%)	8 (100%)	49 (100%)

3.6: Molecular detection of ESBL genes by PCR

Results in table (3-6) showed that bla_{TEM} was the most prevalent gene in the tested isolates at rate (100%) followed by bla_{CTX-M} and bla_{OXA} genes (91.83%) for each.This result is an agreement with Kiratisin, (2007) who reported highest prevalence of bla_{CTX-M} among several *bla* genes in

all isolates which was 99.6%. Also the current result was similar to some previous result such as some studies in which the majority (over two thirds) of the ESBL producing Enterobacteriaceae reported was *E. coli* and most of them (≥ 85%) expressed CTX-M ESBL enzymes (Coque *et al*., 2008; Rossolini *et al*., 2008), In recent study, out of ESBL producing *E. coli,* most of them expressed a CTX-M enzyme (6/7) with a predominance of CTX- M-15 (6/6). The most frequently encountered mechanism of resistance to β-lactams found in their study was the production of CTX-M type β- lactamase as reported by Fatana *et al*., (2011). But disagreement with several studies such as Al-Hilli (2010) who found that 50% of the phenotypic ESBL-producing *E. coli* isolates had CTX-M type in Hilla, In Najaf, Al-Muhannak (2010) reported that 17 (27.4%) of the 62 potential ESBL-producing clinical enteric isolates carried gene of bla_{OXA}, and Hadi (2008) who found that 12 isolates (57.1%) of 21 *E. coli* harbored bla_{TEM} gene.

In other study, Al-Hilali (2010) reported that the PCR assay revealed that, 17 (77.3%) of the 22 *E. coli* isolates obtained from children with diarrhea in Najaf carried bla_{CTX-M}.

According to Al-Hilli (2010), who found that 50% of the phenotypic ESBL-producing *E. coli* isolates had CTX-M type.

In the Middle East area, reports from Lebanon and Kuwait pointed out that CTX-M is predominant ESBL in Enterobacteriaceae (Moubareck *et al.,* 2005; Poirel *et al.,* 2005a).

In the another study by Al Muhanak (2010) who revealed that CTX-M β-lactamase was the most prevalent (38.7%) among the ESBL

producing Enterobacteriacea isolates; while, TEM and OXA β-lactamases were less (27.4% for each). Morever other studies like yan *et al*., (2000) in Taiwan and Goyle *et al*., (2009) in India, who reported that CTX-M β-lactamases were the commonest enzymes than TEM in clinical Grame negative isolates.

In a related study from Brazil, bla_{CTX-M} were detected in 33.9% of ESBL-producing Enterobacteriaceae isolated from inpatients and outpatients at a public teaching hospital (Dropa *et al*., 2009). However in 2006, CTX-M enzymes were detected in 70% of the isolates in the University Health System in San Antonio, the United States (Lewis *et al*., 2007).

In other study reported by Rodriguez *et al*., (2006) who found that 70% of the ESBL producing *E. coli* obtained from bacteremia cases in Spain had CTX-M types. Morever In Korea, Park *et al*., (2009) found that PCR experiments detected the bla_{CTX-M} genes in 84.7% of *E. coli* isolates with an ESBL phenotype and they concluded that CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates.

In the Middle East area, reports from Lebanon and Kuwait point out that CTX-M is the predominant ESBL in *E. coli* (Moubareck *et al*., 2005; Poirel *et al*., 2005). The CTX-M type β-lactamases constitute a novel group of enzymes that have a typical ESBL resistance phenotype, are capable of hydrolyzing broad-spectrum cephalosporins and are inhibited by clavulanic acid. They also confer a high level of activity against ceftazidime (Tzouvelekis *et al*., 2000).

Ellem *et al*., (2011) in their study have reported that majority of ESBL producers were *E. coli* isolates, which carried 64.3% of bla_{CTX-M}-1genes.

Other local study such as AL-Muhannak (2010) who reported that bla_{CTX-M} genes were detected in 8 (40%) out of 20 *E. coli* isolates. Hawkey (2008) reported that all ESBL producing *E. coli* isolates in his study harboured bla_{CTX-M}. In most countries, there are mixtures of CTX-M ESBL types, with ESBL isolates from India being completely dominated by the presence of CTX-M-15 (Hawkey, 2008; Mathai *et al*., 2009). AL-Hilli (2010) who reported that 5/10 (50%) of *E. coli* isolates gave PCR product with CTX-M specific primers.

The elevated rate of CTX-M β-lactamases in *E. coli* isolates suggest that the horizontal transfer of bla_{CTX-M} genes, mediated by plasmid and/ or mobile genetic element, contributes to ease with which these enzymes are spreading in *E. coli* isolates and the dissemination of CTX-M enzymes. Morever, in hospital environment, plasmid could be transferred easily between patients through the health care workers due to hand carriage and antimicrobial selection pressure (AL-Hilli, 2010).

CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates compared with SHV and TEM enzyms (Yongjung *et al*., 2009). Since then, an increase in the CTX-M β-lactamases has been seen in many countries in Europe and Asia (Livermore, 2007). The bla_{CTX-M} was reported the most prevalent *bla*-gene in Korea (Kiratisin *et al*., 2008).

In particular, the CTX-M family of ESBLs appears to be associated with community-acquired infections (Rodriguez *et al.,* 2006; Al-Hilali,

2010).However CTX-M is the predominant ESBL in *E. coli* (Moubareck *et al.,* 2005; Poirel *et al.,* 2005).

The main reason for the prevalence of CTX-M β-lactamases in Najaf may be the wide spread use of certain third generation cephalosporins. Antibiotic selective pressure probably contributes to the increasing prevalence of cefotaxime and ceftriaxone hydrolyzing CTX-M β-lactamases in clinical setting (Wei *et al*., 2005).

Currently, there are more than 90 gene sequences designated as *bla* $_{CTX-M}$ in the National Center for Biotechnology Information GenBank database. Based on percent sequence similarities, bla_{CTX-M} genes can be clustered into five different groups based on their amino acid sequence (Bush and Jacoby, 2010). Group I includes CTX-M 1, 3, 10 to 12, 15 (UOE-1), 22, 23, 28, 29, and 30. Group II includes CTX-M 2, 4 to 7, and20 and Toho-1.Group III includes CTX-M 8. Group IV includes CTX-M 9, 13, 14, 16 to 19, 21, and 27 and Toho-2. Finally group V includes CTX-M 25 and 26. The members of these groups exhibit > 94% amino acid identity within the group and ≤90% amino acid identity between groups (Bonnet, 2004).

TEM-type ESBLs are the first plasmid-mediated β-lactamasethat is often found in genera of Enterobacteriaceae (Mulvey *et al*., 2004).

However, the transfer of bla_{TEM} gene on mobile plasmid led to spread rapidly to members of the same species or organisms of different genera (Lartigue *et al*., 2007).

Several studies such as Shahcheraghi *et al.*, (2009) in Iran found that the frequency of bla_{TEM} gene among the ESBL Gram-negative

isolates were 9%, In Thailand, pongpech *et al.* (2008) found that *bla*$_{TEM}$ gene was present in 78% of the confirmed ESBL producers, and In Portugal, *bla*$_{TEM}$ gene was identified in 101 (85%) (Mendonca *et al*., 2007). A study conducted by Bradford (2001) reported that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. This enzyme has the ability to hydrolyze penicillin's and early cephalosporins.

Additionally, Al-Hilali (2010) reported that the PCR assay revealed 18/22 (81.8%) of *bla*$_{TEM}$ gene positive enteropathogenic *E. coli* recovered from children with diarrhea in Najaf.

Hadi (2008) found that, 14 (66.6%) out of 21 *E. coli* isolates were able to yield amplification products with TEM-PCR specific primers in study conducted in Najaf.

another local study in Hilla, AL-Asady (2009) reported that only 1 (20%) out of 5 *E. coli* isolates was possess *bla*$_{TEM}$ genes . AL-Muhannak (2010) in Najaf show that 5 (25%) of *E. coli* can revealed show *bla*$_{TEM}$ gene.

In previous study originated from geographically diverse locations, suggest that local antibiotic usage and practices may play an active role in promoting the selection of point mutations in *bla*$_{TEM}$-type genes (Huseyin and Bahar, 2005; Tomanicek *et al.,* 2010).

On the other hand, TEM-1, which is responsible for most of the ampicillin resistance in; 94% of *E. coli* strains isolated in Spain, 89% of *E. coli* strains isolated in Hong Kong, and in 78% of *E. coli* strains isolated in London (Livermore, 1995). However, Bradford (2001) reported that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-

1.TEM-2 β-lactamase is widespread in *E. coli*, although they are rare than TEM-1.The classical TEM-1 and TEM-2 enzyemes have manimal activity against newer cephalosporins (Sirot, 1995).

The OXA enzymes are regarded as OXA-type ESBLs and have been discovered mainly in *P. aeruginosa* in specimens from Turkey and France (Harada *et al.,* 2008). These β-lactamases are classified into class D in the Ambler scheme and were placed in group 2d in the Bush-Jacoby-Medeiros functional scheme (Walther-Rasmussen and Hoiby, 2006). Some OXA-type β-lactamases have carbapenemase activity, augmented in clinical isolates by additional resistance mechanisms, such as impermeability or efflux (Jacoby and Munoz-Price, 2005).

In a study done in Thailand, bla-genes encoding OXA were found in 8.1% of ESBL-producing *E. coli* isolates (Kiratisin *et al.,* 2008). However, Al-Hilali (2010) noticed that only 1 (4.5%) of enteropathogenic *E. coli* isolates recovered from children with diarrhea in Najaf was harbored bla_{OXA} gene.

Likewise, Karami and Hannoun (2008) reported that out of 32 ampicillin resistant *E. coli* isolates in Sweden, only one isolate (3%) harboured the bla_{OXA}.

However, in 2008, Bhattacharjee *et al.* reported that of the 361 ESBL-positive enterobacterial isolates from different clinical specimens in India, only one isolate harbored the OXA-10. Whereas in a study done in New York, Jones *et al*., (2009) found that over 40% of the Gram-negative clinical isolates collected carried genes encoding an OXA-type β-lactamase.

In a study done in Tailand, *bla*-genes encoding OXA were found in 8.1% of ESBL producing *E .coli* isolates (Kiratisin *et al*., 2008). Lower rate also recorded in Spain (3.8%) (Tenover *et al*., 2003), Germany (15%) (Svard, 2007) and Malaysia (5%) (Lim *et al*., 2009). Morever Lower prevalence has been reported in Sweden, only one isolates (3%) harboured the bla_{OXA} out of 32 ampicillin resistance *E. coli* isolates (Karami and Hannoun, 2008).

Another study also detected high prevalence of bla_{OXA} in Slovenia (41%) (Istinic, 2008) and in USA (40%) (Jones *et al*., 2009). AL-Hilali (2010) revealed that, only one isolates (4.54%) of *E. coli* carried bla_{OXA} in Najaf.

In a previous study by AL-Muhannak (2010) who revealed that the OXA was detected in 5 (25%) of *E. coli* isolates. However, AL-Hilli (2010) found that all *E. coli* isolates from Merjan teaching hospital in Hilla city were negative in OXA-PCR.

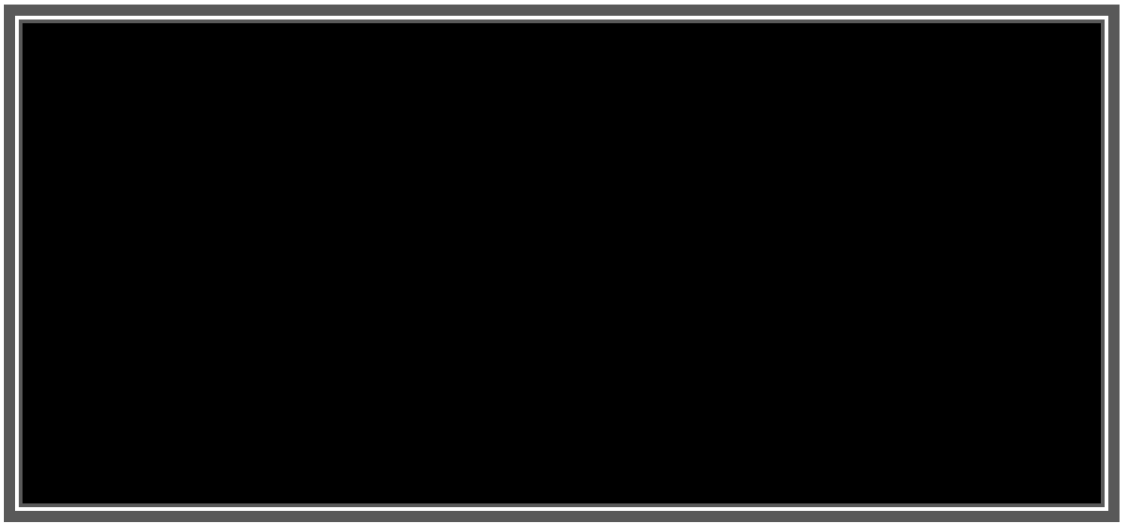

Figure (3-2): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 47 isolates) amplified with diagnostic bla_{CTX-M} gene, show positive results at 550 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (50 bp ladder).

Figure (3-3): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 48 isolates) amplified with diagnostic *bla-*$_{OXA}$ gene, show positive results at 619 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (50bp ladder).

Figure (3-4): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 44 isolates) amplified with diagnostic *bla-*$_{TEM}$ gene, show positive results at 822 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

Table (3-7): Prevalence of plasmid mediated quinolones resistance genes of *E. coli* according to infection site (n=28)

Genes	Urine Total Isolates n=16	Stool Total Isolates n=5	Blood Total Isolates n=7	Total (28)
qnrA	16 (100%)	5 (100%)	7 (100%)	28 (100%)
qnrB	16 (100%)	5 (100%)	7 (100%)	28 (100%)
qnrS	16 (100%)	5 (100%)	7 (100%)	28 (100%)

3.7: Molecular detection of plasmid mediated quinolones resistance in *E. coli* isolates (PMQR)

Results show in table (3-7) that *qnrA*, *qnrB*, and *qnrS* genes in the test isolates have the same percentage (100%). Present study showed that the spreading of PMQR genes among clinical isolates of *E. coli* in Najaf is high and that it is much higher than that reported in other areas, such as the United States (Robicsek *et al.,* 2006d), Taiwan (Wu *et al.,* 2007) and China (Yang *et al*., 2008b).The current result is disagrrement with some previous study such as Low prevalence of *qnr* (A,B,S) genes has also been reported from Seoul, Korea where the prevalence of *qnr* (A,B,S) genes was less than 1% of *E. coli* isolates from a collection of clinical isolates (Minggui *et al*., 2003), In China, Wang *et al.*, (2008a) reported that the prevalence rates of *qnr* among the clinical isolates of ciprofloxacin resistance in *E. coli* and *K. pneumoniae* were 7.5% (11 of

146) and 11.9% (8 of 67), respectively, and In France, the prevalence of *qnr*(A,B,S) genes was 1.6% (2/125) *E. coli* and *Klebsiella* spp. isolates (Poirel *et al*., 2006; and Cattoir *et al*., 2007a).

In the other study, An investigation on 47 ceftazidime-resistant *E. coli* isolates from humans showed that *qnrA* and *qnrB* were both detected in 1 (2%) during the study, no isolates were shown to carry *qnrS*, and none had both *qnrA* and *qnrB* (Robicsek *et al*., 2006b).

Previous studies suggested that *qnr* genes have rarely been found in clinical strains from other areas of the world (Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2004; Mammeri *et al*., 2005; Jacob *et al.,* 2009). However After evaluating a total of 91 *E. coli* isolates from the United States, Jacoby *et al.*, (2003) could identify only one isolate harboring the *qnr* gene. Karah, (2008) found that the *qnr* genes were 1.6% (8/487) in the *E. coli* and *Klebsiella* spp clinical isolates collected from Norway and Sweden.

According to Crémet *et al.* (2009) found that PMQR determinants detected in 13 isolates (27.7%) were either *qnr* gene or *aac(6´)-Ib-cr* gene identified alone or in combination

Other in Canada only about 1% (5/550) of ciprofloxacin and/or tobramycin resistant *E. coli* and *Klebsiella* spp isolates were *qnr*-positive (Pitout *et al*., 2008).

However, studies have shown that plasmid-mediated resistance mechanisms also play a significant role in fluoroquinolone resistance, and its prevalence is increasing worldwide (Robicsek *et al.,* 2006b).

Additionally, moderate prevalence has also been detected in other parts of the world such as Spain (5%) (Lavilla *et al.,* 2008), China (8%) (Jiang *et al.,* 2008), and the United States (15%) (Robicsek *et al*., 2006d).

qnrA is the PMQR gene encoding a 218 amino acid protein of the pentapeptide family that protects DNA gyrase from quinolone inhibition (Tran *et al.,* 2005). Several studies showed a worldwide dissemination of *qnrA* determinants among enterobacterial isolates (Cheung *et al.,* 2005; Mammeri *et al.,* 2005). It has been suggested that the *qnrA* gene may contribute to the increase in quinolone resistance in human *E. coli* (Wang *et al.,* 2003).

Accordiong to Jeong *et al*., (2005) reported that the prevalence of *qnrA* in Korea was 0.8% in *E coli* isolates (ciprofloxacin susceptible and resistant) between 2001 and 2003.

Although the *qnrA* gene seem to be uncommon among human clinical isolates of *E. coli* from the United States and Europe (Nordmann and Poirel, 2005; Robicsek *et al.,* 2006d) the gene is prevalent in fluoroquinolone-resistant *E. coli* from Asia (Wang *et al.,* 2003).

qnrA gene was highly detected in a selected collection of blood culture isolates of Enterobacteriaceae resistant to both ciprofloxacin and cefotaxime in Liverpool (Corkill *et al*., 2005).

However, *qnrA* was recently detected in an *E. coli* clinical isolates from Denmark (Cavaco *et al*., 2007). Furthermore, *qnrA* was the most prevalent *qnr* gene in a most recent Spanish study (Lavilla *et al.*, 2008)

where *qnrA* was detected in 14 of 305 ESBL-producing enterobacterial isolates, whereas only one *qnrS* and no *qnrB* were detected.

A novel PMQR gene, *qnrB*, has been discovered in a plasmid encoding the CTX-M-15 β-lactamase from a *K. pneumoniae* strain isolated in South India. It has less than 40% amino acid identity with the original *qnr* (now *qnrA*) gene or with the recently described *qnrS* but, like them, codes for a protein belonging to the pentapeptide repeat family. Strains with demonstrated low-level resistance to all quinolones tested (George *et al.,* 2006). However, no previous nationwide survey has evaluated clinical isolates of *E. coli* with resistance to quinolones in Iraq for the presence of *qnrB*. Therefore, the present study screened the 28 quinolone resistance *E. coli* isolates for the presence of the *qnrB* gene by using previously described primers and amplification conditions (George *et al*., 2003; Park *et al*., 2006; Thi *et al*., 2007; Koichiro *et al.*, 2009).

In general, present study revealed that, the *qnrB* gene seems to be common in quinolone resistance clinical *E. coli* isolates and may contribute to the alarming rates of quinolone resistance. However, present study indicates that *E. coli* may be representing the greatest reservoir and source of dissemination of plasmid-mediated antimicrobial resistance genes, such as *qnrB*, in Najaf hospitals.

In Korea, Shin *et al*., (2008). reported that 5.6% of *E coli* ciprofloxacin-resistant isolate only *qnrB* (*qnrB*2, *qnrB*4 and/or *qnrB*6).

In previous study by Karah (2008) who found that only 0.7% (3/422) of nalidixic acid-resistant *E. coli* isolates was *qnr*-positive in Norway and Sweden.

In Kuwait, out of 64 Enterobacteriaceae isolates, only 3 (4.7%) isolates were positive for a *qnrB*-like gene (Cattoir *et al.,* 2007b). Morever, In Japan, Ashraf *et al.* (2007) previously identified six *qnrB* genes in *E. coli*, *K. pneumoniae, K. oxytoca, C. freundii, P. mirabilis* and *P. fluorescence* from zoo reptiles and falcons.

Present study revealed that, this is the first detection of a *qnrS* gene in Najaf hospitals, while in other countries such as Spain, France and Korea, other authors have already described *qnrS* genes (Poirel *et al*., 2006; Park *et al*. 2007; Lavilla *et al.,* 2008).

However, present study suggested that the *qnrS* gene was common among quinolones and fluoroquinolones resistance *E. coli* clinical isolates, but its identification might indicate the emergence of this mechanism of quinolone resistance in Najaf hospitals.

In the other local stydy in Morocco, the *qnrS* gene was found in only one (6.2 %) *E. coli* isolate (Bouchakour *et al.,* 2010).

According to Kim *et al*., (2009) determined that 0.5% of *E coli* and 5.9% of *K pneumoniae* (ciprofloxacin susceptible and resistant) isolates in Korea contained *qnr* (*qnrB or qnrS*).

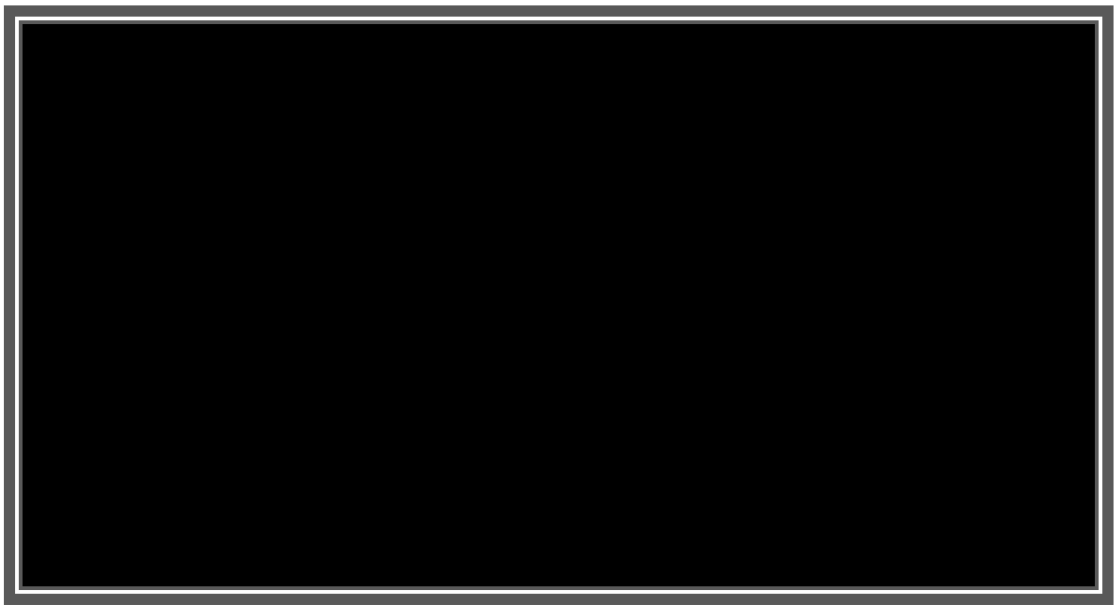

Figure (3-5): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 26 isolates) amplified with diagnostic *qnrA* gene, show positive results at 625 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (50bp ladder).

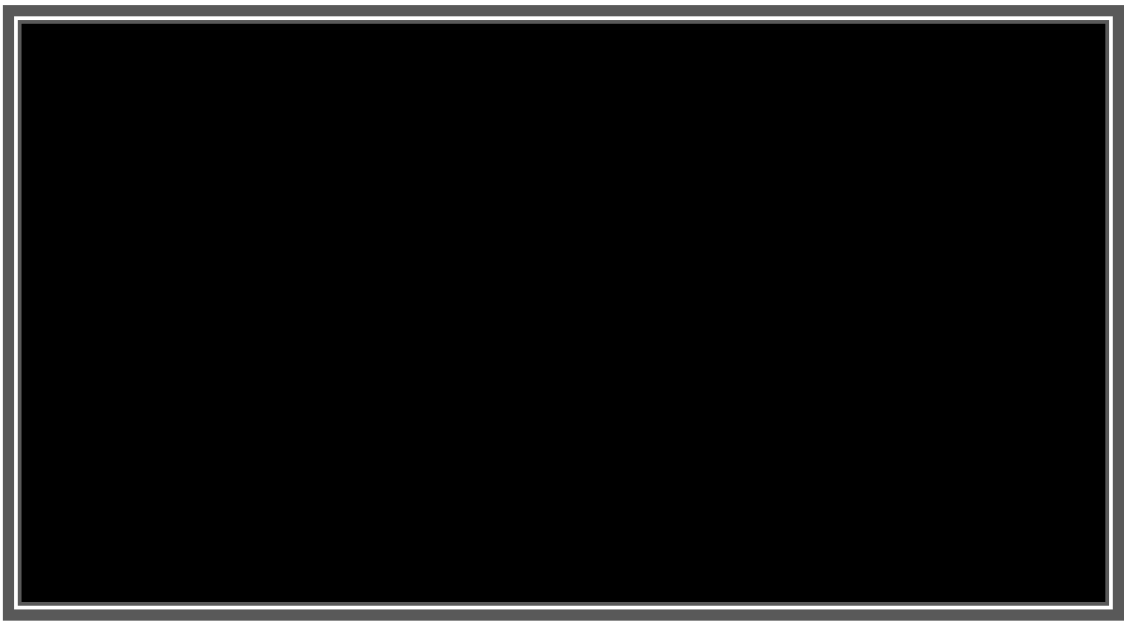

Figure (3-6): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 28 isolates) amplified with diagnostic *qnrB* gene, show positive results at 468bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

Figure (3-7): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 28 isolates) amplified with diagnostic *qnrS* gene, show positive results at 417bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

3. Results and Discussion

3.1: Total specimens

This study was carried out at Al-Hamza Hospital in Al-Diwaniya City and Al-Sadder Medical City in Al-Najaf Governorate-Iraq. A total of 400 specimens from different clinical sources (urine, stool, and blood) were collected.

Out of the 400 total specimens there were 220 specimens (55%) were diagnosed as gram negative bacteria, while there were150 specimens (37.5%) were diagnosed as gram positive bacteria and 30 specimens (7.5%) with no any growth (Table 3-1).

Table (3-1): Numbers and percentage of total specimens. (N=400).

Bacterial growth	No.	Percentage %
Gram negative	220	55
Gram positive	150	37.5
No growth	30	7.5
Total	400	100

Table (3-2): Morphological and biochemical tests for *E. coli* isolates.

Test	Result
Gram stain	Gram negative Cocoobacilli
Growth on MacConky agar	Pink and small colony
Lactose fermenter	+
Oxidase	-
Motility	+
Indole production	+
Methyl red	+

Voges-Proskauer	-
Simmons citrate	-
Urease	-
Triple Sugar Iron Agar	Acid / Acid with Gas
Growth on chrome agar	Metallic blue colonies

3.2: Identification of bacterial isolates

3.2.1: Morphological and biochemical test for *E. coli*

The positive results for cultural, microscopically and biochemical characteristics of *E. coli* were 49 isolates only, (Table 3-2). Primary identification of *E. coli* isolates was based on cultural, morphological characteristics, microscopically diagnosis and biochemical tests , while the confirmatory identification test was based on selective Chrome agar medium (Figure 3-1) and according to Vitek®2 system. Bacterial colonies developed on plate agar, were studied; on MacConkey agar Small, round, entire, opaque, flat, non viscous, rose pink colour, lactose fermenter colonies (lactose fermenter) colonies , on chrome agar the colonies were metallic blue, while on blood agar *E. coli* were rounded, smooth, convex and beta hemolytic colonies, on EMB agar Black, Dark colour colonies with green Metallic sheen. Microscopic examination showed that the isolates were gram negative Coccobacilli.

3.2.2: Identification of *E. coli* isolates according to Vitek®2 system

Identification of all 49 suspected isolates of *E, coli* was confirmed by Vitek®2-automated system. The results demonstrated that there

were full similarity between biochemical tests results, chrome agar and Vitek2 system results.

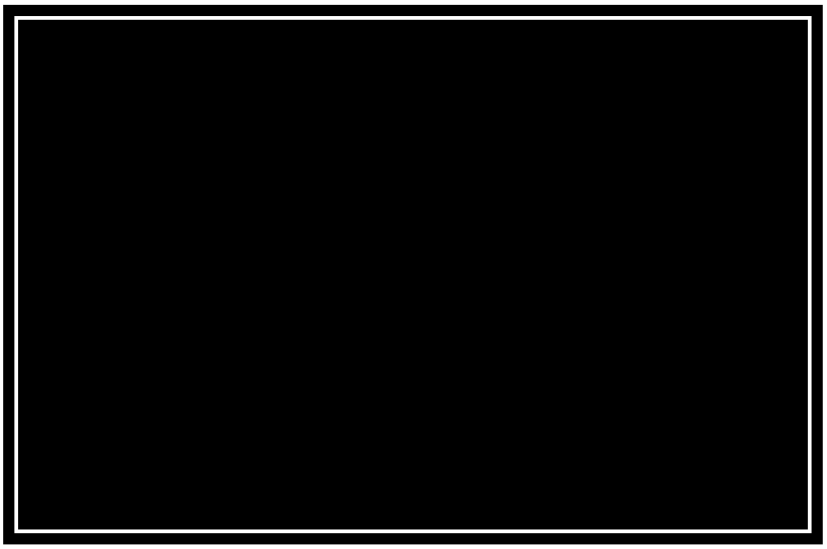

Figure (3-1): *E. coli* showing metallic blue colonies on chrome agar

Table (3-3): Numbers and percentage of *E. coli* isolates according to total gram negative bacterial isolates and sites of infection. (N=220).

Site of infection	Numbers of Gram negative bacteria	*E. coli*	Percentage (100%)
Urine	100	30	30
Stool	75	11	14.66
Blood	45	8	17.77
Total	220	49	22.27

3.3: Numbers and percentage of *E. coli* isolates according to total gram negative bacterial isolates and site of infection

A total of 220 gram negative bacterial isolates were collected from different clinical sources (100 from urine, 75 from stool, 45from blood) there were 30 isolate of *E. coli* (30%) from urine , 11 isolates (14.66%) from stool, 8 isolates (17.77%) from blood (Table 3-3). The results of the present study are in agreement with Akram *et al*., (2007) who showed that the urinary tract was the most common site of infection by *E. coli* strains. But a disggrement with Ziad and Claude (2011) who reported in their study on urinary tract infections in Lebanese patients that *E. coli* was the most frequent isolate (60.64%) among all isolates throughout the duration of study(ten years). Also differ to those reported by Aiyegoro *et al*., (2007) who showed that *E. coli* was the most common organism isolated from patients with significant bacteriuria (52.6%).

However, Al-Husseiny (2004) who isolated *E. coli* (40%) from urine of kidney patients and (50%) from stool of colon patients. Hadi (2008) who isolated *E. coli* at percentage (42.6%) from urine samples with significant bacteriuria in Najaf city.

E. coli accounts for more than 90% of the more than 7 million cases of cystitis and 250,000 of pyelonephritis estimated to occur in otherwise healthy individuals every year in the United States (Ryan and Ray, 2004).

Table (3-4): Antibiotics susceptibility test for 49 clinical isolates of *E. coli*

Antibiotics	Concentration (µg)	NO. (%) of Sensitive Isolates	NO. (%) of Intermediate Isolates	No. (%) of Resistant Isolates
Ampicillin	10	0 (0 %)	2 (4.08 %)	47 (95.91)
Amoxicillin +Clavulinic acid	30	4 (8.16)	4 (8.16)	41 (83.67)
Azteronam	30	11 (22.44)	4 (8.16)	34 (69.38)
Nalidixic acid	30	16 (32.65)	7 (14.28)	26 (53.06)
Ciprofloxacin	5	12 (24.48)	16 (32.65)	21 (42.85)
Imipenem	10	42 (85.71)	5 (10.20)	2 (4.08)
Chloramphenicol	30	36 (73.46)	5 (10.20)	8 (16.32)
Cefotaxime	10	0 (0)	0 (0)	49 (100)
Ceftazidime	30	5 (10.20)	6 (12.24)	38 (77.55)
Ceftriaxone	10	3 (6.12)	7 (14.28)	39 (79.59)
Cefoxitin	30	23 (46.93)	9 (18.36)	17 (34.69)
Gentamycin	10	24 (48.97)	4 (8.16)	21 (42.85)
Tobramycin	10	1 (2.04)	1 (2.04)	47 (95.91)
Nitrofurantoin	300	31 (63.26)	12 (24.48)	6 (12.24)

3.4: Antimicrobial susceptibility testing

The results of the present study demonstrated that *E. coli* isolates showed different level of resistance to antibiotics (Table 3-4).They were resistant to cefotaxime with percentage 100%. It was resistant to ampicillin and tobramycin with percentage 95.91 % while amoxicillin/ clavulanic acid and ceftriaxone were 83.67 % and 79.59% respectively. On the other hand *E. coli* isolates were resistant to other antibiotics with different percentage; it was resistant to ceftazidime and aztreonam with percentage 77.55 % and 69.38 % respectively. nalidixic acid 53.06 %, gentamycin 42.85 %, ciprofloxacin 42.85 %, cefoxitin 34.69 %, chloramphenicol 16.32 %, nitrofurantoin 12.24 %, and imipenem 4.08 %. The present result is in agreement with some previous results such as Kargar *et al*., (2009) who noted that *E. coli* was resistance to several antibiotics like: nalidixic acid (66.67%), gentamicin (44.93%) and chloramphenicol(14.49), Mokracka *et al*., (2012), Oliveira *et al*., (2011), and Rezaee *et al*., (2011) have reported that 0%, 0.5%, and 1.4% of *E. coli* isolates were observed resistance to imipenem . Also our result is similar to other results reported by Allen *et al*., (1999), and Navaneeth *et al*., (2002) have reported increasing resistance in *E. coli* strains to ampicillin other countries, and also to Aiyegor *et al*., (2007) who found that *E. coli* was the principal pathogen isolated from patients with significant bacteriuria, show high resistance to ampicillin. However the current result is similar to other result reported by Al-Hilli (2010) who identified that 75.0% of isolates belonging to *E. coli* isolated from hospital environment were aztreonam resistant.

Morever the current result is close to a study from EsterNepal showed a high prevalence of *E. coli* strains resistance to nalidixic acid, ampicillin, (Srinivasa *et al*., 1999). The high resistance of *E. coli* isolates to ampicillin, may be due to produce β-lactamases enzymes. but its disagreement with Jones *et al*., (2003) reported a resistance to ampicillin 100%, chloramphenicol 84%, gentamicin 79%, and nalidixic acid 68%, Murshed *et al*., (2010) noted resistance rates to, gentamicin, chloramphenicol, , nalidixic acid, and ampicillin were noted as, 54%, 50%, 83%, and 96%, respectively, and with Poovendran *et al*., (2013), who revealed, 95% of *E. coli* isolates resistance to amoxicillin-clavulanic acid. Since clavulanate inhibits the ESBLs, dipping the level of resistance.

However, many studies found the major mechanism of resistance in *E. coli* strains causing clinically significant infection is the expression of β-lactamases of which there are several classes including plasmid encoded and chromosomally encoded enzymes (Medeiros and Jacoby, 1986; Shlaes *et al*., 1986; Livermore, 1995).

Morewever, the excessive and uncontrolled use of fluoroquinolones in clinical and veterinary medicine led to the increasing resistance of bacteria, which reduces the effectiveness of these drugs for future use (Curtis, 2007).

Research in Split-Dalmatia Country, showed that the rate of resistance in the five year period to ciprofloxacin was increased from 2.48% in 1999 to 7.28% in 2004 (Barišić *et al*., 1999-2004).

According to Al-Hilali (2010), Hadi (2008) and Al-Hilli (2010), demonstrated that all (100%) *E. coli* isolates were susceptible to

imipenem. However Al-Hilali (2010) who revealed 4.5% were resistance to gentamycin.

In previous study in Najaf, Hadi, (2008) reported that 42.1%, 36.8% and 55.3% of *E. coli* isolated from patients with significant bacteriuria were resistant to cefotaxime, ceftazidime and ceftriaxone, respectively. However, it might be possible that this high level of resistance to third generation cephalosporins in current study was most probably due to acquisition of β-lactamase, which encode by *bla*-genes possibly during therapy. These antibiotics usually used for treating of urinary tract, respiratory tract and burn wound infections caused by Enterobacteriaceae (Nasser, 2008).

A study accomplished in Najaf by Al-Hilali (2010) who indicated that aztreonam resistance was confirmed in 59.1% of *E. coli*.

The resistance against β-lactamase inhibitors occurs mainly by numerous mechanisms: hyperproduction of β-lactamases, production of β-lactamases resistant to inhibitors, and chromosomal cephalosporinases (Espinasse *et al*., 1997).

Morever Khanal *et al*., (2013) who reported that 66.7% of isolates were resistant to ciprofloxacin. However, Khadgi *et al*., (2013) reported 100% resistance to ciprofloxacin.

Moreover, a high potency of the fluoroquinolones (ciprofloxacin) against uropathogenic *E. coli* was observed by Akram *et al*., (2007) at Aligarh, India.

Bacterial resistance to antibiotics is now widespread and possesses serious clinical threats. The organisms develop resistance to

antibiotics by any of the following mechanisms: selection, mutation, phage transduction, and transference. Microbial resistance can be either hereditary in the organism or acquired through the environment (Ibezim, 2005).

The resistance to third generation cephalosporins was caused mainly by mutations in the common group of class A β-lactamases, which consisting of TEM, SHV and CTX-M β-lactamases that has extended hydrolytic spectrum activity on cephalosprins. The high rate resistance to ceftazidime and cefotaxime in this study may be related with production of enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity towards cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby 2004).

Reduced susceptibility to cefoxitin in the *E. coli* may be an indicator of AmpC activity, but cefoxitin resistance may also be mediated by alterations to outer membrane permeability (Tan *et al*., 2009).

nitrofurantoin remains the drug of choice for treatment and management of asymptomatic bacteriuria in pregnant women and symptomatic UTI in general (Onifade *et al*., 2005; Aiyegoro *et al*., 2007). The lack of resistance may be related to the fact that nitrofurantoin has multiple mechanisms of action, requiring organisms to develop more than a single mutation in order to develop resistance. In addition, limited usage of nitrofurantoin for treating uncomplicated cystitis, may also be a contributing factor to the lack of development of widespread resistance to this drug (Ladhani and Gransden, 2003; Gupta, 2003).

Table (3-5): Numbers and percentage of resistance type in 49 clinical isolates of *E. coli* according to site of infection.

3.4.1: Multidrug resistance (MDR), extensive drug resistance (XDR), and pandrug resistance (PDR) of *E. coli* isolates

The results of the current study reported that 39 isolates (79.59%) of *E. coli* were MDR (bacterial isolate can resist to a minimum at least 3 different classes of antibiotics). Also the result shows that out of 49 isolates of *E. coli* there were 9 isolates (isolate No 1, 3 isolated from blood and isolate No 12, 13, 26, 27, 30 isolated from urine and isolate No 19, 22 isolated from stool, with percentage (18.36%) as XDR (bacterial isolate remain susceptible to only one or two class of antibiotics).On the other hand the result demonstrated that there was no isolate with percentage (0%) was PDR(bacterial isolate resistance to all sub classes in all classes of antibiotics), (Table 3-5). The present study is an agreement with some local study such as Hadi (2008) also reported

Type of resistance	Urine Total Isolates:	Stool Total isolates:	Blood Total isolates:	Total (49)
MDR	25 (51.02%)	8 (16.32%)	6 (12.24%)	39 (79.59%)
XDR	5 (10.20%)	2 (4.08%)	2 (4.08%)	9 (18.36%)
PDR	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ESBL	0 (0%)	1 (2.04%)	0 (0%)	1 (2.04%)

that 82.6% of *E. coli* isolates were MDR in Najaf, In Tabriz, northern west of Iran, 84.2% of tested strains were observed as MDR(Rezaee *et al*., 2011). But it differed with that obtained by other local studies such as Al-Mohana (2004) found that 56.8% of clinical *E. coli* isolates in Najaf were resistant to more than five antimicrobial agents, while Hadi (2008) reported that all 60(100%) of the β-lactam resistant *E. coli* isolates obtained from patients with significant bacteriurea were resistant to at least five antimicrobial agents in Najaf, and MuKherjee *et al*., (2013) revealed, MDR strains was detected in 92.5% of isolates obtained from hospitalized patients in Kolkata, India.

In the another study byAl-Hilli (2010) revealed that all *E. coli* isolate obtained from Merjan Teaching Hospital in Hilla, Iraq, were considered as MDR. The level of MDR amongst the UTI isolates was found to vary from country to country.

According to Ibrahim *et al*., (2012) who founded that a high incidence of MDR *E. coli* in Sudan is (92.2%).

In the another study by D'Agata, (2004) defined multi-drug resistance as resistance to 3 or more antimicrobial classes. He has reported that, multi-drug resistant for all gram negative bacilli increased from 1% to 16% and 0.2% to 4% for *E. coli*.

According to Livermore, (2007) revealed that, the continuous used was observed in Najaf of even a single antibiotic over a period of weeks or months will select bacteria resistant to a different type of antibiotics in addition to the one in use.

Previous studies have indicated lower percentages of MDR compared to this study (Baquero *et al*., 2008; Figueira *et al*., 2011; Ham *et al*., 2012).

However, One study described bacteraemia due to MDR gram-negative bacilli in cancer patients and found the incidence to be 13.7% and out of these 49% episodes were due to MDR *E.coli* (Gudiol *et al*., 2011).

Morever, MDR rates vary from one country to another. Over all Gram-negative isolates from Latin American countries revealed the lowest susceptibility rates to all antibiotics followed by Asian-Pacific isolates and European strains. Strains from Canada exhibit the best global susceptibility testing results (Gales *et al*., 2001). Widespread use of antimicrobial therapy has often been held responsible for co-resistance to four or more unrelated families of antibiotics and the occurrence of multiply resistant *E. coli* strains in hospitals (Maynard *et al*., 2003). A strain of *E. coli* considered as an MDR if it was resistant to at least three classes of antibiotic (Magiorakos *et al.*, 2012).

3.5: Phenotyphc detection of extended spectrum β-lactamases (ESBL)

3.5.1: Confirmatory test for extended spectrum beta lactamase (ESBL)

This test was carried out according to Double Disk Synergy Test (DDST).The results demonstrated that 1 isolate (2.04%) were ESBL producers (Table 3-5).The result of these study is aggrement with some study such as data from the European Antimicrobial Resistance Surveillance System (EARSS), 2.6% of *E. coli* and 1.7% of *K. pneumonia* strains in Sweden were resistant to third-generation cephalosporins in

2010 (EARSS, 2011), In more than 13000 isolates of *E. coli*, the percentages expressing the ESBL phenotype were as follow: in Latin America, 8.5%; in the Western Pacific, 7.9%; in Europe, 5.3%; in the United States 3.3%, and in Canada, 4.2% (Winokr *et al*., 2001), and In a large study performed in 2001, it was demonstrated that about 5.3% of *E. coli* in the United States harbored ESBLs (Winokur *et al*., 2001). Also, the present result is similar to previous study conducted in 2009 showed that 9% of *E. coli* isolates in Texas were ESBL producers (Bhusal *et al*., 2011). This lower prevalence of ESBL may be due to the selection of only tracheal isolates from ICU(Khanal *et al*., 2013). But a dissageement with some previous studies such as in the United States, ESBL production was detected in 57.69% *E. coli* isolates tested by the Clinical Microbiology Laboratory in Rochester (Robberts *et al*., 2009), Tankhiwalae *et al*, (2004) reported 48.3% and Aggarwal., (2009) reported 40 % ESBL producers *E. coli* isolated from the urine at Rohtak.

In Saudi Arabia, although the exact prevalence of ESBL is currently unknown, the PEARLS study (2001- 2002) reported that the overall ESBL production rate from Enterobacteriaceae was 18.6% (Kader *et al*., 2004).

In the another study, 35.2% of *E. coli* and *K. pneumoniae* isolates obtained from nosocomial blood stream infections were ESBL producer (Superti *et al*., 2009).

However, in a survey of nosocomial isolates of Enterobacteriaceae collected from different wards in Pakistan, 28.57% of *E. coli* isolates were ESBL producers (Ali Shah, 2003).

The prevalence of ESBLs in India has now reached epidemic proportions, ranging from 62% to 100% in *E. coli* as observed in the 10 Indian medical centres sentry study (Mathai *et al*, 2009). Overall the ESBLs rate in the India sentry surveillance was 84%. This higher prevalence of ESBL may be because of the selection of various types of clinical samples from the ICU patients (Altun *et al*., 2013).

In previous Study from Lucknow, Delhi and Nagpur have reported that there is an increased percentage of ESBL positive isolates (Hawser, 2009).

In Turkey, several studies have shown that ESBL was found in high ratio in clinical isolates in Enterobacteriaceae (Tasli and Bahar, 2005).

However, the prevalence of ESBLs among clinical isolates varies from country to another country and from institution to another (Bradford, 2001).

In study for clinical isolates of ESBL-producing bacteria carried out in Najaf, the prevalence of ESBL-producing *E. coli* isolates was 18.3% (Hadi, 2008).

Other studies reported that a high endemic of ESBL- producing Enterobacteriaceae have been determined in Latin American countries especially in Brazil (Marry *et al*., 2006; Martins *et al*., 2006).

According to Fatemeh *et al*., (2012) found that 26.5% of *E. coli* and 43% of *K. pneumonia* were ESBL positive in their study conducted at the Imam Reza hospital of Mashhad, Iran.

In the another study by Majda *et al*., (2013) reported that 72% of *E. coli* and 65.8% of *K. pneumoniae* were ESBL producers at the Microbiology laboratory of Shalamar Medical College, Lahor.

According to a study conducted at the National Public Health Laboratory (NPHL), Kathmandu, Nepal reported that 31.57% of *E. coli* were confirmed as Extended Spectrum β-lactamase producers, these isolates further exhibited co-resistance to several antibiotics (Thakur *et al*., 2013).

Although strains that produce ESBL are characteristically resistant to new cephalosporins and/or aztreonam, many strains producing these enzymes appear susceptible or intermediate to some or all of these agentsin vitro, while expressing clinically significant resistance in infected patients (Paterson and Bonomo, 2005).

Table (3-6): Prevalence of extended spectrum beta lactamases genes of *E. coli* according to infections site (n=49)

Genes	Urine Total Isolates (n=30)	Stool Total Isolates (n=11)	Blood Total Isolates (n=8)	Total (49)
bla_{CTX-M}	29 (96.66%)	9 (81.81%)	7 (87.5%)	45 (91.83 %)
bla_{OXA}	29 (96.66%)	9 (81.81%)	7 (87.5%)	45 (91.83 %)
bla_{TEM}	30 (100%)	11 (100%)	8 (100%)	49 (100%)

3.6: Molecular detection of ESBL genes by PCR

Results in table (3-6) showed that *bla*$_{TEM}$ was the most prevalent gene in the tested isolates at rate (100%) followed by *bla*$_{CTX-M}$ and *bla*$_{OXA}$ genes (91.83%) for each.This result is an agreement with Kiratisin, (2007) who reported highest prevalence of *bla* $_{CTX-M}$ among several *bla* genes in all isolates which was 99.6%. Also the current result was similar to some previous result such as some studies in which the majority (over two thirds) of the ESBL producing Enterobacteriaceae reported was *E. coli* and most of them (≥ 85%) expressed CTX-M ESBL enzymes (Coque *et al*., 2008; Rossolini *et al*., 2008), In recent study, out of ESBL producing *E. coli,* most of them expressed a CTX-M enzyme (6/7) with a predominance of CTX- M-15 (6/6). The most frequently encountered mechanism of resistance to β-lactams found in their study was the production of CTX-M type β- lactamase as reported by Fatana *et al*., (2011). But disagreement with several studies such as Al-Hilli (2010) who found that 50% of the phenotypic ESBL-producing *E. coli* isolates had CTX-M type in Hilla, In Najaf, Al-Muhannak (2010) reported that 17 (27.4%) of the 62 potential ESBL-producing clinical enteric isolates carried gene of *bla*$_{OXA}$, and Hadi (2008) who found that 12 isolates (57.1%) of 21 *E. coli* harbored *bla*$_{TEM}$ gene.

In other study, Al-Hilali (2010) reported that the PCR assay revealed that, 17 (77.3%) of the 22 *E. coli* isolates obtained from children with diarrhea in Najaf carried *bla*$_{CTX-M}$.

According to Al-Hilli (2010), who found that 50% of the phenotypic ESBL-producing *E. coli* isolates had CTX-M type.

In the Middle East area, reports from Lebanon and Kuwait pointed out that CTX-M is predominant ESBL in Enterobacteriaceae (Moubareck *et al.,* 2005; Poirel *et al.,* 2005a).

In the another study by Al Muhanak (2010) who revealed that CTX-M β-lactamase was the most prevalent (38.7%) among the ESBL producing Enterobacteriacea isolates; while, TEM and OXA β-lactamases were less (27.4% for each). Morever other studies like yan *et al*., (2000) in Taiwan and Goyle *et al*., (2009) in India, who reported that CTX-M β-lactamases were the commonest enzymes than TEM in clinical Grame negative isolates.

In a related study from Brazil, bla_{CTX-M} were detected in 33.9% of ESBL-producing Enterobacteriaceae isolated from inpatients and outpatients at a public teaching hospital (Dropa *et al*., 2009). However in 2006, CTX-M enzymes were detected in 70% of the isolates in the University Health System in San Antonio, the United States (Lewis *et al*., 2007).

In other study reported by Rodriguez *et al*., (2006) who found that 70% of the ESBL producing *E. coli* obtained from bacteremia cases in Spain had CTX-M types. Morever In Korea, Park *et al*., (2009) found that PCR experiments detected the bla_{CTX-M} genes in 84.7% of *E. coli* isolates with an ESBL phenotype and they concluded that CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates.

In the Middle East area, reports from Lebanon and Kuwait point out that CTX-M is the predominant ESBL in *E. coli* (Moubareck *et al*., 2005; Poirel *et al*., 2005). The CTX-M type β-lactamases constitute a

novel group of enzymes that have a typical ESBL resistance phenotype, are capable of hydrolyzing broad-spectrum cephalosporins and are inhibited by clavulanic acid. They also confer a high level of activity against ceftazidime (Tzouvelekis *et al*., 2000).

Ellem *et al*., (2011) in their study have reported that majority of ESBL producers were *E. coli* isolates, which carried 64.3% of bla_{CTX-M}-1genes.

Other local study such as AL-Muhannak (2010) who reported that bla_{CTX-M} genes were detected in 8 (40%) out of 20 *E. coli* isolates. Hawkey (2008) reported that all ESBL producing *E. coli* isolates in his study harboured bla_{CTX-M}. In most countries, there are mixtures of CTX-M ESBL types, with ESBL isolates from India being completely dominated by the presence of CTX-M-15 (Hawkey, 2008; Mathai *et al*., 2009). AL-Hilli (2010) who reported that 5/10 (50%) of *E. coli* isolates gave PCR product with CTX-M specific primers.

The elevated rate of CTX-M β-lactamases in *E. coli* isolates suggest that the horizontal transfer of bla_{CTX-M} genes, mediated by plasmid and/ or mobile genetic element, contributes to ease with which these enzymes are spreading in *E. coli* isolates and the dissemination of CTX-M enzymes. Morever, in hospital environment, plasmid could be transferred easily between patients through the health care workers due to hand carriage and antimicrobial selection pressure (AL-Hilli, 2010).

CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates compared with SHV and TEM enzyms (Yongjung *et al*., 2009). Since then, an increase in the CTX-M β-lactamases has been seen

in many countries in Europe and Asia (Livermore, 2007). The *bla*$_{CTX-M}$ was reported the most prevalent *bla*-gene in Korea (Kiratisin *et al*., 2008).

In particular, the CTX-M family of ESBLs appears to be associated with community-acquired infections (Rodriguez *et al.,* 2006; Al-Hilali, 2010).However CTX-M is the predominant ESBL in *E. coli* (Moubareck *et al.,* 2005; Poirel *et al.,* 2005).

The main reason for the prevalence of CTX-M β-lactamases in Najaf may be the wide spread use of certain third generation cephalosporins. Antibiotic selective pressure probably contributes to the increasing prevalence of cefotaxime and ceftriaxone hydrolyzing CTX-M β-lactamases in clinical setting (Wei *et al*., 2005).

Currently, there are more than 90 gene sequences designated as *bla* $_{CTX-M}$ in the National Center for Biotechnology Information GenBank database. Based on percent sequence similarities, *bla*$_{CTX-M}$ genes can be clustered into five different groups based on their amino acid sequence (Bush and Jacoby, 2010). Group I includes CTX-M 1, 3, 10 to 12, 15 (UOE-1), 22, 23, 28, 29, and 30. Group II includes CTX-M 2, 4 to 7, and20 and Toho-1.Group III includes CTX-M 8. Group IV includes CTX-M 9, 13, 14, 16 to 19, 21, and 27 and Toho-2. Finally group V includes CTX-M 25 and 26. The members of these groups exhibit > 94% amino acid identity within the group and ≤90% amino acid identity between groups (Bonnet, 2004).

TEM-type ESBLs are the first plasmid-mediated β-lactamasethat is often found in genera of Enterobacteriaceae (Mulvey *et al*., 2004).

However, the transfer of *bla*$_{TEM}$ gene on mobile plasmid led to spread rapidly to members of the same species or organisms of different genera (Lartigue *et al*., 2007).

Several studies such as Shahcheraghi *et al.*, (2009) in Iran found that the frequency of *bla*$_{TEM}$ gene among the ESBL Gram-negative isolates were 9%, In Thailand, pongpech *et al.* (2008) found that *bla*$_{TEM}$ gene was present in 78% of the confirmed ESBL producers, and In Portugal, *bla*$_{TEM}$ gene was identified in 101 (85%) (Mendonca *et al*., 2007). A study conducted by Bradford (2001) reported that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. This enzyme has the ability to hydrolyze penicillin's and early cephalosporins.

Additionally, Al-Hilali (2010) reported that the PCR assay revealed 18/22 (81.8%) of *bla*$_{TEM}$ gene positive enteropathogenic *E. coli* recovered from children with diarrhea in Najaf.

Hadi (2008) found that, 14 (66.6%) out of 21 *E. coli* isolates were able to yield amplification products with TEM-PCR specific primers in study conducted in Najaf.

another local study in Hilla, AL-Asady (2009) reported that only 1 (20%) out of 5 *E. coli* isolates was possess *bla*$_{TEM}$ genes . AL-Muhannak (2010) in Najaf show that 5 (25%) of *E. coli* can revealed show *bla*$_{TEM}$ gene.

In previous study originated from geographically diverse locations, suggest that local antibiotic usage and practices may play an active role in promoting the selection of point mutations in *bla*$_{TEM}$-type genes (Huseyin and Bahar, 2005; Tomanicek *et al.,* 2010).

On the other hand, TEM-1, which is responsible for most of the ampicillin resistance in; 94% of *E. coli* strains isolated in Spain, 89% of *E. coli* strains isolated in Hong Kong, and in 78% of *E. coli* strains isolated in London (Livermore, 1995). However, Bradford (2001) reported that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1.TEM-2 β-lactamase is widespread in *E. coli*, although they are rare than TEM-1.The classical TEM-1 and TEM-2 enzyemes have manimal activity against newer cephalosporins (Sirot, 1995).

The OXA enzymes are regarded as OXA-type ESBLs and have been discovered mainly in *P. aeruginosa* in specimens from Turkey and France (Harada *et al.,* 2008). These β-lactamases are classified into class D in the Ambler scheme and were placed in group 2d in the Bush-Jacoby-Medeiros functional scheme (Walther-Rasmussen and Hoiby, 2006). Some OXA-type β-lactamases have carbapenemase activity, augmented in clinical isolates by additional resistance mechanisms, such as impermeability or efflux (Jacoby and Munoz-Price, 2005).

In a study done in Thailand, bla-genes encoding OXA were found in 8.1% of ESBL-producing *E. coli* isolates (Kiratisin *et al.,* 2008). However, Al-Hilali (2010) noticed that only 1 (4.5%) of enteropathogenic *E. coli* isolates recovered from children with diarrhea in Najaf was harbored bla_{OXA} gene.

Likewise, Karami and Hannoun (2008) reported that out of 32 ampicillin resistant *E. coli* isolates in Sweden, only one isolate (3%) harboured the bla_{OXA}.

However, in 2008, Bhattacharjee *et al.* reported that of the 361 ESBL-positive enterobacterial isolates from different clinical specimens in India, only one isolate harbored the OXA-10. Whereas in a study done in New York, Jones *et al*., (2009) found that over 40% of the Gram-negative clinical isolates collected carried genes encoding an OXA-type β-lactamase.

In a study done in Tailand, *bla*-genes encoding OXA were found in 8.1% of ESBL producing *E .coli* isolates (Kiratisin *et al*., 2008). Lower rate also recorded in Spain (3.8%) (Tenover *et al*., 2003), Germany (15%) (Svard, 2007) and Malaysia (5%) (Lim *et al*., 2009). Morever Lower prevalence has been reported in Sweden, only one isolates (3%) harboured the bla_{OXA} out of 32 ampicillin resistance *E. coli* isolates (Karami and Hannoun, 2008).

Another study also detected high prevalence of bla_{OXA} in Slovenia (41%) (Istinic, 2008) and in USA (40%) (Jones *et al*., 2009). AL-Hilali (2010) revealed that, only one isolates (4.54%) of *E. coli* carried bla_{OXA} in Najaf.

In a previous study by AL-Muhannak (2010) who revealed that the OXA was detected in 5 (25%) of *E. coli* isolates. However, AL-Hilli (2010) found that all *E. coli* isolates from Merjan teaching hospital in Hilla city were negative in OXA-PCR.

Figure (3-2): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 47 isolates) amplified with diagnostic bla_{CTX-M} gene, show positive results at 550 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (50 bp ladder).

Figure (3-3): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 48 isolates) amplified with diagnostic $bla\text{-}_{OXA}$ gene, show positive results at 619 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (50bp ladder).

Figure (3-4): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 49 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 44 isolates) amplified with diagnostic *bla-*$_{TEM}$ gene, show positive results at 822 bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

Table (3-7): Prevalence of plasmid mediated quinolones resistance genes of *E. coli* according to infection site (n=28)

Genes	Urine Total Isolates n=16	Stool Total Isolates n=5	Blood Total Isolates n=7	Total (28)
qnrA	16 (100%)	5 (100%)	7 (100%)	28 (100%)
qnrB	16 (100%)	5 (100%)	7 (100%)	28 (100%)
qnrS	16 (100%)	5 (100%)	7 (100%)	28 (100%)

3.7: Molecular detection of plasmid mediated quinolones resistance in *E. coli* isolates (PMQR)

Results show in table (3-7) that *qnrA*, *qnrB*, and *qnrS* genes in the test isolates have the same percentage (100%). Present study showed that the spreading of PMQR genes among clinical isolates of *E. coli* in Najaf is high and that it is much higher than that reported in other areas, such as the United States (Robicsek *et al.,* 2006d), Taiwan (Wu *et al.,* 2007) and China (Yang *et al*., 2008b).The current result is disagrrement with some previous study such as Low prevalence of *qnr* (A,B,S) genes has also been reported from Seoul, Korea where the prevalence of *qnr* (A,B,S) genes was less than 1% of *E. coli* isolates from a collection of clinical isolates (Minggui *et al*., 2003), In China, Wang *et al.*, (2008a) reported that the prevalence rates of *qnr* among the clinical isolates of ciprofloxacin resistance in *E. coli* and *K. pneumoniae* were 7.5% (11 of 146) and 11.9% (8 of 67), respectively, and In France, the prevalence of *qnr*(A,B,S) genes was 1.6% (2/125) *E. coli* and *Klebsiella* spp. isolates (Poirel *et al*., 2006; and Cattoir *et al*., 2007a).

In the other study, An investigation on 47 ceftazidime-resistant *E. coli* isolates from humans showed that *qnrA* and *qnrB* were both detected in 1 (2%) during the study, no isolates were shown to carry *qnrS*, and none had both *qnrA* and *qnrB* (Robicsek *et al*., 2006b).

Previous studies suggested that *qnr* genes have rarely been found in clinical strains from other areas of the world (Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2004; Mammeri *et al*., 2005; Jacob *et al.,* 2009).

However After evaluating a total of 91 *E. coli* isolates from the United States, Jacoby *et al.*, (2003) could identify only one isolate harboring the *qnr* gene. Karah, (2008) found that the *qnr* genes were 1.6% (8/487) in the *E. coli* and *Klebsiella* spp clinical isolates collected from Norway and Sweden.

According to Crémet *et al.* (2009) found that PMQR determinants detected in 13 isolates (27.7%) were either *qnr* gene or *aac(6´)-Ib-cr* gene identified alone or in combination

Other in Canada only about 1% (5/550) of ciprofloxacin and/or tobramycin resistant *E. coli* and *Klebsiella* spp isolates were *qnr*-positive (Pitout *et al*., 2008).

However, studies have shown that plasmid-mediated resistance mechanisms also play a significant role in fluoroquinolone resistance, and its prevalence is increasing worldwide (Robicsek *et al.,* 2006b).

Additionally, moderate prevalence has also been detected in other parts of the world such as Spain (5%) (Lavilla *et al.,* 2008), China (8%) (Jiang *et al.,* 2008), and the United States (15%) (Robicsek *et al*., 2006d).

qnrA is the PMQR gene encoding a 218 amino acid protein of the pentapeptide family that protects DNA gyrase from quinolone inhibition (Tran *et al.,* 2005). Several studies showed a worldwide dissemination of *qnrA* determinants among enterobacterial isolates (Cheung *et al.,* 2005; Mammeri *et al.,* 2005). It has been suggested that the *qnrA* gene may contribute to the increase in quinolone resistance in human *E. coli* (Wang *et al.,* 2003).

Accordiong to Jeong *et al*., (2005) reported that the prevalence of *qnrA* in Korea was 0.8% in *E coli* isolates (ciprofloxacin susceptible and resistant) between 2001 and 2003.

Although the *qnrA* gene seem to be uncommon among human clinical isolates of *E. coli* from the United States and Europe (Nordmann and Poirel, 2005; Robicsek *et al.,* 2006d) the gene is prevalent in fluoroquinolone-resistant *E. coli* from Asia (Wang *et al.,* 2003).

qnrA gene was highly detected in a selected collection of blood culture isolates of Enterobacteriaceae resistant to both ciprofloxacin and cefotaxime in Liverpool (Corkill *et al*., 2005).

However, *qnrA* was recently detected in an *E. coli* clinical isolates from Denmark (Cavaco *et al*., 2007). Furthermore, *qnrA* was the most prevalent *qnr* gene in a most recent Spanish study (Lavilla *et al.*, 2008) where *qnrA* was detected in 14 of 305 ESBL-producing enterobacterial isolates, whereas only one *qnrS* and no *qnrB* were detected.

A novel PMQR gene, *qnrB*, has been discovered in a plasmid encoding the CTX-M-15 β-lactamase from a *K. pneumoniae* strain isolated in South India. It has less than 40% amino acid identity with the original *qnr* (now *qnrA*) gene or with the recently described *qnrS* but, like them, codes for a protein belonging to the pentapeptide repeat family. Strains with demonstrated low-level resistance to all quinolones tested (George *et al.,* 2006). However, no previous nationwide survey has evaluated clinical isolates of *E. coli* with resistance to quinolones in Iraq for the presence of *qnrB*. Therefore, the present study screened the 28 quinolone resistance *E. coli* isolates for the presence of the *qnrB* gene

by using previously described primers and amplification conditions (George *et al*., 2003; Park *et al*., 2006; Thi *et al*., 2007; Koichiro *et al.*, 2009).

In general, present study revealed that, the *qnrB* gene seems to be common in quinolone resistance clinical *E. coli* isolates and may contribute to the alarming rates of quinolone resistance. However, present study indicates that *E. coli* may be representing the greatest reservoir and source of dissemination of plasmid-mediated antimicrobial resistance genes, such as *qnrB*, in Najaf hospitals.

In Korea, Shin *et al*., (2008). reported that 5.6% of *E coli* ciprofloxacin-resistant isolate only *qnrB* (*qnrB*2, *qnrB*4 and/or *qnrB*6).

In previous study by Karah (2008) who found that only 0.7% (3/422) of nalidixic acid-resistant *E. coli* isolates was *qnr*-positive in Norway and Sweden.

In Kuwait, out of 64 Enterobacteriaceae isolates, only 3 (4.7%) isolates were positive for a *qnrB*-like gene (Cattoir *et al.,* 2007b). Morever, In Japan, Ashraf *et al.* (2007) previously identified six *qnrB* genes in *E. coli*, *K. pneumoniae, K. oxytoca, C. freundii, P. mirabilis* and *P. fluorescence* from zoo reptiles and falcons.

Present study revealed that, this is the first detection of a *qnrS* gene in Najaf hospitals, while in other countries such as Spain, France and Korea, other authors have already described *qnrS* genes (Poirel *et al*., 2006; Park *et al*. 2007; Lavilla *et al.,* 2008).

However, present study suggested that the *qnrS* gene was common among quinolones and fluoroquinolones resistance *E. coli*

clinical isolates, but its identification might indicate the emergence of this mechanism of quinolone resistance in Najaf hospitals.

In the other local stydy in Morocco, the *qnrS* gene was found in only one (6.2 %) *E. coli* isolate (Bouchakour *et al.,* 2010).

According to Kim *et al*., (2009) determined that 0.5% of *E coli* and 5.9% of *K pneumoniae* (ciprofloxacin susceptible and resistant) isolates in Korea contained *qnr* (*qnrB or qnrS*).

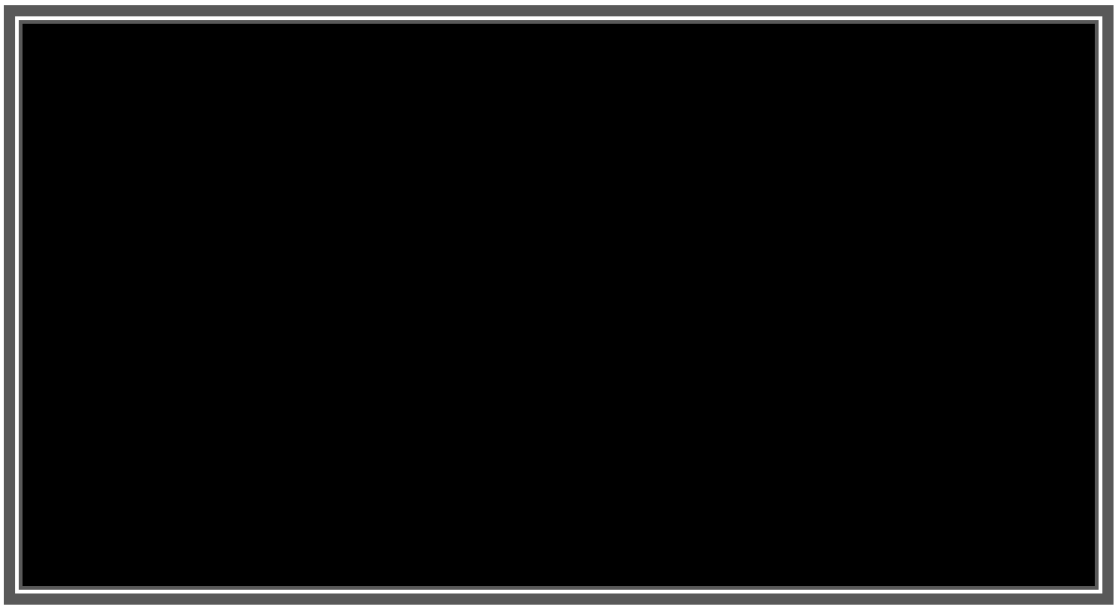

Figure (3-5): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 26 isolates) amplified with diagnostic *qnrA* gene, show positive results at 625 bp . The electrophoresis was performed at 80 volt for 95

minutes. (L): DNA molecular size marker (50bp ladder).

Figure (3-6): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 28 isolates) amplified with diagnostic *qnrB* gene, show positive results at 468bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

Figure (3-7): Ethidium bromide-stained agarose gel electrophoresis of monoplex PCR amplified products from extracted total DNA of 28 *E. coli* isolates isolated from different clinical specimens. Lane: (1 to 28 isolates) amplified with diagnostic *qnrS* gene,

show positive results at 417bp . The electrophoresis was performed at 80 volt for 95 minutes. (L): DNA molecular size marker (100bp ladder).

Conclusions and Recommendations

Conclusions:

1- There was a relationship between phenotypic and genotypic detection of genes responsible for antibacterial resistance in *E. coli*.

2- *E. coli* became highly resistant to antibiotics especially to the 3rd generation cephalosporins (cefotaxime).

3- *E. coli* isolates that are isolated from urine were more resistant to antibiotics (cefotaxime, ampicillin, tobramycin, amoxicillin+clavulanic acid) than isolated from other site of infections.

4-Most tested *E. coli* isolates are multidrug resistant (39 isolates 79.59%) ; therefore, such organisms represent a serious therapeutics challenge in patients.

Recommendations:

1- Using phenotypic and genotypic methods to identify the presence of an ESBL should be carried out in all hospitals laboratories routinely in our country.

2- Use the modern genetic methods like (PFGE, RFLP, Sequencing…etc) to study the molecular features of *E. coli* isolates.

3- Avoiding the use of third generation cephalosporins against pathogenic bacteria that appear resistant to these antibiotics (i.e. ESBL-producers), and it is important to determine the most effective antibiotic for treating infections caused by ESBL-producing isolates in patients.

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الخلاصة

صممت هذه الدراسة لتشخيص انتشار بعض الجينات المقاومة للمضادات الحيوية لبكتريا الاشريشيا القولونية *Eschirichia coli* ألمعزولة من عينات مختلفة بواسطة الطرائق المظهرية والتقنية الجزيئية (بواسطة تفاعل انزيم البلمرة). جمعت 400 عينة مختلفة من الادرار(100عينة), الخروج (75عينة), والدم (45عينة) من من مرضى أدخلو الى مستشفى الحمزة في مدينة الديوانية ومدينة الصدر الطبية في محافضة النجف الاشرف.

أظهرت النتائج وجود 220 عينة (55%) شخصت على انها بكتريا سالبة لصبغة كرام. من مجموع العينات 220 كانت هناك 49 عزله (22.27%) شخصت على انها بكتريا *E. coli* بواسطة الصفات الزرعية و الكيموحيوية ونظام Vitek2® .

اختبرت حساسية عزلات *E. coli* ل14 نوع من المضادات الحيوية بأستخدام طريقة انتشار القرص. أظهرت النتائج بأن جميع العزلات (100%) كانت مقاومة للسيفوتاكزيم, 39 عزلة (79.59%) كانت متعددة المقاومة للمضادات الحيوية, 9 عزلات (18.36%) كانت حساسة فقط الى نوع واحد او نوعين من اصناف المضادات الحيوية. اثبتت هذه الدراسة ان 47 عزلة (95.91%) كانت مقاومة للأمبيسيلين والتوبراميسن, 41 عزلة (83.67%) كانت مقاومة للأموكسيسيلين+كلافولانك أسد. اظهرت نتائج الدراسة الحاليه ان 39 عزلة (79.59%) و 38 عزلة (77.55%) لبكتريا *E. coli* كانت مقاومة للسيفترياكزون وألسيفتازيديم على التوالي لذا فهي بكتريا تقاوم مضادات البيتالاكتاميز واسعة لطيف. بينت الدراسة الحالية ان 34 عزلة من *E. coli* (69.38%) كانت مقاومة للأزترونيم, 26 عزلة (53.06%) كانت مقاومة للناليديكسك أسد, 21 عزلة (42.85%) كانت مقاومة للسيبروفلوكساسين والجينتاميسين, 17 عزلة (34.69%) كانت مقاومة للسيفوكزيتين, ثمان عزلات (16.32%) كانت مقاومة للكلورامفينيكول, ست عزلات (12.24%) وعزلتين (4.08%) كانت مقاومة للنيتروفيورونتوين والأميبينيم على ألتوالي.

اختبرت العزلات بعد ذلك لقابليتها على انتاج انزيمات اليتا-لاكتاميز باستخدام الطريقة التوكيدية (أختبار تأزر القرص المزدوج), أظهرت النتائج بأن عزلة واحدة فقط (2.04%) من مجموع 49 عزلة لبكتريا *E. coli* كانت منتجة فعلا لأنزيمات البيتا-لاكتاميز واسعة الطيف.

أستخدمت تقنية تفاعل أنزيم البلمرة لتحديد بعض الجينات التي تشفر عوامل لمقاومة المضادات الحيوية لعزلات بكتريا *E. coli*. بلنسبة للجينات المسؤلة عن بعض انزيمات البيتا-لاكتاميز واسعة الطيف (bla_{TEM} and bla_{OXA} ,$bla_{\text{CTX-M}}$), اثبتت الدراسة الحالية ان جين

bla_{TEM} كان يمتلك نسبة عالية (100%),يليه جيني (bla_{CTX-M} and bla_{OXA}) بنسبة (91.83 %).

أخيرا, كشفت هذه الدراسة عن بعض الجينات المسببة لمقاومة الكوينولون (*qnrA,qnrB, qnrS*), اظهرت النتائج بأن هذه الجينات كانت تمتلك نفس النسبة (100%).

وزارة التعليم العالي والبحث العلمي

جامعة القادسية / كلية الطب

فرع الأحياء المجهرية

التوصيف الجزيئي للجينات المقاومة للمضادات الحيوية في بكتيريا أشريشيا القولون المعزولة من عينات سريريه

رسالة مقدمة ألى

مجلس كلية الطب- جامعة القادسية

وهي جزء من متطلبات نيل درجة الماجستير علوم

في علم الأحياء المجهرية الطبية

من قبل

علاء حمزه جابر الجليحاوي

بكالوريوس- علوم تحليلات مرضية / جامعة الكوفة (2014)

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