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Phenotypic and Genotypic Characterization of β-lactamases Enterobacteriaceae Isolated from Bacteremia in Babylon Province, Iraq

A Thesis
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بسم الله الرحمن الرحيم

(إِنَّ فِي خَلْقِ السَّمَاوَاتِ وَالْأَرْضِ وَاخْتِلَافِ اللَّيْلِ وَالنَّهَارِ وَالْفُلْكِ الَّتِي تَجْرِي فِي الْبَحْرِ بِمَا يَنْفَعُ النَّاسَ وَمَا أَنْزَلَ اللَّهُ مِنَ السَّمَاءِ مِنْ مَاءٍ فَأَحْيَا بِهِ الْأَرْضَ بَعْدَ مَوْتِهَا وَبَثَّ فِيهَا مِنْ كُلِّ دَابَّةٍ وَتَصْرِيفِ الرِّيَاحِ وَالسَّحَابِ الْمُسَخَّرِ بَيْنَ السَّمَاءِ وَالْأَرْضِ لَآيَاتٍ لِقَوْمٍ يَعْقِلُونَ ﴿١٦٤﴾)

صدق الله العلي العظيم

سورة البقرة (الآية ١٦٤)

Dedication

To who gave me the endurance to complete my road, my father

To the best woman in the universe, my mother

To my brothers and sisters…

To my close friends

To my Supervisor

My beloved country Iraq

The martyrs of Iraq with all the love and appreciation

Douaa Hussein

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Doaa

summary:

The present study include 163 blood samples collected from patients with bacteremia in hospitals in the Babil province during the period from October 2020 to April 2021. Only 63 bacterial isolates (38.65 %) as positive results. Of these, 35 bacterial isolates (21.47%) are identified as *Enterobacteriaceae* and 28 bacterial isolates (17.17) belonged to following species (*Streptococcus.spp, Staphylococcus.spp* and *Pseudomonas aeruginosa*). And the study reveal that *Klebsiella pneumoniae* (22.85%), *Proteus mirabilis* (20%), *Salmonella typhi* (20%) and *Escherichia.coli* (17.14%) are the most common isolated bacteria as compared with other members of the *Enterobacteriaceae*. The study shows that the susceptibility of males (51.42%) and females (48.57%) to blood stream infection is nearly the same.

The disk difussion test is used to determine the antibiotic susceptibility patterns of 35 bacterial isolates, which reveals that the majority of these isolates are highly resistant to the antibiotics tested, particularly beta-lactam antibiotics, with carbapenems (meropenem) having the lowest rate of resistance (17.14%). Phenotypic ESBL detection was carried out by disk approximation method, 9 isolates (25.71%) were beta lactamase producers.

Polymerase Chain Reaction (PCR) results reveals that among the 35 bacterial isolates, 19 isolates had the TEM enzyme type, 21 isolates had the OXA-10 enzyme type, and 23 isolates had the CTX-M-1 enzyme type. Results of PCR show that TEM, OXA-10, and CTX-M-1 enzymes are highly prevalent among species of *Enterobacteriaceae* that are responsible for blood stream infections. In addition to that, the PCR test is considered an accurate genotypic method used for detection of the ESBL enzyme type.

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List of Abbreviations

Abbreviations	**Description**
ESBLs	extended-spectrum β-lactameses
PCR	polymerase chain reaction
ECA	Enterobacterial common antigen
EHEC	Enterohemorrhagic *E coli*
UTI	urinary tract infection
CPS	capsular polysaccharides

LPS	lipopolysaccharides
SCD	soybean-casein digest broth
BHI broth	brain heart infusion broth
BSI	Bloodstream infections
ESBL-P-PM	extended-spectrumβ-lactameses -producing *P.mirabilis*
KPC	*Klebsiella pneumoniae* carbapenemase
MBLs	metallo-β-lactamases
OXA	oxacillinase types
CTX-M	cefotaxime first isolated at Munich
PBPs	Penicillin Binding Proteins
IP	isoelectric point
pCMB	p-chloromercuribenzoate
Blas	β-lactamases
MICs	minimum inhibitory concentrations
CLSI	The Clinical and Laboratory Standards Institute
TEM	Temoneira
SHV	Sulfhydryl Variable
NCCLS	National Committee for Clinical Laboratory Standards
EMB	Eosin Methylin blue medium
S-S agar	Salmonella shigella agar
MR-VP	Methyl-red Voges -Proskauer medium
DDST	Double-Disc Synergy Test
CN	Gentamicin
MEM	Meropenem
CAZ	Ceftazidime
C	Chloramphenicol
AMC	Amoxicillin
AM	Ampicillin
NDM-1	New Delhi metallo-beta-lactamase1
CTX	Cefotaxime
AZM	Azithromycin
CRO	Ceftriaxone
F	Nitrofurantion
EMB	Eosin Methylene Blue medium
EDTA	Ethylene Di-amine Tetra acetic Acid
TE buffer	Tris- EDTA buffer
SDS	Sodium Dodecyl Sulfate
TBE	Tris-Borate-EDTA Buffer

التوصيف المظهري والوراثي لإنزيمات البيتالاكتاميز لعزلات البكتريا المعوية المسببة لإنتان الدم في محافظة بابل، العراق

رســـالــــة مقدمـــة

الى مجلس كلية العلوم للبنات في جامعة بابل

كجزءٍ من متطلبات نيل درجة الماجستير

في العلوم / علوم الحياة

من قبـــل

دعاء حسين عبدعلي مصيبح

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بإشراف

أ.م.د. فاطمة معين عباس

الخلاصة:

تضمنت هذه الدراسة، جمع ١٦٣ عينة دم من مرضى مصابين بتجرثم الدم في مستشفيات محافظة بابل للفترة من تشرين الأول/٢٠٢٠ إلى نيسان/٢٠٢١. أظهرت النتائج بان ٦٣ عزلة (38.65 %) فقط اعطت نتائج ايجابية للنمو البكتيري من اصل (١٦٣) عزلة، منها ٣٥ عزلة بكتيرية (21.47%) تم تشخيصها كأنواع تابعة للعائلة المعوية enterobacteriaceae و ٢٨ عزلة بكتيرية (١٧.١٧%) كانت تابعة للانواع التالية (Streptococcus.spp و Staphylococcus.spp و Pseudomonas aeruginos). واضهرت الدراسة بأن العزلات التابعة للانواع klebsiellae pneumoniae (٢٢.٨٥%)، Proteus mirabilis (٢٠%)، Salmonella typhi (٢٠%)، Escherichia.coli (١٧.١٤%) هي أكثر الأنواع التي تم عزلها من عينات دم المرضى مقارنة بالأنواع البكتيرية المعزولة الأخرى التابعة للعائلة المعوية، كما أوضحت الدراسة حساسية الذكور (47.1%) والاناث(52.9%) للاصابة بتجرثم الدم كانت نفسها تقريبا.

تم دراسة تأثير مجموعة من المضادات الحيوية على العزلات البكتيرية (٣٥عزلة) باستخدام طريقة انتشار القرص (disk diffusion) ووجد بان غالبية العزلات كانت تمتلك مقاومة عالية للمضادات الحيوية وخاصة مجموعة البيتالاكتام، بينما لوحظت نسبة منخفضة من المقاومة في مجموعة ال carbapenems مثل ال meropenem (17.14%). أظهر الفحص المظهري لإنزيمات البيتالاكتاميز بطريقة (disk approximation test) بأن ٩ عزلات كانت منتجة لهذه الانزيمات.

كشفت نتائج طريقة ال Polymerase Chain Reaction (PCR) بان من بين ال ٣٥ عزلة بكتيرية، ١٩ عزلة تمتلك إنزيم من نوع TEM، ٢١ عزلة تمتلك انزيم من نوع OXA-10، ٢٣عزلة تملك إنزيم من نوع CTX-M-1. وأظهرت النتائج التي تم الحصول عليها بطريقة ال PCR بان الانزيمات TEM و OXA-10 و CTX-M-1 كانت واسعة الانتشار بين الأنواع العائدة لعائلة Enterobacteriaceae. بالإضافة إلى ذلك، تعتبر طريقة ال PCR من الطرق الجينية العالية الدقة في تحديد أنواع إنزيمات البيتالاكتاميز واسعة الطيف (ESBLs).

Chapter One

Introduction

1.1 Introduction:

Bacteremia is defined as the presence of bacteria in circulating blood whether continuously or transiently. It may be asymptomatic and resolve without treatment, or it may cause serious and immediate consequences including shock, organ failure and death (Tsai *et al*., 2014). In individuals with decubitus ulcers, Gram-negative bacteraemia is a condition caused by infection that generally begins in the gastrointestinal system or the skin (Brook, 2010, Montazeri *et al.,*2020).

Many of the most commonly used antibiotics in clinical practice belong to the β-lactam family. Because of their usefulness and relatively low toxicity, they have been essential historically and currently. Beta-lactamases production, which breaks down the structural beta-lactam ring of penicillin-like drugs is thought to be a major mechanism of bacterial resistance to β-lactam antibiotics, particularly in bacteria that causes clinically significant infections, and there are several classes of these enzymes, such as plasmid-encoded and chromosomally-encoded enzymes (Garca-Solache *et al.,* 2016).

Extended-spectrum β-lactameses (ESBLs) produced by *Enterobacteriaceae* are a huge global problem (Pitout *et al.,* 2008). These microorganisms are resistant to all penicillins and cephalosporins, as well as numerous additional antibiotic classes (Ben-Ami *et al.,* 2006). As a result, treatment options for infections caused by these bacteria are limited (Pitout *et al.,* 2008). ESBL-producing *Enterobacteriaceae* have been classified as pathogens by the Infectious Diseases Society of America, demanding the rapid development of new and innovative therapies(Talbot *et al*., 2006; Kern *et al.,*2020).

Extended-spectrum β-lactams have become commonly employed in the treatment of Gram-negative bacteria infections. Resistant to the extended-spectrum β-lactam has been an increasing concern among

Enterobacteriaceae species over the world (Padmini *et al.,* 2016). transferrable plasmid-encoded extended-spectrum β-lactamases are frequently linked to such resistance (ESBLs). Also most ESBLs from *K.pneumoniae* and *E. coli* are generated from SHV or TEM type β-lactamase and have one or more amino acid changes that confer resistance to broad-spectrum β-lactam antibiotics (Villegas *et al.,* 2008).

In hospital practice, ESBLs in Gram-negative bacillary infections become an increasing concern. The prompt selection of the right antibiotic therapy as well as the establishment of sanitary precautions to avoid further dissemination of the strains implicated are dependent on the successful discovery of the ESBL mechanism (Newbigging, 2019).

The Polymerase Chain Reaction (PCR) with oligonucleotide primers specific for a β-lactamase gene is the simplest and most popular molecular approach for redetecting the presence of β-lactamase enzyme belonging to a family of enzymes (Al-Jubori *et al.,* 2012).

1.2 The Present Study Aims for:

1. Isolation and identification of enterobacterial species from blood sample of patients attending diffrent hospitals in Babylon province

2. Determining antibiotic suspectibility pattern of bacterial isolates towards different antibiotics.

3. Phenotypic detection of extended-spctrum β-lactameses (ESBLs) producing by a disk approximation method.

4. Genotypic detection of ESBL enzymes ($bla_{CTX-M-1}$, bla_{TEM} and bla_{OXA-10} genes) by Polymerase Chain Reaction (PCR) technique.

Chapter Two

Letratuer Review

2.1 *Enterobacteriaceae*:

The *Enterobacteriaceae* Family are a Gram-negative, non-spore-forming rod and facultatively anaerobic bacteria. With the exception of few species, this family's general traits include motility. The Embden-Meyerhof pathway for metabolism of sugar and fermentative acid production from glucose, oxidase negative, catalase positive except in *Plesiomonas*. The geometry of the cell, oxidase production, arrangement of flagellar, salt needs, and they are recognized from other Gram-negative rod-shaped bacteria by the presence of enterobacterial common antigen (ECA). (Francino *et al.,* 2006).

However, the classic morphological and biochemical characterizations, which previously defined this family, are no longer unequivocal. 16S rDNA and additional gene sequences are necessary to differentiate members of this family (Francino *et al.,* 2006). *Escherichia*, *Enterobacter*, *Shigella*, *Salmonella Serratia,*, *Yersinia*, *Klebsiella*, *Citrobacter*, *Raoultella*, *Proteus*, *Edwardsiella*, *Hafnia*, *Providencia*, and *Morganella* are among the *Enterobacteriaceae* genera that regularly colonize human intestine or linked with human diseases (Forbes *et al.,* 2007).

Most of the genera described above are opportunistic pathogens that cause a variety of diseases in addition to nosocomial infections. However, the clinically most important species are briefly described below:

2.1.1 *Escherichia coli:*

Theodor Escherich, who is the first to isolate the type species of the genus, is given the name *Escherichia coli* (*E. coli*). (Castellani and Chalmers, 1919). *E. coli* is a well-known human and animal pathogen that lives in the intestine and it is a common cause of intestinal and extraintestinal diseases in humans and other animals. *E. coli* has been

found in nearly every system and tissue of the human body. enterohemorrhagic, enteropathogenic, enteroaggreative, enterotoxigenic, enteroinvasive, and extraintestinal pathogenic *E. coli* (EHEC, EPEC, EAEC, ETEC, EIEC, and ExPEC) are the different pathogenic classifications of *E. coli* based on their method of pathogenesis (Croxen and Finlay, 2010). *E. coli* is found in the environment as a result of contamination by feces and may also be a long-term resident, since environmental *E. coli* has been identified as unique (Walk *et al.,* 2009).

Bacterial conjugation, transduction, and transformation have all been documented in *E. coli* and similar bacteria, allowing genetic material to propagate horizontally across an existing population. This procedure resulted in the transmission of the Shiga toxin gene from Shigella to *E. coli* O157:H7 through a bacteriophage (Brussow *et al.,* 2004).

Cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveler diarrhea, and other clinical illnesses such as newborn meningitis and pneumonia can all be caused by virulent *E. coli* strains. hemorrhagic colitis or hemolytic-uremic syndrome (HUS) are caused by enterohemorrhagic *E coli* (EHEC) (Harrington *et al.,* 2006).

2.1.2 *Klebsiella* Species:

Klebsiella is a rod-shaped bacteria with unique polysaccharide capsules that are non-motile, oxidase-negative, and positive Voges-Proskauer test (Ryan and Ray, 2004). *Klebsiella spp.* are often identified in the gut microbiota of animals and humans (Bilinski *et al.,* 2016). They infect a broad range of animals and plants (Wyres and Holt, 2018), and they can survive in both soil and water (Rock *et al.,* 2014). *Klebsiella spp.* are opportunistic microorganisms in general (Wyres and Holt, 2018).

Klebsiella spp. cause urinary tract infection (Sewify *et al.,* 2016), infections of soft tissue, pneumonia, septicemia, bacteremia, meningitis,

and pyogenic (Youssef *et al.,* 2012). Because *Klebsiella spp.* have developed resistance to a variety of antibiotics, treating these infections has grown more difficult (Bengoechea and Sa Pessoa, 2019).

Capsular polysaccharides (CPS), lipopolysaccharides (LPS), siderophores, fimbriae (alternatively, pili), a type VI secretion system, outer-membrane proteins, porins, efflux pumps, an iron transport system, biofilms, and allantoin metabolism are all used by *Klebsiella spp.* to defend themselves from the immunological response of the host (Hsieh *et al.,* 2019). Among these, LPS, CPS, fimbriae, and siderophores are well-characterized virulence factors of *Klebsiella spp*. (Paczosa and Mecsas, 2016).

2.1.3 *Enterobacter* Species:

Enterobacter species can survive well on any medium, glucose and lactose may both create acid and gas, Carbon dioxide is created in two times the amount of hydrogen produced by glucose. The methyl red test is negative for the majority of species, whereas the Voges–Proskauer test is positive. H2S generation and phenylalanine deaminase are both negative (Hoffmann *et al.,* 2005b).

Enterobacter species are opportunistic bacteria that can cause nosocomial epidemics and invasive illnesses like bloodstream infections in hospitals (Qureshi *et al.,* 2011). *Enterobacter* can be detected in the intestines of humans and other animals (Hoffmann *et al.,* 2005a).

Gram-negative bloodstream infection is caused by *Enterobacter spp.* which represents the fourth most common cause for bacteremia (Al Hasan *et al.,* 2011). The *Enterobacter cloacae* complex, which includes *E. cloacae* and *E. Hormaechei*, is one of the most frequent *Enterobacter spp.* that cause nosocomial bloodstream infection (Mezzatesta *et al.,* 2012). Antibiotic resistance is also on the rise as a result of this (Al-Hasan *et al.,* 2011).

2.1.4 *Proteus* **Species:**

This genus contains small rods that can vary in length and motility. Acid production from D-xylose and glucose, tyrosine utilization, phenylalanine deaminase, H2S generation, gelatin hydrolyses and urea hydrolyses are all positive, whereas arginine dihydrolase, lysine decarboxylase, and Voges–Proskauer are all negative (O'Hara *et al.,* 2000a).

Proteus spp. are abundant and found in the intestines of reptiles, mammals, and birds. The flora of the human gastrointestinal system includes *P. mirabilis* and *P. vulgaris*. Both species may cause urinary tract infections in humans, although *P. mirabilis* is more common (Manos and Belas, 2006).

P. mirabilis is a significant nosocomial pathogen. It can also cause other infections including bacteremia, neonatal meningoencephalitis, empyema, osteomyelitis, and endocarditis (Kalra *et al.,* 2011). It is also implicated in rheumatoid arthritis with Proteus urinary tract infections as a triggering factor (Ebringer and Rashid, 2006**).**

2.1.5 *Salmonella* **Species:**

Salmonella spp. is a well-known foodborne bacterial disease that causes a significant number of illnesses and deaths across the world (CDC, 2007a). The majority of *Salmonella* infections are caused by consuming contaminated food and are characterized by gastroenteritis (Harish and Menezes, 2015). Invasive infection, such as bacteremia and meningitis, is more prevalent in people with weakened immune systems (Dhanoa and Fatt, 2009) such as individuals with HIV (human immunodeficiency virus) infection (Graham, 2010), infants (Jones *et al.,* 2018), and older adults who may have increased risk of complications, including death (Chen *et al.,* 2012).

There are other species of *Enterobacteriaceae*, that include: *Shigella*, *Raoultella*, *Yersienia* (pathogenic), *Citrobacter*, *Serratia*, *Providencia*, and *Morganella* (apportunistic) (Forbes *et al*., 2007).

2.1.6 *Shigella* Species

*Shigella*e are Gram-negative, non-motile, facultative anaerobic pathogens that are closely related to *Escherichia coli* but have evolved specific traits of pathogenicity, physiology and serology (Ud-Din and Wahid, 2014). that are positive for Catalase, Methyl Red, Nitrate Reduction and Gas production, and negetive for Oxidase, Voges Proskauer, Citrate utilization, Gelatin Hydrolysis, H2S production(Shimamoto *etal.,*2015).

Shigella is a common food-borne bacterial cause of dysentery and rarely causes bacteremia in the immunocompromised host (Keddy *et al.,*2012).

2.2 Bacteremia Caused by *Enterobacteriaceae*:

Blood is normally sterile so, culturing of such blood does not normally lead to isolation of the bacteria. Bacteremia is most usually diagnosed by blood culture (Thomson *et al.,* 2004).

Soybean-casein digest (SCD) broth is the most often used medium for aerobic blood culture bottles. Brain heart infusion (BHI) and enriched peptone broths are two other media used to recover fastidious bacteria. Anaerobic blood culture bottles are filled with a range of media, including SCD, Columbia, and peptone broths. The majority of commercial media contain patented mixes of nutrients and other growth agents (Lin *et al.,*2013).

Growth in media is indicated by the presence of turbidity, gas bubbles, and haemolysis of the blood in culture bottle or even by observation of bacterial colonies suspended in the bottle (Wayne, 2009).

Gram-stained smears should be made of any broth that shows visible signs of growth, cultured characteristics, biochemical, serological, and genetic analysis which are widely used to confirm the diagnosis of bacterial isolates (Isenberg, 2007).

In the general population, sepsis have a considerable influence on morbidity and death. (Goto and Al-Hasan, 2013). Effective antibiotic therapy is critical for reducing the burden of bloodstream infection, since incorrect antimicrobial therapy has been linked to higher fatality rates (Retamar *et al.,* 2012).

Several studies have looked at the risk factors, clinical results, and treatment outcomes of blood stream infection caused by extended spectrum beta-lactamase -positive isolates of *K. pneumoniae* and *E. coli* (Cordery *et al.,* 2008), but blood stream infection caused by *Proteus mirabilis* strains is a clinical entity that is not very common, and its importance has received very little attention (Endimiani *et al.,* 2015).

 P. mirabilis, on the other hand, is a less prevalent cause of bloodstream infection. (1 to 2% of all cases, ranking fourth among Gram-negative bacteria after *K. pneumoniae*, *E. coli*, and *Enterobacter spp.*). The rising prevalence of ESBL in strains of this species is now making this problem more interesting (Tumbarello *et al.,* 2012). ESBL-producing *P.mirabilis* (ESBL-P-PM) strains have been shown to be resistant to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (Winokur *et al.,* 2001).

Enterobacter cloacae and *Enterobacter aerogenes* have gained clinical relevance as opportunistic bacteria and have appeared as nosocomial infections in critical attention patients, specially those on mechanical ventilation. *E. cloacae* is common in nature, but it is also cuase diseases. the most common *E. cloacae* and *E. hormaechei* isolates from human clinical samples, in the recent decade, *E. cloacae* has

become one of the most prevalent *Enterobacter spp.* producing only nosocomial infections, and much has been written on their antibiotic resistance characteristics (Randolph and McCulloh, 2014). Despite *E. Cloacae* importance as a nosocomial pathogen, the pathogenic processes and factors involved in illness caused by the *E. cloacae* complex are still unknown. Its pathogenicity is dependent on its capacity to build biofilms and release different cytotoxins (enterotoxins, hemolysins, pore-forming toxins) (Mezzatesta *et al.,* 2012).

Low production of the natural inducible cephalosporinase makes *E. cloacae* naturally resistant to amoxicillin–clavulanic acid, cephalothin, cefoxitin, and ampicillin. They are capable of overproducing AmpC β-lactamases by blocking the repression of a chromosomal gene or by the acquisition of a transferable ampC gene on plasmids conferring the resistance to third-generation cephalosporins (Davin-Regli, 2015).

In the last decade, *E. cloacae* has emerged as the third most common *Enterobacteriaceae* resistant to third generation cephalosporins with enteric *E. coli* and *K. pneumoniae* (Jarlier and INVS, 2014). Bloodstream infections also involve other members of *Enterobacteriaceae* such as *Salmonella, Serratia and Citrobacter* (Winokur *et al.,* 2001).

Sepsis is the leading cause of mortality in newborns and children across the world (Carcillo, 2005). When pediatricians say "sepsis," they typically mean an infection that overwhelms the host, resulting in capillary leak, respiratory failure, and/or hypotension. Children must have a proven or suspected illness, as well as symptoms of a systemic reaction to that infection, to be septic. end organ system involvement must be diagnosed in severe sepsis. Cardiovascular dysfunction is required for septic shock (Goldstein *et al .,* 2005). It also causes death in children under the age of five, particularly in developing countries, where a

combination of   poor sanitation, indoor air pollution, crowding, contaminated water, inadequate immunization, low birth weight, and nutrition allow pathogens to invade and multiply relatively unchecked in the body (Rudan *et al* ., 2008).

If certain germs are present after delivery, Early sepsis in newborns is a possibility. If some germs are introduced to newborns before delivery, they can develop early-onset sepsis. The following are some of the risk factors for early sepsis: infection in the mother, such as chorioamnionitis (presence of group B *streptococcus* in the mother), and premature rupture of the fluid-filled membranes that surround the embryo (Randolph and McCulloh, 2014).

Childrens exposure to diseases might rise as they become older, as they attend child care, go to school, and participate in activities like sports. Bacterial infections in children and adults include urinary tract infections, skin infections, pneumonia, appendicitis, and meningitis. All of these can lead to sepsis if left untreated (Liang, 2016). *Enterococcus spp. ,Klebsiella spp. ,Escherichia coli* and GBS, were most commonly identified in infants and children (Bhat *et al* ., 2011).

2.3 Bacteremia Caused by Gram Positive Bacteria:

Bacteremia have become more common among hospitalized patients in the recent decade. Gram-positive bacteria have become major pathogens in the community and hospitals in recent years. To make the problem worse, Gram-positive bacteria are increasingly exhibiting antibiotic resistance, which was formerly thought to be the realm of Gram-negative bacteria (Howe *et al.,* 2016).

Three etiologic variables have contributed significantly to the rise in the relative prevalence of Gram-positive BSI across all nosocomial infections.changes in demography of patients, increased use of intravascular and other prosthetic devices, as well as increased use of

broad-spectrum drugs such as cephalosporins. Since the early 1980s, the proportion of blood stream infection because of Gram-positive microorganisms has grown, while the proportion of blood stream infections in total nosocomial infections has been increasing (Pfaller *et al* .,2018).

2.4 β-Lactam Antibiotics:

The history of β-lactam antibiotics began in 1929 when Alexander Fleming described antibacterial activity of a substance produced by *Penicillium* mould and gave it the name penicillin. However, the phenomenon of antibiotic is already well known at this time (Letek, 2020).

Antibiotics are chemical substance produced by a living organism, generally a microorganism, that is detrimental to other microorganisms. Microorganisms that produce antibiotics useful in preventing or treating disease include bacteria and fungi illness. They may either kill or inhibit the growth of bacteria(John Wiley and Sons Inc, 2012).

The development of many additional antibiotics was sparked by the introduction of penicillin into therapeutic use. The invention of semi-synthetic penicillins, followed by the creation of semi-synthetic cephalosporins and other β-lactam antibiotics, ushered in a huge expansion of the β-lactam field in the early 1960s (Letek, 2020). Antibiotics classified as β-lactams comprise a wide range of drugs.
 β-lactam antibiotics include cephalosporins, penicillins, and carbapenems. Monobactams (aztreonam) are structurally identical to other β-lactams, but they lack one of the two rings seen in other beta-lactams and have little to no cross-allergenicity (Romano *et al.,* 2016). The most common mechanism of bacterial resistance to β-lactam antibiotics is the formation of β-lactamase. Extended spectrum

β-lactamases (ESBLs), which are largely plasmid mediated, are able to inactivate newer cephalosporins (Queenan and Bush, 2017).

Members of *Enterobacteriaceae* are increasingly resistant to currently available antibiotics (Tzouvelekis *et al.,* 2012). In particular, resistance to β-lactams and fluoroquinolones is widespread among the *Enterobacteriaceae* and has emerged as major challenges as these antibiotics are important drug classes used to treat infections caused by *Enterobacteriaceae.* The newer β-lactamases encountered in *Enterobacteriaceae* include AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs) [e.g., cefotaxime first isolated at Munich (CTX-M) types, carbapenemases (e.g., *Klebsiella pneumoniae* carbapenemase (KPC) types, the metallo-β-lactamases (MBLs) and the oxacillinase type (OXA)] (Marsik and Nambiar, 2011).

AmpC β-lactamases, conferring resistant to most cephalosporins and monobactams, are normally chromosomally encoded and constitutively expressed in *E. coli* and inducible in *Enterobacter, Citrobacter freundii, Morganella, Providencia, and Serratia.* Plasmid-encoded AmpC has been found in *E. coli and Klebsiella* (Denton, 2007). ESBLs confer resistance to penicillins, cephalosporins, and monobactams. The common ESBLs are of TEM, SHV, and CTX-M types and are widely distributed among *Enterobacteriaceae* (Pitout, 2008) .

In the *Enterobacteriaceae* family, KPC β-lactamases are common. Penicillins, carbapenems, cephalosporins, and monobactams confer resistance, while commercial β-lactamase inhibitors, like tazobactam and clavulanic acid, suppress them (Davin-Regli, 2015). The effectiveness of β-lactamases in hydrolyzing the β-lactam ring, which is required for antibiotic action, varies greatly, and the enzymes may be considered of as a family with a wide range of activity.

Penicillinases are enzymes that have a high affinity for penicillins (6-aminopenicillanic acid derivatives) and was the first β-lactamase to be discovered; it was isolated from *E. coli* by Abraham and Chain in 1940, long before penicillin was used in clinical practice (Jarlier *et al.,* 2019).

New Delhi metallo-beta-lactamase1(NDM-1), which was initially identified in 2009 (Yong *et al,.* 2009) and is currently present in practically all clinical *Enterobacteriaceae* species and in many regions of the world, is the most recent addition to the MBLs. The bla$_{NDM-1}$ gene may be found on the chromosome as well as on a variety of plasmids (Walsh and Toleman, 2011).

In *Enterobacteriaceae*, OXAtype carbapenemases are less common. OXA-48 was discovered in *C. freundii* and *E. coli* after it originally appeared in *K. pneumoniae* (Tzouvelekis *et al.,* 2012). Quinolone resistance is mainly caused by chromosomal mutations in topoisomerase IV and DNA gyrase in *Enterobacteriaceae*. Quinolone resistance mediated by plasmids has been reported in a wide range of bacteria, including *Klebsiella*, *E. coli*, *Providencia*, *Enterobacter*, and *C. freundii stuartii*. Quinolone resistance genes are frequently found in combination with ESBL genes (Strahilevitz *et al.,* 2009).

Table (1-1): Groups and Examples of β-Lactam Antimicrobial Agents (modified from Samaha-Kfoury and Araj, 2003).

β-Lactam Groups	Antimicrobial Agents Example
Penicillins	• Penicillin,VpenicillinG • Penicillinase resistant penicillins: nafcillin,methicillin, cloxacillin, oxacillin. • Carboxypenicillins:ticarcillin,carbenicillin. • Aminopenicillins:amoxicillin,ampicillin. • Ureidopenicillins: piperacillin, mezlocillin.
Cephalosporins	• First generation: cephalothin, cefazolin,cephalexin

	• Second generation:cefamandole, cefaclor, Cefuroxime • Third generation**:** cefotaxime, ceftriaxone,ceftizoxime Cefoperazone, ceftazidime. • Fourth ,generation: cefpirome, cefepime. • Fifth generation: ceftobiprole Cephamycin: cefoxitin, cefotetan, cefmetazole
ß-Lactamase inhibitor	Ampicillin + sulbactam, Amoxicillin + clavulanic acid, Piperacillin + tazobactam.
Carbapenems	Imipenem, meropenem, ertapenem.
Monobactams	Aztreonam.

2.5 Bacterial Resistance to β-Lactam Antibiotic:

Resistance to β-lactam antibiotics in bacteria could be due to four mechanisms (Lingzhi *et al.,* 2018).

2.5.1 Destruction the Active Drug by the β-Lactamase:

Beta-lactamases are enzymes produced by bacteria that provide multi-resistance to β-lactam antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems (ertapenem), although carbapenems are relatively resistant to beta-lactamase. Beta-lactamase provides antibiotic resistance by breaking the antibiotics' structure. These antibiotics all have a common element in their molecular structure: a four-atom ring know n as a β-lactam. Through hydrolysis, the enzyme lactamase breaks the β-lactam ring open, deactivating the molecule's antibacterial properties (Jarlier *et al.,* 2019).

2.5.2 Decreased Affinity of the Target Penicillin Binding Proteins (PBPs):

When PBPs are inhibited, cell wall metabolism becomes unbalanced, leading in inhibition of growth or lysis. The relationship between PBP inhibition and biological outcomes is still unknown. [e.g. *Staphylococcus aureus* (Giesbrecht *et al.,* 1998), *Escherichia coli* (Santos*.,* 2012),

Enterococcus hirae (Pucci *et al.,* 1986), *Streptococcus pneumoniae* (Mascher *et al.,* 2006)].

Despite the ignorance of the detailed consequences of β-lactam treatment, various means of resistance have been uncovered and investigated, such as a decreased permeability of the outer membrane, export of the antibiotics (these mechanisms are restricted to gram-negative bacteria), degradation of the antibiotic by β-lactamases or utilization of PBPs with low affinity for the β-lactams.

2.5.3 Decreased Permeability of Drug into the Cell:

Gram-negative bacteria have an outer membrane that acts as extra barrier and preventing harmful chemicals as antibiotics from penetrating. Porins are proteins found in the outer membrane of cells that regulate cellular permeability and antibiotic resistance (Choi *et al.,* 2019).

Polar antibiotic drugs, including β-lactams, floroquinolones, and aminoglycosides, enter the periplasmic space through porin channels. Specificity of the outer membrane protein for the antibiotic drug is important. Because porins mediate the passive diffusion of antibiotics across the outer membrane , they are closely associated with antibiotic resistance in the Gram-negative bacteria. For example, β-lactams and fluoroquinolones were known to penetrate the outer membrane through the non-specific porin OmpF (Delcour, 2009). Therefore, the ompF mutant was resistant to several β-lactam antibiotics in some Gram-negative pathogens, including *Escherichia coli* (Ziervogel and Roux, 2013), *Klebsiella pneumoniae* (Sugawara *et al.,* 2016), *Serratia marcescens* (Moya-Torres *et al.,* 2014), and *Enterobacter aerogenes* (Bornet *et al.,* 2000).

2.5.4 Efflux Pumps:

In Gram-negative and/or Gram-positive bacteria, many kinds of pumps have been discovered that remove one or more antibiotics from the bacterial cell. They might be very selective or have a broad substrate specificity. The majority of these pumps are found in the cytoplasmic membrane and drive drug efflux via proton motive force. *Pseudomonas aeruginosa* is one example of this resistance mechanism (Strateva and Yordanov, 2009).

2.6 Occurance of β-Lactamase Resistance:

Abraham *et al.,* (1940) were the first to describe β-Lactamase as a penicillin-degrading enzyme found in bacteria. The synthesis of these hydrolytic enzymes, either constitutive or inducible, is the first factor that contributes to bacterial resistance. The International Union of Biochemistry Special Commission classified these enzymes as amide and cyclic amide bond cleaving enzymes (Webb, 1984) .

Throughout the 1960s and 1970s, a relentless raise in reports of resistance to β-lactam as consequence of selection of bacteria that produce β-lactamase (Heritage *et al.,* 1999), while in early 1980s, a level of 30%-40% in both hospitals and community in the United Kingdom and in many other countries was elevated (Verbist and Piot, 1986).

Many novel β-lactam antibiotics have been created in the last 20 years that were especially created to be resistant to β-hydrolytic lactamases activity. Bacteria, on the other hand, created a novel class of β-lactamases with broad range activity (Haller *et al.,* 2018).

2.7 Mechanisms of Action of β-Lactamase:

Antibiotics have five different modes of action: inhibition of cell wall formation, inhibition of nucleic acid synthesis, cytoplasmic membrane impairment, metabolic antagonist activity, and inhibition of protein

synthesis. In general, there are four basic mechanisms by which resistance to drug may occur in bacteria: alteration of the antimicrobial target that can be due to the complete loss of affinity or simple reduction of it; reduction in the amount of the antimicrobial that reaches the target by entrance reduction caused by a decrease permeability due to porin mutation or by an exit increase caused by the pumping out by an efflux transporter; the presence of an enzymatic mechanism that totally or partially destroys the antimicrobial molecules; and the development of an alternative metabolic pathway involving precursors (Harbottle *et al.,* 2016).

The four-membered β-lactam ring of cephalosporin and penicillin antibiotics, such as the carbapenem series, is hydrolyzed by β-lactamases. They deactivate the chemical characteristics of the drug molecule, which is the chemically reactive acylating group for changing the active site serine side-chains in PBPs, and so remove the antibacterial action. The activity of the β-lactase enzymes was discovered a number of years before penicillins were used clinically in humans, indicating its presence in soil bacteria that combat the natural product penicillins (Livermore and Woodford, 2000) .β-lactamases inactivate the β-lactam antibiotics by breaking the amide bond in β-lactam ring which leads to the destruction of antibiotic molecule, turning the antibiotic into an inactive compound (Harbottle *et al.,* 2016).

2.8 Classification of β-Lactamases:

Many Gram-negative and Gram-positive bacteria generate the β-lactamases. They differ in isoelectric point (IP), substrate profile, sensitivity to inhibitors, molecular weight, and genetic determination (Bush and Jacoby, 2010). Such classification was first proposed by Jack and Richmond in 1970 (Jack and Richmond, 1970) and was expanded by Richmond and Sykes in 1973 (Richmond and Sykes, 1973). The enzymes

were categorized into five classes using this system, Class I enzymes are mostly cephalosporinases, class II enzymes are penicillinases, Class III enzymes have a broad spectrum of activity and are susceptible to cloxacillin inhibition, also they have a high resistance to p-chloromercuribenzoate (pCMB), class IV enzymes are broad-spectrum but resistance to cloxacillin inhibitions and susceptible to pCMB, and lastly, Class V enzymes are represented by penicillinases that can hydrolyze cloxaci (Jack and Richmond, 1970).

Classes A and B were proposed by Sykes and Matthew (1976), Class A enzymes are classed as (a) cephalosporinases, (b) penicillinases, and (c) broad-spectrum ß-lactamases and are chromosomally mediated. Class B enzymes are determined by R-plasmids and subclassification leads to isoxazolyl-non-hydrolysing, isoxazolyl-hydrolyzing, and other β-lactamases.

The Ambler classification classifies β-lactamases into four groups (A, B, C, and D) according to their amino acid sequences. This type of categorization is stable because it represents underlying connections and is unaffected by mutations (Ambler, 1980).

Bush proposed a categorization scheme for β-lactamases in 1989, which was modified in 1995 and than in 2010 (Bush and Jacoby, 2010), categorised them by their substrate preferences and susceptibility to clavulanate inhibition (Bush *et al.,* 1995). The updated system of Bush includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases and serine carbapenemases; and group 3 metallo-β-lactamases. Several new subgroups of each of the major groups are described, based on specific attributes of individual enzymes. A list of attributes is also suggested for the description of a new β-lactamase, including the requisite microbiological properties, substrate and inhibitor profiles, and

molecular sequence data that provide an adequate characterization for a new β-lactam-hydrolyzing enzym (Bush and Jacoby, 2010).

Phenotypic classification face the problem that point mutation can greatly alter substrate specificity and inhibitor susceptibility (Vedel *et al.,* 1992) changing the group to which an enzyme is assigned. On contrast, sequence-based classification of Ambler is more stable because it cannot be destroyed by mutations (Jacoby *et al.,* 2016).

2.9   Genetic Factors Controlling the Production of β-Lactamases:

2.9.1 Chromosomally-Mediated β-Lactamases:

In Gram-positive bacteria, no chromosomal β-lactamases have been detected in *staphylococci*, *streptococci*, and *enterococci*. Chromosomally mediated resistance to β-lactam antibiotics in members of these genera is as a result of other processes, such as alternations in PBPs (Rice, 2006). However, many *Bacillus spp.* and some *closteridia* produce inducible chromosomal β-lactamase (Shahid *et al.,* 2009).

In Gram-negative bacteria, Except for *Salmonellae*, chromosomal β-lactamases are almost ubiquitous in *enterobacteria*. They vary considerably in amount, mode of action, production, and contribution to resistance (Spafford *et al.,* 2019).

Molecular class A enzymes are found in certain species, whereas class C enzymes are found in a higher number. The expression of chromosomal β-lactamases can be inducible, low or high level constitutive in *Enterobacteriaceae*, Depending on the strain and the species. *E. coli* presents the simplest case, because they usually have a low quantity of inducible molecular class C enzymes, often known as AmpC types (Jacoby and Munoz-Price, 2015). *Klebsiella spp.* has a class A (SHV-1,TEM-1, TEM-2) chromosomal β-lactamases, which are much

different from class C types. SHV-1(from sulfhydryl variab) β-lactamases are found in the majority of *K. pneumoniae* isolates and they are normally generated in little amounts that are sufficient to defend against ampicillin, amoxicillin, carbenicillin, and ticarcillin (Bonomo, 2017).

In Gram-negative bacteria, over 200 different plasmid-mediated β-lactamases have been reported. The first plasmid mediated β-lactamase, TEM-1 was discovered in an *E. coli* isolate from a patient named Temoneira (thus the name TEM) in Athens, Greece, in 1965 (Datta and Kontomichalou, 1965). Because its plasmid and transposon mediated, TEM-1 has spread to additional *Enterobacteriaceae* species, such as *P.aeroginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*. (Fre`re, 1995; Bush, 2010).

Another plasmid-mediated β-lactamase is SHV-1 found in *E. coli* and *K. pneumoniae*. The SHV-1 β-lactamase is chromosomally encoded in the majority of *K. pneumoniae* strains, but it is mostly plasmid-mediated in *E.coli* (Cantón *et al.,* 2012). chromosomal types differ from plasmid-mediated β-lactamases in general, however there are some overlaps exist, such as SHV-1 β-lactamase, which is both a plasmid type and a chromosomal origin (Shobha *et al.,* 2009).

2.10 Extended-Spectrum β-Lactamases (ESBLs):

Antibiotic-resistant bacteria have emerged as a severe danger to public health. β-lactamases (Blas) Expression (a type of enzyme able of cleaving the β-lactam ring of antibiotics), leading to the absence of antibiotic activity, is one of the most prevalent mechanisms in resistance (Bonomo, 2017). Furthermore, considerable numbers of these bacteria, categorized as expanded spectrum betalactamase (ESBL) producers, are found to show resistance not only to one type of drugs but to various β-lactam-based antibiotics (Shaikh *et al.,* 2015).

The commonest plasmid-mediated β-lactamases encountered in *Enterobacteriaceae* are TEM-1, TEM-2, SHV-1, and OXA-1 enzymes, which have a weak activity against first generation cephalosporins (Naas *et al.*, 2017).

Bacteria resistant to both cefotaxime (CTX) and ceftazidime (CAZ), the third-generation oxyimin-cephalosporins, at the same time are generally regarded as strong candidates for ESBL producers.Third-generation cephalosporins are more effective against Gram-negative bacteria, and they are commonly used to treat Gram-negative bacterial infections.The acquisition of various forms of ESBL and/or plasmid-mediated AmpC-lactamases (PABL) is primarily responsible for gram negative bacilli resistance to third-generation cephalosporins (Thai *et al.,* 2015). Despite their capacity to hydrolyze third-generation cephalosporins, they have different antibiotic preferences depending on the kind of β-lactamases. The process of developing the next generation of antibiotics to treat drug-resistance bacteria is time-consuming and expensive (Dortet *et al.,* 2014).

Bacteria continue to modify enzymes that render newly discovered antibiotics ineffective. As a result, separated of infected patients according to early and sensitive detection of antibiotic resistant, regardless of the type of β-lactamases, is an alternate option to deal with the problem and preventing the spread of drug-resistant bacterial infections (Dortet *et al.,* 2014). Because of their increased spectrum of activity, those enzymes were called extended-spectrum β-lactamases (ESBLs).They were first recognized in a single strain of *K. pneumoniae* isolated in Germany (Yazdansetad *et al.,* 2019).

Besides *K. pneumoniae* and *E. coli*, TEM-type ESBLs have been reported in *Morganella morganii*, *Salmonella spp.*, *Enterobacter aerogenes,* and *Proteus mirabilis.* The majority of SHV-type ESBLs are

found in *K. pneumoniae* strains (Doi Y *et al.*, 2007). ESBLs were also found in other Gram-negative bacteria.

Most of ESBL-expressing bacteria are sensitive to cephamycins (cefoxitin and cefotetan). Martinez-Martinez *et al.,* (1996), on the other hand, found that ESBL-producing bacteria can develop cephamycin resistance owing to the deletion of an outer membrane porin protein. Porin deficiency has been linked to antibiotic resistance in both laboratory mutants and clinical isolates (Jacoby *et al.,* 2004).

2.11 ESBL Detection Methods:

ESBLs can be detected in the laboratories by two main methods; clinical microbiology method and molecular method. The two methods are shortly described below:

2.11.1 Clinical Microbiology Techniques:

Jarlier *et al.,* (1988) described the disk approximation test, also referred to as "double disk synergy" test, which uses a clavulanic acid-containing disk (usually amoxicillin-clavulanate) in the close proximity to aztreonam and disks included extended-spectrum cephalosporins to prove that the test strain possesses ESBL and its action is inhibited via clavulanic to demonstrate that the test. ESBL production is inferred by enhancement of the zone of inhibition between the clavulanate-containing disk and one or more of the antibiotic-containing disks (Jorgensen *et al.*, 2011).

Because practically all ESBLs exhibit clear resistance to ceftazidime, screening with this chemical is the most practical option. Testing with ceftazidime with and without clavulanate, as an inhibitor, allowing for the detection of ESBL formation using zone comparison (Wayne, 2009).

• The Vitek ESBL test which makes use of an autoamated growth moniatoring system, and E-test which uses a plastic drug-impregnated strips are two examples of such tests (Essack, 2000).

• It was also suggested that determination of minimum inhibitory concentrations (MICs) of extended-spectrum cephalosporins with and without clavulanate, could be used for discovery of ESBLs in clinical *Klebsiella* isolates. In this test, MICs for cefotaxime, ceftazidime, ceftriaxone, and aztreonam with and without clavulanic acid are determined by a two-fold microdilution technique. The test is considered positive if the MICs of the ß-lactams reduced $\geq$ 8-folds by clavulanate (Rawat and Nair, 2010).

CLSI (The Clinical and Laboratory Standards Institute) proposed an initial screening test in broth medium with 1 g/ ml of one of five expanded-spectrum β-lactam antibiotics for β-lactamase-producing isolates. The presence of ESBL was suspected when a positive result was obtained. Following this, a phenotypic confirmation test is performed, which involves calculating the MICs of cefotaxime or ceftazidime with and without clavulanic acid. In the presence of clavulanate, a drop in the MICs of 8 two-fold dilutions is considered an ESBL existence evidence (CLSI, 2020).

The existence of ESBLs in a bacteria does not necessarily create a resistant phenotype when applying the usual MIC and disk diffusion interpretative criteria established by the NCCLS, making identification of organisms generating ESBLs challenging. However, the tests mentioned above were described presumptively identity the presence of ESBL, without identifying ESBL in clinical isolates specifically, hence molecular methods are described as quite satisfactory (Coudron *et al.*,1997).

2.11.2 Molecular Methods:

Molecular methods use the DNA probes that specific for TEM and SHV enzymes (Croxen.2010). The first molecular method for the identification of β-lactamase was the oligotyping method developed by Ouellette *et al*., (1988), which was used to discriminate between TEM-1 and TEM-2 enzymes. Several additional methods for detecting and identifying ESBLs have been developed since, but nucleaotide sequeancing remains the standarad for determining the particular β-lactamase geane presaent in a bacterial strain (CLSI, 2020).

Chapter Three
Material and Methods

3.1 Material:

3.1.1 Equipments:

The following are the Equipments that were utilized in this study (**Table 3-1**).

Table (3-1): Equipments used in this research

No	Instrument	Company \ Origane
1.	Autoclave	HIRAYAMA \ Japan
2.	Beakers	Volca \ Iraq
3.	Buener	Volca \ Iraq
4.	Disposable plastic petri dishes	Bio-Hit, Finland
5.	Distillator	Memmert \ Germany
6.	Eppendorf centrifuge	MIKRO 120 \ Germany
7.	Electrophoresis power supply	American science and surplus \ France
8.	Gel photo documentation system	American science and surplus \ France
9.	Hood	Bio Lab \ Korea
10.	Horizontal Electrophoresis unit	Pelex \ France
11.	Incubator	Memmert GmbH \ Germany
12.	Light Microscope	Olympus\ Japan
13.	Micropipettes5-50 ml, 100-1000 ml, 0.5-10 ml	Slamid \ USA
14.	Microwave oven	American science and surplus \ USA
15.	Nanodrop device	Thermo ScientificInc \ USA
16.	Oven	Olympus \ Japan
17.	PCR divice	Applied bio system \ USA
18.	PCR Tubes	Eppendorf \ Germany
19.	Plastic Test tubes 10ml	AFCO, Jordan
20.	Refrigerator	Ishtar \ Iraq
21.	Sensitive balance	American science and surplus \ USA
22.	Tips (Different sizes)	Jippo \ Japan
23.	Uv-Visible Spectrophotometer	Shimadzu -1800 \ Japan
24.	Ultra violet light transiluminator	American science and surplus \ USA
25.	Vortex Mixer	Griffin \ England

3.1.2 Biological Materials:

Table (3-2): The Biological Meadia Used in This Study

NO.	Culture Media	Company \ Origane
1.	MacConkey agar	Himedia Accumax \ India
2.	Blood agar base	Himedia Accumax \ India
3.	Nutrient agar	Oxoid \ England
4.	Brain heart infusion broth (BHI)	Prondisa \ spain
5.	Salmonella shigella agar(SSA)	Oxoid \ England
6.	Eosin Methylin blue (EMB) medium	Himedia Accumax \ India
7.	Kligler iron agar medium	Biolife \ Italy
8.	Simmons citrate agar	Himedia Accumax \ India
9.	Urea agar	Himedia Accumax \ India
10.	(MR-VP) medium	Himedia Accumax \ India
11.	Peptone water media	Mast\ U.K
12.	Muller-Hinton agar	Oxoid \ England

3.1.3 Chemical Materials:

Table (3-3): The Chemicals Used and Their Sources

No.	Chemical Materials	Company \ Origane
1.	Agarose	Froggabio ,Canada
2.	Crystal violates	BDH/England
3.	Catalase reagent	Prondisa \ spain
4.	DNA ladder marker (1500&5000bp)	Biolab, UK
5.	Distilled water	Oxoid \ England
6.	DNA loading dye	Promega/USA
7.	Ethanol 70%	BDH/ England
8.	Ethidium Bromaide	Sigma/USA
9.	Genomic DNA extraction kit	favorgen/taiwan
10.	Iodine	BDH/England
11.	Kovac's reagent	Oxoid \ England
12.	Methyl red reagent	Prondisa \ spain
13.	Nuclease-Free- Water	Promega/ USA
14.	Oxidase reagent	Oxoid \ England
15.	PCR pre mix (master mix)	Bioneer/Korea
16.	Primers	Bioneer/Korea
17.	Safranine	BDH/England
18.	Tris Borate EDTA buffer(10 x)	Sigma/USA
19.	Voges-Proskauer reagent	Biolife \ Italy

3.1.4 Antibiotic Disks:

Table (3-4): Antibiotics Discs Used in This Study

NO	Antibiotic Discs	Symbol	Disc potency (µg)	Origane
1.	Gentamicin	CN	10	
2.	Meropenem	MEM	10	
3.	Ceftazidime	CAZ	30	
4.	Chloramphenicol	C	10	
5.	Amoxicillin	AMC	30	Bioanalyse/Turkey
6.	Ampicillin	AM	30	
7.	Cefotaxime	CTX	30	
8.	Azithromycin	AZM	30	
9.	Ceftriaxone	CRO	30	
10.	Nitrofurantion	F	300	

3.1.5 Primers in PCR:

Tabale (3-5) Primers Used in This Study.

Primer Name	Sequence	Size (bp)
TEM(F)	5'- ATAAAATTCTTGAAGAAGACGAAA-3'	1080
TEM(R)	5'- GACAGTTACCAATGCTTAATC -3	
OXA-10(F)	5'-GTC TTT CGA GTA CGG CAT TA-3'	720
OXA-10(R)	5'-ATT TTC TTA GCG GCA ACT TAC- 3'	
CTX-M-1(F)	5'-AAAGTGATGGCCGTGGCC -3'	522
CTX-M-1(R)	5'-GATATCGTTGGTGGTGCCA-3'	

3.1.6 DNA lader marker Used in PCR:

Tabale (3-6) DNA Leader Marker Used in This Study.

DNA Leader Wight	Company \ Origane
1500bp	Biolab, UK
5000bp	Biolab, UK

3.1.7 Master Mix Used in PCR (Promega, USA):

2x Green Tag reaction buffer pH 8.5 is included with Tag DNA polymerase, 400 m dGTP, 400 m dATP, 400 m dTTP, 400 m dCTP, and 3 mM MgCl2.

3.1.8 Genomic DNA extraction kit (G-spin™ genomic DNA extreaction kit ,korea):

Tabale (3–7): Genomic DNA Extraction Kit

Material Name	Size
G-buffer	20 ml
Binding buffer	15 ml
Washing buffer A	9 ml
Washing buffer A	10 ml
Elution buffer	20 ml
Columns	50 columns
Collection tubes	50 tubes
Rnase A	3 mg
Proteinase K	1.76 mg
Lysozme	20 mg

3.1.9 Preparation of Reagents:

The following reagents were prepared as described in MacFaddin (2000):

3.1.9.1 Catalase Reagent:

3% H_2O_2 solution was made using 97 ml D.W and 3ml H_2O_2 and kept in a dark container .

3.1.9.2 Oxidase Reagent:

In a dark bottle, dissolve 0.1 g of Tetramethyl-p-phenylene-diamine dihydrochloride in 10 ml of D.W.

3.1.9.3 Kovac's Reagent:

It was made by dissolving 5 gm of P-dimethylamine benzy laldehyde in 75 ml of amyle alcohole and 35 ml of concentrate H Cl acid in 75 ml of amyle alcohole. It can be used to detect the formation of Indole.

3.1.9.4 Methyl Red Reagent:

The solution was used to measure the acidity in the medium, which was formed as a consequence of complete carbohydrates fermentation. A weight of (0.01gm) of methyl red was mixed in (30ml) of ethanol (99%) and then the final volume was made to (50ml) distilled water.

3.1.9.5 Voges-Proskauer Reagents (Bareritt's Reagent):

It is utilized to detect acetyl-methel carbinol synthesis. It is made up of the following two soloutions:

A. 5 gm of α-nephthol added to 99 % ethanol (100 ml), then kept in a derk conteiner.

B. 40 gm of potassium hydroxide (KOH) mixed in 100 m l of distilled water.

3.1.10 Preparation of Culture Media:

3.1.10.1 Blood Agar Medium:

The manufacturer recommendations procedure for preparing blood agar media was followed**.** This medium was used to promote bacterial isolation and to assess bacteria's ability to determine the type of Hemolysis (Russell *et al*., 2006).

3.1.10.2 MacConkey Agar Medium:

The manufacturer recommendations procedure for preparing MacConkey agar medium was followed. It was utilized to distinguish Gram negative bacteria from Gram positive bacteria (MacFaddin, 2000).

3.1.10.3 Nutrient Agar Medium:

The manufacturer recommendations method for nutrient agar medium preparation was used. When necessary, it was utilized for the culture of bacterial isolates(MacFaddin, 2000).

3.1.10.4 Peptone Water Medium:

The manufacturer recommendations procedure for preparing this medium was followed. It was used to demonstrate the capacity of bacteria to convert the amino acid tryptophan to indole. The creation of a red ring at the upper of the broth was a positive result(MacFaddin, 2000).

3.1.10.5 Eosin Methylene Blue (EMB) Medium:

The manufacturer recommendations method for nutrient agar medium preparation was used.. It was used to distinguish *Escherichia coli* from other *Enterobacter* which has a green metallic sheen in light reflected (Russell *et al*., 2006).

3.1.10.6 Maintenance Medium:

The maintenance medium consisted as a basal medium of brain heart infusion broth, enhanced with (15 percent) glycerol , Following autoclaving for (15) minutes at (121 ° C) and cooling at (45 ° C). It was dispersed in 5 ml sterile test tube. This medium had been used to maintain long-term of bacterial isolates at (-20 ° C).(Van Bodegom, 2007)**.**

3.1.10.7 Motility Medium:

The manufacturer recommendations method for nutrient agar medium preparation was used.The purpose of this medium was to identify bacterial movement(Russell *et al*., 2006).

3.1.10.8 Urea Agar Medium:

The manufacturer recommendations method for urea agar medium preparation was used. The medium was placed in to sterile test tubes and

allowed to solidify in a slant shape after the pH was adjusted to 7.1.This madium was used to examine the capacity of bacteria to form the urease enzyme (Forbes *et al.,* 2007).

3.1.10.9 Simmon's Citrate Agar:

The manufacturer recommendations procedure for preparing this medium was followed. It was used to demonstrate the ability of bacteria to utilise citrate as their only source of energy. The change of color from green to blue was a positive outcome(Forbes *et al.,* 2007).

3.1.10.10 Kligler Iron Agar:

Kligler iron agar was preparered according to the manufacturing company. This media is used to determine bacterial capacity to ferment glucose and lactose, as well as probable hydrogen sulfide (H2S) generation(Forbes *et al.,* 2007).

3.1.10.11 Muller-Hinton Agar Medium:

Muller-Hintoin agar medium was produced according to the manufacturer's instructions. It was used to determine the sensitivity of bacterial isolates to antibiotic(MacFaddin, 2000).

3.1.11 Soluotions Used in DNA Exatraction:

The following soluotions were prepared as desicribed by Posipiech and Neuimann, (1995):

3.1.11.1 Lyophilized RNase A Solution :

For each vial, mix the RNase A with 300 ul of pure D.W. Lyophilized RNase A can be kept at room temperature (15-25°C) until the expiry date and can be stable at -20°C for up to 24 months.

3.1.11.2 Lyophilized Proteinase K:

Mix the Proteinane K with 88 ml of pure D.W. for each vial and dissolved Proteinase K should be refrigerated at -20 °C right away.

3.1.11.3 G-Butfer :

Mix 250 ul of RNase A solution and 40 ul of Proteinase K solution before using. Mix RNase A and Proteinase K after that. The G-buffer temperature should then be maintained between 2 and 8°C.

3.1.12 Solutions Used in Gel Electrophoresis:

The solutions were made according to Sambrook and Rusell instructions (2001).

3.1.12.1 Tris-Borate-EDTA Buffer (TBE):

Boric acid (0.08 M), Tris-OH (0.08 M), and EDTA (0.02 M) (0.08 M). The pH was fixed to 8.0, then autoclaved and stored at 4° C.

3.1.12.2 Ethidium Bromide Solution:

The stock solution (5 mg/ml) was made by mixing 0.005 gm ethidium bromide in 10 ml D.W and storing it in a dark reagent vial.

3.1.12.3 DNA Loading Buffer:

Table (3-8): DNA Loading Buffer Content

No	Material Name	Wieght
1.	Xylene xyanol	25 mg
2.	Sucrose	4 mg
3.	Bromophenol blue	25 mg
4.	D.W	10 ml

3.2 Methods:

3.2.1 Sample Collection:

A total of 163 blood samples (2-5 ml) were taken from patients and withdrawn using a disposable syringe as soon as possible. The blood samples were inoculated into a sterile blood culture vial with brian heart infusion broth (BHI broth) (each 1 ml of blood was added to 9 ml of BHI

broth). After that, the blood culture bottle was incubated at 37 ℃ for 24-72 hours. If any sign of bacterial growth is indicated , the bacteria is further cultured on MacConkey and blood agar, Gram-stain procedure. Other morphological and biochemical tests should be done to confirm the diagnosis of bacterial isolates.

3.2.2 Patients:

Between October 2020 and April 2021, a total of 163 patients hospilized or visited Hillah hospitals (Marjan teaching hospital, Imam sadiq hospital and Babylon teaching hospital for maternity and pediatric). of all ages and sexes were included in this study in Hilla city. The patient's name, age, and sex were recorded.

3.2.3 Laboratory Diagnosis:

According to Maccfaddin's suggested diagnostic techniques (2000). The following procedures were used to isolate and identify G+ve and G-ve bacteria.

3.2.4 Microscopic Examination and Colonial Morphology:

Each positive culture yielded a single colony, which was identified based on its morphological traits (size of colony, color, shape, translucency, edge, elevation, natural of pigments, and texture). Gram staining was used to examine colonies for their unique form, gram reactions staining, cell organization, and specific intracellular components. Traditional biochemical tests were used to identify bacterial isolates to the species level, and later rapid identification tools were used to validate the results, as indicated by (Himedia/ India).

3.2.5 Biochemical Tests:

For the identification of *Enterobacticeae* isolates, the following biochemical tests were used:

3.2.5.1 Indole Test:

This test was carried out by inoculating peptone water media with growth of bacteria from the loop for 24-48 hours at 37 °C. The indole test was performed by putting 6-8 drops of Kovac's reagent to the Indole solution. The formation of a red color ring at the top of the broth indicated a positive reaction (McFaddin, 2000).

3.2.5.2 Methyl-red Test:

Select bacterial colonies were inoculated into the tubes of MR-VP broth medium, then incubated at 37°C for 24-48 hours. The methylred reagent was then added in five drops. The presence of red color indicated a positive outcome (McFaddin, 2000).

3.2.5.3 Vogues-Proskauer Test:

The bacterial colonies were inoculated into the tubes of MR-VP broth and incubated at 37oC for 24-48 hours. The presence of pink color after 2-5 minutes was taken to indicate a positive result after adding 0.6 ml of alpha-nephthol (reagent A) and 0.2 ml of 40% KOH solution (reagent B) to the result (McFaddin, 2000).

3.2.5.4 Oxidase Test:

The colony to be tested was picked up with a sterile wooden stick and smeared on a strip of filter paper soaked in a little newly prepared 1% solution of tetramethyl-P-pheneylene-diaminic dihydrochloride. A deep purple color occurred within 5-10 seconds, indicating a positive result (McFaddin, 2000).

3.2.5.5 Urease Test:

By inoculating urea medium with growth of bacteria, this test was carried out. At 37°C, the tubes were incubated for 24-48 hours. A positive result was shown by the medium becoming pink (McFaddin, 2000).

3.2.5.6 Motility Test:

This test was carried out by stabbing the tube containing semisolid medium with the tested bacteria and incubating it at 37°C for 24-48 hours. The spread of growth outside the stab line was an indicator of a poisitive outcome (McFaddin, 2000).

3.2.5.7 Catalase Test:

This test was performed using a sterile needle. On a clean glass slide, a 24-hour-old bacteria colony was placed with a drop of 3% H_2O_2 solution. The presence of oxygen babbles immediately suggested positive(McFaddin, 2000).

3.2.5.8 Kligler Iron Agar Test:

A thick inoculum was smeared across the slope's surface and stabbed into the bottom , then incubated for 24 hours at 37°C. Results were unformatted according to MacFaddin (2000) as follows:

Slant / Butt	Color
Alkaline / Acid	Red / yellow
Alkaline / Alkaline	Red / Red
Acid / Acid	Yellow / Yellow
H_2S production	Black precipitate
Gas production	Breaking or hole in the medium

3.2.6 Antibiotic Disk Susceptibility Test:

A pure culture of bacterial isolate was used in the experiment. The inoculum for this test was made by mixing 5 ml of Nutrient broth with growth from 5 isolated colonies grown on blood agar plates and incubating at 37°C for 18 hours. An inoculum was obtained from the bacterial suspension using a sterilized swab. This inoculum was smeared

and dried on a Muller Hinton agar plate. With flamed forceps or a disc applicator, the antibiotic disks (listed in table 3-4) were equally dispersed on the surface of the medium and incubated for 18 hours at 37°C. The inhibition zones were measured with a ruler and compared with guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI,2020).

3.2.7 ESBL Production Detection:

The method used to detect ESBLs in isolates that cause bacteremia. This test as follows:

3.2.7.1 Double-Disc Synergy (Approximation) Test (DDST):

The following procedure was used, as modified by Coudron *et al.,* (1997),

As suggested for the standard disk diffusion susceptibilities test, a Muller Hinton agar plate was inoculated with an overnight blood agar culture of the test bacterial isolate. 30 μg of ceftazidime, ceftriaxone, cefotaxime, and aztreonam disks were put 15 mm (edge to edge) from a disk of augmentin (20 μg amoxicillin plus 10 μg clavulanate) and incubated at 35 °C for 16-20 hours. Any increase in the zone of inhibition between the β-lactam and augmentin disks indicated that the test isolate contained ESBLs whose activity was reduced by clavulanic acid (Rolinson, 1994).

3.2.8 DNA Extraction from Gram Negative Bacteria:

This methodology was introduced using a genomic DNA purification kit provided by the manufacturer (G-spin™ genomic DNA extreaction kit ,korea).

1. Harvest 1-2 ml of cells by centrituging at 13.000rpm for 1min. and Remove supematant.

2. Add 300 ul of G-Buffer solution, and invert-mix wel.

3. Incubate at 65 C for 15 min

4. Add 250 ul of Binding Buffer. and completely mix well by pipeting (at least 10 times) or ganty vorlezing

5. Cel lysates loading on column and centrifuge at 13,000 rpm for 1 min.

6. To wash, add 500 ul of Washing Bufer A to column and centrifuge for 1 min at 13,000 pm

7. Remove soluton. Add 500 ul of Washing Buffer B to column and centrifuge for 1 min at 13.000 rpm

8. Remove solution. and centrituge for 1min at 13.000 rpm

9. Place the G-spin™ column in a clean 1.5 ml   microcentrifuge tube (not provided), and add 50-200 ul of Elution Bufer directly into the membrane.

10. incubate at RT for 1 min, and then centrituge for 1 min at 13,000 pm.

3.2.9 Detection of Resistance Genes by Polymerase Chain Reaction (PCR):

3.2.9.1 Preparing the Suspension of Primers:

The DNA primers were resuspended as stock solution by dissolving the lyophilized product after spinning it down momentarily in TE buffer molecular grad according to the manufacturers instructions. Diluted with TE buffer molecular grade, a working primer tube was created. The final picomoles were determined using the primer's procedure.

3.2.10 Polymerase Chain Reaction Protocols:

3.2.10.1 PCR Mixture:

DNA (extracted from bacterial cells) is used as a template in specific PCR for the detection of resistance gene (TEM, OXA-10, CTX-M-1). DNA was purified from bacterial cells using G-spin™ genomic DNA

extreaction kit ,(korea). A pair of specific primers is used for the amplification of a fragment gene, as shown in table (3-5).

To produce a total volume of 20μl, a single reaction mixture contains 1 μl of upstream primers, 1 μl of downstream primers, 2 μl of DNA extraction, 5 μl of master mix, and 6 μlof nuclease free water. The PCR products were then run on a 1.5 % agarose gel.

Tabal (3-9): Protocols of PCR Reaction Mixture Volumes

NO.	PCR Reaction Mixture	Size
1.	Upstream primer	1 μl
2.	Downstream primer	1 μl
3.	Green master mix	10 μl
4.	Nuclease free water	6 μl
5.	DNA	2 μl

Under sterile conditions, all PCR components were combined in a PCR tube and mixed on an ice bag.

Table (3-10): PCR Condition for TEM Gene

Step	Time	Temperature C°	Cycles
Initial denaturation	5min.	94	1
Denaturation	30 sec.	94	
Annealing	30 sec.	50	30
Extension	1min.	72	
Final extension	5 min.	72	1

Table (3-11): PCR Condition for OXA-10 Gene

Step	Time	Temperature C°	Cycles
Initial denaturation	5min.	94	1
Denaturation	30 sec.	94	
Annealing	30 sec.	55	35
Extension	1min.	72	
Final extension	5 min.	72	1

Table (3-12): PCR Condition for CTX-M-1 Gene

Step	Time	Temperature C°	Cycles
Initial denaturation	5min.	94	1
Denaturation	30 sec.	94	
Annealing	30 sec.	63	35
Extension	1min.	72	
Final extension	5 min.	72	1

3.2 10.2 Agarose Gel Electrophoresis:

All technical and preparatory conditions for agarose gel electrophoresis for DNA detection and analysis were performed (Bartlett and Stirling, 1998).

3.2.10.3 Preparation of Agarose Gel and DNA Loading:

The agarose gel was made by mixing 1 gram of agarose powder with 100 m.l of TBE buffer that had already been prepared (90 ml D.W were added to 10 ml TBE buffer l0X, the final concentration was 1 X and pH 8). The mixture was put in a boiling water bath until it became transparent, then allowed to cool to 50°C before adding 0.5 ml ethidium bromide.The agaros poured easily into the equilibrated gel tray, which had been set up with two combs in the end and centre with the two ends of the gel tray closed. The agarose was allowed to solidify for 30 minutes at room temperature. The seal and combs were carefully removed from the tray. The comb created wells that were used to load DNA samples. The agarose gel wells were loaded with 5 µl of amiplified PCR product, after by a DNA marker (ladder) in one of thes wells. The gele tray was placed in the electrophoresis chamber, and IX TBE buffer was added until the whole surface of the gel was covered. The electric current was run for 1.5-2 hours at 70 volts.

3.2.10.4 Gel documentation:

The amplified PCR products were identified using agarose gel electrophoresis and stained with ethidiuim broemide for visualization. The gel documentation systems was used to identify the electrophoresis outcome. Positive findings weres identified when the samples DNA band base pairs were equivalent to the desired product size. Finally, utilizing the E. graph documentation system, the gel was photographed (Bartlett and Stirling, 1998).

Chapter Four
Results and Discussion

4.1 Isolation of Bacterial Isolates:

In this study, blood samples are collected from (163) patients suffering from bacteremia infection with diffrent ages from both sexes during the period of study extended from October 2020 to April 2021. A total of 163 samples from patients with bacteremia infection were included in this study, only 63 (38.65%) patients with positive bacterial culture and 100 (61.34%) patients with negative bacterial culture. These results were shown in table (4-2). Isolation of *Enterobacteriaceae* . Specimens were collected from Babylon province hospitals shown in table (4-1) from patients of both ages and sexes.

Table (4-1): Distribution of *Enterobacteriaceae* in Different Teaching Hospital in Babil Province.

Hospital name	No(%). Of samples	No(%) of *Enterobacteriaceae* isolates
Marjan teaching hospital	61(37.5)	17(48.57)
Babylon teaching hospital for maternity and pediatric	52(31.99)	6(17.14)
Imam sadiq hospital	50(30.67)	12(34.28)
Total	163(100)	35(100)

The results shows, high percentage (48.57%) in Marjan teaching hospital while low percentage was recorded for Imam sadiq hospital and Babylon teaching hospital for maternity and pediatric , they were reached to (34.28%) , (17.14)%) respectively.

Table (4-2): Types of Bacterial Isolates Collected from Patients

Bacterial Isolates	No. of Isolates	Percentage (%)
Enterobacteriaceae	35	21.47%
Streptococcus.spp	14	8.59%
Staphylococcus.spp	6	3.68%
Pseudomonas aeruginosa	8	4.9%
positive growth	63	38.65%
No growth (negative)	100	61.34%
Total	163	100%

According to the findings of this study, Gram-negative bacilli account for roughly half of all bloodstream infections, accounting for 55.55 % of all positive patients (out of 63 bacterial isolates obtained, 35 isolates diagnosed as Gram-negative bacilli). These results were similar to those reported by Pereira *et al.,* (2013).

Of these 163,100 bacterial isolates, 61.34% were negative to blood culture, 28 bacterial isolates were distributed among *Streptococcus.spp* (8.59%), *Pseudomonas aeruginosa* (4.9%) and *Staphylococcus.spp* (3.68%), and 35 bacterial isolates (21.47%) were identified as members of the *Enterobacteriaceae* family, as reported in table (4-3). Various members of *Enterobacteriaceae* have been reported to be the major pathogens in blood infection (Réjiba and Kechrid, 2007). The most prevalent *Enterobacteriaceae* isolates were *Klebsiella pneumoniae* (22.85%), *Salmonella typhi* (20%), *Proteus mirabilis* (20%), and *E. coli* (17.14%), according to the findings. This could be due to the fact that these bacteria have a variety of antibiotic resistance mechanisms that allow them to enter the bloodstream.

Table (4-3): The Percentage of the Members of Bacterial Isolates Belongs to the *Enterobacteriaceae* Family.

Bacterial Isolates	No. of isolates	Percentage (%)
Klebsiella pneumoniae	8	22.85%
proteus mirabilis	7	20%
salmonella typhi	7	20%
Escherichia coli	6	17.14%
Enterobacter aerogenes	3	8.57%
shigella flexneri	2	5.72%
proteus vulgaris	2	5.72
Total	35	100

A study of bacteremia by Muro *et al.,* (2012), who showed that among 90 isolates of *Enterobacteriaceae* were detected. Of these, *Klebsiella peumoniae* (61.11%), *Enterobacter cloacae* (15.55%),

Escherichia coli (15.55%), and *Serratia marcescens* (7.77%). Also close with the results reported by Al Zamil, (2018) which found that among 65 isolates of *Enterobacteriaceae*. Of these *Klebsiella pneumoniae* (26.15%), *proteus mirabilis* (23.07), *salmonella typhi* (20%), *proteus vulgaris*(15.38%), and *Escherichia coli*(15.38).

In Pakistan, a study was performed by Fayyaz *et al.,* (2015). They involved 465 blood samples and found that of these 465, only 115 (24.73%) bacterial isolates can be identified as *Enterobacteriaceae*. Of these, *Salmonella spp.* (37.39%), *E. coli* (34.78%), and *Acinetobacter* (37.39%) were isolated.

4.2 The Distribution of Patients with Bacteremia According to Sex and Age:

Table(4-4): Distribution of *Enterobacteriaceae* According to Sex

Patient profile	Status	No. (%) Of samples	No(%) of *Enterobacteriaceae* isolates
Gander	Male	84(51.53)	18 (51.42)
	Female	79 (48.46)	17 (48.57)
Total		163(100)	35(47.1)

In the present study, the prevalence of bcteremia by bacterial isolates belonging to the family *Enterobacteriaceae* among female patients was 17/35 (48. 57%), while among male patients it was 18/35 (51.42%). The results indicated that the susceptibility of both males and females to blood stream infection was nearly similar. This result was in accordance with that reported by Omoregie *et al.,* (2017), who stated that the prevalence of bacteremia in females (46.5%) was not significantly different from that in males (53.5%), and this finding disagreed with that of Mugalu *et al.,* (2016), who reported a significantly higher prevalence of bacteremia in the females than in the males. The reason for this difference is unclear, but may be due to geographical location and type of infection.

Table(4-5): Distribution of Patients According to Age

Age group	No(%). Of samples	No(%) of Enterobacteriaceae isolates
1-19	85 (53.12%)	11(31.42**%**)
20-39	34 (21.25%)	19 (54.28**%**)
40 -59	24 (15.00%)	3 (8.57**%**)
60-79	20 (10.65%)	2 (5.73**%**)
Total	163 (100%)	35 (100**%**)

The study found that 1 to 19 years old patients made up 11 (31.42%), 20-39 years old patients made up 19 (54.28%), 40-59 years old patients made up 3 (8.57%), and 60-79 year old patients made up 2 (5.73%). The results show the higher percent of infection is among patients aged 20–39 (54.28%) years old. and this finding close with that of *Bokaeian et al.,* (2015), who reported the higher percent of infection is among patients aged 25–45(59.21%).

4.3. Identification of Gram Negative Bacteria:

Enterobacteriaceae isolates were identified using traditional morphological and biochemical diagnostic tests according to the methods of Macfaddin, (2000) as shown in table (4-6).

Table (4-6): Morphological and Biochemical Tests for Identification of *Enterobacteriaceae*

Biochemical Tests and characterization	Type of Bacteria							
	Escherichia coli	*Proteus mirabilis*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*	*salmonella typhi*	*shigella flexneri*	*proteus vulgaris*
Morph	Rode	Rode	Rode	Rode	Rode	Rode	Rode	Rode
Flagella	+	+	-	+	-	+	-	+
Gram Staining	-	-	-	-	-	-	-	-
Lactose Fermenter	+	-	+	-	-	-	-	-
Oxidase	-	-	-	+	-	-	-	-

Catalase	+	+	+	+	+	+	+	+
Gelatin Hydrolysis	-	+	-	+	-	-	-	+
Indole	+	-	-	+	-	-	-	+
Methyl Red	+	+	-	-	-	+	+	+
Voges-Proskr	-	-	+	+	+	-	-	-
CitrateUtilization	-	+	+	+	+	-	-	+
H_2S	-	+	-	-	-	+	-	+
Urease	-	+	+	-	+	-	-	+
Motility	+	+	-	+	+	+	-	+
Kligler iron agar	A/A	K/A	A/A	K/K	A/A	K/A	K/A	K/A

(+) positive; (-) negative; (K) alkaline; (A) acid:(KIA) Kligler Iron Agar (MR) methyl red; (VP) voges-proskauer.

4.4 Antibiotics Susceptibility Test:

4.4.1 Disk Diffusion Method:

A total of 35 bacterial isolates were discovered. The disk diffusion method was used to assess the susceptibility of all 35 isolates to a number of antibiotics (listed in table 3-4), all of these isolates were found to be resistant to at least 5 antibiotics out of a total of 10 drugs utilized in the study, as shown in table (4-7). As a result of these findings, all of these isolates were classified as multi-drug resistant. Antibiotic resistance in bacteria is common nowadays, and it presents a severe clinical danger (Kaye and Pogue, 2015). Antibiotic resistance in bacteria could be hereditary (natural), such as the outer membrane in Gram-negative bacteria, which serves as a permeability barrier against antibiotics, or acquired (due to two genetic processes in bacteria: vertical evolution

(mutation) and horizontal evolution) (gene exchange between strains and species) (Todar, 2011).

Table (4-7): Antibiotics Susceptibility Prophiles of Bacterial Isolates

Type of antibiotic	Symbol	Sensetive		intermediate		Resistant	
		No	%	No	%	No	%
Ceftazidime	CAZ	0	0	0	0	35	100
Azithromycin	AZM	18	51.42	2	5.71	15	42.85
Chloramphenicol	C	8	22.85	6	17.14	21	60
Gentamicin	CN	13	37.14	0	0	22	62.85
Nitrofurantion	F	12	34.28	1	2.85	22	62.85
Ceftriaxone	CRO	7	20	1	2.85	27	77.14
Amoxicillin-clavulanate	AMC	7	20	0	0	28	80
Meropenem	MEM	25	71.42	4	11.42	6	17.14
Ampicillin	AM	1	2.85	0	0	34	97.14
Cefotaxime	CTX	0	0	0	0	35	100

Results from table (4-7) shows that all isolates (100%) are found to be resistant to ceftazidime and cefotaxime (third generation, extended-spectrum cephalosporins). These findings are agreed with those obtained by the study by Kitamura, (2019), which found a high level of resistance to cefotaxime and ceftazidime. This result is agreed with the study carried out by Hu *et al*., (2020) which found that resistance to cefotaxime and ceftazidime was (100%). In this study, resistance to Ceftriaxone (also belonging to the third generation, extended-spectrum cephalosporins) was (77.14%). These findings were agreed with those of Spafford *et al.,* (2019), in which the rate of (81.%) and Tohamy *et al*., (2018), which found that the resistance to Ceftriaxone was (80.6%).

The ability of β-lactam antibiotics, which include cephalosporins, to interfere with the formation of the peptidoglycan component of the bacterial cell wall by binding to and inactivating penicillin-binding proteins contributes to their bactericidal effect (PBPs). PBPs are enzymes

that aid in the formation of cell walls. There are three main mechanisms of bacterial resistance to cephalosporins: (1) alteration of PBPs that reduces the PBP affinity for the antibiotic; (2) production of beta-lactamases, which inactivates the drug; and (3) modifications in the outer cell wall, especially loss of porin channels, which reduce the antibiotic ability to reach the PBPs. To render an organism resistant, several resistance mechanisms may act in combination (Asbel and Levison, 2017).

Antibiotic resistance was found in significant percentages in these isolates, indicating that they have an enzymetic mechanism of resistance characterized by the synthesis of β-lactamase enzymes (Haller *et al*., 2018). Cefotaxime, Ceftriaxone and Ceftazidime resistance, mainly, are an important indicator for the presence of extended-spectrum β-lactamases (ESBLs) (Saravanan *et al*., 2018).

The relatively high rates of antimicrobial resistance may also attributed to additional factors such as poorly directed therapy, drug overuse and over-the-counter sales (Mathur *et al*. 2002). Antimicrobial resistance was also reported to be high among ESBL-producers. The expression of β-lactamase enzymes, of which there are various kinds including plasmid-encoded and chromosomally encoded enzymes, was discovered to be the principal mechanism of antibiotic resistance in Gram-negative bacteria that cause clinically relevant illness (El Salabi *et al.,*2013).

Penicillins and early cephalosporins like cefaclor, cephalothin, and cefazolin are hydrolyzed by the parental β-lactamase enzymes (SHV-1, TEM-1, and TEM-2) but do not hydrolyze the later, more stable cephalosporins, cephalosporins as cefixime, cefuroxime, ceftriaxone, cefotaxime, cefepime, monobac, and the ceftazidime. The mutations

broaden the substrate profiles, making it possible to hydrolyze at least some of the more stable antibiotics (Bush *et al.,* 1995).

So many of bacterial isolates had demonstrated resistance to the antibiotic group β-lactam. Amoxicillin's effect on bacteria is expected, as seen in the findings showing that isolates (97.14%) were resistant to this antibiotic agent. Such findings are partially agreed with those of Mercuro *et al.,* (2018) who noted that 91% of the isolates were amoxicillin resistant. While Fernandes, (2008) reported that all *Enterobacteriaceae* isolates appeared resistant to amoxicillin (100%).This resistance to amoxicillin was identified because generation of β-lactamase enzymes which hydrolyze of the β-lactam ring and rendered inactive, antibiotic failure to bind to the target site of Penicillin Binding Protein (PBP) and low-affinity antibiotic binding to (PBP) also acquire resistance to these antibiotics (Ang *et al*., 2004).

As shown in table (4-7)**, (** 62.85%) of bacterial isolates were found to be resistant to gentamicin, which belongs to aminoglycosides. genetic studies indicate that resistance genes of gentamycin are present on mobile genetics element that can facilitate their horizontal transfer and that genes are found on R plasmid of the *Enterobacteriaceae* (Diazz *et al*., 2006). Gentamycin   had a medium activity on *Enterobacteriaceae* isolates (62.85%), Because it belong to Aminoglycosides, which are effective broad spectrum antimicrobials, which were protein synthesis inhibitors in prokaryotes.

the present study was nearly from a study done by Saeed *at el.,*(2019) shows that most of isolated bacteria (67.3%) were resistance to gentamicin also Fernandes,(2008) it was found in his study to be resistant to gentamicin was (58%).

General aminoglycoside resistance was caused by the generation of manipulating enzymes that change and prevent the aminoglycoside from

binding to ribosome (Llano-Sotelo *et al*., 2002). aminoglycosides invade the bacteria, first, the absorption of aminoglycosides into the bacteria is an important process for their biological function, secondly, the aminoglycoside binds to the ribosome within the bacterial cell and inhibits protein synthesis(Vakulenko and Mobashery, 2003).

The results of the current study show that the sensitivity ratio of *Enterobacteriaceae* to Meropenem is (71.42%). While the findings of Shortridge *et al.,* (2018) show that (98.4%) are susceptible to Meropenem, along with other findings such as those reported by Li,C *et al.,* (2019), *Enterobacteriaceae* isolates were found to be (77.77%) sensitive to these antibiotics. Also, a study by Villegas *at el.,* (2016) found that the sensitivity to meropenem is (78%).

Meropenem is a broad-spectrum antibiotic of carbapenem. It is active against Gram-positive and Gram-negative bacteria, exercises its activity by efficiently penetrating bacterial cells and interference with the synthesis of essential cell wall components, that is resulting in cell death (Narala and Saraswathi, 2011).

In addition, some macrolide antibiotics have also been used, such as azithromycin and erythromycin, and the result showed that azithromycin has a (42.85%) resistance rate against *Enterobacteriaceae* isolates (42.85%). These results are close to those obtained by Nusrat *et al.*, (2020), where the resistance to azithromycin was at a rate of (47.25%). Also, Lusa and Mukhtar, (2019) found that the resistance to azithromycin was (45.99%).

The macrolide antimicrobials exert their antibacterial effects by reversibly binding to the 50s subunit of the bacterial ribosome. This interaction inhibits RNA-dependent protein synthesis by preventing transpeptidation and translocation reactions (Sturgill and Rapp, 1992). Resistance to macrolide antibiotics can arise from a variety of processes,

which are divided into two categories: resistance caused by a mutation in a ceIl character, and resistance caused by the presence of a foreign gene not involved in the ceIl's regular metabolism (Wittman *et at.,*1973). Azithromycin, like other macrolide antibiotics, works by attaching to and interference with the synthesis of the 50S large subunit of the ribosome and the formation of the nascent polypeptide chain, which suppresses bacterial protein synthesis (Hansen *et al.,* 2019).

The study reported resistance to nitrofurantoin (62.85%). These result is closly with a published study by Zhang *et al.*, (2019), who found that the resistance of *Enterobacteriaceae* isolates to this antibiotic was (53.7%). Gunasekaran *et al.*, (2020) found that (56.5%) of the population was resistant to nitrofurantoin.

Nitrofurantoin (nitrofuran class) is a unique antibiotic that belong to the antimicrobial class of antibiotics. It is distinguished by a hydantoin ring with a nitro-substituted furanyl side chain that is metabolized within bacteria to form bactericidal reactive compounds. Antibacterial activity is unknown, but it is thought to be mediated through changes in ribosomal proteins and other intracellular structures (Long *et al.,*2017).

In this study, (60%) of the isolates were resistant to chloramphenicol (belongs to the phenicol class). Abera *et al.*, (2016) and Murni *et al.*, (2016) reported that the resistance to this antimicrobial was (61.25%) and (56.82%), respectively. Malek *et al.*, (2016) found in their study that the resistance of *Enterobacteriaceae* isolates to chloramphenicol was (53.5%).

Chloramphenicol acts as a bacteriostat by suppressing protein synthesis. The peptidyl transferase activity of the bacterial ribosome is inhibited, which limits protein chain elongation. It prevents the formation of peptide bonds by attaching to A2451 and A2452 residues in the 23S rRNA (Schifan *et al.*, 2016). In contrast to macrolides, which inhibit the

progress of the developing peptide, chloramphenicol immediately interacts to substrate binding in the ribosome (Jardetzky,1963; Wolfe and Hahn, 1965).

This study reports that (80%) of *Enterobacteriaceae* isolates were resistant to amoxicillin-clavulanate with respect to the β-lactamase inhibitor combination. This result was close to that of Tohamy *et al.*, (2018) and Teklu *et al.*, (2019) where they documented that resistacet to amoxicillin-clavulanate was (74.16%) and (85.4%), respectively.

Amoxicillin is a bactericidal antibiotic that inhibits the formation of the peptidoglycan layer in the bacterial cell wall and kills bacteria, this layer is responsible for the cells structural integrity and makes the outermost portion of the cell wall. The facilitation of DD-transpeptidases (a kind of penicillin-binding protein), is essential for peptididoglycan synthesis (PBP). Amoxicillin acts by binding to these PBPs and blocking peptidoglycan synthesis, which disrupts the cell wall formation and leads inevitably to bacterial death, or lysis (Kharrat *et al.,* 2018).

Clavulanic acid is a β-lactamase inhibitor that is frequently used with amoxicillin to broaden the antibiotic spectrum and prevent resistance. It has limited antibacterial activity and instead operates by stopping bacteria from destroying β-lactams. Through the synthesis of enzymes known as beta-lactamases, several bacteria have evolved resistance to typical beta-lactam antimicrobials over time(Brogden *et al.,*1981).The β-lactam ring which is required for penicillin-like antimicrobials to work, is targeted and hydrolyzed by these enzymes and clavulanic acid inhibits this destruction by attaching to and deactivating beta-lactamases, restoring amoxicillin's antimicrobial effects (Wyres *et al.,*2018).

β-Lactam antibiotics are bactericidal agents that interfere with the production of bacterial cell walls by binding covalently to penicillin-binding proteins (PBPs), enzymes involved in the last steps of

peptidoglycan cross-linking in Gram-positive and Gram-negative bacteria. Every bacterial species has its own set of PBPs, which can include anywhere from three to eight enzymes (Georgopapadakou and Liu, 1980). The antibiotic might then be progressively hydrolyzed to generate a microbiologically inactive entity as a result of the inactive acyl enzyme (Graham, 2010).

4.5 The Production of Extended-Spectrum β-Lactamases:

Extended spectrum β-lactamase (ESBLs), produced by Gram negative organisms, are enzymes capable of hydrolyzing extended spectrum cephalosporins, penicillins and monobactam but inactive against cephamycin and imipenem, and are generally derived from TEM and SHV-type enzymes (Biswas *et al.,* 2013). ESBLs are frequently encoded by genes found on large plasmids, which also contain resistant gene to trimethoprim, aminoglycosides, sulphonamides, chloramphenicol, and tetracyclines (Pitout *et al.,* 2008).

Single amino acid alterations in three non-ESBL parental β-lactamase enzymes, TEM-1, TEM-2, and SHV-1, are used to make the majority of ESBLs. Since β-lactamase inhibitors are equally effective against TEM- and SHV-ESBLs (e.g. sulbactam, clavulanic acid and tazobactam), the use of inhibitor /ß-lactam combination was considered to be an alternative (AL-Tememy *et al.,* 2015).

4.5.1 Approximation Disk Method:

Jarlier *et al.* (1988) were the first to describe this method, which is also known as the double disk synergy test. Any increase in the zone of inhibition between the β-lactam and augmentin disks in this test indicated that the test strain includes ESBLs whose activation is inhibited by clavulanic acid (Figure 3-2). By using the approximation disk method, it

was found that only 9 bacterial isolates (25.71%) were detected as ESBL-producers, out of 35 as shown in table (3-5).

Table (4-8): Bacterial Isolates Producing and Non-Producing ESBLs Among *Enterobacteriaceae* Members Approximation Disk Method

Types of Bacterial Isolates	No. of ß-Lactamase Producers (%)	No. of ß-Lactamase-Non Producers (%)
Klebsiella peumoniae	0(0)	8(22.85)
Escherichia coli	2(5.72)	4(11.42)
proteus mirabilis	2(5.71)	5(14.28)
proteus vulgaris	0(0)	2(5.71)
Enterobacter aerogenes	3(8.57)	0(0)
salmonella typhi	0(0)	7(20)
shigella flexneri	2 (5.72)	0(0)
Total	9(25.71)	27(74.26)
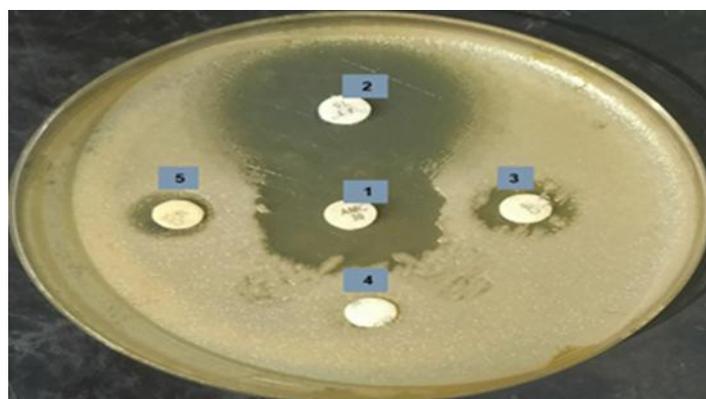
Figure (4-1): Disk Approximation Method for Detection of ESBL-Producing Isolates.

1-Amoxicillin-clavulanate disk (20/10 μg). 2- Aztreonam disk (30 μg).

3- Ceftriaxone disk (30 μg). 4- Ceftazidime disk (30 μg). 5- Cefotaxime disk (30 μg).

Tables (4-8) show that the results are approximately in accordance with a study by Hadi, (2008). Only 11 isolates (18.3%) were discovered as ESBL-producers by the approximation disk method out of 60 ß-lactam

resistant *K. pneumoniae subsp. pneumoniae* and *E. coli* isolates. In another study by Vaidya, (2011), he found that out of 70 bacterial isolates of *Enterobacteriaceae*, only 24 isolates (28.5%) were recognized as ESBL-producers using a double disk synergy test.

For many years, approximation disk method has been the standard method for detecting ESBL-producing bacteria. One of the most difficult problems in clinical microbiology laboratories is detecting ESBL-mediated resistance in Gram-negative bacteria (Shaikh *et al.*, 2015). Furthermore, many studies have shown that rapid bacterial identification and antibiotic susceptibility assessment have therapeutic and financial benefits. (Oviano *et al.*, 2018).

4.6 Detection of *bla*$_{TEM}$, *bla*$_{OXA-10}$ and *bla*$_{CTX-M-1}$ Genes by PCR:

Detection of genes (bla$_{TEM}$, bla$_{OXA-10}$ and bla$_{CTX-M-1}$) for 35 bacterial isolates of *Enterobacteriaceae* was done by the PCR. DNA extracted from bacterial cells was used as a template in specific PCR technique for detection of *bla*$_{TEM}$,*bla*$_{OXA-10}$ and *bla*$_{CTX-M-1}$ genes. This study has been designed to detect ESBLs distribution among Gram negative bacteria isolates from people who infected by bacteriama. Many mechanisms of resistance to β-lactam antibiotics exist among members of the *Enterobacteriaceae* family but, β-lactamases are the most frequent cause of resistance among this bacterial group (Wax *et al.*,2018). Three types of ESBLs are detected in this study, namely: bla$_{TEM}$, bla$_{CTX-M-1}$, and bla$_{OXA-10}$ type. PCR assays are performed on the 35 bacterial isolates. The frequency of TEM, OXA-10and CTX-M-1 enzymes type among β-lactamase producing isolates was summarized in table (4-9).

Table (4-9).The Frequency of TEM, OXA-10 and CTX-M-1 Enzymes Type According to PCR Technique.

Type of Bacteria	No.	TEM		OXA-10		CTX-M-1	
		No. (+)	No. (-)	No. (+)	No. (-)	No. (+)	No. (-)
Klebsiella peumoniae	8	8	0	5	3	7	1
proteus mirabilis	7	3	4	2	5	3	4
salmonella typhi	7	2	5	6	1	7	0
Escherichia coli	6	3	3	4	2	3	3
Enterobacter aerogenes	3	3	0	3	0	2	1
shigella flexneri	2	0	2	1	1	1	1
proteus vulgaris	2	0	2	0	2	0	2
Total	35	19	16	21	14	23	12

Results from table (4-9) show that the prevalence of the TEM gene was (100%) (8/8), OXA-10 was (62.5%) (5/8) and the CTX-M-1 gene was (87.5%) (7/8) in *Klebsiella peumoniae, while Proteus mirabilis* was (42.8%) (3/7) for the TEM gene, the OXA-1O gene (28.5%) (2/7) and the CTX-M-1 gene was (42.8%) (3/7). The TEM gene for *salmonella typhi* was (28.5%) (2/7), OXA-10 was (85.7%) (6/7) and CTX-M-1 was (42.8%).

Table (4-9) shows that *Escherichia coli* was (50%) (3/6) for the TEM gene, the OXA-10 gene was (66.6%) (4/6), and the CTX-M-1 gene was (50%) (3/6). *Enterobacter aerogenes* was (100%) (3/3) for the TEM gene, the OXA-10 gene was (100%) (3/3), and the CTX-M-1 gene was (66.9%) (2/3). *Shigella flexneri* was (0%) (0/2) for the TEM gene, (50%) (1/2) for the OXA-10 gene, and was (50%) (1/2) for CTX-M-1. *Proteus vulgaris* was (0%) (0/2) for TEM, OXA-10, and the CTX-M-1 genes.

Results from table (4-9) shown that the TEM gene was positive for 19 bacterial isolates, which is equal to a product size of 1080 bp, while the OXA-10 gene was positive for 16 bacterial isolates, which is equal to a product size of 720 bp, and finally, the CTX-M-1 gene was positive for 23 bacterial isolates that are equal to a product size of 720 bp.

The study shows that the most common gene is CTX-M-1 (65.7%), followed by OXA-10 (60%) and the TEM gene (54.2%). These results closely to the study carried out by Kharrat *et al.,* (2018) which showed the predominance of the blaCTX-M-1 gene (55%). Also, the results of Engel *et al.,*(2017) were similar for the *E. coli* genotype, the CTX-M-1 gene was (62.5 %) and the results of Frazee *et al.,*(2018) study were similar to our study, which showed that the most common gene is the CTX-M-1 gene (66.6%), followed by the OXA-10 gene (50%) and then the TEM gene (33.3%). However, the CTX-M-1 gene was previously reported in Hiallah hospitals by Abbas, (2019).

In July 1984, the cefotaximase TEM-3/CTX-M-1 was discovered in *K. pneumoniae* at the teaching hospitals of Clemont-Ferrand, France, as the first ESBL (Sirot *et al*., 1987).The prevalence of ESBLs genes differs among patient groups and clinical and geographic settings (Livermore, 2012). This transition may have occurred not just as a result of the widespread distribution of the appropriate ESBLs genes in highly mobile genetic platforms like as plasmids and transposons, but also as a result of successful clones containing these platforms. Antibiotic use, as well as distinct risk factors in different geographical locations and patient groups, as well as the peculiarities of different compartments, all contributed to the global dissemination of ESBL genes (Cantón *et al.,* 2012). Worldwide, Antimicrobial resistance may be increasing as a result of irrational antibiotic usage and transmission via numerous mechanisms such as antibiotic resistant plasmids, bacteriophages, transposons, and integrons (Livermore *et al.,*2012). Continuous antibiotic exposure in patients with bacteriama is the principal source of high resistance rates, which results in persistence and a rise in the prevalence of Gram negative organisms (DeBusscher *et al.,* 2009).

Genes expressing ESBLs are typically found on plasmids that also contain genes encoding resistant to trimethioprim and aminoglycosides. There have been an increasing number of reports of plasmid-encoded reductions in quinolone susceptibility, sometimes in conjunction with plasmid-mediated cephalosporin resistance. Even when there is no plasmid-encoded reduction in quinolone susceptibility, there is a substantial link between quinolone resistance and the generation of ESBLs (Paterson and Bonomo, 2015).

The elevated rate of ESBLs genes among Gram negative bacteria isolates suggest that the horizontal transfer of ESBLs genes, mediated by plasmids and /or mobile elements, contributes to ease with which these enzymes are spreading in Gram negative bacteria isolates and the dissemination of ESBLs enzymes. Moreover, in hospital environment, plasmids could be transferred easily between patients through health care workers due to hand carriage and antimicrobial selection pressure (Al-Hilali, 2010).
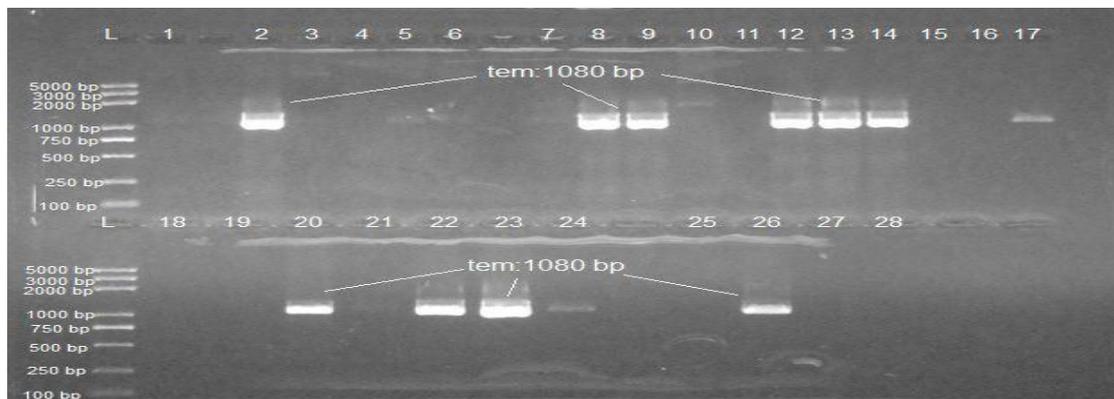

Figure (4-2): Electrophoresis of PCR Test to Determine the Frequency of bla$_{TEM}$ Gene in Isolates of *Enterobacteriaceae*

Lane: (L), DNA Ladder size .marker (5000bp ladder).

Lanes: 2, 8,9, 12,13,14,17, 20, 22, 23,24, 26,29,30, 31, 32, 33, 34, and 35 isolates positive for TEM gene.

Lanes: 1,3,4, 5,6,7,10,11,15,16,18,19, 21,25,27, and 28 isolates are nenative for TEM gene.
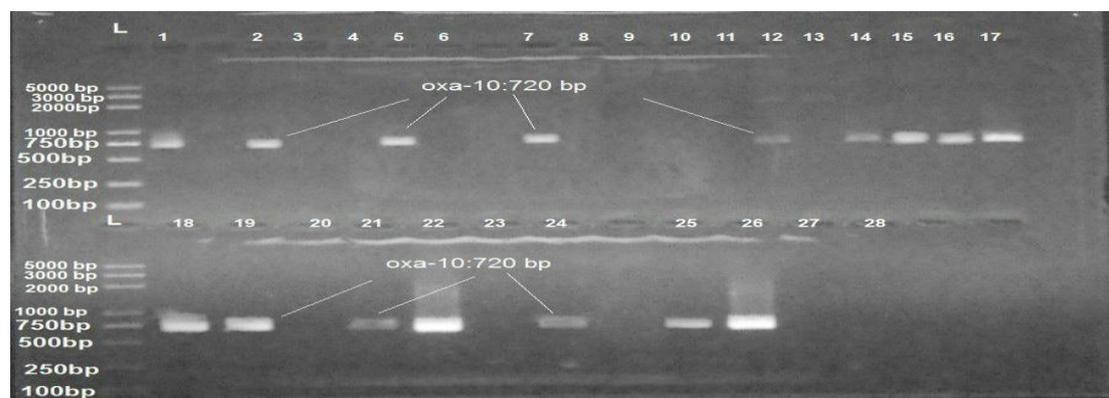

Figure (4-3): Electrophoresis of PCR Test to Determine the Frequency of bla$_{OXA-10}$ Gene in Isolates of *Enterobacteriaceae*

Lane: (L), DNA Ladder size .marker (5000bp ladder).

Lanes: 1,2,5,7 12,14, 15,16,17,18,19,22, 24,25,26,28,29, 31,32, 33 and 34 isolates are positive for OXA-10 gene.

Lanes: 3,4,6,8,9,10,11,13,20,21,23,27,30, and 35 isolates aer nenative for OXA-10 gene).
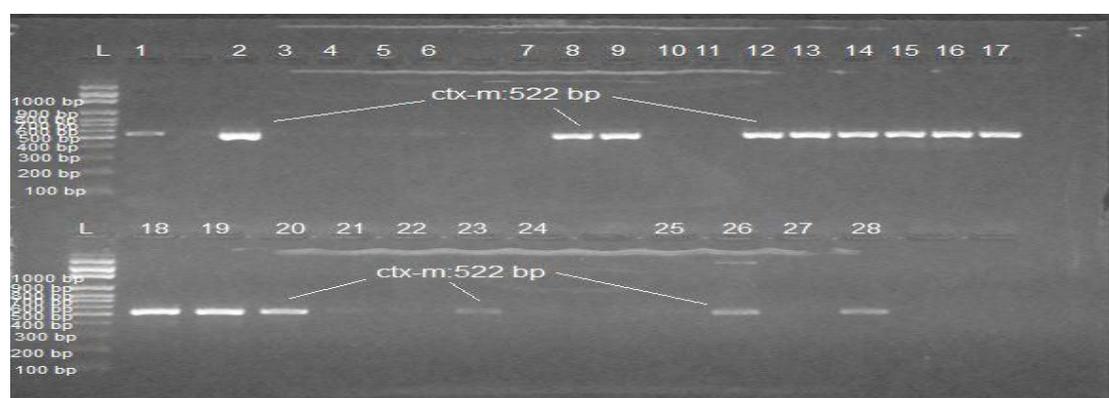

Figure (4-4): Electrophoresis of PCR Test to Determine the Frequency of bla$_{CTX-M-1}$ Gene in Isolates of *Enterobacteriaceae*

Lane: (L), DNA Ladder size .marker (1500bp ladder).

Lanes: 1,2,8,9, 12, 13,14,15,16,17,18,19,20, 23,26,28,29,30, 31,32,33,34, and 35 isolates are positive for CTX-M-1 gene.

Lanes: 3,4,5,6,7,10,11,21,22,24,25, and 27 isolates aer negative for CTX-M-1 gene.

PCR test is very sensitive and accurate, As a result, it is recommended from practical view to use them in routine laboratories. It can serve as an important detector of TEM and SHV β-lactamase enzymes in the epidemiological studies. Regarding these results, many risk factors may be associated with the development of blood stream infection by multi-drug resistant Gram-negative bacteria in paediatrics, such as: the presence of underlying diseases, the duration of hospitalization prior to the occurrence of bloodstream infection, severity of the clinical conditions, and the presence of central venous catheter (Kang *et al*., 2005)

Conclusions:

1- The most prevalent infections found in patient blood were Gram-negative bacilli (*Enterobacteriaceae*).The study showed that patients were at increased risk for blood stream infections by *Enterobacteriaceae*, in comparison to other types of bacteria.

2- The risk of blood stream infection by *Enterobacteriaceae* members was higher in the 20-39 age group than in other age groups.

3- According to the results, males and females were approximately equally susceptible to bloodstream infection.

4- The most common species detected in the blood of patients were *Klebsiella pneumonia* and *proteus spp.* (*proteus mirabilis* and *proteus vulgaris*).

5- The lower rate of resistance was detected for carbapenems (meropenem) with (17.14%).

6- β-lactamase producers made up about 9 of the 35 examined isolates, and the majority of these 35 bacterial isolates had increased antibiotic resistance to β-lactam antibiotics.

7- By the use of PCR technology, β-lactam enzymes(TEM, OXA-10 and CTX-M-1 genes that studied in this research) are present in the majority of *Enterobacteriaceae* isolates

Recommendations:

1- It is important to make blood culture and subsequently, antibiotic susceptibility pattern for any patient not responds to antibiotic treatment especially third generation cephalosporins.

2- Carbapenems (meropenem) was the one we recommended for treating bacterial infections caused by ESBL-producing isolates.

3- Tests used for detection of ESBL-producing isolates should be carried out, if possible, in all hospital laboratories in Hilla.

4- Other Gram-negative bacteria, as well as Gram-positive bacteria, are being studied for their ESBL enzymes.

5- Other types of extended spectrum beta lactamase (ESBL) enzymes should also be investigated for their presence and distribution.

6- Using more advance techniques in addition to PCR, such as DNA sequencer and isoelectric focusing for detection and characterization of the gene expression of β-lactamases.

7- Further studies must done to make continous surveillance of β-lactamases-producing species of G-ve bacteria which will be helpful in monitoring antimicrobial resistance and to guide intervention to minimize it's occurrence

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