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DETECTION OF ANTIBIOTIC RESISTANCE GENES AMONG ENTEROBACTERIACEAE ISOLATED FROM CLINICAL AND DIFFERENT SITES CLOSED OF IRAQI HOSPITALS

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December 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Rasha Ali Hussein AL-KHAFAJI

ABSTRACT

DETECTION OF ANTIBIOTIC RESISTANCE GENES AMONG ENTEROBACTERIACEAE ISOLATED FROM CLINICAL AND DIFFERENT SITES CLOSED OF IRAQI HOSPITALS

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Master of Science in Biology

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December 2022

For the purpose of obtaining pure isolates of the Enterobacteriaceae and for the evaluation of sensitivity to antibiotics and identification of resistance genes, a total of 134 clinical isolates were collected, 80 clinical isolates from multiple laboratories in Baghdad and 54 environmental isolates from nearby Tigris River. Samples were collected and studied during 6 months for the period from November 2021 to April 2022. Each isolate was identified using morphological, biochemical, and VITEK-2 System confirmatory tests. Where the environmental results showed *Eschericha coli* was a rate 44.44%, *Klebsiella pneumoniae* 31.48%, *Proteus mirabilis* 7.40%, *Citrobacter freundii* 1.85%, *Citrobacter koseri* 1.85%, *Enterobacter aerogenes* 12.96%. While the clinical results showed *E. coli* 33%, *K. pneumoniae* 28%, *P. mirabilis* 19%. The antibiotic susceptibility test results indicated that all clinical 47 bacteria were unsusceptible to Penicillin, Amoxicillin, Cephalexin, Ceftazidime and Tetracycline by 100%, Amoxicillin–clavulanic acid 80.85%, Cefotaxime 65.95%, Cefixime 59.57, Cefoxitin 57.44%, Aztreonam 61.7%. While all isolates were sensitive to Meropenem 10.6% and Impenem 14.89%. On the other hand, the Antibiotic susceptibility test results for environmental 22 bacteria revealed unsusceptible to Penicillin, amoxicillin, Cephalexin, Ceftazidime, and Tetracycline by 100% Amoxicillin-clavulanic acid 77.27%, Cefotaxime 77.2%, Cefixime 63.63%, Cefoxitin 59.09%, Aztreonam 54.5% while all isolates were sensitive to Meropenem was rate 18.18% and Impenem 13.6%. Replication of the DNA sequence by PCR was performed for 69 clinical and

environmental isolates of Enterobacteriaceae. The results showed that for the environmental isolates, all isolates contained the gene *bla*$_{TEM}$ and the *bla*$_{SHV}$ at a rate of 40.47%. While the 47 clinical isolates, all isolates contained the gene *bla*$_{TEM}$ was rate 85.10% and *bla*$_{SHV}$ 82.97%. The results showed that *E. coli* contained the *bla*$_{TEM}$ gene by 15 (31.91 %), *K. pneumoniae* 14 (29.78%), and *P. mirabilis* 11 (23.40 %). *bla*$_{SHV}$ *E. coli* 14 (29.78%), *K. pneumonia* 14 (29.78%) and *P. mirabilis* 11 (23.40%). Based on the findings, clinical isolates had a greater virulence factor than environmental isolates. This is so because they have resistance genes (*bla*$_{TEM}$ and the *bla*$_{SHV}$) at higher rates than environmental isolates

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Keywords: Antibiotics resistance, Enterobacteriaceae, Resistance genes

ÖZET

IRAK HASTANELERİNİN YAKININDAKİ KLİNİK VE FARKLI YERLERDEN İZOLE EDİLEN ENTEROBACTERIACEAE'LERDE ANTİBİYOTİK DİRENÇ GENLERİNİN TESPİTİ

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Enterobacteriaceae saf izolatlarını elde etmek, antibiyotiklere duyarlılığı değerlendirmek ve direnç genlerini belirlemek için Bağdat ilindeki birden fazla laboratuvardan 80 klinik izolat ve Dicle Nehri yakınlarından 54 çevresel izolat olmak üzere toplam 134 klinik izolat toplandı. Numuneler, Kasım 2021'den Nisan 2022'ye kadar 6 ay boyunca toplandı ve incelendi. Her izolat, morfolojik ve biyokimyasal sistem doğrulama testleri ve VITEK-2 kullanılarak tanımlandı. Çevresel sonuçlar *Escherichia coli* %44,44, *Klebsiella pneumoniae* %31,48, *Proteus mirabilis* %7,40, *Citrobacter freundii* %1,85, *Citrobacter koseri* %1,85, *Enterobacter aerogenes* %12,96 gösterirken, klinik sonuçlar *E. coli* %33, *K. pneumoniae* %28, *P. mirabilis* %19 olarak bulundu. Antibiyotik duyarlılık testi sonuçları, klinik 47 izolatta Penisilin, Amoksisilin, Sefaleksin, Seftazidim ve Tetrasiklin'e %100 ve sırasıyla Amoksisilin-klavulanik asit %80,85, Cefotaksim %65,95, Cefixime 59,57, Sefoksitin %57,44, Aztreonam %61,7 oranında dirençli olduğunu göstermiştir. Tüm izolatlar Meropenem'e %10,6 ve Impenem'e ise %14,89 oranında duyarlılık göstermiştir. Öte yandan, çevresel 22 izolat için yapılan antibiyotik duyarlılık testi sonuçlarında Penisilin, Amoksisilin, Sefaleksin, Seftazidim ve Tetrasiklin'e %100, Amoksisilin-klavulanik asit %77,27, Cefotaksim %77,2, Cefixime %63,63, Sefoksitin %59,09, Aztreonam'a %54,5 oranında dirençli olduğunu ortaya çıkmıştır. Tüm izolatlar Meropenem'e %18,18 oranında duyarlı iken, bu oran Impenem için %13.6 olarak bulunmuştur. DNA dizisinin PCR ile replikasyonu, Enterobacteriaceae'nin 69 klinik ve çevresel izolatı için gerçekleştirilmiştir. Sonuçlar,

çevresel izolatlar için tüm izolatların %40,47 oranında bla$_{TEM}$ ve bla$_{SHV}$ geni içerdiğini göstermiştir. 47 klinik izolatta (bla$_{TEM}$) gen oranı %85,10 ve bla$_{SHV}$ %82,97 iken, sonuçlar *E. coli*'nin bla$_{TEM}$ genini 15 (%31,91), *K. pneumoniae'*nin 14 (%29,78) ve *P. mirabilis'*in 11 (%23,40) şeklinde içerdiğini göstermiştir. bla$_{SHV}$ *E. coli* için 14 (%29.78), *K. pneumonia* 14 (%29.78) ve *P. mirabilis* 11 (%23.40) olarak tespit edilmiştir. Bulgulara göre, klinik izolatlar çevresel izolatlardan daha büyük bir virülans faktörüne sahiptir. Bunun nedeni, çevresel izolatlardan daha yüksek oranlarda direnç genlerine (*bla$_{TEM}$* ve *bla$_{SHV}$*) sahip olmalarıdır.

2022, 100 sayfa

Anahtar Kelimeler: Antibiyotik direnci, Enterobacteriaceae, Direnç genleri

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CONTENTS

LIST OF SYMBOLS

%	Percentage
°C	Degrees celsius
mg	Milligram
mL	Milliliter
mm	Millimeter
μL	Microliter

LIST OF ABBREVIATIONS

BA	Brain adhesions
BL	Beta-lactam
CA	Cerebral abscesses
EHEC	Enterohemorrhagic *E. coli*
ESBLs	Extended spectrum β-lactamases
GE	Gastroenteritis
GN	Gram-negative
GP	Gram-positive
HAIs	Healthcare associated infections
HAPs	Hospital-acquired pneumonias
IAIs	Intra abdominal infections
ICU	Intensive care units
IE	Infective endocarditis
LPS	Lipopolysaccharides
LRT	Lower respiratory tract infections
MDR	Multi drug resistant
PCR	Polymerase chain reaction
SA	Septic arthritis
SSTI	Soft-tissue infections
TPC	Total plate count
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
XDR	Extensively drug resistance

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The rise of bacteria that are resistant to antibiotics poses a major threat to global health, nutrition security, and evolution today, it may afflict anyone of any age in any region. It happens naturally. Overuse and misuse of antibiotics in humans and animals increased the development of multidrug resistance (MDR), and the emergence and global dissemination of novel resistance mechanisms pose a threat to our ability to treat prevalent infectious diseases effectively (Aslam *et al.* 2018).

Previous research has found antibiotics and MDR bacteria in hospital effluents, particularly Enterobacteria that generate beta-lactamases capable of hydrolyzing penicillin, cephalosporin, Monobactams, carbapenems and beta-lactamase inhibitors (Soriano-Moreno *et al.* 2021).

MDR strains are those that are resistant to several drugs. MDR literally means "resistant to more than one antimicrobial agent," although the majority describes it as "resistant to three or more antimicrobial classes" (Basak *et al.* 2016).

Beta-lactam (BL) antibiotics are the first-line treatment for a variety of diseases (Romano *et al.* 2020), including penicillin, cephalosporins, carbapenems, and monobactams (Felix *et al.* 2021).

β-lactamases are Gram-negative bacteria's major protection against β-lactam antibiotics (Bush 2018)

Extended spectrum β-lactamases (ESBLs) are plasmid-mediated beta-lactamases that make bacteria resistant to penicillin and cephalosporins. They can be stopped by beta-lactamase inhibitors like clavulanic acid, sulbactam, and tazobactam (Ansari *et al.* 2015).

Enterobacteriaceae gram-negative (GN), non-spore-forming, and facultatively anaerobic are prevalent in hospitals and the general society. *Klebsiella* and *Escherichia coli* are the two much more prevalent types of ESBL-producing bacteria; many other pathogens can also produce them. These include *Enterobacter spp., Proteus spp., Morganella spp., Salmonella spp.* and *Providencia spp.,* to cause many infections (Kiros *et al.* 2021).

Enterobacteriaceae earn ESBL genes by horizontal plasmid transfer or mutation, resulting in oxyimino-cephalosporin resistance. *blaOXA, blaCTX-M, blaTEM,* and *blaSHV* are the most dominant ESBL-encoding genes (Abrar *et al.* 2019). The existence of these genes in enteric bacteria causes these organisms to be more resistant to certain beta-lactam medicines (Adamski *et al.* 2015)

The *bla*SHV and *bla*TEM families of ESBL enzymes are mutations of beta-lactamases, whereas the *bla*CTX-M family originated from bacterial populations in environment. Furthermore, numerous variants of bla CTX-M have emerged owing to point mutants in the gene. ESBL-producing bacteria can release CTX-M, SHV, and TEM enzymes containing over 450 variations (Ejaz *et al.* 2021).

Aim of study

The present study aims to isolate antibiotic-resistant bacteria from normal and clinical environments that cause serious infections like urinary tract inflammation and beta-lactam antibiotic genes studies, including bla_{TEM} and bla_{SHV}. Through:

- Isolating and microscoping identification of Enterobacteriaceae isolates from hospital environmental (water samples) and clinical samples.
- Biochemical test of Enterobacteriaceae isolates as secondary identification methods.
- Evaluating the susceptibility antibiotics sensitivity of Enterobacteriaceae isolates.

- Molecular detection with (polymerase chain reaction (PCR) technique) to detection the extended-spectrum beta-lactamase genes (*bla*$_{TEM}$ and *bla*$_{SHV}$) among beta-lactam-resistant Enterobacteriaceae isolates.

2. LITERATURE REVIEW

2.1 Enterobacteriaceae

Enterobacteriaceae are GN rods measuring between 1 and 3 μm in length. They are elective anaerobes, negative for oxidase and positive for catalase, and they can be grown on MacConkey agar. The human and animal intestines are a natural host for them. The Enterobacteriaceae family has 44 genera and over 176 species. This family is classified between fermented lactose species like *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Citrobacter* and non-fermented lactose species like Proteus, *Yersinia*, *Shigella*, and *Salmonella*. They are gut flora, including *Escherichia coli, Enterobacter sp., Morganella sp., Providencia sp., Proteus sp., Klebsiella sp.*, and *Serratia sp*.; in addition, there are three obligate human pathogens, involving *Yersinia sp., Shigella*, and *Salmonella* (Ibrahim and Hameed 2015).

Enterobacteriaceae develop at 37°C and inhabit several environmental, animal, and human habitats, acting as plant, water, soil, as well as the mammalian intestines (Barco *et al*. 2014). In intensive care units (ICU), these organisms can drive various diseases, such as urinary tract infection (UTI), infections of wound (sepsis/abscesses), and pneumonia (Aman *et al*. 2022).

Several Enterobacteriaceae members can create endotoxins that, when released into the bloodstream after cell lysis, induce a systemic inflammatory and vasodilator response. The most intense of this response, known as endotoxic shock and causes a deathly infection (Augusto *et al*. 2021).

2.1.1 Epidemiology

Enterobacteriaceae are among the most frequent pathogens that infect humans globally. They are in charge of community-gained, hospital-gained, and healthcare setting diseases; It is also worth noting that, as resident gastrointestinal tract flora,

Enterobacteriaceae isolate may signify colonizing rather than an actual disease (Zahar *et al*. 2017). This is applied to species derived from urine, respiratory secretions, or rectal swabs. Although the majority of diseases caused by Enterobacteriaceae are sporadic, outbreaks of infections can happen. This is more prevalent among long-term care facility and hospital residents infected with *Klebsiella* species. Less usually, outbreaks may be caused by other *Enterobacter* species. Outbreaks are typically caused by many resistant strains, which complicates infection control (Kaye and Pogue 2015).

Some kinds are naturally parts of the "normal flora" of animals, even human. However, most are usually connected with diarrheal diseases and extra-intestinal infections.

The Enterobacteriaceae family contains a number of the most significant pathogens in human history, such as the plague *Yersinia pestis* agent, and other species that are currently a big public health concern (such as *Shigella*, *Salmonella enterica serotype Typhi*, *E. coli*) (Mariam 2019).

2.2 Virulence Factors in Pathogenic Bacterial

2.2.1 Hemolysin

Hemolysins which are very important virulence factors, there are produced by a various kinds of bacteria; these substances cause membrane disruption, cell lysis, and destruction of neighboring cells and tissues to supply nourishing, mostly iron, for bacteria that produce toxins (Pérez *et al*. 2020). Hemolysin is attached to specific receptors on the erythrocytes blood cell wall, poring in the cell wall so that the erythrocytes lyses (Koo *et al*. 2019).

Hemolysin is a prototype calcium dependent repeat in toxin-produced protein usually located in uropathogenic *E. coli* strains as opposed to fecal strains. This toxin is active against multiple cell types, involving uroepithelial cells, and inserts itself into the membranes of host cells. Hemolysin, which is also involved in invasions, stimulates the

synthesis of cytokines and induces an inflammatory response. Although hemolysin is not needed for colonizing in experimental urinary tract infection (UTI), it adds to pathogenicity by causing epithelial injury and bleeding in the bladder (Nagamatsu *et al*. 2015).

2.2.2 Adhesion

Adhesion to host cells is the first step in interactions between bacteria and host cells after the epithelial layer comes into contact with bacteria. There are several mechanisms that bacteria use to overcome the host's defense mechanisms and attach to the host cells. Microbial adherence to host cells can occur via fimbria or polysaccharides and pili. All of these factors can found in the same pathogen and contribute to bacterial adhesion (García *et al*. 2016).

Bacteria with GN collect various surface structures, containing pili and fimbriae, hair-like organelles. Typically, pili are engaged in sticking and facilitate reaction with various surfaces, microorganisms, and other cell types, including host cells. Pili plays an essential part in *E. coli* adhesion to its contact hosts (Werneburg and Thanassi 2018). Following adhesion, microbial pathogens can initiate biochemical processes that lead to infection, such as toxin secretion and host cell invasion (Flores-Mireles *et al.* 2015).

2.2.3 Formation of biofilm

Biofilms of bacteria are colonies of bacteria stuck to one surface either another and immersed in a matrix that they manufacture themselves. The matrix of a biofilm is composed of polysaccharides, proteins, and eDNA. Furthermore, biofilm-embeded bacteria can also employ several other survival methods to be shielded from the host's defenses by the matrix. Remaining motionless and concealed from the body's defenses can induce near tissue harm and severe infection (Vestby *et al*. 2020).

Biofilm-associated infections are classified into two types; the first involves the formation of biofilms on host's tissues (e.g., epithelial, mucosal surfaces, teeth). The second type of infection is caused by non-living area microbial colonization, like those on installed medical equipment (e.g., dental prostheses, central venous and/or urinary catheters, and many others) (Høiby *et al*. 2015, Römling *et al*. 2014).

A critical characteristic of biomaterial associated illnesses is that microbial cells can separate from biofilms and spread to encircling tissues or the circulation, worsening the infection's clinical upshot (Batoni *et al.* 2016).

2.2.4 Capsular polysaccharides

Capsular polysaccharides (K antigen) Many genera of the Enterobacteriaceae family contain capsular and microcapsular polysaccharides. In addition, bacteria of the same family, acting as *Serratia sp., Enterobacter sp.*, and *E. coli*, possess capsules with comparable features. Most commonly, the term capsule refers to an outer material that covers the whole bacterial surface and is so securely attached to other surface constructural that it cannot be completely dissolved in a fluid medium. The Quellung reaction is the most commonly used process for determining K antigen, referred to as the Neufeld interaction. It is a biochemical process in which antibodies connect to the bacterial capsules of *N. meningitidis*, *B. anthracis*, *Salmonella* sp., *E. coli*, *H. influenzae*, *K. pneumoniae*, and *S. pneumoniae*. Antibody interaction allows visualization of these species under the microscope. The capsule gets gloomy and seems largely due to the positive reaction (Dougnon *et al*. 2020).

2.2.5 Toxins

Lipopolysaccharides (LPS), also known as endotoxins, Endotoxins are complex lipopolysaccharides located in GN bacteria cell walls, such as *E. coli* and *Salmonella* (Bertani and Ruiz 2018). Endotoxin could enhance to transmission of Enterobacteriaceae through the intestinal tract and blood brain barrier, which may

increase the danger of neonatal infection (Collado *et al.* 2015). Exotoxins (proteinaceous toxins) are mostly enzymes. These toxins are classified into two groups based on how they are delivery to eukaryotic cells.

- Excretion into the cell of the host
- Injection into a host cell cytoplasm (Sastalla *et al.* 2016).

Exotoxins from GN bacteria and enteropathogenic bacteria have important roles in pathogenesis. These toxins cause excessive secretion of fluids and the death of intestinal muscle tissue, which are associated with digestive illnesses such as vomiting and diarrhea (Hernández-Cortez *et al*. 2017).

2.2.6 Fimbriae

The filamentous filaments known as fimbriae (pili) protrude from the surface of bacteria. Anderson firstly mentioned it (1949). Fimbriae are discovered in each of the genera of Enterobacteriaceae. The growing conditions are critical to their development. Film production is often connected with fimbriation, and these microrganisms do best in liquid cultures, but they often reach a mature state when grown on standard agar. Studies have shown that numerous Urinary tract infections (UTI) and Gastro Intestinal Tract (GIT) infection Enterobacteriaceae isolates carry type 1 fimbriae (Dougnon *et al.* 2020).

2.2.7 O–antigen

Lipopolysaccharide (LPS) is a glycolipid that is a significant and distinguishing ingredient of the outer membrane of most GN bacterial cell envelopes that aid in the avoidance of innate immune responses (Bi *et al*. 2018).

Lipopolysaccharide antigens withstand temperatures up to 100°C. This antigen is made up of three components: "polysaccharide, core and lipid wall" (Dougnon *et al.* 2020).

2.2.8 Motility

The flagellum is a bacterial organelle that take control of motility. The flagellum is made up of three parts: the filament, the hook, and the basal body. Motility-related functions in the bacterial pathogenesis include bacterial movement to the site of infection, colonization or invasion, and post-infection dispersal (Acharya 2019).

2.3 Most Important of Genera

2.3.1 *Escherichia coli*

In 1885, German scientist Theodor Echerich found this bacterium and isolated it from the feces of healthy people and dubbed it bacterium coli it was considered as unsatisfactory at the time (Farré-Maduell and Casals-Pascual 2019). It is responsible for a variety of illnesses acting as (diarrhea, meningitis, septicemia, soft tissue infection, and urinary tract infection) and in these conditions, it is named to an opportunistic bacteria (Li *et al.* 2019).

Escherichia coli is a type of (GN) bacterium that is only (0.35 μm) in diameter and (1.0 μm) in length and belongs to the family Enterobacteriaceae. The major habitat of this isolate in human and warm-blooded animals is the bottom section of the intestinal tract (Schönheit *et al.* 2016). *E. coli* capacity to grow and flourish in the ecosystem is possible due to inconstancy in energy gain, because *E. coli* is a heterotrophic bacterium that requires just simple nitrogen and carbon sources, in addition to phosphorus, sulfur, and other effective components in their growth. This bacteria may also decompose various aromatic compounds (such as benzoic acid and phenylacetic acid) to get energy. *E. coli* may grow aerobically and anaerobically and at a broad range of temperatures (18-45°C), with optimal growth at 37°C (Noor *et al.* 2013).

2.3.1.1 Pathogenicity of *E. coli*

Pathogenic *E. coli* strains drive a variety of illnesses in human, including gastroenteritis, newborn meningitis, uropathogenic *E. coli*, and mastitis septicemia (Adefisoye and Okoh 2016). Numerous *E. coli* strains drive the disease UTI is common among women (Lee *et al*. 2018). The most prevalent cause of Enterohemorrhagic *Escherichia coli* (EHEC) infection was O157:H7; however, subsequent conditions induced by strains of EHEC were linked to serogrouped aside from O157, like O145, O26, O111, and O103. One of the utmost prevalent bloody diarrhea reasons is *E. coli* O157, which can resolve on its own or progress to life-threatening Hemolytic Uremic Syndrome (HUS) (Sah *et al*. 2017). UTI are caused by the same bacteria and are more common in women than males because of the shorter length of the female urinary system. When bacteria colonize the urethra, they can go to the "bladder and kidneys", where they can cause pyelonephritis by producing toxins that destroy the cells lining the urinary tract (Flores-Mireles *et al*. 2015). To boost their pathogenicity and antibiotic resistance, Uropathogenic *E.coli* (UPEC) bacteria can form biofilms to hide from the human immune system (Tajbakhsh *et al*. 2016).

2.3.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae was initially reported by Carl Friedlander in 1882. The bacterium was found in the pulmonary of people who had a death of pneumonia (Friedländer 1882). Antibiotic resistance and high pathogenicity of the bacterium can appear once they have entered the human (Lenchenko *et al.* 2020). About 3–8% of all bacterial diseases are induced by *K. pneumoniae*, making it the much more prevalent cause of hospita- contracted pneumonia (Yu *et al*. 2020).

K. pneumoniae is a GN, facultative anaerobic shaped like a rod bacteria that can be found single, in doubles, or straight chains. *K. pneumoniae* is lactose-fermenting, non-motile, non-spore-forming, and oxidase negative, with a conspicuous polysaccharide capsule of substantial thickness that gives colonies on agar plates a mucoid look. *K. pneumoniae* grows swiftly on standard medium, and its colonies look big, pinkish, and

spherical on MacConkey agar, marking fermentation of lactose and acid generation (Sharma *et al*. 2015).

2.3.2.1 Pathogenicity of *Klebsiella pneumoniae*

Hospital-acquired pneumonias (HAPs) caused by *K. pneumoniae* are significantly more widespread than community-acquired pneumonia. This species is responsible for 12.8% of HAPs worldwide (Ashurst and Dawson 2018). *K. pneumonia* is a major contributor to patient mortality and morbidity due to its role as the causative agent of (HAP) and ventilator-associated pneumonia (VAP) (Feng *et al.* 2019).

This isolate is considered as normal flora in the intestine, and not with an elevated concentration that threatens human health (Manges *et al*. 2016). They are also present in the skin and mouth; these bacteria infect the lungs, leading to necrosis, inflammation, and bleeding (Ashurst and Dawson 2018).

K. pneumoniae it is an opportunistic bacterial species of the intestinal family, and it causes urinary tract infections (UTI). It is characterized by its possession of a polysaccharide bag, which is a virulence factor, which helps it avoid host defenses, and it produces urease, which helps bacteria to withstand the acidity of urine (Huynh *et al*. 2017). *Klebsiella pneumoniae* is the second-most prevalent reason for community-and hospital-associated bacteremia after *E. coli.* An increased risk of death is connected with a variety of patients factors, involving patients accepted to intensive care units which are between 65 -70 years old, have cancer, or display pneumonia, require urinary catheters or mechanical ventilation, and are alcoholics (Zammit *et al*. 2014).

K. pneumoniae isolates can cause myositis, endophthalmitis, cellulitis, necrotizing fasciitis, and abscesses in the kidneys, lungs, and neck (Maramraj *et al.* 2020). In contrast to other pyogenic liver abscesses generated by polymicrobial sources, *K. pneumoniae* isolates appear to cause preliminary liver abscesses in patients with no liver disease. Abscesses of liver are associated with an elevated risk of hematogenous spread

to other organs such as the eye and lung, as well as relatively high rates of morbidity and death (4–8 %) (Cheong *et al*. 2017).

2.3.3 Citrobacter species

In 1932, Werkman and Gillen proposed this genus. This genus is GN, straight, facultatively anaerobes that typically swim by peritrichous flagellum (Werkman and Gillen 1932). Bacteria can use citrate as their only source of carbon and produce gaseous H_2S through fermentation (Hashim and AlKhafaji 2018). *Citrobacter* genus contains 14 species, and the most common pathogen bacteria in humans are *C. youngae, C. amalonatic, C. farmeri, C. koseri,* and *C. braakii* (Poonam *et al*. 2019).

Citrobacter is present in soil, water, human and animal digestive systems. This bacterium seldom drives disease, but certain types could give kids brain infections (meningitis), blood infections (sepsis), and urinary tract infections (UTIs). These isolates have a significant role in public health as contagious opportunists in food borne diseases (Hidayatullah *et al.* 2020). Neonatal (especially preterm), as well as immunocompromised, old, or weakened patients, are at an elevated risk of infection (Tollkuci and Myers 2021).

2.3.3.1 Pathogenesis of citrobacter

Nosocomial contagions, especially those of the urinary and respiratory tracts, are usually caused by Citrobacter species, which inhabit the human alimentary tract and are often discovered in human excreta and the habitat of hospitals (Praharaj *et al*. 2016).

C. freundii is responsible for nosocomial, bloodstream, wound, brain abscesses (BA), urinary tract infections (UTIs), infective endocarditis (IE), and gastroenteritis (GE) (Hayder and Aljanaby 2019). *C. koseri* infection causes brain adhesions (BA), cerebral abscesses (CA), pneumocephalus, encephalitis, and ventriculitis (Bonasoni *et al*. 2022).

2.3.4 *Enterobacter* species

Enterobacter is a GN, fermentative, Enterobacteriaceae family bacterium. Hormaeche and Edwards were the first to define this genus (1960a). These bacteria are facultative, anaerobic rod-shaped bacteria that move through a peritrichous flagellum (typically 4–6) and ferment glucose to produce gas and acid (Ghazvini and Farsiani 2020). The capacity of the *Enterobacter* spp. to remain alive in a variety of habitat circumstances results in the spread of numerous illnesses in medical settings (Chavda *et al.* 2016). Up to now, numerous species of *Enterobacter* have been discovered *E. pulveris, E. mori, E. cloacae, E. radicincitans, E. aerogenes, E. oryzae, E. taylorae, E. dissolvans, E. turicensis, E. asburiae, E. gergoviae, E. pyrinus and E. soli*. Between these isolates, nomenclature for some species that make up the *E. cloacae* complicated (*E. ludwigii, E. hormaeche, E. cloacae, E. nimipressuralis, and E. asburiaes*) is founded on the fact that they all share similar phenotypic and, more importantly, genotypic characteristics, as determined by entire genome hybridizations. In fact, the genome of *E. cloacae* shares 60% commonality leastwise with those of the other four members of this association (Davin-Regli *et al*. 2019).

Many strains, but not all, are contagious and induce opportunistic illness in hosts with immunodeficiency (Keller *et al.* 1998). *Enterobacter* caused (UTI) disease and bacteremia, skin and soft-tissue infections (SSTI), and it was also identified as a pathogen in immunosuppressed patients; this infection depended on the health state of the patients (Yang *et al*. 2017).

2.3.4.1 Pathogenesis of Enterobacter

Enterobacter spp. is opportunism bacteria that drive diseases in the pulmonary, gastrointestinal, blood, and urinary systems, involving *Enterobacter aerogenes* and *E. cloacae* (Munny *et al.* 2021).

The bacterium *Enterobacter* is closely linked to HAIs and is the highly prevalent isolate, following *E. coli* and *Klebsiella* species (Bae *et al.* 2016).

Enterobacter is a risky pathogen, such as *E. aerogenes*, which causes urinary tract infections (Shantiae *et al*. 2022).

E. cloacae is a microbe that can generate infective endocarditis (IE), septic arthritis (SA), urinary tract infections (UTIs), intra abdominal infections (IAIs), bacteremia, and skin infections (Yadav *et al.* 2016).

2.3.5 *Proteus* species

In 1885, German microbiologist Gustav Hauser described bacteria of the genus *Proteus* and discovered that they might swarm on inanimate surfaces. *P. mirabilis* and *P. vulgaris* are two species identified by Hauser. The name comes from Homer's "Odyssey" and the character *Proteus*, who could morph endlessly (Drzewiecka 2016). *Proteus* is a rod-shaped gram-negative, facultatively anaerobic bacterium. It possesses swarming move and urease activity. Because it is considered part of Enterobacteriaceae family, known characteristics apply to this isolate: "it is actively motile, non-spore-forming, oxidase-negative, but catalase and nitrate positive, not capsulated." Certain assays, such as positive urease and deaminase tests, are required to identify *Proteus* (Ahmed 2015).

There are five named species inside the genus Proteus (*P. myxofaciens, P. hauseri, P. mirabilis, P. vulgaris, and P. penneri*), and these species are opportunism pathogens (Passat 2016).

Proteus on blood agar forms concentric zones or a film. MacConkey agar colonies are unpainted, plane, and swarm somewhat (this is specific to *P. mirabilis* and *P. vulgaris*). *Proteus* bacteria are widespread in the habitat and mammalian intestines, including humans (Abduladheem 2019).

2.3.5.1 Pathogenesis of *Proteus*

Many *Proteus* species exist, with Proteus mirabilis being one of the most important. It is classified as opportunism, which causes several diseases such as UTIs and nosocomial infections in immunodeficiency cases suffering from diverse illnesses (Hasan *et al*. 2021).

Proteus isolate, especially *P. mirabilis*, is the most significant reason of disease belonging to kidney-stone, a serious complication of problem bacteria (Norsworthy and Pearson 2017). Meningitis, Gastroenteritis (GE) are just few of the diseases linked to *P. mirabilis*. In addition, it drives infections UTI (46%), lower respiratory tract LRT (30%), and surgical injuries (24 %). Among the rarer causes of bacteremia, *Proteus* species typically affect older people (Bahashwan and El Shafey 2013).

Proteus mirabilis is common in environmental, due to its ability to degrade organic compounds; it is present in polluted soil, water, and other environments. *P. mirabilis* causes two category of UTIs: ascending and hematogenous, in which the bacteria penetrate the urinary system from the urethra to the kidney. The second category of these bacteria infection is much more typical in people with urinary catheters after UTI surgery. Following *K. pneumoniae* and *P. mirabilis, E. coli* is the frequent reason for a severe UTI (Hasan *et al*. 2021).

2.4 Resistance of Antibiotic

Resistance of antibiotic is the capacity of a microbial strain to withstand bactericidal dosages that would normally kill the majority of the microorganisms used against. Even though the importance of antibiotics, developing resistance mechanisms limits their efficacy, a severe public health problem (Abushaheen *et al*. 2020).

For decades, antibiotic resistance in morbific bacteria has been a big issue in medicinal. The evolution of defiance of antibiotic by microorganism pathogens due to of natural

selection or acquisition of resistance determinative, even when organisms were not exposed directly to antibiotics. Furthermore, both prospective and obligate pathogens gain resistance to determiners diffuse among microorganisms (Omoregbe and Fagade 2020).

2.4.1 Mechanism of antibiotic resistance

Because of the wide availability of antibiotics, multidrug resistance has been developed via several different routes of resistance. An example, Figure 2.1 bacteria can acquire resistance through various mechanisms, including de novo mutation, horizontal gene transfer (transposons, plasmids, conjugation, transformation, and transduction), and developed resistance. In overall, the mutation change antibiotics function via modify antibiotic aim reducing antibiotic absorb, or efflux pumps activation or altering the metabolic route via regulatory operons (Adhikari 2020).

The resistance genes to an antibiotic could direct bacterial isolate to construct antibiotic inhibitory enzymes or efflux techniques that stop the medication from accessing the desired target, alter the drug's target place, or create an alternate path to avoid the drug's action (Douafer *et al*. 2019).

Resistance genes from other bacteria can be acquired by conjugation, which makes use of motail genetic components similar to transposons and plasmids. Mobile gene cassettes called integrons are a site specific-recombination system that can recruit open reading frames and are a significant approach for accumulating resistance genes (Adhikari 2020).

The evolution of antibiotic defiance mechanisms and the proliferation of microorganisms with genetic determinants of resistance are considered an important danger to global public health (Tang *et al.* 2014) (Figure 2.1).

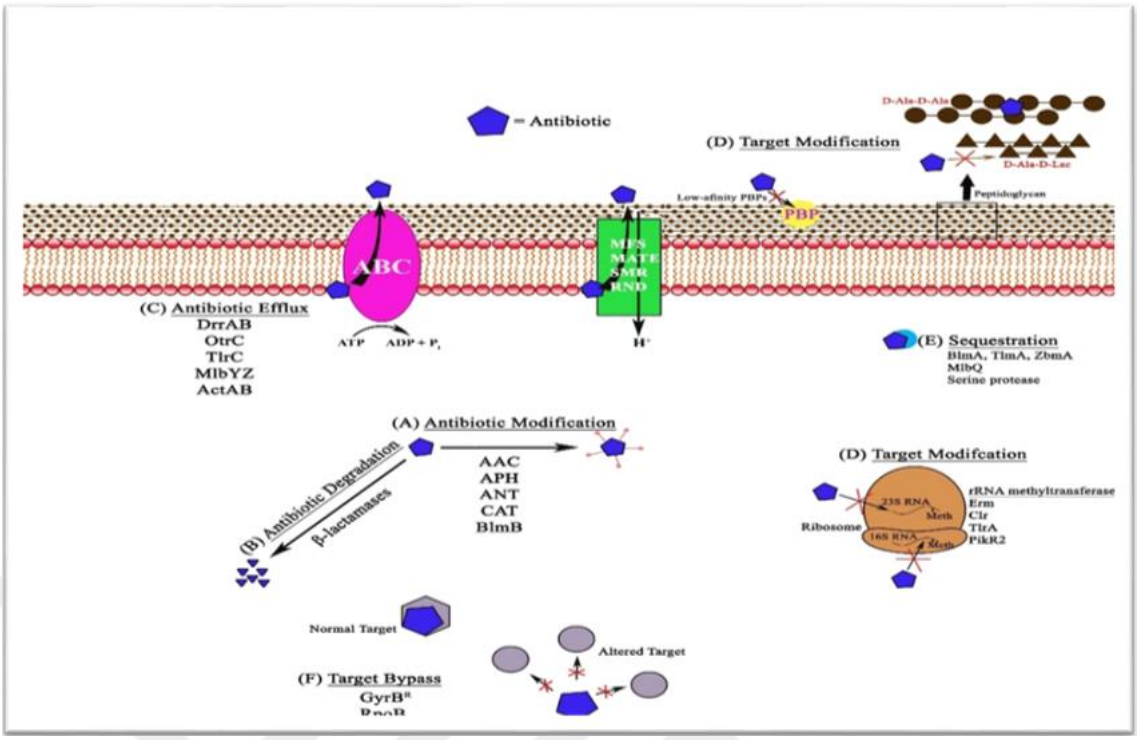

Figure 2.1 Mechanisms antibiotic resistance in bacteria (Peterson and Kaur 2018)

2.4.2 Beta-lactam antibiotics and β–lactamase

Antibiotics are organic materials generated with microorganisms that prohibit the development of different microorganisms by killing or limiting the inhibition (Coumes-Florens *et al.* 2011). Antibiotics with beta-lactam rings in their structures belong to the broad-spectrum antibiotic class known as β-lactams. It consists of (derivatives of penicillin, carbapenems, cephalosporin, and monobactams) (Caruso *et al.* 2021). Antibiotics with β -lactam work by discouraging cell wall biogenesis of bacteria; these medications are the best used for acting against bacterial diseases. By producing beta-lactamases, enzymes that hydrolyze the beta-lactam ring, bacteria develop defiance to beta-lactam antibiotic. To counteract the effects of antibiotic resistance, substances with beta-lactams, acting as clavulanic acid, can be used as a beta-lactamase inhibitor (Pandey and Cascella 2022).

Antibiotics of beta-lactam and penicillin are often recognized as the most essential medications because of their capacity to treat bacterial infections and thereby improve world health (Lobanovska and Pilla 2017).

β-lactams can kill a wide range of bacteria, and their harm to human is very vile, meaning that impedance to β -lactam antibiotics is a severe danger (Pandey and Cascella 2022).

This enzyme is encoded by genes found on the chromosome of bacteria or gained through plasmid transport (plasmid-intercede resistance) or by inducible production of the beta-lactamase gene when bacteria are exposed to beta-lactams (Bush and Bradford 2020)

To prevent the β-lactamase enzyme from affecting amoxicillin, clavulanic acid act as an inhibiter and overpower all β-lactamase enzymes. Thy have found widespread use as a treatment for healthcare-associated diseases driven by β-lactamase productive microorganisms (Tooke *et al*. 2019).

In all situations where infected beta-lactamase productive microorganism are supposed, the selection of suitable β-lactam antibiotics should be meticulously considered prior therapy. It is critical, to select effective β-lactam antibiotic for species that have any level of β-lactamase expression (Bush and Bradford 2020).

Most microorganisms that drive diseases in hospitals are immune to one or more of the antibiotics that are, statistically speaking, the most widely used. It has been discovered that some bacteria are unsusceptible to nearly all the accredited antibiotics, and the just drugs that can be used to treat them are potentially toxic. There is an ominous rise in antibiotic antagonism in bacteria that induce society-acquired diseases, such as staphylococci and pneumococci, two of the commonplace reasons of diseases and death (Kollef *et al*. 2017).

It was recently noticed high spread of bacterial impedance to diseases like *Pseudomonas, E. coli, Acinetobacter*, and *Klebsiella* (Singh *et al*. 2017).

2.4.3 Group of beta-lactam antibiotics

A- Penicillins

Penicillin and related antibiotics of beta-lactam are among the oldest and utmost used antibiotic groups. It has long been understood that part of their method of the act is to prevent the proper formation of the cell wall in bacteria (Park and Strominger 1957).

Penicillins are categorized as natural or else synthetical. Biological penicillins are extracted from cultivation solutions of Penicillium. It inhibits the growth of Gram-positive (GP), penicillinase-sensitive cells and is pH sensitivity, making penicillin G a prototype (Zango *et al.* 2019).

The antibiotics Amoxicillin, Piperacillin, Ampicillin, Methicillin, Nafcillin, Ampicillin, Penicillin V, Carbenicillin and Penicillin G belong to the group of Penicillin. As the first antibiotic ever created, penicillin G. It was first found and extracted by Alexander Fleming from the *P. notatum* fungi. Due to its limited sensitivity, penicillin G is only helpful against certain strains of GP bacteria (streptococci) and a few strains of GN bacteria (including Treponema pallidum, the bacterium responsible for syphilis) and meningococci (Etebu and Arikekpar 2016).

Penicillin structures have a thiazolidine ring that is linked to a beta-lactam ring that is connected to a side chains. Penicillins are derivatives of 6-amino-penicillanic acid; nevertheless, their side-chain structures are what set them apart. Penicillins are categorized as natural or else semi-synthetic. Growing penicillium in a lab and synthesizing just the nucleus yields semi-synthetic penicillins. To get the required R group, the penicillium is grown, raw penicillin is extracted, the R group is removed, and

the desired R group is attached in the lab. This class of penicillin has a wider range, is adequate contra GN bacteria, and is not immune to Penicillinase (Zango *et al*. 2019).

Penicillinase-resistant penicillins: This class of drugs works by adding a large side chain to the penicillin molecule, which inhibits penicillinase generated by *Staphylococcus* of entering or incoming the penicillin molecule and adhering the beta-lactam ring. Methicillin is the model for these drugs, and nafcillin is only available intravenously. Cloxacillin and dicloxacillin are only occure as orally agents, These drugs are appropriate for the cure of moderate infections of the soft tissue and skin, particularly when penicillinase productive staphylococci are identified as acting as causal factors. It has been shown or hypothesized that they are effective against penicillin-resistant *Staphylococcus aureus* infections, making them potentially useful for treating hemolytic streptococcal pharyngitis and pneumonia (Cag *et al*. 2016).

Ampicillin and amoxicillin are penicillinase sensitized, orally motivated antibiotics in whose the phenylacetic acid moiety is substituted by phenylglycine in the D-configuration. These antibiotics have a greater range than penicillin G, however they are very vulnerable to b-lactamase. To prevent enzymatic degradation, they are mostly administered with clavulanic acid (Fernandes *et al*. 2013).

B- Cephalosporins

Cephalosporins are closely related in structure to Penicillin and are by-products of 7-amino-cephalosporanic acids. They include a beta-lactams ring. They are extremely unsusceptible to penicillinase and stable in dilute acid relatively well. All cephalosporins are effective versus utmost GN cocci; the first generation includes cephalothin, cefazolin, and cephalexin.

Antimicrobial efficacy against GN bacteria is improved in the second generation, and they are more influential against GP bacteria. The third generation characterises the maximum effectiveness against GN bacteria and minimal efficacy against GP bacteria.

Because they are zwitterions, fourth-generation cephalosporins can break through the outer membrane of GN bacteria. They are also more resistant to β-lactamases than third-generation cephalosporins (Zango *et al*. 2019).

C- Monobactams

Aztreonam is an individual example of this class of drugs; it was initially extracted from *Chromobacterium violaceum* and is now produced as a synthetic antibiotic. Aztreonam demonstrates practical and distinctive vivacity versus a wide range of GN aerobic pathogens such as *P. aeruginosa*, the bactericidal impact of aztreonam because to inhibition of bacterial cell wall synthesis imputable to the increased attraction of aztreonam for penicillin tying protein (Lomovskaya *et al.* 2017).

Patients with immunoglobulin-E-arbitrated penicillin allergy could bear it without needed to interaction, and no significant toxicity has been registered, aside from skin rashes and petty aminotransferase disorders. Super-contagions by staphylococci and enterococci can happen (Fernandes *et al*. 2013).

Aztreonam, which varies from most beta-lactam antibiotics, prevent beta-lactamase activity and its molecular structure provides an elevated level of defiance to hydrolysis by beta-lactamases (like cephalosporinases and penicillinases) constructed via prevailing GN and GP microorganisms (Vandavasi *et al*. 2017).

D- Carbapenems

Carbapenem is regarded as the most effective antimicrobial agent with the widest range of activity. They are unsusceptible to hydrolysis by most β-lactamases. Carbapenems, which include Imipenem, Meropenem, and Ertapenem, are rapidly bactericidal and that cover GN bacteria that included ESBL and anaerobic microorganisms. All carbapenems are widely diffusion, and penetrate body tissues (Reddy 2022).

2.5 Extended Spectrum B-Lactamases (ESBLs)

ESBLs are groups of plasmid-interceded, various, complicated, and quickly developing enzymes that open the beta-lactam ring, leading to antibiotic deactivation. These enzymes could hydrolyze extended-spectrum β-lactams antibiotics, for instance, cephalosporin, penicillin, and monobactams (Aztreonam). These enzymes hydrolyze the (amide bond) between the carbon and nitrogen of a four-membered or (beta-lactam ring) (Figure 2.1). Still, they are controlled by death inhibitors (enzyme inhibitors) for example sulbactam, clavulanic acid, and tazobactam. The Enterobacteriaceae are produced particularly for ESBLs, especially *E. coli* and *Klebsiella* (Shaikh *et al.* 2015).

ESBLs are belonging to genes limited-spectrum beta-lactamase (*blaTEM-1, blaTEM-2, or blaSHV-1*) through modifications that change the structure of amino acidc for the active site of an enzyme. Which are naturally coded via plasmids that it is possible easily or quickly swap across bacterial types; plasmids with genes encoding ESBLs frequently also contain genes coded resistant to antimicrobial drugs, for instance, quinolones and aminoglycosides. ESBLs are organized into various configurational and developmental groups depending on amino acids sequence contrast, including *SHV, OXA, TLA, GES, BES, TEM, CTX-M, PER,* and *VEB* (Gonzalez-Padilla *et al*. 2015).

Moreover, more than (600) ESBL varieties are described, including TEM, the first plasmid-mediated beta-lactamase discovered in Greece in the 1960s and generated by gram-negative bacteria; it was termed *(TEM*) according to the patient's name (Temoniera), the associated but least common enzyme was named SHV, Because the effect of sulfhydryl reagents on substrate specificity are varied, whereas (*CTX-M*) is associated to hydrolyze cefotaxime capabilities, all *CTX-M* kinds enzymes are ESBLs, whil the *TEM* and *SHV* emerge from (point mutations) at particular residues in the natural *SHV1* and *TEM-1/TEM-2* β-lactamase (Tamma *et al.* 2015).

Beta-lactamases are categorized into four categories (Ambler classification) depending on their elementary structure: A, B, C, and D enzymes. Class A, C, and D enzymes have serine found in the active site, while category B enzyme is zinc-metalloenzyme. The

most important beta-lactamase is (*TEM-1*). It has been discovered that the existence of *TEM-1* is associated with above 90% of ampicillin resistance in *E. coli*; *TEM-1* is qualified for hydrolyzing penicillin and initial-generation cephalosporins. *TEM-2* is coming from *TEM-1* with a sole change or exchange of amino acid. The Bush–Jacoby–Medeiros classification was used to classify beta-lactamases based on enzyme functional features, such as substrate and inhibitor profiles (Al-Agamy *et al.* 2016) (Figure 2.2).

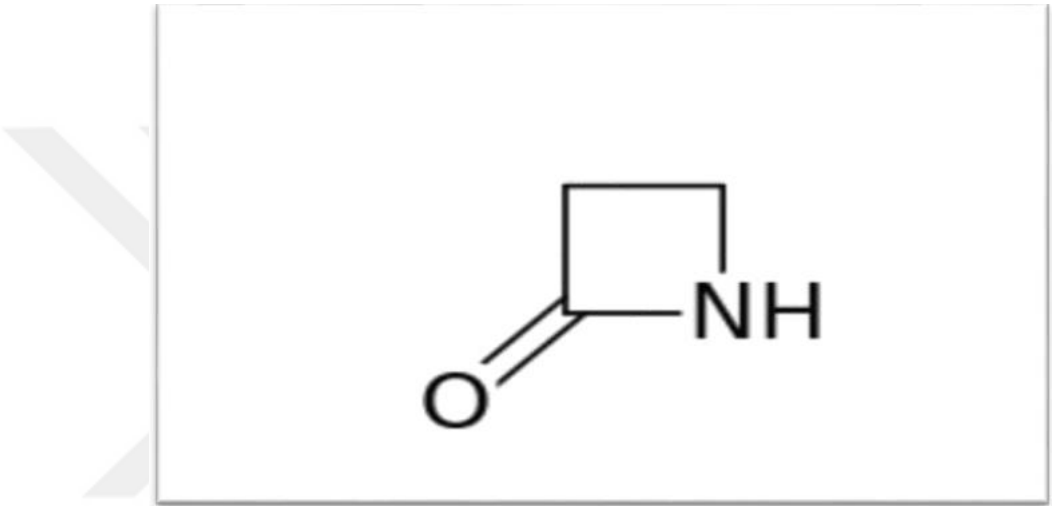

Figure 2.2 Chemical structure of a beta-lactam ring (Etebu and Arikekpar 2016)

2.5.1 Kinds of extended-spectrum β-lLactamases (ESBLs)

- **TEM**

ESBLs of the TEM kind are developed from TEM -1 and TEM -2. In 1965, an Escherichia coli strain designated TEM-1 was found in a sample taken of a sick person in Athens, Greece, called Temo Neira (hence the designation TEM) (Datta and Kontomichalou 1965). Hydrolysis of ampicillin by TEM-1 is much above cephalothin, carbenicillin, or oxacillin; however, TEM-1 is inhibited to a lesser extent by clavulanic acid; therefore, its impact on extended-spectrum cephalosporins is minimal. TEM-2 has the same hydrolytic profile as TEM-1 but a different promoter and isoelectric point (5.6 compared to 5.4) (Nagshetty *et al*. 2021).

In 1987, French researchers discovered TEM-3 in *K. pneumoniae*. The protein was first referred to as CTX-1 because of its ability to inhibit the action of cefotaxime (Nagshetty *et al*. 2021). It is now known as TEM-3, and as different to TEM-2, which is a replacement from two amino acid (Ali *et al.* 2018).

There are exceeding one-handed TEM type beta-lactamases. Besides the peculiarity of *TEM-1* and *TEM-2*, they are all ESBLs. The much frequent TEM kind ESBL is discovered in *E.coli* and *K.pneumoniae*. They may, however, be located in other GN bacteria as well as in various Enterobacteriaceae genera such as "*E.aerogenes, E. cloacae, Morganella morganii, P. mirabilis* and *Salmonella spp."*. They are found in *P. aeruginosa* of the Non-Enterobacteriaceae (Ghafourian *et al*. 2015).

- **SHV Type of (ESBLs)**

The SHV is more common in clinical isolates of bacteria than the other types of ESBLs (Ghafourian *et al*. 2015). In 1983, SHV-2 was isolated *Klebsiella ozaenae* isolate in Germany has been discovered to have a b-lactamase that is competently hydrolyzed cefotaxime and to a smaller degree, ceftazidime (Messaoudi *et al*. 2019).

In contrast to TEM kind beta-lactamase, fewer SHV kinds of beta-lactamase originated from SHV-1. Most strains have SHV on their plasmid to exchange serine for glycine at location 238. Similarly, at place 240, some strains change lysine to glutamate. Ceftazidime hydrolysis requires the presence of a serine at position 238, and cefotaxime hydrolysis involves the presence of lysine at position 240. Over a hundred distinct types of SHV have been identified thus far. Multiple species of the family Enterobacteriaceae produce ESBLs of the SHV type. Including *Acinetobacter spp.* and *P. aeruginosa* (Ghafourian *et al*. 2015).

2.6 Polymerase Chain Reaction (PCR)

In 1983, scientist Kary Mullis conceived the PCR; it uses fundamental biochemistry to amplify a bit or small bits of DNA. The objective is to amplify a particular portion of DNA that includes diagnostic or healing data. DNA amplification happens following the PCR reaction mix, which includes the DNA template and primers (short DNA nucleotides of four nitrogenous bases and the existence of the enzyme DNA polymerase thermally steady and magnesium chloride, in addition to nuclease-free water eppendorf tube) (Khalil 2021).

The basic steps of the reaction of the PCR including:

- **Denaturation:** an incubation at high temperatures (90-98°C) that melts the double-stranded DNA that contains the target region into separate strands.
- **Annealing:** temperature reduction (usually to 37-65°C) to permit two synthetic oligodeoxyribonucleotides (primers) to anneal to locations flanking the area of DNA to be amplified. Primers are counted for the reaction admixture extremely from molars to promote the configuration of the primer-target complex instead of renaturation of the target DNA, primers anneal into opposed DNA strands, with their 3' ends facing each another.
- **Extension:** The ideal temperature (72°C) for the DNA polymerase enables the separation of deoxynucleotides (dNTPs) from the interaction admixture of the 5' to 3' synthesized DNA headed from the primers and uses the target DNA serving as a template. The primers are integrated into the PCR direct product through this process (Cahová *et al.* 2016).

Types of Polymerase Chain Reaction (PCR)

- Standard PCR
- Variants Reverse Transcription-PCR (RT-PCR)
- Real time-PCR or quantitative PCR (qPCR)

- RT-PCR/qPCR combined (Singh *et al.* 2014).

3. MATERIALS AND METHODS

3.1 Materials

The main equipment and laboratory apparatus utilized in this study are illustrated in (Table 3.1).

Table 3.1 Equipment and laboratory apparatus

NO.	Apparatus/Equipment	Manufacturing
1	Autoclave	Japan
2	Incubator	Optima / Japan
3	Air laminar hood	German
4	Refrigerator & Freezer	Ishtar/ Iraq
5	Shaker incubator	Optima /Japan
6	Micro centrifuge /centrifuge	Eppendorf/ Germany
7	Microscope	England
8	Electrical balance	Switzerland
9	Eppendorf tubes	U.S.A
10	Microwave	England
11	PCR analysis unite	Bio-Rad/ USA
12	Gel analyzer	Bio-Rad/ USA
13	Shaker incubator	Optima /Japan
14	Pyrex bottles	England
15	Petri dishes	India
16	pH meter	German
17	Microcentrifuge tubes	Eppendorf
18	Loop	Himedia India
19	Cotton Swap	India
20	Vitek 2 Compact System	Biomeriux / France
21	Micro pipette	China
22	Flask, Beaker glass, Cylinder	Germany
23	Vortex	German
24	water-bath	Japan

The main biological and chemicals substances utilized in this study (Table 3.2)

Table 3.2 Chemicals and biologicals substances

No.	Chemicals and biological materials	Manufacturing Origin
1	Agarose	Promega (USA)
2	Glycerol Millipore	Sigma(German)
3	Buffered Solutions (PH 4,7,10)	Cryson(German)
4	DNA ladder 100bp	Promega USA
5	Nuclease free water	Promega / USA
6	Blue loading dye 6X	Promega / USA
7	Ethyl alcohol	BDH / England
8	Ethidium Bromide dye	Promega / USA
9	Isopropanol	Sigma Aldrich(German)
10	Distel water	England
11	Grame stain	BDH- England
12	Deionized Distilled water (ddH2O)	Bioneer -Korea

3.1.1 Media culture

Culture media employed in this study (Table 3.3)

Table 3.3 All cultural media using in the study

No.	Media culture	Manufacturing company
1	MacConky Agar	HiMedia–India
2	Eiosin Methylene Blue (EMB)	HiMedia–India
3	Brain Heart Infusion Broth	HiMedia–India
4	Nutrient Agar, broth	HiMedia–India
5	Muller-Hinton agar, broth	HiMedia–India
6	Xylose – Lysin – Deoxycholate (XLD)	HiMedia–India
7	Salmonella – shigella agar (SS) agar	HiMedia–India
8	Simmon Citrate agar	HiMedia–India
9	Methyle Red/Vogas proskaur	HiMedia-India
10	Urea agar base	HiMedia–India
11	Pepton water medium	England (Oxoid)
12	Kligler's iron agar	HiMedia–India

3.1.2 Antibiotic disk

The antibiotic discs used in the study are shown in Table 3.4.

Table 3.4 Antibiotics instrument used in the study

No.	Beta lactam group	Antimicrobial Agent	Symbol	Disk Concentration (µg/disk)	Company And Origin
1	**Penicillins**	Penicillin	G P	10	Liofilchem (Italy)
		Amoxicillin	AML	25	Liofilchem (Italy)
		Amoxicillin–clavulanic acid	AUG	30	Liofilchem (Italy)
2	**Cephalosporin**	Cephalexin	CL	30	Liofilchem (Italy)
		Cefixime	CFM	5	Liofilchem (Italy)
		Cefotaxime	CTX	30	Liofilchem (Italy)
		Ceftazidime	CAZ	30	Liofilchem (Italy)
		Cefoxitin	FOX	30	Liofilchem (Italy)
3	**Carbapenems**	Imipenem	IMP	10	Liofilchem (Italy)
		Meropenem	MRP	10	Liofilchem (Italy)
4	**Monobactams**	Aztreonam	ATM	30	Liofilchem (Italy)
5	**Tetracycline**		TC	45	Liofilchem (Italy)

3.1.3 Kits

The main kits used in the study are shown in Table 3.5.

Table 3.5 The main kits used in this study are listed

No.	Type of Kits	Company / Country
1-	Wizard DNA extraction kit	Geneaid /Taiwan
2-	VITEK-2 system	BioMerieux (France)

3.1.4 Primers

The main Primers used in the study are shown in Table 3.6.

Table 3.6 The main Primers used in this study are listed

Genes	Primer name	Sequence 5'-3	Size (bp)	Reference
bla_{TEM}	TEM-F	AGTGCTGCCATAACCATGAGTG	431	(Kim *et al.* 2009)
	TEM-R	CTGACTCCCC GTCGTGTAGATA		
bla_{SHV}	SHV-F	GATGAACGCTTTCCCATGATG	214	(Kim *et al.* 2009)
	SHV-R	CGCTGTTATCGCTCATGGTAA		

3.2 Methods

3.2.1 Collection of bacterial isolates

This study was accomplished in the laboratories of the food contamination research center/environmental and water directorate/ministry of high education and Scientific research and science and technology. A total of 80 isolates of clinical and 54 of environmental isolates of raw water were taken from the Tigris river and from various sources (Baghdad Medical City, Ghazi Hariri General Hospital, Karkh Maternity Hospital, and Nuaman General Hospital) and clinical samples were obtained from different laboratories in Baghdad for a period of six months (from November 2021 to April 2022) (APPENDIX 1).

3.2.2 Clinical samples

Eighty clinical isolates were obtained from some laboratory in Baghdad province in Iraq. All isolates were obtained from diverse clinical specimens of hospitalized patients with different diseases (urine infection and burn infection). Isolates were activated by nutrient broth and then isolates were distributed on Nutrient agar, MacConkey agar, and

Eosin-methylene blue agar (EMB), The dishes were put in an incubator overnight at 37°C for 24 hrs behind incubation cultures were inspected for different colonies.

3.2.3 Environmental samples

Raw water samples were taken from 20 to 30 cm below the surface of the Tigris river, and put in clean, sterilized glass bottles with a capacity of 1000 mL. All of these bottles were meticulously sealed and taken to the lab on ice, where they were stored at 4°C and used separate sterile pipettes, decimal dilutions of 10^{-1}, 10^{-2}, 10^{-3} were prepped, of raw water by moving 10 mL of sample to 90 mL of sterile water.

To identify Enterobacteriaceae bacteria, taken 1 mL from water diluted and used a sterile L-shaped glass rod (spreader) was used to disseminate different dilutions of water samples and distributed on MacConkey, after that working a sub-cultures on the Nutrient agar, and Eosin-methylene blue agar (EMB), Xylose – Lysin–Deoxycholate (XLD) and (SS) agar to determine the bacteria. The dishes were put in an incubator overnight at 37°C for 24 hrs, following incubation, cultures were inspected for different colonies, and incubated at 44°C for 24–48 h to fecal coliform bacteria (FCB).

3.2.4 Methods of sterilization

3.2.4.1 Moist heat sterilization

The all culture media, tips, Eppendorf tubes and solution were sterilized via autoclaving at 121°C for 15 minutes beneath 1.5 bar pressures.

3.2.4.2 Dry heat sterilization

Glassware was sterilized in an oven at 180°C for two hours.

3.2.4.3 Direct sterilization

The forceps tip and inoculating loop have been sterilized using a Bunsen burner flame.

- **Preparation of Culture Media**

Ready to used media

All media employed in this study were prepped in the following manner: pH was adjusted as directed; autoclaved at 121°C for 15 minutes at 15 pounds/square; and then distributed as needed in Petri dishes or tubes, put in an incubator at 37°C for 24 h to guarantee sterility, and stored at 4°C until use.

MacConkey agar

This media was created by solvating 49.53 g in 1000 mL of D.W. It was heated for five minutes to dissolve thoroughly before being sterilized in an autoclave at 121°C for 15 minutes. The prepped medium was chilled to 45°C to 50°C. The medium was then placed into a sterile petri plate until it hardened.

Eosin methylene blue (EMB) agar

37.46 g of this medium were dissolved in 1000 mL to D.W. heated, and sterilized by autoclaving at 121°C for 15 minutes. After cooling to 50°C, pour onto a sterile petri dish and let aside to harden.

Nutrient agar

These media were prepared as reported by the manufacturer's instructions before being autoclaved at 121°C for 15 minutes.

Nutrient broth

It was made in accordance with company-processed instructions. It is utilized for bacterial isolate growth and preservation.

Salmonella and Shigella agar (SS agar)

Dissolve 63.02 g in 1000 mL purified/distilled water. To completely disband the media brings to a boil with continual stirring. Do not overheat or autoclave. Overheating the medium may reduce its selectivity. Allow cool to around 50°C. After mix transfer to sterile Petri dishes.

Xylose–Lysin–Deoxycholate (XLD) agar

Dissolve 56.93 g in 1000 mL purified/distilled water. To completely disband the medium brings to a boil with continual stirring. Do not overheat or autoclave, Transfer immediately to a 50°C water bath. Pour into sterilized Petri plates after chilling.

Brain heart infusion broth

A specified amount of BHI broth was dissolved in distilled water and stirred with the heat until it was completely dissolved. It was then separated into 10 mL flat tubes for autoclave sterilization at 15 psi and 12°C. After then, it was chilled and stored refrigerated before use. This medium was used to test the activation of bacterial culture.

Kligler's iron agar

57.52 g in 1000 mL purified/distilled water bring to a boil to thoroughly disband the media. Mix nicely and distribute into tubes. Autoclave for 15 minutes at 15 pounds pressure (121°C). Permit the tubes to cool in a tilted position to form slopes with 1 inch butts.

Medium of peptone water broth

It was made by solvating 20g of peptone and 5g of sodium chloride in 85 0mL of distilled water, then increasing the volume to 1000 mL following adjusting the PH to 7.2; 5mL of the media was divided into autoclavable screwcap test tubes, which were utilized to notice indole produced (MacFaddin 2000).

Urea agar base media

This medium was prepped as reported by the factory instructions for it by disbanding 24 g of urea agar in 950 mL of distilled water, then adjusting the pH to 7.2, then sterilizing the mixture in an autoclave then letting it cool to a temperature of 50°C, then adding 50 mL of 40% solution of urea using. Millipore filter with a diameter of 45.0 μm, then 15 mL was poured into each tube diagonally, and after solidification, it was kept at a temperature of 4°C. The potential for urease production in this medium was studied (Forbes *et al*. 2016).

Simmons citrate test agar

This medium was made as reported by the manufacturer's instructions by disbanding 28.24 g in a liter of distilled water, sterilizing with a sterilizer, then cooling to 45-50°C, pouring into clean, sterilized test tubes at an angle, and storing in the refrigerator until use. Bacteria tolerance to citrate as a sole carbon provider was evaluated in this media (MacFaddin 2000).

Methyl red (Voges-proskauer media)

For our preparations, we followed the package guidelines and disbanded 7 grams of peptone, 5 g of glucose, and 5 g of K_2HPO_4 in 950 mL of distilled water, before correcting the pH to 7.2 and raising the volume to 1000mL. The disinfected medium was transferred to 5 mL sterile test tubes **(**Hemraj *et al*. 2013).

- **Bacterial Isolates Maintenance**

The preservation of bacterial isolates was accomplished in accordance with (Atlas *et al*. 1995).

Short-term storage

MacConkey agar medium was streaked with Enterobacteriaceae isolates and put in an incubator for 24 h at 37°C before being kept at 4°C for two weeks.

Intermediate storage

Nutrient slant agar was inoculated by Enterobacteriaceae isolates and put in an incubator at 37°C for 24 h before being kept at 4°C.

Long term storage

The bacterial isolates were kept at -20°C for an extended period of time in Luria Bertain (LB) broth containing 20% glycerol at -20 °C (deep freezing). The final amount was poured into a tiny screw-capped container sterilized by autoclaved. After chilling, 500 µL of the preceding solution is added to Eppendorf tubes, and the medium was injected with pure isolated colonies of each isolate. The Eppendorf tubes were then carefully wrapped in paraffin film and kept at -20°C.

- **Laboratory Diagnosis**

Morphological examination

Studied of colonies on diverse media (Nutrient agar, EMB, and MacConkey agar) is researched in terms of colony form, color, and margin, as stated by (Begum *et al*. 2006).

Microscopic examination

Gram staining is employed to detect the distinctive morphology of the bacteria in the suspected colonies by using light microscope. Gram stain is applied to bacterial isolates in order to study their form under a light microscope. A little part of the reported positive culture colony was put on a clean, microscopic glass slide. The gram staining process is used, and all slides are then examined with oil emersion (Froböse *et al*. 2020).

Biochemical tests

According to (MacFaddin 2000) done as follows:

Indol test

Bacteria are added to a tryptophan broth medium and incubated for 24 h at 37°C. When a few drops of Kovac's reagent are applied to the tube, the creation of a pink-wine colored ring indicates a favorable result.

Methyl red test

MR-VP is prepared in a plane tube as reported by the manufacturer's instructions and injected with bacterial culture and put in an incubator for 24 h at 37°C and added 5 drops of methyl red reagent. A vivid red color formation indicated a favorable result; the result was read shortly after mixing.

Voges-proskauer test

Bacterial culture is put into MR-VP broth medium and then put in an incubator at 37°C for 24 h. The alpha-naphthol solution and the KOH solution were each placed in small

amounts and well combined. After 15 minutes, there was still no indication of success in the form of a pinking of the medium.

Urease test

This test has been performed to determine these isolates' capacity to divide urea into two ammonia molecules by the activity of the enzyme urease, and resulting alkalinity. Furthermore, a pure culture of 18-24 h has been streaked onto the urea agar slants, and the tubes have been put in an incubator for 24 h at 37°C. The medium's hue changing from yellow to pink indicates the presence of urease.

Simmon citrate test

Slant are contain simmons citrate medium that are inoculated with overnight to activate bacteria Then it was kept at 37°C for two or three days. It is considered a favorable outcome when the media color changes to blue (Cheng *et al*. 2012).

Triple sugar iron test (TSI)

TSI slants were inoculated with bacteria using the stabbing and streaking technique (the loop stab must reach the tube bottom), the isolates were labeled, and they were put in an incubator overnight at 37°C. If the phenol present in the TSI medium was red, the entire medium became yellow. It indicates that the bacteria examined can ferment entire sugars like glucose, sucrose, and lactose. When bubbles and fissures appeared in the medium, it was indicated producing the gas. Furthermore, when the medium became black, it indicated the possibility of H_2S production (Islam *et al*. 2017).

- **Antibiotic Susceptibility Testing**

Mueller-hinton agar preparation

MHA was prepared from a dehydrated as reported by the manufacturer's directions. The medium was chilled to 50°C before being poured into the plates. The plates were dried for immediate use at 35°C for 30 minutes after the agar is solidified by placing them in an upright situation in the incubator with the lids inclined.

Inoculums preparation (Turbidity standard)

3-5 nutrient agar colonies are transferred to 0.85% NaCl tubes, mixing well, and comparing to McFarland tube to get culture with 1.5×10^8 Cell/mL, and adjusting to McFarland 0.5 turbidity standard, suspensions were utilized within 30 minutes after preparation.

Test plates inoculation

- Inoculate Mueller-Hinton agar with the bacterial suspension using a sterile cotton swab, and let the plates dry.
- Used sterile forceps and transferred the antimicrobial discs to the surface of agar.
- The plates were then put in an incubator for 24 h at 37°C

Reading the results

The diameter of each antibiotic disc's inhibitory zone has been measured using a ruler, and the findings are classified as resistant (R), susceptible (S), or intermediate (I), in comparison to the standard inhibition rate recommended by the standard decided by the clinical and laboratory standards (Table 3.7).

Table 3.7 Zones diameter interpretation standards

No	Antimicrobial Agent	Symbol	Disk Concentration (µg/disk)	Diameter of Inhibition Zone (mm)		
				R*	I*	S*
1	Penicillin	G P	10	≤ 13	14-16	≥17
2	Amoxicillin	AML	25	≤ 13	14-17	≥ 18

3	Amoxicillin–clavulanic acid	AUG	30	≤ 14	15-19	≥ 20
4	Cephalexin	CL	30	≤ 14	15-17	≥ 18
5	Cefixime	CFM	5	≤ 15	16-18	≥ 19
6	Cefotaxime	CTX	30	≤ 22	23-25	≥ 26
7	Ceftazidime	CAZ	30	≤ 20	-	≥ 21

Table 3.7 Zones diameter interpretation standards (Continued)

No	Antimicrobial Agent	Symbol	Disk Concentration (µg/disk)	Diameter of Inhibition Zone (mm)		
				R*	I*	S*
8	Cefoxitin	FOX	30	≤ 14	15-17	≥18
9	Imipenem	IMP	10	≤ 20	21-24	≥ 25
10	Meropenem	MRP	10	≤ 14	15-17	≥ 18
11	Aztreonam	ATM	30	≤ 17	18-20	≥ 21
12	Tetracycline	TC	45	≤ 11	12-14	≥ 15

- **VITEK2 compact system**

The Vitek 2 compact has been utilized to verify the collaboration of Enterobacteriaceae isolates. Vitek 2 is an autonomous technology that represents a smarter technique for diagnostic testing in microbiology. It allows automated, quick, and consistent validation of test findings.

Materials:

- Normal saline.
- Polystyrene test tube (kan tube).
- Vitek 2 GN ID Card.
- Sterile sticks.
- DensiCHEK plus (turbidity meter).
- Vortex

Method

- Using an applicator stick, morphologically identical single colonies are transferred from pure bacterial culture and suspended in 3mL of normal saline (0.45% NaCl, 4.0-7.0 PH).
- The turbidity was measured with a Vitek2 turbidity meter called the DenseCHEK plus to attain the requisite turbidity.

The suitable reading range from 0.50 to 0.63.

- A Kan tube containing microorganism suspension containing was placed in the cassettes in the well in front of the ID Card, and the transfer tube unit was inserted into the Kan tube.

Vitek2 cassette is able to accommodate up to ten tests.

- Finally, the filled cassette was placed in the cassette chamber station.

The result is available within 4–6 hours.

Determination of *TEM* (bla_{TEM}) and *SHV* (bla_{SHV}) genes using multiplex PCR among Enterobacteriaceae isolates:

The multiplex PCR was adopted to detection *TEM* (bla_{TEM}) and *SHV* (bla_{SHV}) the genes of Enterobacteriaceae isolates in the DNA samples of these isolates.

Extraction of genomic DNA

Utalizing Wizard® Genomic DNA Extraction Kit supplemented by (Geneaid / Taiwan) to extract genomic DNA.

DNA extraction protocol

The genomic DNA is abstracted from the *Enterobacteria* isolates utilizing a commercial wizard genomic DNA purification kit (Geneaid/Taiwan) using the instruction of the manufacturer, with little modifications as the following protocol:

- Transfering bacteria cells (up to 1×109) to a 1.5 mL microcentrifuge tube.
- Centrifuge for 1 minute at 14.000 -16.000 × g, after that toss the supernatant.
- Add 180 µL of GT Buffer afterwards re-suspend the cell pellet by vortex or pipette.
- Adding 20 µL of Proteinase K (make sure dd H2O was add). Incubate at 60°C for at least 10 minutes. Amidst incubation invert the tube every 3 minutes.
- Adding 200 µL of GT Buffer to the specimen and mix by vortex for 10 seconds. Incubate at 70°C for at a minimum10 minutes to insure the sample lysate is obvious. Amidst incubation, invert the tube every 3 minutes.
- Pre-heat the required Elution Buffer (200 µL pre-sample) to 70°C (for step 5 DNA Elution).
- Following 70°C incubation, add 5 µL of RNase A (50mg/mL) to the clear lysate after that shake vigorously. Incubate at room temperature for 5 minutes.
- Placing 200 µL of pure ethanol to the specimen lysate and mixed directly by shaking vigorously. If precipitate shows, fragment it up as much as possible with pipette.
- Add GD column in a 2 mL collection tube. transport of admixture (containing any insoluble precipitate) to the GD column after that centerfuge at 14-16.000×g for 2 minutes.
- Toss the 2 mL Collection Tube having the flow–through afterwards place the GD Column in a new 2 mL Collection tube.
- Putting in 400 µL of W1 Buffer to the GD Column. Centrifuge at 14-16.000×g for for 30 second after that toss the flow-through. Add the GD Column back in the 2 mL Collection tube.
- Placing 600 µL of Wash Buffer (make sure ethanol was placed) to the GD column. Centrifuge at 14-16.000×g for 30 seconds, and after that toss the flow-through. Add the GD Column back to the 2 mL Collection tube. Centrifuge also for 3 minutes at 14-16.000×g to dry the column matrix

- Transmitting the dried GD Column to a clean 1.5 mL micrcentrifuge tube. Place 100 µL of pre-heated Elusion buffer, TE buffer or water into the center of the column matrix. Allow stand for at least 3 minutes to permit Elusion buffer. TE buffer or water to be fully absorbed. Centrifuge at 14-16.000× g for 30 seconds to elute the purified DNA.

Measurement of DNA concentration and purity

The Nanodrop technology has been identified in the assessment of DNA concentration and purity employing 2µL of both DNA samples. The purity was determined using the following procedure: DNA purity=O.D260/O.D280. The determined ratio was obtained from the operating software and shown by the Nano-drop spectrophotometer (Gallagher 2017).

Multiplex PCR

Genes from Enterobacteriaceae isolates were amplified by PCR using forward primer and reverse primers sequences. Table 3.8 contains the primer sequences that were employed in this work. Lyophilized primers were provided, dissolved in nuclease-free water to provided final solution concentration of 100 pmol/µL as recommended by the provider, that is a stock solution, then made a worked solution by transferring 10 µL from the stock solution to 90 µL of nuclease-free water to make a concentration of 10 pmol/µL and stored in a freezer until use.

Preparation of Multiplex PCR mixture

The target fragment gene was amplified utalizing PCR using particular primers, and DNA (previously extracted) was utilized as a template in addition to master mix and Nucleas free water. The mix final up to 25 µL as shown in the Table 3.8.

Table 3.8 The 25 µL final volume of the Multiplex PCR reaction admixture contains all necessary constituents

NO.	Components	Volume
1	Green Master mix (2X)	12.5 µL
2	Forward primer	1.25 µL
3	Reverse primer	1.25 µL
4	DNA template	5 µL
5	Nuclease-free water	5 µL
6	Final volume	25 µL

Multiplex PCR programs

The following program has been operated to amplify the *bla$_{TEM}$-bla$_{SHV}$* genes as illustrated in Table 3.9.

Table 3.9 Conditions multiplex PCR reaction after optimization used in genetic diagnosis using the initiator *bla$_{TEM}$-bla$_{SHV}$*

Gene name	Optimization	Temp. °C	Time	No. of cycle
bla$_{TEM}$-bla$_{SHV}$	Initial denaturation	95	3 min	
	denaturation	95	30 sec	30
	Annealing	58	30 sec	
	Extension	72	60 sec	
	Final extension	72	10 min	
	Hold	4°C	10 min	

- **Agarose Gel Electrophoresis**

Gel electrophoresis has been utilized to define DNA and PCR outcome, which were then visualized with an assisted ethidium bromide and a (UV transilluminator) documentation system.

- **1X TBE Electrophoresis Buffer**

1X buffer has been made by diluting 100 mL of 10X stock to water to 900 mL with deionized water.

Agarose Gel Preparation (Ezeokoli *et al*. 2016)

- The volume of a 1X TBE 100 mL was taken in the beaker.
- Powder of Agarose (0.7 g for genomic DNA detection and 1.5 g for PCR product detection) is placed to the buffer.
- The solution was microwaved to boiling till all of the gel particles were disbanded.

- Added Ethidium Bromide 5 µL of 10mg/mL to the agarose solution. Because ethidium is a potent mutagen, gloves and a mask should be worn at all times during the job.
- The mixture is transferred on a tray with a good comb and left at room temperature for 30 minutes to polymerization.
- The agarose is swirled to mix it and avoided bubbles from forming.
- The solution was left to cool at 50–60°C the comb was removed when the agarose has been solid.

3.3 Statistical Analysis

The Statistical Analysis System (SAS 2018) program was used to detect the effect of difference factors in study parameters. Least significant difference–LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

4. RESULTS AND DISCUSSION

The results in this study revealed high level of pollution at different sites, it includes the first site near Karkh Maternity Hospital, the second site near Ghazi Hariri General Hospital, the third site near the Baghdad Medical City, and the fourth site near Nuaman General Hospital. In location 3 was the highest pollution while in location 1, 2 and 4 is the lowest average of pollution, also we notice raise of total plate count (TPC) average value in autumn and spring season. Where there was a high significant difference ($P \leq 0.01$) between the third site and the other sites and we did not find a significant difference between the first, second and fourth site ($P \leq 0.05$), and the difference in results between seasons is due to difference in temperature. The tempreture is most important factor in an ecosystem is temperature, and water temperature is an important factor in river ecology because of its impact on water quality (Abbas and Hassan 2018).

The reason for the high rate of (TPC) in the third site is due to the high rate of water for hospital sewage. Hospital sewage poses a threat to public health because of its highly infectious potential and role in pathogen dissemination into the environment, These effluents transport drugs, antibiotics, disinfectants, anaesthetics, heavy metals, and nonmetabolized pharmaceuticals into the environment, as well as pathogenic microorganisms like viruses, bacteria, fungi, protozoans, and helminthes (Santoro *et al.* 2015) (Table 4.1)

Table 4.1 The monthly dilution of raw water obtained from different sites near Iraqi hospitals during the study period

Month	Monthly total plate count ($\times 10^3$) Cell/100 mL				LSD value
	Set.NO1	Set NO.2	Set NO.3	Set NO.4	
Nov.2021	29 ±2.5	14±0.73	200±12.5	14±0.78	37.52**
Dec.2021	21±1.8	13±0.81	130±8.9	10±0.53	17.67**
Jen.2022	19±0.87	9±0.47	150±11.4	11±0.61	22.70**
Feb.2022	70±4.9	23±1.7	170±14.1	12±0.77	27.54**
Mar.2022	35±2.8	10±0.76	157±11.7	13±0.85	19.02**
Apr.2022	45±3.1	13±0.85	138±9.2	11±0.63	16.44**
LSD value	13.47 **	8.54 *	20.07 **	5.17 NS	---
*(P≤0.05), **(P≤0.01).					

4.1 Isolation and Identification

The current study included investigation of the genera of intestinal bacteria in 134 different environmental and clinical isolates for the duration from (November 2021-April 2022).

The specimens are cultured according to bacteriological methods in the Petri dishes and used different agars (MacConky Agar-Eiosin Methylene Blue (EMB)-Nutrient Agar-Xylose–Lysin–Deoxycholate (XLD) and (S-S) agar). The dishes were incubated upturned aerobically at 37-44°C for 24 hours.

4.1.1 Clinical samples

In this work, Eighty clinical isolates were collected from different samples that included (urinary and burns) of different ages from laboratories in the city of Baghdad, Samples (urine and burns) were obtained by transferring urine samples through transferred media (Brain Heart Broth) and samples burns by circulating a sterile swab in tanks and placing it in transport media then collecting it in an ice box and diagnostic culture tests were performed on them.

The investigated types are diagnosed as preliminary depending on the phenotypic characteristics and the microscopic examination. And the biochemical properties, then the diagnosis were verified using the VITEK-2 System for Identification of the isolates.

The results showed in Table 4.2 that *E. coli* and *K. pneumonia* were common bacteria among the urinary tract infection (UTI) isolates with rates of 41.25% and 25%, respectively, followed by *P. mirabilis* with a rate of 23.75%. We find a high significant difference ($P \leq 0.01$) in the urine compared to the burn sampels.

E. coli is the most common type of bacteria isolated from UTIs with a rate of 41.25%, and the results by Seifu and Gebissa were similar to the results of this study, as the rate of *E. coli* isolation is 39.3% (Seifu and Gebissa 2018).

As for the results of (Duffa *et al*. 2018), they are similar to the outcome the current study in terms of the rate of isolates of *E. coli* and *K. pneumonia*, at a rate of 45.9% and 27.9%, respectively, and different with the rate of isolates of *P. mirabilis*, where the rate of 4.9% (Duffa *et al*. 2018).

The results of (Ejerssa *et al*. 2021) show similar results to the results of the present work with to *E. coli* and *P. mirabilis* at a rate of 45.2%, 22.6%, respectively, and different with to isolate *K. pneumoniae* by 16.1%. As for the isolation of Burn swabs, the consequences of this work were comparable to the consequences of (Richcane *et al.* 2017) and regarding the isolation of *K. pneumonia* at a rate of 14.0% and did Not compatible with the consequences of the (Akaa *et al.* 2020), where the average of isolates 3.03% (Table 4.2).

Table 4.2 Different bacterial isolates were obtained from different clinical specimens

Type of Bacterial isolets	**Types of clinical specimen**				**Chi-Square (χ^2)**	**Total of bacterial isolates NO**.
	Urine	**%**	**Burn swabs**	**%**		
E.coli	33	41.25	0	0	12.630 **	33
Klebseilla pneumonia	20	25.00	8	10	5.493 *	28
Proteus mirabilis	19	23.75	0	0	8.021 **	19
Total	72	90	8	10	16.59 **	80
Chi-Square (χ^2)	--	5.037 *	--	4.33 *	---	---
*(P≤0.05),** (P≤0.01)						

The cause for the higher rate of isolation of *E. coli*, which causes (UTI), is higher than that of the rest of the Enterobacteriaceae. This bacteria is part of the normal flora in the intestine, and this enables it to enter different parts of the body as well as places of infection for instance, urinary tract infection and wound infection. And respiratory tract infection due to its virulence factors such as production of β- hemolysin and biofilms

and resistance to beta-lactam antibiotics and this enables it to persist and induce many diseases (Kukanur *et al*. 2015).

The cause for the presence of *K. pneumonia* in the urine that causes urinary tract infections in the elderly is the use of catheters for a long period of time, especially in patients who stay in hospitals for a long time (Liu *et al.* 2020).

Proteus mirabilis, the reason for its diffusion is due to the phenomenon of (motility swarming) and the activity of the enzyme urease, which often causes urinary tract infection associated with catheters, and the infection of the urethra may develop into the formation of kidney stones or bladder stones (Yuan *et al*. 2021).

4.1.2 Environmental samples

Fifty-four of environmental isolates of raw water have been taken from the Tigris River and from various sources (Baghdad Medical City, Ghazi Hariri General Hospital, Karkh Maternity Hospital, and Numan General Hospital).

The studied species were diagnosed as a preliminary diagnosis based on the phenotypic characteristics, the biochemical properties, and the microscopic examination, then the diagnosis was affirmed utilizing the VITEK-2 System for Identification of the isolates.

The results shown in Table 4.3 *E. coli* was the highest species, with a total of 24 isolates at 44.44%, followed by *K. pneumonia* with 31.48%, *P. mirabilis* with 7.40%, *E. aerogenes* rate was 12.9%, and both (*C. freundii* and *C. kosri*) at 1.85%. This shows a high significant difference ($P < 0.01$) between *E. coli* with the rest of the isolates.

The outcomes of this work are in accordance with a work conducted in India on hospital water that isolated different bacterial types and included *E. coli* with a rate of 28.6% and *K. pneumonia* 25.7% (Bardhan *et al*. 2020), and in a previous local study in Baghdad Governorate (Al-Bayatti *et al*. 2012). Enterobacteriaceae family constituted the highest

percentage of the total diagnosed and isolated isolates from the waters of the Tigris River, and the proportions of these genera were *E. coli* a rate 27%, *K. pneumoniae* a rate 30%, and *Citrobacter freundii* rate was 14%.

A study conducted in Japan (Hata *et al.* 2015) revealed the existence of several genera of intestinal bacteria in water, including *Enterobacter* spp. 39.1%, *Klebsiella* spp. 13.8%, and *Pantoea* spp. 12.5%, *Citrobacter* spp. 7.4%, *Serratia* spp. 6.1%, *Escherichia* spp. 5.4%, *Raoultella* spp. 2.6% and *Buttiauxella* spp. by 1.6% and other types by 11.5% (Table 4.3).

Table 4.3 Different bacterial isolates obtain from different sites near Iraqi hospitals

NO.	Types of bacteria isolates	Set NO.1	Set NO.2	Set NO.3	Set NO.4	Total bacterial isolates NO.	%
1	***E. coli***	6	5	8	5	24	44.44
2	***Klebseilla pneumoniae***	5	3	6	4	17	31.48
3	***Proteus mirabilis***	1	-	2	1	4	7.40
4	***Citrobacter freundii***	1	-	-	-	1	1.85
5	***Citrobacter kosri***	-	-	1	-	1	1.85
6	***Enterobacter aerogenes***	1	1	3	2	7	12.96
Total		15	10	21	12	54	100
Chi-Square (χ^2)		--	--	--	--	--	15.407**
** ($P \leq 0.01$).							

The high rates of these intestinal bacteria isolated from the raw waters of the Tigris River reflect the high level of water pollution with these bacteria, most of which are caused by fecal contamination of humans, animals and sewage. The rate of coliform bacteria is a signal of fecal contamination, which means the presence of other dangerous intestinal types that can lead to serious health problems.

The reason is owing to the high percentage of *E. coli* bacteria in wastewater because it is part of the natural flora of the intestines of humans and warm-blooded animals, and most of them are able to adapt to different temperatures and PH values. Some species may be pathogenic, especially diseases of the digestive system (Ibrahim *et al*. 2014, Anderson *et al*. 2005).

E. coli is one of the important types of antimicrobial-resistant bacteria that originate from human and animal faeces, because it has antibiotic resistance genes, which can exert severe pressure to spread resistance to antibiotics (Adefisoye and Okoh 2016).

GN bacteria, particularly Enterobacterales individuals for instance, *E. coli, K. pneumonia*, and *Enterobacter cloacae*, are widespread inhabitants of the intestinal microbiome and among the most prevalent causes of hospital-acquired diseases (Aranega-Bou *et al*. 2021).

4.2 Identification of Isolated Bacteria

All Gram-negative bacteria are identified using the gram stain, morphological characteristics on culture medium, and biochemical tests, and the bacterial isolates were submitted to identification utalizing the VITEK 2 compact system for further confirmation.

4.2.1 Microscopic characteristic

All isolated bacteria are submitted to gram stain for microscopic analysis to discriminate Gram-negative and Gram-positive bacteria according to the Gram stain reaction as outlined in the Gram stain technique. The bacteria appeared as gram-negative, bacilli, short rods, and single-cell bacteria under a light microscope (100X), and the bacteria considered as gram negative bacteria as seen in the Gram stain results shown in Figure 4.1.

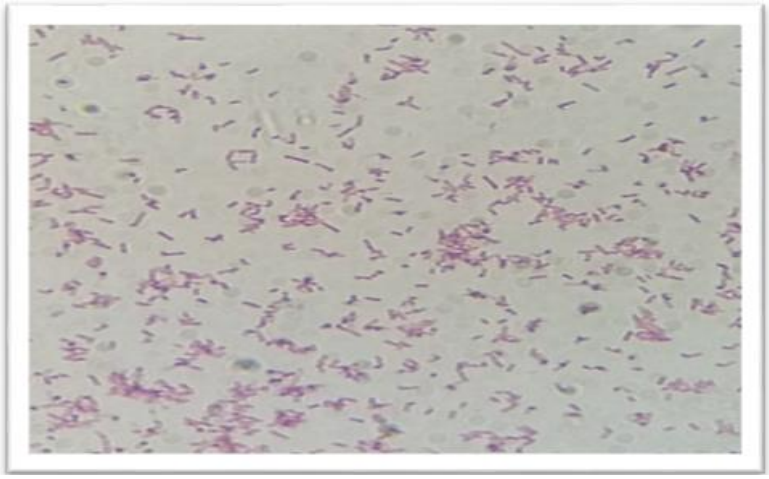

Figure 4.1 The characteristics of a microscope to gram-negative bacteria

4.3 Cultural Characteristics

This study included five types of Gram-negative bacteria and as shown in Table 4.2 and Table 4.3 the bacteria were initially diagnosed on the medium of MacConkey, Nutrient Agar, and Eosin Methylene Blue (EMB),and based on the differential diagnostic characteristics of each type of bacteria was diagnosed *E. coli*, *K. pneumoniae*, *P. mirabilis*, *E. aerogenes*, *Citrobacter* spp. After being isolated and purified from different environmental and clinical samples, depending on the microscopic characteristics of bacterial cells, morphological and cultivation characteristics, including the size, color, edge and height of bacterial colonies, and biochemical tests used to isolate and diagnose each bacteria and their conformity as stated in the approved diagnostic systems (Cowan 2004).

E. coli type was specified relative to its physical characteristics. When the isolate is grown on MacConkey agar, the colonies were bright pink, whereas the colonies on EMB medium appeared with a green metallic sheen. This is because the crystal violet and bile salts in MacConkey agar promote GN bacteria growth while inhibiting GP bacteria growth. Also located that the bacteria fermented sugar as displayed in Figure 4.2 (A). While on Eosin Methylene Blue (EMB). It was a selective medium for *E. coli* that was utilized to distinguish it from other Enterobacteriaceae individuals. Sheen green metallic colonies were discovered, indicating that the colonies made organic acids

as a result of glucose and lactose fermentation, which gave the colonies a sheen green metallic hue in the presence of eosin and methylene as illustrated in Figure 4.2 (B) (Singh and Prakash 2008).

When Gram-negative bacteria colonies are grown on nutrient agar, the form and features of developing isolates appeared on a plate as a smooth creamy colony as shown in Figure 4.2 (C).

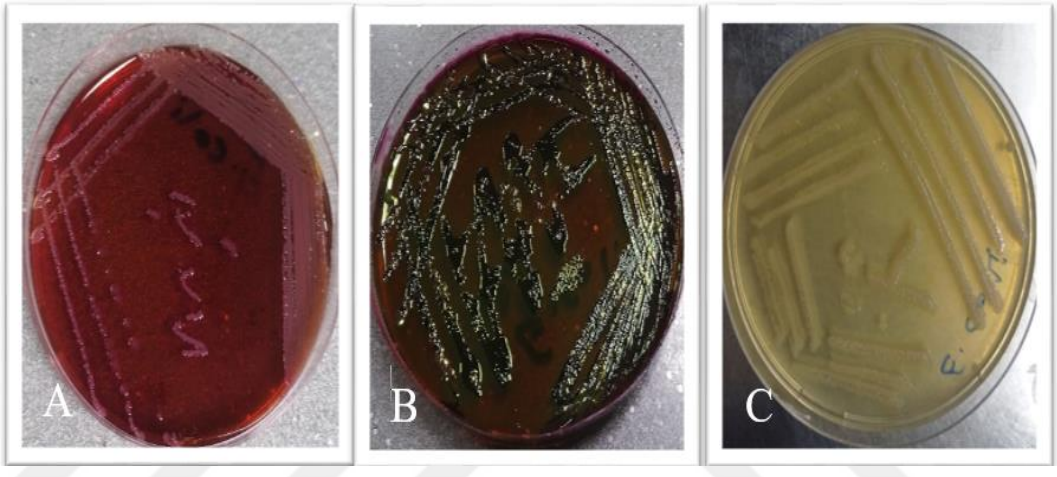

Figure 4.2 Bacterial growths on three various media. **A:** The bright pink colonies are bacterial colonies grown on MacConkey agar. Colonies **B:** that has a metallic green sheen on EMB medium. **C:** Bacterial colonies on nutrient agar plates, smooth creamy colony

K. pneumoniae on MacConkey agar plates, appeared mucoid, large, smooth and pink because to lactose fermentation according to capsule structure (Liu *et al*. 2014), to identify *K. pneumoniae* colonies from *E. coli* colonies, all isolates were grown on EMB, and colonies were large, purple, and mucoid as shown in Figure 4.3.

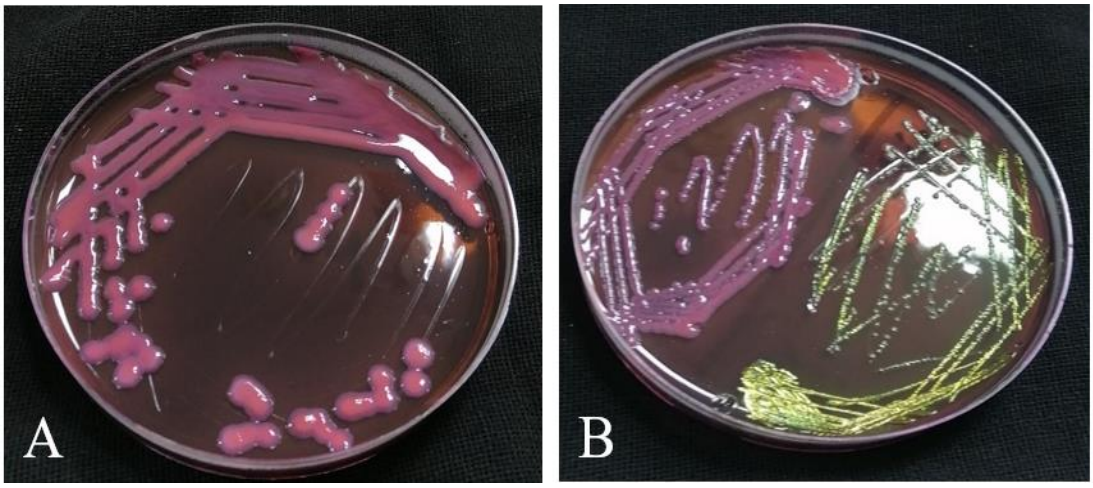

Figure 4.3 Bacterial growth on Eosin Methylene Blue (EMB) **A:** *K. pneumoniae* colonies on EMB agar large, purple, and mucoid, **B:** *E. coli* and *K. pneumoniae* on EMB media

Proteus mirabilis on MacConkey agar plates pale because unable to ferment lactose and its consumption of peptone as a nitrogen source and the production of metabolites that increase the pH value of the medium, which in turn affects the reagent (neutral red), causing the color of the colonies to become pale and revealed their swarming motility on plate agar which characterized them from other Enterobacterciea (Gazel *et al*. 2021).

Enterobacter spp. appeared small in size and semi-mucosa with a pink color due to the fermentation of lactose sugar on the medium of the MacConkey, while *Citrobacter* spp. colonies appeared circular and pink because of the fermentation of the sugar lactose on the medium of MacConkey.

4.4 Diagnosis Tests

All Isolates of Gram-negative were subjected to different biochemical tests as the results showed in Figure 4.4, and Table 4.4.

4.4.1 *Escherichia coli* diagnosis

All isolates of *E.coli* are positive to indole and methyl red tests and it is negative to Voges–Proskauer and Citrate utilization tests and ureas when tested for traditional biochemical tests (IMVIC test) and lactose fermenting (Tonu *et al*. 2011, Sarba *et al*. 2019)

4.4.2 *Klebseilla pneumoniae* diagnosis

K. pneumonia is negative to indole and methyl red. It is positive to Voges–Proskauer, Citrate utilization, lactose fermenting (Stella and Halimi 2015).

4.4.3 *Proteus mirabilis* diagnosis

It is negative to indole, Lactose, and Voges–Proskauer, positive to methyl red and ureas, a variable to Citrate utilization (Schaffer and Pearson 2017).

4.4.4 *Enterobacter aerogenes* diagnosis

E. aerogenes is negative to indole and methyl red, positive to Voges–Proskauer and Citrate utilization, does not lactose fermenting, and negative to ureas (Stella and Halimi 2015).

4.4.5 *Citrobacter spp.* diagnosis

Citrobacter spp are positive to indole and methyl red tests and it was negative to Voges–Proskauer and ureas, positive to Citrate use tests and lactose non fermenting (Nayar *et al*. 2014) (Table 4.4).

Table 4.4 The results of biochemical tests for the Enterobacteriaceae

Types of	IMVIC Test	KIA Test

bacteria isolates	I	MR	VP	C	Slant	Butt.	Gas	H_2S
E. coli	+	+	-	-	**A**	**A**	+	-
K. pneumoniae	-	-	+	+	**A**	**A**	+	-
P. mirabilis	-	+	-	**V**	**K**	**A**	+	+
E. aerogenes	-	-	+	+	**A**	**A**	+	-
Citrobacter spp.	+	+	-	+	**A**	**A**	+	+

(+): positive result, (-): negative result, (V): variable result-KIA: Kligler iron agar, K= alkaline slant (red), A=acid butt (yellow)

When Kovac's reagent was applied to the Indole test, it determines if the isolates produced indole (red ring, positive result) or does not create indole (brown ring, a negative result) (Akter *et al*. 2013). The urease test is employed to distinguish microorganisms depending on their capacity to hydrolyze urea using the enzyme urease; positive enzyme urease will conquer the buffer in the media and alter the color of the indicator phenol-red from yellow (negative) to pink (positive) (Brink 2010). Methyl red test recognized the fermentation of glucose and the production of acid, resulting in a pH less than 5 for this production interaction with the methyl red indicator and the production of red color (positive result). The Vogas-Proskauer test recognized the fermentation of glucose and acetone production, resulting in a pH less than 5 for this production interaction and the production of red color (positive result) (McDevitt 2009). Simmon citrate test is utilized to identify microorganisms based on their capacity to consume citrate (carbon source) and alter the color from green (negative result) to blue (positive result), to include this medium on the indicator Bromothymol blue (MacWilliams 2009) as shown in Figure 4.4.

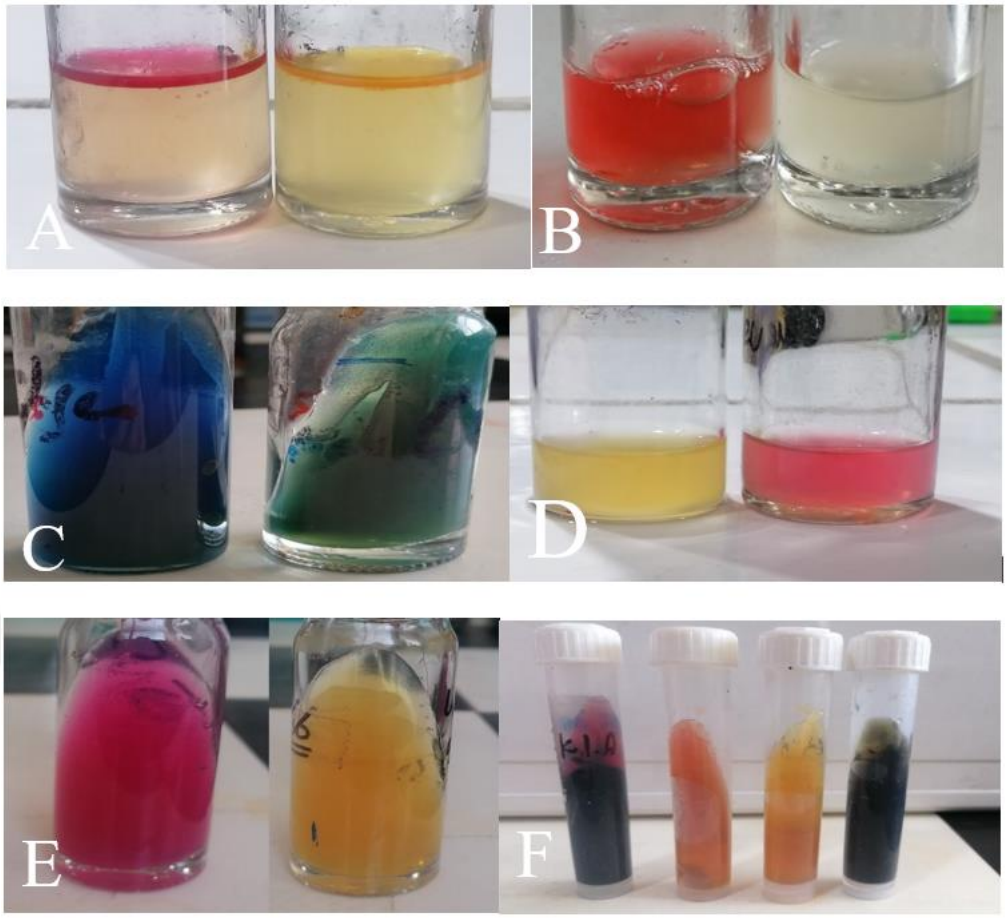

Figure 4.4 Biochemical tests

(A) Indol test (red ring is positive, yellow ring is negative), **(B) Vogas -Proskauer test:** red color is positive, yellow color is negative. **(C) Simmon citrate test:** Green color is negative, blue color is positive. **(D) Methyl red test:** red color positive, yellow color is negative. **(E) Urease test:** red color is positive and yellow color is negative (**F) Kligler iron agar test:** black precipitate is glucose fermentation only, gas produced, H_2S produced, Yellow/Yellow with bubbles is glucose and lactose and or sucrose fermentation. Red/Yellow with bubbles is glucose fermentation only, gas produced Red/Yellow with black precipitate is glucose fermentation only, H_2S produced

4.5 VITEK-2 System for Identification of Enterobacteriaceae

This system has been used to prove a final identification of Enterobacteriaceae. The system revealed bacteria efficiently, quickly, and out of the contamination that may

preclude disclosure of the pathogen, by using identification gram-negative Bacteria (ID-GNB) cards and showed results that (134) isoletes belonged to Enterobacteriaceae, 54 isolates from Environmental samples and 80 isolates from clinical samels. The GN card for gram-negative bacteria was used which consists of 47 biochemical tests.

4.6 Sensitivity of Antibiotices to Enterobacteriaceae isolate

In this study, the sensitivity patterns of 134 isolates from clinical and environmental of Enterobacteriaceae were achieved against 12 different antibiotics. An agar disk diffusion test was utalized to detect the antibiotic susceptibility of isolates (Hudzicki 2009). It is based on measuring the diameter of the inhibition zone to collect the antigens used in the study compared to what was mentioned. The results indicated an apparent dissimilarity in the response of the isolates undergoing study to the antibiotics employed. The antibiotics utilized including, Penicillin, Amoxicillin, Amoxicillin–clavulanic acid, Cephalexin, Cefixime, Cefotaxime, Ceftazidime, Cefoxitin, Imipenem, Meropenem, Aztreonam, and Tetracycline. The results are a obvious variation in the extent among the isolates in the response to antibiotics used in this study. That is sensitive, intermediate, and resistant shown in Figure 4.5.

Figure 4.5 Antibiotic sensitivity test of sampels of Enterobactereaceae isolates

4.6.1 Antibiotics resistance of environmental samples

The results showed varying percentages of antibiotic resistance of bacterial isolates collected from water. As 22 isolates of the total of 54 environmental isolates of Enterobacteriaceae showed resistance to a group of beta-lactam antibiotics The results are shown in Table 4.5 as an apparent variation in the range among the isolates in the response to antibiotics used in a study, and the highest results of resistance among family Enterobacteriaceae were recorded against Penicillin 100%, Amoxicillin 100%, Cephalexin 100%, Ceftazidime 100% and Tetracycline 100% followed by Amoxicillin–clavulanic acid 77.27%, Cefotaxime 77.2%. Moderate resistance appeared against some antibiotics Cefixime 63.6%, Cefoxitin 59.09%, Aztreonam 54.5%. The lowest resistance was recorded against Meropenem 18.18% and Imipenem 13.6%.

Carbapenems antibiotics group (Impenem, Meropenem) environmental isolates showed sensitivity against the Carbapenems group with a percentage of 81. This study agree with (Hamed *et al.* 2016) that the rate is 95.93%.

The present study outcomes agreement with what has been located by (Anayochukwu *et al.* 2021), that the resistances rate of isolates to Ampicillin was 95% and Ceftazidime 100 % while differing from what was found (Hata *et al.* 2015) where the percentage of resistance of the isolates to Ampicillin, 52.8% and Ceftazidime 5.1% and Tetracycline 4.2% and also differed from the results of Cefotaxime, 11.9% while agree with results on Imipenem, 5.8%

This study's results agree with what is found by (Odumosu and Akintimehin 2015) in Nigeria, to study on the Enterobacteriaceae, where they found the resistance to Amoxicillin/clavulanic acid 89.71% Cefotaxime 79.47 %, Cefixime 48.53%, while the results on Ceftazidime at 41.08%

In 2016, (Tolessa *et al.* 2016) found that the rate of Amoxicillin resistance was 94.94%, compared with the results of the current work.

Antibiotic resistance among bacteria may be caused by an increase in illogical and haphazard utaliz of antibiotics, sales the antibiotics of substandared, and transfer of drug resistant microorganisms among individuals, Antimicrobial resistance has appeared as a major worldwide public health concern. Infections produced by drug-resistant bacteria are a leading source of morbidity and mortality. Antibiotics for the treatment of bacterial infections should be chosen based on culture and sensitivity data (Neupane *et al*. 2016).

E. coli isolates show resistance to Penicillin, Amoxicillin, Cephalexin, Ceftazidime, Cefotaxime, and Tetracycline that rate 100%. With a difference in the percentage of resistance to the rest of the antibiotics that rate Amoxicillin–clavulanic acid 83.3%, Cefixime 83.3%, Aztreonam 66.6%, Cefoxitin 50%, Imipenem and Meropenem was 16.6%.

This study demonstrated a resistance average in *E. coli* of 100% for Penicillin, Amoxicillin, Cephalexin, Ceftazidime, Cefotaxime, and Tetracycline Lower average were registered in parallel work performed by (Akther *et al.* 2018) in Bangladesh on *E. coli* strains isolated from the Waste Water.

E. coli isolates exhibited resistance to Amoxicillin-clavulanic acid with a percentage 83.3 % and Cefoxitin 50% that agree with what was found by (Mahato *et al.* 2019) t the results were 85.7% and 43% respectively (Tesfaye *et al.* 2019). In Ethiopia reported similar resistance rates 83.3%, 55.6% for Amoxicillin-clavulanic acid and Cefoxitin on *E. coli* strains isolated in wastewater samples and the consequences of the existing study varied with the outcome of (Adegoke *et al.* 2020), where the percentage of resistant isolates reached 35.3%.

Klebsiella pneumonia showed 100% resistance to Ampicillin and Amoxicillin, Ceftazidime 50% to Aztreonam and Cefotaxime was 50% which was in agreement with (Mahato *et al.* 2019) while dissagrement to Ceftazidime and Cefotaxime 66.7%, but higher than the study of (Belachew *et al.* 2018) in Ethiopia to Ampicillin a results was 94%. The percentage of resistance to Cefixime was 62.5%. This result differed with the

results (Tanni *et al.* 2021), as the rate of isolates resistance was 100%. *K. pneumonia* it was sensitivity to (Imipenem, Meropenem) that rate was 75%, that is disagree what found (Abas and Abdulredha 2021) in Basrah they are found all *K. pneumonia* isolates are resistant to (Imipenem, Meropenem) for 100%.

E. aerogenes isolates showed 100% resistance to Amoxicillin antagonists. This result agreement with (Moyen *et al.* 2021), where the antibody resistance was 100%. While *E. aerogenes* showed Cefoxitin 66.6%, Ceftazidime 100%, Aztreonam 33.3%, Imipenem 0% that resulted disagreement with similar study in Bangladesh found by (Munny *et al.* 2021) the results respectively 100%, 50%, 16.67% but consistent with Aztreonam 33.3%.

The results showed that the most resistant antibiotics by *P. mirabillis* were Penicillin, Amoxicillin, Cephalexin, Ceftazidime, Tetracycline, Cefoxitin. It was all resistance 100% followed by Amoxicillin–clavulanic acid and Cefotaxime 66.6%. The lowest resistance rate was Meropenem 33.3%, Imipenem 0% and Aztreonam 33.3%. (Salman *et al.* 2022) found that resistance to Penicillin and Tetracycline is 100%, whereas sensitivity to Aztreonam and Imipenem was also 100%. (Moyen *et al*. 2021) discovered that Amoxicillin resistance was 100%, Ceftazidime resistance was 100%, while Cefotaxime resistance was 0%. The present study results are consistent with these findings.

C. freundii and *C. koseri* isolates showed resistance to all antibiotics that rate 100%, while the isolates showed 100% sensitivity to İmipenem, Meropenem and Tetracycline for 100%. The current study's rate that close to the results found by (Liu *et al*. 2021) in clinical samples the resistance of (Imipenem, Meropenem) in *C. freundii* was 11.5%, 3.8% *and C. koseri* was 0%, 0%.

Resistance to β-lactam antibiotics is increasing in *E. coli* and Enterobacteriaceae because to the output of β-lactamase, which includes cephalopsorinase and pencillinase, which operate on hydrolyzing the beta-lactam ring, inhibiting antibiotics from the penicillin and cephalosporin groups (Schechter *et al*. 2018). Bacteria develop resistance

by a mixture of methods, containing the production of lactamase enzyme, efflux pumps, and a producing an adjusted PBP (penicillin-binding protein) with a lower affinity β-lactams Antibiotics (Worthington and Melander 2013, Giani *et al.* 2017).

The work was accomplished by (Akther *et al.* 2018) in Bangladesh they are found the strains of highly resistant bacteria that are resistant to antibiotics *E. coli* may be responsible for transferring their resistance genes into other highly pathogenic bacteria found in wastewater. Furthermore, bacteriophages from animal feces as well as feces in hospitals can serve as environmental vectors for the horizontal transmission of antibiotic resistance genes.

Scientific reports in the countries of the Middle East confirmed that the resistance of the Enterobacteriaceae to group antibiotics Carbapenems is rare. In the Indian subcontinent and in the city of New Delhi, it was proven that bacterial isolates possess carbapenems enzymes responsible for resistance to antibiotics Carbapenems (El-Herte *et al*. 2012) (Table 4.5).

Table 4.5 Antibiotic sensitivity profile of environmental Entrobacteriaceae isolates

Isolates	S/R/I	Antibiotics											
		Penicillins Groups			Cephalosporin Groups					Carbapenems Groups		Monobactams Groups	Tetracycline
		P 10	AML 25	AUG 30	CL 30	CFM 5	CTX 30	CAZ 30	FOX 30	IMP 10	MRP 10	ATM 30	TC 45
E. coli	R	100%	100%	83.3%	100%	83.3%	100%	100%	50%	16.6%	16.6%	66.6%	100%
	S	0%	0%	16.6%	0 %	0%	0%	0%	33.3%	83.3%	83.33%	16.6%	0%
	I	0%	0%	0%	0%	16.6%	0%	0%	16.6%	0%	0%	16.6%	0%
K. Pneumoniae	R	100%	100%	75%	100%	62.5%	50%	100%	37.5%	%25	25%	50%	100%
	S	0%	0%	12.5%	0%	12.5%	0%	0%	25%	75%	75 %	12.5%	0%
	I	0%	0%	12.5%	0%	25%	50%	0%	37.5%	0%	0%	37.5%	0%
E. aerogenes	R	100%	100%	66.6%	100%	33.3%	100%	100%	66.6%	0%	0%	33.3%	100%
	S	0%	0%	33.3%	0%	0%	0%	0%	0%	100%	100 %	0%	0%
	I	0%	0%	0%	0%	66.6%	0%	0%	33.3%	0%	0%	66.6%	0%
P. Mirabillis	R	100%	100%	66.6%	100%	33.3%	66.6%	100%	100%	0%	33.3%	33.3%	100%
	S	0%	0%	33.3%	0%	66.6%	33.3%	0%	0%	66.6%	66.6%	66.6%	0%
	I	0%	0%	0%	0%	0%	0%	0%	0%	33.3%	0%	0%	0%
C.Freundii	R	100%	100%	100%	100%	100%	100%	100%	100%	0%	0%	100%	0%
	S	0%	0%	0%	0%	0%	0%	0%	0%	100%	100%	0%	100%
	I	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
C.Koseri	R	100%	100%	100%	100%	100%	100%	100%	100%	0%	0%	100%	0%
	S	0%	0%	0%	0%	0%	0%	0%	0%	100%	100%	0%	100%
	I	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
P. value	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	---
* (P≤0.05), ** (P≤0.01).													

4.6.2 Antibiotics resistance of clinical samples

The results showed varying percentages of antibiotic resistance of bacterial isolates collected from water. As 47 isolates of the total of 80 clinical isolates of Enterobacteriaceae showed resistance to a group of beta-lactam antibiotics. The results are shown in Table 4.6. As an evident interpretation in the range among the isolates in response to antibiotics used in a study, the highest results of resistance among the family Enterobacteriaceae were recorded against Penicillin 100%, Amoxicillin 100%, Cephalexin 100%, and Tetracycline 100% followed by Amoxicillin–clavulanic acid 80.85%, Cefotaxime 65.95%. Moderate resistance appeared against some antibiotics Cefixime 59.57, Cefoxitin 57.44%, Aztreonam 61.7%. The lowest resistance was recorded against Meropenem 10.6% and Imipenem 14.89%.

E. coli isolates exhibited resistance to Penicillin, Amoxicillin, Cephalexin, Ceftazidime and Tetracycline that rate 100%. With a difference in the percentage of resistance to the rest of the antibiotics that rate Amoxicillin–clavulanic acid 77.77%, Cefixime 66.66%, Aztreonam 72.2%, Cefotaxime 83.33%, Cefoxitin 44.44%. Carbapenems antibiotics group Impenem, Meropenem that rate 16.6%, 11.11%

The cause of the resistance of *E. coli* and Enterobacteriaceae to antibiotics is due to the production of enzymes (ESBL) that hydrolyze beta-lactam rings, contining cephalosporins and monobactams (Ceccarelli *et al*. 2019). Also, the production of β-lactamase enzymes is one of the most important mechanisms of antibiotic resistance and constitutes a major problem that threatens the health sector in large parts of the world (Chuma *et al*. 2013). The most clinically important ESBL variats are found in the gene families $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV}, as well as the pAmpC blaCMY gene family (Bush and Fisher 2011). The effective dissemination of ESBL/pAmpC genes is largely owing to their location on plasmids, which allows for simple transfer across bacteria (Rozwandowicz *et al.* 2018). The incidence of MDR (multidrug resistance) ESBL-producing *E. coli* appears to be high, the highest identification bla_{TEM} being the most often gene (Jena *et al*. 2017).

This study is in accordance with (Al-Salmani *et al*. 2020). The proportion of Ampicillin was 100% and Ceftazidime was 100%. And the consequences of the present study disagree with (Assafi *et al.* 2022). The percentage of resistance to the Amoxicillin was 88.89% and for antibiotics Amoxicillin-clavulanic acid, Cephalexin, Ceftazidime, Cefixime was 92.07%, 92.86%, 87.32%,85.71% respectively. this study were close with results found by (Abbas 2015) the rate of sensevity to (Imipenem and Meropenem) was 100% each other.

Klebsiella pneumonia in the study the percent of resistance was showed 100% resistance to Penicillin, Amoxicillin, Cephalexin, Ceftazidime, Tetracycline. This results obtained is agreed with (Ali *et al.* 2019). The resistance to Ampicillin was 100%, and it disagreed with it in the antibiotic Ceftazdime the rate of resistance to antibiotic was 82.05%. (Rhumaid and Al-Mathkhury 2015) reported *K. pneumoniae* clinical isolates were resistant to Cefotaxime 43.3%, Ceftazidime 58.8%, and these results in agreement to the present study with Cefotaxime 43.75% and disagreement with Ceftazidime.

K. pneumoniae is resistant to a variety of antibiotics; this bacterium possesses a number of antibiotic resistance genes that can be horizontally transferred to other gram negative bacteria and it is a associated of nosocomial hospital infections (Aljanaby *et al.* 2018). Resistance to some β-lactams is intrinsic in *K. pneumoniae* species because some of the resistance genes are found on the chromosome. For example, the *SHV* gene is found on chromosomes and confers resistance to Ampicillin (Polse *et al.* 2020).

K. pneumoniae is resistant to a variety of antibiotics; this bacterium possesses a number of antibiotic resistance genes that can be horizontally transferred to other gram negative bacteria

The existing study outcomes agree with what is discovered by (Al-Azawi 2014) that all isolates of *K. pneumoniae* were resistant to Ampicillin, Amoxicillin and Ceftazidime that a rate 100%, and disagreement with Tetracycline the rate was 66.6%. In study by (Mofolorunsho *et al.* 2021) illustrated that the resistance rate of *K. pneumoniae* clinical

isolates was Cefoxitin 62.5%, while the percentage of resistance of Cefoxitin was 37.5%. The outcome of this work differed from (Moghadas *et al*. 2018) where the percentage of Aztreonam was 39.3% and Ceftazidime was 42.7%.

In this study a resistance *K. pneumonia* to Cefixime and Amoxicillin–clavulanic acid is rate 62.5%, 87.5%. While disagreement what found by (Polse *et al.* 2020) where the average of resistance to antibiotics was 82.2%, 39% respectively. The percentage of *K. pneumonia* resistance to Imipenem and Meropenem in this study is %18.75, 12.5%, and this percentage was close to what is found by (Albassam 2015), where the percentage of resistance was 9.3%.

Several mechanisms have been identified that can confer resistance to various antibiotics from different classes. The most important mechanisms discovered in *K. pneumoniae* are the production of extended-spectrum beta-lactamase (ESBL) enzymes, efflux pump overexpression, target modifying enzymes, and mutation (Navon-Venezia *et al.* 2017).

As for *P. mirabilis* bacteria it was highest resistant to Penicillin, Amoxicillin, Cephalexin, Cefoxitin, Tetracycline percentage 100%, Amoxicillin–clavulanic acid 76.92%, Cefixime 42.85%, Ceftazidime 61.53%, Aztreonam 61.53%, Cefotaxime 69.23%.

Antibiotic susceptibility tests revealed that *Proteus* species are resistant to a wide range of antibiotics. This could be due to the extra outer cytoplasmic membrane, which has a lipid bilayer, lipoproteins, polysaccharides, and lipopolysaccharides, and, of course, misuse and overuse of antibiotics may play a role in the development of resistant strains (Mordi and Momoh 2009).

The results of the current study contradict with (Musa *et al.* 2019) who mentioned the rate of resistance of *P. mirabilis* isolated from clinical samples to Amoxicillin at 40%, Ceftazidime 25.6% and Cefotaxime 18.9%.

The results of the current study are in agreement with (Serry *et al.* 2018) who stated that the rate of resistance of *P. mirabilis* isolates to antibiotics Tetracycline 100%, Ampicillin 85.1%. The current study differs with it in the rate of bacterial resistance to antibiotics Cefotaxime 51.1%, Ceftazidime 44.7%, Aztreonam 14.9%.

A study was done by (Salama *et al.* 2021) found the strains resistant to antibiotics Amoxicillin/Clavulanic acid 45.0%, Cefotaxime and Ceftazidime 41.7% and 40.0% respectively that is in disagreement with a current study while agreement with rate of Imipenem 11.7%

Gram-negative bacteria isolates show high sensitivity to carbapenems because production acquired carbapenemases the very clinically important carbapenem-resistance mechanism with the other know resistance mechanisms such as include lower outer membrane permeability and up-organization of t efflux system with/without the produce of extended-spectrum β-lactamases (ESBLs) (Olowo-Okere *et al*. 2020)

The ESBL production is encoded by the genes that are mostly found on large conjugative plasmids ranging in size from 80 to 160 kb. These plasmids are easily transmitted through divers Enterobacteriaceae members, and the accumulation of resistance genes by them results in the evolution of multidrug-resistant strains. As a result, ESBL-producing isolates are resistant to a variety of antibiotic classes. Furthermore, the development of these multiple resistant Enterobacter strains is unfortunately involved by a comparatively high steadiness of the plasmid-encoding ESBLs (Velasco *et al*. 2007). Resistance to third-generation cephalosporins is frequently mediated by TEM- and SHV-type B-lactamases in Enterobacteriaceae (Sharma *et al*. 2008) (Table 4.6).

Table 4.6 Antibiotic sensitivity profile of clinical Entrobacteriaceae isolates

Isolates	S/R/I	Antibiotics												P-value
		Penicillins Groups			Cephalosporin Groups					Carbapenems Groups		Monobactams Groups	Tetracycline	
		P 10	AML 25	AUG 30	CL 30	CFM 5	CTX 30	CAZ 30	FOX 30	IMP 10	MRP 10	ATM 30	TC 45	
E. coli	R	100%	100%	77.77%	100%	66.66%	83.33%	100%	44.44%	16.6%	11.11%	72.2%	100%	0.0001**
	S	0%	0%	22.22%	0%	27.77%	16.66%	0%	38.88%	83.33%	77.77%	16.66%	0%	0.0001**
	I	0%	0%	0%	0%	5.55%	0%	0%	16.66%	0%	11.11%	11.11%	0%	0.033*
K. Pneumoniae	R	100%	100%	87.5%	100%	62.5%	43.75%	100%	37.5%	18.75 %	12.5%	50%	100%	0.0001**
	S	0%	0%	0%	0%	31.25%	43.75%	0%	62.5%	68.75%	81.25 %	18.75%	0%	0.0001**
	I	0%	0%	12.5%	0%	6.25%	12.5%	0%	0%	12.5%	6.25%	31.25%	0%	0.0085**
P. Mirabillis	R	100%	100%	76.92%	100%	42.85%	69.23%	61.53%	100%	7.69%	7.69%	61.53%	100%	0.0001**
	S	0%	0%	23.07%	0%	53.84%	30.76%	38.46%	0%	84.61%	84.61%	38.46%	0%	0.0001**
	I	0%	0%	0%	0%	0%	0%	0%	0%	7.69%	7.69%	0%	0%	0.294 NS
P-value														----
*(P≤0.05), ** (P≤0.01).														

Note; P: Penicillin, AML: Amoxicillin, AUG: Amoxicillin–clavulanic acid, CL: Cephalexin, CFM: Cefixime, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, IMP: Impenem, MRP: Meropenem, ATM: Aztreonam, TC: Tetracycline

There is a high significant difference (P <0.01) between the resistance trait for all isolates

4.7 Multiple Antibiotic Resistance

The results show that 24 total isolates of 9 environmental and 15 clinical are multi-drug resistant (MDR) to three different groups of Antibiotic shown in Table 4.7 from the sum of the different antibiotics used in the study, which includes biologics as a group of beta-lactam antagonists as an antagonist (Penicillin) and group of Antibiotic such as (Cephalosporin) and group of Antibiotic such as (Monobactams) The majority of the isolates under study showed the character of multiple resistance to the antibiotics used in treatment, owing to a mutation in the chromosome, or the resistance may be carried on a plasmid that encodes multiple antibiotic resistance.

The outcomes of the work demonstrat the presence of multiple resistance within the isolates, as it showed 6 of the total 134 isolates resistant to 5 groups of the antibiotics used under study that extensively drug resistance (XDR), while 62 isolates show sensitivity towards (carbapenem groups) of the antibiotics in this research. That the presence of the resistance trait among the isolates confirms the existence of multiple resistance within the local isolate as a result of the random use of antibiotics. The isolates under study were divided into three groups depending on the number of antibiotics that those isolates were resistant to.

The first group is resistant to 1-3 and less of the antibiotics by 17.91%, the second group is resistant to 4-5 antibiotics, Most of the isolates of bacteria under study fall within it, which included 45 isolates resistant to (4-5) groups of antibiotic and by 33.58 % of the total isolates resistant to antibiotics under study, and that the isolate of (*E. coli 3, E. coli 13, E. coli 17, K 12, K 26, K 33*) resisted all the groups of antibiotics used in the current study.

The emergence of multiple antibiotic resistance has evolved a global concern, because bacteria have become multiple mechanisms of antibiotic resistance, due to the indiscriminate utalize of antibiotics and lack of health awareness, which has drive to the development of resistant strains with their own flow and systems and the ability to alter the permeability barrier and produce beta-lactamase enzymes, which made it a

constantly increasing problem, which made it difficult to choose the appropriate treatment for it (Table 4.7).

Table 4.7 Multiple antibiotic resistance of the isolates of clinical and environmental under study

Types of bacteria isolates	The number of groups of antibiotics that the isolates resisted	Number and kinds of isolates	Classification of isolates
E. coli	1-3 resisted group of antibiotics	*E1 - E1- E6- E11- E14*	MDR
K. pneumoniae		*K7 - K10- K11- K19-K23- K25- K29-K30-K31- K32*	
P.mirabillis		*P19 – P39- P43- P44- P46*	
E. aerogenes		*E16 – E17*	
C. freundii		*C -21*	
C. koseri		*C-22*	
E. coli	4-5 resisted group of antibiotics	*E2 – E3- E4- E5-E6- E2- E3- E4- E5- E7-E8 -E9 -E10 -E12- E13 -E15 -E16 -E17-E18*	XDR
K. pneumoniae		*K8-K9-K12-K13-K14-K20- K21-K22-K24-K26 -K27- K28-K33-K34*	
P. mirabillis		*P18-P20-P35-P36-P37-P38- P40-P41-P42-P45-P47*	
E. aerogenes		*E15*	

4.8 Molecular Study

4.8.1 Extraction of DNA and measuring the purity

DNA was extracted from 69 (22 enviromental isolates and 47 clinical isolates) different samples depending on their multidrug antibiotic resistance (MDR). The total DNA of 69 bacterial isolates was successfull extracted according to the wizard kit's protocol, and a procedure has been proceeded to extract genomic DNA from each isolate of Enterobacteriaceae Concentration and purity of the extracted DNA were confirmed with nanodrop, the purity of DNA ranged between 1.61-2.03, while the concentration of DNA template was ranged between (25-112 ng/µL), determining the DNA concentration is important because it represents the main requirement for PCR reaction (Figure 4.6).

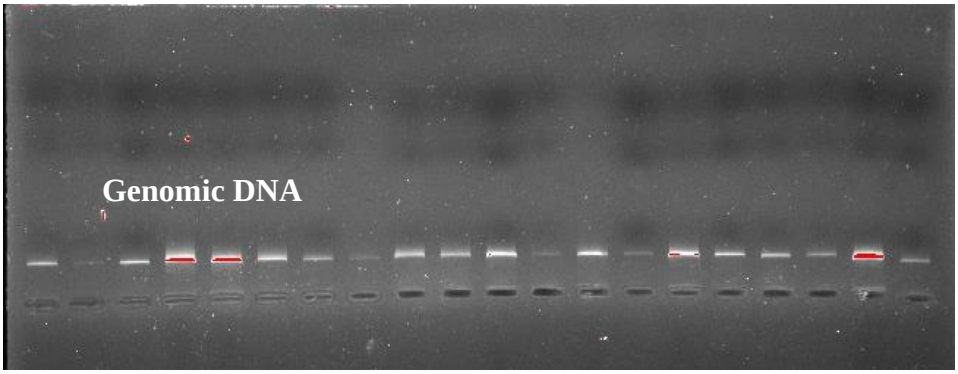

Figure 4.6 Gel electrophoresis of DNA with loading dye, from Enterobacteriaceae Agarose (0.7) %, 75 V/cm for 1 h, and visualized on a UV transilluminator

4.8.2 Molecular detection of (*bla*$_{TEM}$ and *bla*$_{shv}$) among clinical and environmental isolates of Enterobacteriaceae

4.8.2.1 Detection of the *bla*$_{TEM}$ gene and *bla*$_{shv}$ in environmental isolates of Enterobacteriaceae

The results show that 22 environmental isolates of Enterobacteriaceae contained the gene (*bla*$_{TEM}$) and depending on the appearance of a band of size 800 base pairs and gen (*bla*$_{shv)}$ appearance of a band of size 730 base pairs. Whereas the isolates of *E. coli* 5 (24) and *K. pneumonia* was 8 (17). *E. aerogenes* was 3(7), *P. mirabilis* 3(4), *C. freundii* 1(1) *and C. kosri* was 1(1) showen in Figure 4.7 and Figure 4.8.

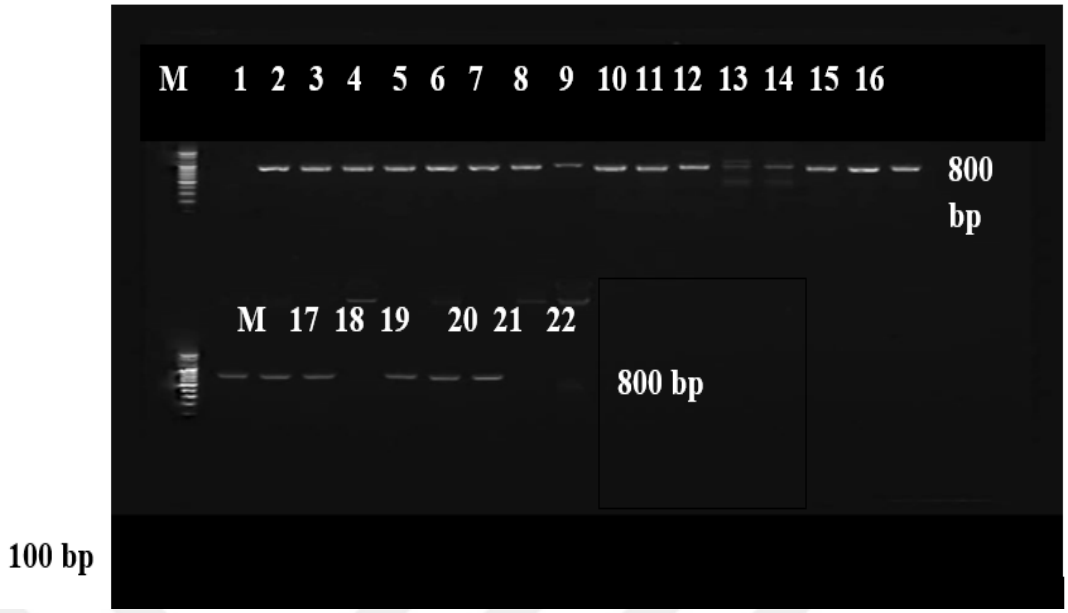

Figure 4.7 PCR amplification of *bla*$_{TEM}$ genes in environmental Enterobacteriaceae isolates. Electrophoresis was done in agarose gel (1.5%) at (75V/cm) for 90 min

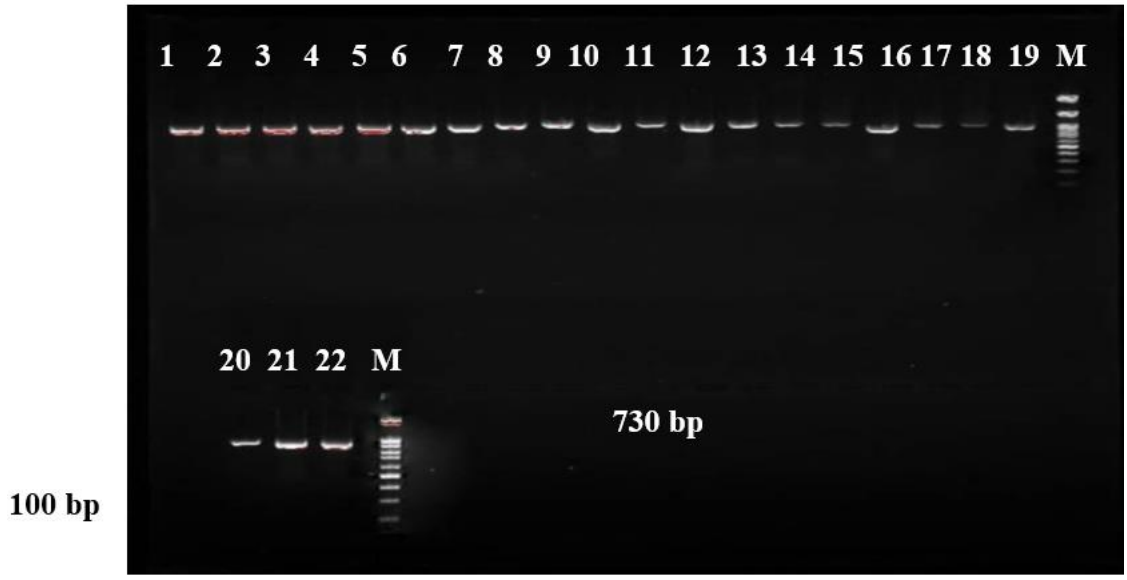

Figure 4.8 PCR amplification of *bla*$_{SHV}$ genes in environmental Enterobacteriaceae isolates. Electrophoresis was done in agarose gel (1.5%) at (75V/cm) for 90 min

The results show that contained the genes of the bla$_{TEM}$ and the bla$_{SHV}$ with a percentage of 40.74% depending on the appearance of a band size of 730 and 800 base pairs this results are compatible with (Zagui *et al.* 2020) where the proportion of the *bla*$_{TEM}$ was

17.8% and the *bla*$_{SHV}$ was 13.3 % in gram-negative bacteria in hospital sewage. The products of the existing work agreed with (Ojer-Usoz *et al.* 2014) where the proportion of *bla*$_{TEM}$ in Enterobacteriaceae was 47%, and it differed in *bla*$_{SHV}$ results of 17.4%.

A study done by (Amador *et al.* 2015) the proportion of the prevalent gene bla$_{TEM}$ is 24.1% of the isolates of Enterobacteriaceae obtained from wastewater which results in disagreement with the current study.

The outcome of a work in Singapore by (Haller *et al.* 2018) of antibiotic-resistant bacteria in hospital wastewater for *Klebsiella* spp and *Enterobacter* spp. and *Citrobacter* spp. and the result was that bla$_{SHV}$ was the most dominant gene 41.1%.

The reason for the difference in the outcomes between the existing work and earlier works is owing to the contrast in the number of isolates, the primers utilized, and the place and nature of the diverse isolates.

4.8.2.2 Detection of the *bla*$_{TEM}$ gene and *bla*$_{shv}$ in clinical isolates of Enterobacteriaceae

The results show that 47 clinical isolates of Enterobacteriaceae contained the gene (*bla*$_{TEM}$) and depending on the appearance of a band of size 800 base pairs and gen (*bla*$_{SHV}$) appearance of a band of size 730 base pairs.

Detection of *bla*$_{TEM}$ gene in clinical Enterobacteriaceae

The *bla*$_{TEM}$ gene is determined in *E. coli* and its appearance is in 15 isolates with a percentage of 83.3% out of the total 18 isolates Figure 4.9. This result is similar to results with (Alipour and Jafari 2019) where was the rate *bla*$_{TEM}$ was 89%. Study has indicated (Adwan and Abu Jaber 2016) that rate of *bla*$_{TEM}$ was 82.9%. While this percentage differed with the work of (Fattouh *et al.* 2017, Bajpai *et al.* 2017) where the percentage of *bla*$_{TEM}$ in *E. coli* was 53.8% and 48.7%.

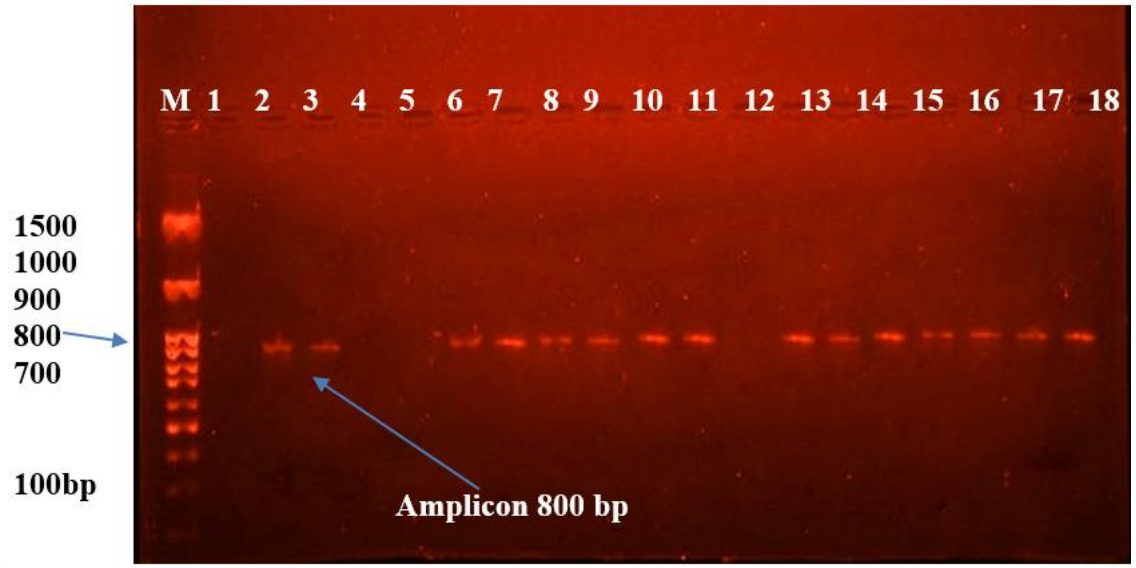

Figure 4.9 PCR amplification of *bla*$_{TEM}$ genes in *E. coli* isolates. Electrophoresis was done in agarose gel (1.5%) with Ethidium bromide in TBE1X solution at (75V/cm) for 90 min, M Standard volumetric guide (100pb DNA ladder)

The percentage of appearance of the *bla*$_{TEM}$ gene from a total of 16 *K. pneumoniae* isolates was 87.5%. These results are in disagreement with (Sarshar *et al.* 2021) where was the rate *bla*$_{TEM}$ in *K. pneumoniae* was 28% while Study has indicated by (Yazdansetad *et al.* 2019) The bla$_{TEM}$ was rate 50%. This result is similar to the current study by (Maleki *et al.* 2018) where was the rate *bla*$_{TEM}$ was 76%.

The percentage of emergence 84.61% of the bla$_{TEM}$ gene from a total of 13 isolates of *P. mirabilis* under study. These outcomes are in variance with (Hamid *et al.* 2020) where the rate *bla*$_{TEM}$ in *P. mirab*ilis was 60% and the study by (Ibrahim *et al.* 2021) the rate of *bla*$_{TEM}$ in *P. mirabilis* was 80% results are similar to the current study while the work by (Abed Gumar *et al.* 2022) the *bla*$_{TEM}$ was rate 90%.

Detection of *bla*$_{SHV}$ gene in clinical Enterobacteriaceae

The *bla*$_{SHV}$ gene is noticed in *E. coli* and its appearance is in 14 isolates with a rate of 77.77% out of the total 18 isolates shown in Figure 4.10. This result is different with a study by (Haji *et al.* 2018) where was the rate *bla*$_{SHV}$ is 28.5%. While the study by

(Sáenz *et al.* 2004) indicated the lack of this gene in *E. coli*, and the study by (Rezai *et al.* 2015) the rate *bla*$_{SHV}$ was 44%.

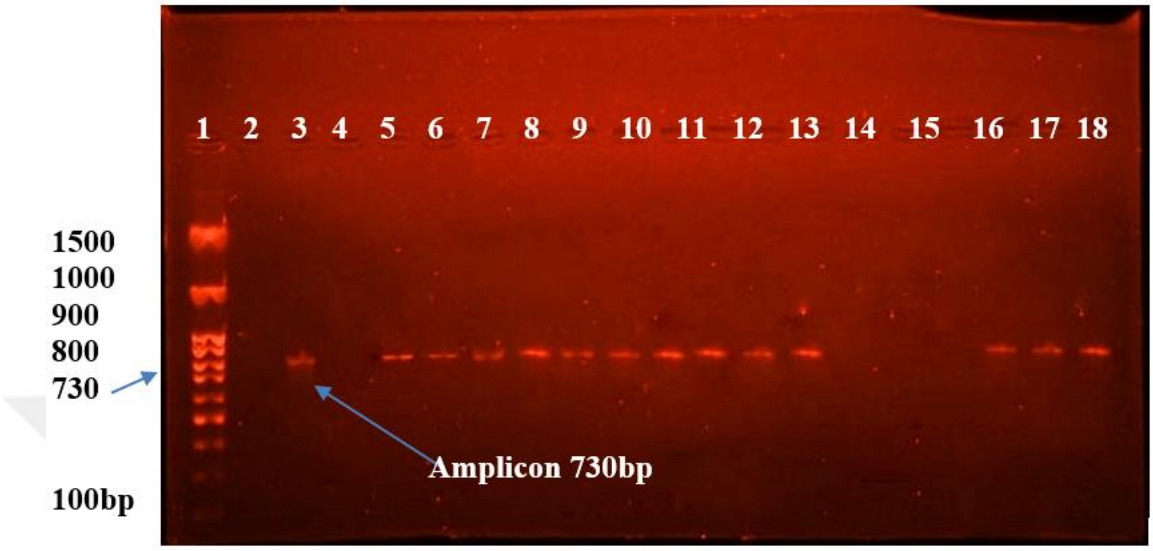

Figure 4.10 PCR amplification of *bla*$_{SHV}$ genes in *E.coli* isolates. Electrophoresis was done in agarose gel (1.5%) with Ethidium bromide in TBE1X solution at (75V/cm) for 90 min, M Standard volumetric guide (100pb DNA ladder)

The percentage of appearance of the *bla*$_{SHV}$ gene from a total of 16 *K. pneumoniae* isolates was 87.5 %. The study by (Pishtiwan and Khadija 2019) found the rate of *bla*$_{SHV}$ to be 35.2%. The study by (Yazdansetad *et al.* 2019) has indicated that rate of *bla*$_{SHV}$ was 70%. The outcomes of the existing work are in agreement with the results of (Dehshiri *et al.* 2018) where the rate of the *bla*$_{SHV}$ gene was 85.5%. The rate of the *bla*$_{SHV}$ gene in the bacteria *P. mirabilis* was 84.61% in 13 isolates. This result was different with the study by (Hamid *et al.* 2020) who indicated the absence of the *bla*$_{SHV}$ gene in *P. mirabilis*. Whereas, the bla$_{SHV}$ gene was diagnosed by a study by (Chinnam *et al.* 2021) in animal samples. These results are in disagreement with (Ram *et al.* 2022, Naidu *et al.* 2020) where the rate bla$_{SHV}$ in *P. mirabilis* was 4.34% and 5.40%.

Differences between the present study consequences and those of identical works are likely attributable to several factors, including differences in the numeral of isolates, primers utilized, study locations, and isolate types.

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

- All environmental and clinical isolates were subjected to sensitivity testing, out of a total of 54 environmental isolates showed 22 that rate 40.7% isolates resistant to antibiotics, and out of a total of 80 clinical isolates, 47 showed a rate of 58.75% isolates resistant to antibiotics.
- The numbers of *E. coli* and *K. pneumoniae* bacteria were recorded as the most common species, both in environmental and clinical samples.
- The highest percentage of resistance shown by the Clinical and Environmental isolates against (Penicillin, Amoxicillin, Cephalexin, Ceftazidime and Tetracycline) and it showed moderate resistance towards Cefixime, Cefoxitin, Aztreonam, and it showed the lowest resistance towards Meropenem and Imipene.
- Of the total environmental isolates, 9 and 15 clinical isolates were resistant to three groups of antibiotics (MDR), while 6 isolates out of a total of 134 isolates were resistant to all antibiotics (XDR).
- Environmental and clinical isolates underwent Polymerase Chain Reaction (PCR) to detect the resistance bla_{TEM} genes and the bla_{SHV} genes of and in the Enterobacteriaceae. Environmental isolates showed that they contain resistance genes bla_{TEM} and the bla_{SHV}, while clinical isolates showed higher rate of resistance genes bla_{TEM} was bla_{SHV}.
- In the current study, the results show the appearance of a high percentage of bla_{SHV} gene in *P. mirabilis* isolates, While the previous studies showed that this gene was a little percentage which confirms the transfer of resistance genes between the species of the Enterobacteriaceae.

5.2 Recommendations

- Control attenuation for hospital wastewater release into Tigris River.

- Increasing studies for virulence genes in environmental and clinical isolates bacteria that have higher antibiotic resistance.
- Increasing the awareness of people about the use of antibiotics, especially the ones that bacteria have shown resistance to, such as (Penicillin, Amoxicillin, Cephalexin, Ceftazidime and Tetracycline).
- Reducing the spread of *E. coli*, *K. pneumoniae* and *P. mirabilis* bacteria, as they are opportunistic pathogens in humans.
- A complementary study should be carried out to confirm the presence of the third gene to antibiotic resistance ($bla_{CTX\text{-}M}$) in environmental and clinical isolates belonging to the Enterobacteriaceae.

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