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Study the third-Generation Cephalosporins Nonsusceptibility (3GC-NS) Among Hospitalized patients with Monomicrobial *Enterobacteriaceae* Bacteremia

A Thesis

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بسم الله الرحمن الرحيم

وَيَسْـَٔلُونَكَ عَنِ ٱلرُّوحِ ۖ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّى وَمَآ أُوتِيتُم مِّنَ ٱلْعِلْمِ إِلَّا قَلِيلًا

صدق الله العلي العظيم

سورة الاسراء (الآية 85)

Supervision Certification

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Dedication

To…

The warm heart that embraces me all the time with the love. To the spirit that lives inside me……. my mother and Father

I wish to thank my wife, who has stood by me through all my travails, my absences, my fits of pique and impatience

Thanks my martyr brothers and sisters

All my best friends …….. With love.

I dedicate this work

Jawad 2022

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List of Abbreviations

Abbreviation	Key
AMR	Antimicrobial resistance
AST	Antibiotics susceptibility
B.S.I	Bloodstream infection
BHI	Brain heart infusion
bp	Base pair
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CTXNS-En	cefotaxime-non-susceptible Enterobacteriaceae
DDT	disc diffussion test
EMB	Eosine methylen blue agar
ESBLs	Extended-spectrum β-lactamases
EDR	Extensively drug resistant
GBD	Global disease burden
3GC-NS	Third-Generation Cephalosporins Nonsusceptibility
H_2O2	Hydroxid Potassium
HAI	Hospital aquaired infection
I	Iodine
LMIC	low- and middle-income countries
MALDI-TOF MS	matrix-assisted laser desorption-ionization time-of flight mass spectrometry
MBC	Minimum bactericidal concentration
MBLs	Metallo-β-Lactamases
MDR	Multi drug resistance
MEB	*Monomicrobial Enterobacteriaceae* Bacteremia
MHA	Muller Hinton agar
NaCl	Sodium Chloride

NDM	New-Delhei MBL
OmpC	Outer membrabe protein C
OmpF	Outer membrabe protein F
OXA	Oxacillinase
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDR	Pand drug resistant
SBL	Serine Beta lactamases
USA	United state of America
VP	Voges proskaur
WHO	World Health Organization

Summary

A total of 266 blood samples were collected from different patients from both sexes suffering from bacterimia admitted to different hospitals of medical city in Baghdad City. The results found that only 46 (17.3%) blood samples were positive culture, of these cultures, 25 (55.6%) females and 20 (44.4%) males.

After observing the cultural and morphological characteristics of bacterial isolates and performing the traditional biochemical tests, only 45 (16.9%) bacterial isolates were recovered including 25 (55.6%) *Escherichia coli* and 20 (44.4%) *Klebsiella pneumoniae* isolates. After that, The identification of all *E.coli* and *K. pneumoniae* isolates was confirmed by VITEK-2 automated identification system. These results revealed that there was monomicrobial enterobacteriaceae Bacteremia (mEB) infection among patients with bloodstream infections. The results found that most cases of enterobacteriacaeae infections (28.9%) out of 45 cases were occurred among age groups (1-19) years while the frequencies and percentage of enterobacteriaceae infections were decreased among the patients at the sixth to eighth decade of age.

The antimicrobial susceptibility test was conducted for both species using both of VITEK-2 automated system and the disc diffusion method, including Ampicillin, Ceftriaxone, Cefotaime, Cefuroxime, Cefepime, Ceftazidime, Meropenem, Imipenem, Ampicillin-Sulbactam, Ciprofoloxacin, Amikacin and Gentamicin. Regarding to resistance of isolates to penicillins, the results showed that 80% of *E. coli* isolates were resistant to ampicillin, while 90%. *K. pneumoniae* isolates were resistant to this antibiotic. For Cephalosporins, all isolates of *E. coli* isolates and *K. pneumoniae* were resistant (100%) to CXM and CTX, while they showed different degrees of resistance to other types Cephalosporin. For Carbapenems, *E. coli* isolates showed high susceptibility to Imipenem and Meropenem (12%), while *K. pneumoniae* isolates have shown intermediate susceptibility (70% and 55%) for the these antibiotics, respectively.

For Aminoglycosides Amikacin, Gentamicin, both species *E. coli* isolates and *K. pneumoniae* were highly susceptible for these antibiotics.

For ciprofloxacin, 75% of *K. pneumoniae* isolates were resistant while only 60% of *E. coli* were resistant to this antibiotic. Regarding the molecular studies, only 24 out of 45 isolates were subjected to detect the presence of five commone types of ESBLs genes including *blaTEM, blaSHV, blaOXA, blaCTX-M* and *AmpC.* Amplified PCR-products for the five genes among both *E. coli* and *K. pneumoniae* exhibited different and molecular weights (867.930.564.561.634) bp respectively. Genotypically, 85.7%, 85.7%, 85.7%, 92.8% and 85.7% of K. pneumoniae (N=14) isolates *blaTEM, blaSHV, blaOXA, blaCTX-M* and *AmpC* resistance genes respectively, while phenotypically ESBL producing *E.coli* (N=10) isolates were harbouring *blaTEM, blaSHV, blaOXA, blaCTX-M and AmpC* with less prevalence rate except the SHV genes which were more prevalent among *E.coli* than *K. pneumoniae.*

Chapter one

Introduction and Literature Review

Chapter one: Introduction and Literature Review

1.1. Introduction:

Enterobacteriaceae isolates are responsible for a wide variety of nosocomial and community-acquired infections and third-generation cephalosporins (3GCs) are administered as the main choice for the treatment of infections caused by these microorganisms (Syue *et al*., 2019). However, along with the over-prescription of 3GCs by clinicians, reducing the therapeutic efficacy of these antimicrobial agents for Enterobacteriaceae infections (Hung *et al*., 2019), and the increasing trend in nonsusceptibility (NS) to 3GCs were evidenced (Martelius *et al.,* 2016). Moreover, infections caused by 3GC-resistant Enterobacteriaceae were significantly associated with the increasing hazard of death and excess length of stay and costs (Stewardson *et al.,* 2016). Taking *E. coli* as an example, the total cost attributable to excess hospital stays for bloodstream infections caused by 3GC-resistant isolates was estimated up to 18.1 million Euros each year in Europe furthermore, for patients with Enterobacter bacteremia, the 30-day mortality rate of patients infected by 3GC-resistant isolates was significantly higher than those by 3GC-susceptible isolates, regardless of whether stratified by infection sites or by the initial presence of septic shock (De Krake *et al.,* 2011). The species *K. pneumoniae*, within the family *Enterobacter-iaceae*, includes opportunistic pathogens, with ubiquitous distribution (Pendleton *et al*., 2013,Wyres and Holt 2018).

The ubiquity and clinical relevance of *K. pneumoniae* is due, in part, to the genome plasticity, in which genes acquisition, such as those encoding antibiotic resistance (Paczosa and Mecsas, 2016). Indeed,

genes acquired by horizontal gene transfer, encoding resistance against aminoglycosides, 3rd generation cephalosporins, carbapenems and fluoroquinolones (Wyres *et al.*,2020) or metals such as arsenic, copper, tellurium and mercury are frequent in *K. pneumoniae* (Bialek-Davenet *et al.*, 2014). The ubiquity and clinical relevance of *K. pneumoniae* is also due to a wide array of genes that encode functions related with adhesion, protection (capsules) or siderophore production (Paczosa and Mecsas, 2016). The combination of these features and ubiquitous distribution make *K. pneumoniae* an important opportunistic pathogen, responsible for one third of the hospital infections caused by Gram-negative bacteria (Navon-Venezia *et al.*, 2017).

In 2010, the Clinical and Laboratory Standards Institute (CLSI) published the revised minimum inhibitory concentration interpretive criteria for Enterobacteriaceae. Routine ESBL reporting is no longer recommended if the new breakpoints are adopted (Wayne *et al.*, 2015). Resistance to third-generation cephalosporins (3GCs), especially Cefotaxime, has been proven to be a suitable surrogate marker for identifying ESBL- or AmpC β-lactamase-producing *E. coli* strains (Wang *et al.*, 2015). It is now believed that antibiotic treatment can be reliably guided through antimicrobial susceptibility testing without information regarding ESBL or AmpC β-lactamase production. However, studies on bacteremia caused by 3GC-resistant *E. coli* based on the revised breakpoints remain relatively few (Bidell *et al.*, 2016).

Aim of the study:

In Iraq, little information are available on prevalence of monomicrobial *Enterobacteriaceae* that are resistant to 3GC among hospitalized patients with bacteremia. In addition there is little information regarding their virulence and antibiotic resistance patterns. So the aim of this study is to investigate the incidence of monomicrobial *Enterobacteriaceae* that are resistant to 3GC among hospitalized patients with bacteremia and study their antimicrobial susceptibility patterns.

Objective of the study:

To achieve this aim, the following objectives should be followed:

1. Collection of blood samples from patients with bacteremia.
2. Isolation and identification of bacterial isolates of Enterobacteriaceae from blood cultures.
3. Determination of antibiotic susceptibility (AST) and antibiotic resistance patterns of the Enterobacteriaceae isolates using disk diffusion test (DDT) and VITEK -2 system. The interpretation of AST results will be according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2021.
4. Genotypic detection of antibiotic resistance genes among Enterobacteriaceae isolates including Rio/Toronto (*bla*$_{TEM}$), sulfhydryl reagent variable (*bla*$_{SHV}$), oxacillinase (*bla*$_{OXA}$), Cefotaximase - Munich (*bla*$_{CTX-M}$) and ampicillinase C (*bla*$_{AmpC}$) among both *E. coli* and *K. pneumoniae* bacterial isolates.

1.2 Literature review

1.2.1. General Characteristic of *Enterobacteriaceae*

Enterobacteriaceae is a heterogeneous group of gamma proteobacteria, that are straight rod-shaped and non-sporulated; they are also non-motile or motile by means of peritrichous flagella, facultative anaerobes, oxidase-negative, catalase-positive, nitrate-to-nitrite reducers, glucose-fermentors producing various final products, having simple nutritional requirements. On average they measure 2-4 μm in length by 0.4-0.6 μm in width, with rounded ends, and in vitro generation time between 20 and 30 minutes (Strockbine *et al.*,2015, Octavia and Lan, 2014).

Members of *Enterobacteriaceae* are widely distributed in nature, and many of their species live in the gut of humans and animals, including insecta, where they can cause enteric diseases or remain as commensal organisms. The members of this family play a role as plants pathogens, and biotechnological microorganisms for the heterologous production of proteins (Octavia and Lan, 2014). However, not all genera o species within a same genus are pathogen, only a small group of species are considered strict pathogens (Murray *et al.*,2014).

Historically, the differentiation of members of this family has been based on biochemical features; with the use of miniaturized tests that contain various carbon and nitrogen sources; and more recently, with the analysis of the sequence of the 16S RNAr gene. However, in some cases, the low discriminatory power of 16S RNAr sequence analysis (Naum *et al.*, 2008), make it necessary to use additional identification techniques

(Konstantinidis and Tiedje, 2005), as a result, the taxonomy of the *Enterobacteria* have undergone repeated changes in recent decades.
Recent re-classification of organisms can cause confusion in clinicians and microbiologists, as well as in the regulatory authorities responsible for the monitoring and control of these microorganisms (Strockbine NA *et al.*,2015, Forsythe JM *et al.*,2015).

But the main change in its taxonomic classification came in 2016, when Adelou *et al*. (Adeolu *et al.,* 2016) based on phylogenetic analyzes and conserved molecular characteristics analysis, proposed that the *Enterobacteriales* order, which until then, had a one single *Enterobacteriaceae* family (Ewing WH *et al.,* 2008), changed its name to order Enterobacterales, and it would be divided into seven families: *Enterobacteriaceae* (*Erwiniaceae* fam nov., *Pectobacteriaceae* fam. nov., *Yersiniaceae* fam. nov., *Hafniaceae* fam. nov., *Morganellaceae* fam. nov. and *Budviciaceae* fam. nov. (Adeolu *et al.,* 2016). The order *Enterobacterales* contains the type genus *Enterobacter*, and its description is the same as the *Enterobacteriaceae* family.

1.2.2. Major Clinical and Public Health Aspects of the Family

Food borne disease and outbreaks, Infectious diarrhea is a leading cause of morbidity and mortality on a worldwide basis. The most recent figures released by the Global Disease Burden (GBD) Diarrheal Diseases Collaborators (Troeger *et al.,* 2018). Estimate that there were 2.39 billion episodes of diarrheal disease in 2015, almost 1 billion of which occurred in children under 5 years of age. The World Health Organization (WHO) further estimates that from 550 to 600 million of these cases of diarrhea

are foodborne infections (Chlebicz and Sli_zewska, 2018, Holland *et al*., 2020). Projections that 70% of all diarrheal disease are food related translates to between 350 to 420 million episodes of gastroenteritis being of bacterial etiology (Chlebicz and Sli_zewska, 2018; Holland *et al.*,2020) .The figures are staggering. Among major foodborne bacterial pathogens, the family Enterobacteriaceae is well represented by several groups, including Salmonella, Escherichia coli (O157, non-O157), *Shigella*, and *Yersinia enterocolitica* (Chlebicz and Sli_zewska, 2018; Buzby and Roberts, 2009; Bintsis, 2017; Farmer, 2015). The CDC Foodborne Diseases Active Surveillance Network (FoodNet) provides active surveillance and epidemiologic studies in conjunction with 10 state health departments regarding the above-listed agents (Buzby and Roberts, 2009).

In addition to its association with neonatal meningitis and powdered infant formula (Henry and Fouladkhah, 2019; Laupland and Church, 2014), *C. sakazakii* has been implicated in a large-scale foodborne outbreak of gastroenteritis in high school students and school employees (Laupland, 2013).

1.2.3. Bloodstream infection

Bloodstream infection is a major cause of morbidity and mortality, comparable with major traumatic injury, myocardial infarction, and stroke with an annual incidence of 140–160 per 100,000 populations (de Kraker *et al*., 2011; Laupland, 2013,). Antimicrobial resistance (AMR) leads to increased mortality, length of hospital stay, and hospital costs associated with bloodstream infections, including both community-onset and nosocomial infection bacteremia (Paterson, 2006; Lim *et al*., 2016) Gram-negative bacteria belonging to the family Enterobacteriaceae are

associated with infections ranging from minor urinary tract infections to life-threatening bacteremia. Enterobacteriaceae, especially *Escherichia coli* and *K. pneumoniae*, are becoming a major threat to public health because of their ability to acquire resistance to most current antibiotics, especially third-generation cephalosporins, such as ceftriaxone, through the production of extended-spectrum beta-lactamases (ESBLs) (Tacconelli *et al*., 2018). The WHO has listed ESBL-producing Enterobacteriaceae (ESBL-E) as critical priority pathogens for research and development of new antibiotics (Hawser *et al*., 2009).

1.2.4. Third generation cephalosporin non-susceptible Entero-bacteriaceae and bacteremia

Third generation cephalosporins (3GC), such as Cefotaxime, form an important part of empirical antimicrobial therapy for infections caused by members of the *Enterobacteriaceae* family, such as *E. coli* and *K. pneumoniae*. (3GC) can be a reasonable choice even for patients with nosocomial infections who have non-severe illness. However, a recent increase in the prevalence of third-generation cephalosporin-resistance has challenged the use of this therapy (Courpon-Claudinon *et al.*, 2011).

β-Lactamases have been recognized as the main cause of cephalosporin resistance among Enterobacteriaceae. The most common β-lactamases are extended-spectrum β-lactamases (ESBLs), followed by AmpC β-lactamases (Gürntke *et al.*, 2014). When gram-negative bacteria is grown in blood culture, the type of positive blood culture bottle (aerobic or anaerobic) and the gram stain findings help us to estimate if the bacteria belongs to the Enterobacteriaceae family or are non-fermenting gram-negative bacteria. It is impossible to determine the

exact genus or species without emerging rapid identification technologies, such as matrix-assisted laser desorption-ionization time-of flight mass spectrometry (MALDI-TOF MS). Therefore, we usually determine a regimen of empiric therapy targeting Enterobacteriaceae, not specific species (e.g., *E. coli).* However, most studies regarding bacteremia due to (3GC) - susceptible Enterobacteriaceae have focused on *E. coli* and *K. pneumoniae* (Gürntke *et al*., 2014; Rottier *et al*., 2015). A few studies have investigated Enterobacteriaceae as a group. Rottier et al. assessed risk factors for bacteremia by third-generation cephalosporinnon-susceptible Enterobacteriaceae (Vlieghe *et al*., 2015, Rottier *et al*., 2015) and two studies analyzed resistance mechanisms (Castanheira *et al*., 2010; Asensio *et al*., 2010).

The prevalence of cefotaxime-non-susceptible Enterobacteriaceae (CTXNS-En) varies across different geographic regions. A study from Spain revealed that 9.7, 12.5 and 29.1% of third-generation cephalosporin resistance in bloodstream infections were caused by *E. coli*, *K. pneumoniae* and *Enterobacter* spp., respectively (Chopra *et al*., 2015). In the SENTRY program study from the United States of America, the prevalence of third-generation cephalosporin-resistant Enterobacteriaceae that caused bacteremia was 6.4% (Asensio *et al*., 2010). In the Asia-Pacific region, approximately 10% of Entero-bacteriaceae were phenotypically positive for ESBL production (Chopra *et al*., 2015).

Infections caused by members of the Enterobacteriaceae family are among the major causes of hospital admission and associated morbidity and mortality in children, particularly in Africa (Berkley *et al*., 2005; Sigaúque, *et al*., 2009). Infections caused by these microorganisms

in low- and middle-income countries (LMIC) have been successfully treated with the inexpensive antibiotics available. Nevertheless, with the widespread development of multidrug-resistant (MDR) strains, the usefulness of the early effective antibiotics has greatly decreased (Mandomando *et al*., 2010; Okeke *et al*., 2005), leading to the introduction of broad-spectrum antibiotics such as fluoroquinolones or third-generation cephalosporins (cefotaxime, ceftriaxone, or ceftazidime). Unfortunately, these agents are often unaffordable in most LMIC, especially in remote rural areas. On the other hand, since their first description in 1983, extended-spectrum β-lactamases (ESBLs) produced by enteric pathogens have spread worldwide (Rossolini *et al*.,2008).

The emergence and spread of ESBLs, especially those included in the CTX-M group, is an important public health problem (Peirano and Pitout, 2010). In fact, it has been considered that ESBL-carrying Entero-bacteriaceae cause >1700 deaths yearly in the USA alone, (CDC, 2013), and these pathogens have had a tremendous impact on the treatment of severe or MDR-associated infections, particularly in LMIC where second-line antibiotics are often unaffordable or unavailable. In addition, few new antibiotics against Gram-negative bacteria have been marketed in the last decades (Fair and Tor, 2014), which may favor the emergence of new resistances, further challenging the management of infectious diseases in this setting. This may play a role in the high morbidity and mortality observed in these countries, particularly in children <5 years of age.

Although different types of ESBLs have been reported among the Enterobacteriaceae family, CTX-M-15, a community-acquired ESBL that was originally described in India in the 1990s, is one of the most frequent type I CTX-M disseminated worldwide (Coque *et al*., 2008). The genes encoding ESBL enzymes are usually located in plasmids but can also be

found in the chromosomal DNA as described elsewhere (Rodríguez *et al*., 2014).

It has been reported that the $bla_{\text{CTX-M-15}}$ gene is usually found downstream from the insertion sequence IS*Ecp1* that may be involved in their dissemination and expression (Chouchani C *et al*., 2012).

Plasmid-mediated ESBL genes are of special interest due to their capability of getting transferred between strains or even species, favoring their dissemination among the bacterial population and from region to region. Moreover, these plasmids usually carry other antibiotic resistance determinants, resulting not only in the spread of ESBL but also in the dissemination of other resistance genes (Carattoli, 2013).

The selection of one resistance gene due to environmental pressure harbored in the same genetic element as another resistance gene or genes is known as the co-selection of resistance genes phenomenon. Since ESBL-producing microorganisms are also often resistant to other commonly available antibiotics, including fluoroquinolones, especially in most LMIC,(Tumbarello *et al*., 2006). Knowledge of their prevalence and characterization is important for defining local empirical stewardship programs for infections caused by MDR organisms.

Klebsiella pneumoniae is one of these pathogens. It is able to cause significant nosocomial outbreaks in high-risk units such as ICUs, onco-haematology or neonatology. The main therapeutic options for treatment of invasive *K. pneumoniae* infections include 3rd generation cephalosporins (cefotaxime, ceftazidime); however, the recent spread of *K. pneumoniae* strains that are resistant to these antibiotics has generated a significant clinical and epidemiological problem because: (i) the therapeutic options are reduced due to co-resistance; (ii) the consumption of carbapenem antibiotics considered last-line antibiotics, i.e., those used when the bacteria is resistant to the rest of antibiotics, increases (Aracil-García *et al*., 2017).

1.2.5. Antimicrobial Resistance of Enterobacteecieae

Misuse, overuse, and underuse of antimicrobials have become the main problem in the evolution of the resistance to an antibiotic. Over the last decade, the evolution and dissemination of antibiotic resistance has given rise to severe clinical and public health effects (Khan *et al.,* 2019). The mechanisms resistance in *K. pneumoniae* to various antibiotics classes included; production of antibiotic- inactivating enzymes, a variation of antibiotic target sites, changing of cell membrane permeability, efflux pump systems, and modification of metabolic pathways (Verma *et al.*, 2015). Certain mechanisms are either intrinsically encoded or acquired by the acquisition of resistance genes (Bialek-Davenet *et al.,* 2014). Among these mechanisms, efflux pump systems and enzymatic degradation play a significant role in increasing MDR *K. pneumoniae*. *K. pneumoniae* was observed high develop antibiotic resistance due to the production of new enzymes that break down antimicrobials more easily than most bacteria (Venkatachalam *et al.*, 2014).

High expression of β-lactamase as carbapenemases and cephalosporinases enzymes in *K. pneumoniae* lead to increased resistant to β-lactam antibiotics. Typically, *K. pneumoniae* producing ESBL displayed resistance to β-lactam antibiotics like penicillins, cephalosporins, and monobactams. *K. pneumoniae* carrying ESBL enzymes are frequently resistant to another class of antibiotics, including aminoglycosides, quinolones, and chloramphenicol (Gruber *et al.*, 2013). Carbapenems are regarded to be effective agents for the treatment of severe infections caused by isolates producing ESBLs. *K. pneumoniae* isolates considered to be one of the common risk factors correlated with carbapenem resistant *K. pneumoniae* infections (Denkinger *et al.*, 2013).

Escherichia coli is one of the leading pathogens causing community-acquired infections. The threat of antibiotic resistance among *E. coli* and other Enterobacteriaceae has caused great concern (Partridge, 2105). Moreover, a significant increase in community-acquired infections caused by extended-spectrum β-lactamase (ESBL)- or AmpC β-lactamase-producing *E. coli* strains has been observed worldwide (Chong *et al.*,2013). In addition to being resistant to most of the cephalosporins, these *E. coli* strains are often coresistant to fluoroquinolones and other first-line antibiotics (Durmaz *et al.*,2015). For patients infected with these drug-resistant *E. coli*, adequate antimicrobial therapy easily gets delayed (Nguyen *et al.*,2015). In 2010, the Clinical and Laboratory Standards Institute (CLSI) published the revised minimum inhibitory concentration interpretive criteria for Enterobacteriaceae.

1.2.6. Epidemiology

Multidrug-resistant Enterobacteriaceae (MDR-E) is thought to have initially arisen in the 1980s, shortly after the widespread use of cephalosporins and other broad- spectrum ß-lactam antibiotics. SHV and TEM were among the first ESBLs found, followed by CTX-M, which have now expanded to become the most common plasmid-mediated ß-lactamases worldwide. Different carbapenemase-producing Enterobacteriaceae are becoming more prevalent in different parts of the world, according to epidemiological data. Carbapenem-resistant strains were initially discovered in the 1980s and rapidly spread over the World (Vink *et al.*, 2020).

New Delhi metallo-ß-lactamase-1 is the most common carbapenemase generating resistance in India, Pakistan, and Sri Lanka. KPC-producing Enterobacteriaceae, on the other hand, are found in the United States, Colombia, Argentina, Greece, and Italy, and OXA-48-like enzyme-producers are found in Turkey, Malta, the Middle East, and North Africa (Bush and Bradford, 2020). Another study from 2011 to 2014 in the United

States found 10% carbapenem resistance in *Klebsiella pneumoniae* and 16–36% third-generation cephalosporin (3rd GC) resistance in *Escherichia coli* (Weiner *et al.*,2016). Another study using clinical isolates in Europe found that *K. pneumonia* is resistant to third GC in 31% of the cases, while *E. coli* is resistant to carbapenem in 8% of the cases and third GC in 12% of the cases (Weist and Högberg, 2016).

Before 2001, the Greek System for Antibiotic Resistant Research showed a carbapenem resistance rate of 1%; by 2008, this had risen to 30% in hospital wards and 60% in intensive care units. According to data from the European Centre for Disease Prevention and Control's EARS-Net, 678 (62.3%) of 1088 Greek *K. pneumoniae* isolates were carbapenem-resistant in 2014 (Souli *et al.,2015*). In 2008 and 2013, two cross-sectional nationwide surveys of CP Enterobacteriaceae (CPE) in Israeli post-acute-care hospitals revealed a considerable drop in the overall incidence of carbapenem resistance among Enterobacteriaceae isolates (184 of 1147 isolates (16%)). KPC-carrying *K. pneumoniae*, on the other hand, remained the most common CPE, with a growing proportion of ST258 *K. pneumoniae* strains (120 of 184 (65%) in 2008 versus 91 of 113 (80%) in 2013) (Adler, 2015).

1.2.7. Mechanisms of Resistance in *Entero-bacteriacea*

1.2.7.1. ESBL Production

Extended spectrum β-lactamases are found primarily in the *Enterobacteriaceae* family, especially *K. pneumoniae* and *E. coli* isolates (Khosravi *et al.*, 2013). These enzymes are also produce by non-fermentative Gram-negative bacteria, like *P. aeruginosa* and *Acinetobacter baumannii* (Jacoby, 2009). ESBLs enzymes are plasmid-mediated and have the ability to hydrolyze monobactams and oxyimino-cephalosporins, but not carbapenems or cephamycins (Bradford, 2004). There are several ESBL genotypes, the most common of which are CTX-M, SHV, and TEM

(Paterson and Bonomo, 2005). When ESBLs were first identified in the1980s, the point mutation of the β-lactamase broad spectrum TEM and SHV was shown to result in resistance to cephalosporin (Kliebe *et al.,* 1985). TEM and SHV genes have been recognize globally and characterized as providing resistance to extended-spectrum cephalosporins (Livermore, 2008). Mutations in these genes cause the development of high catalytic activity (increased affinity) for β-lactams by ESBL enzymes (Knothe *et al.*, 1983). Emergencies of ESBL enzymes are considered to be a major cause of nosocomial infections, particularly inside the ICUS (Alves *et al.*, 2016).

The CTX-M genotype, derived from Kluyvera spps chromosomally encoded β-lactamase enzymes, has increased dramatically, particularly in *E. coli* and *K. pneumoniae* (Livermore, 2008). Thought that *bla*CTX-M genes were inserted into plasmids, and thus facilitate transitions to pathogenic bacteria with having the capacity to move between various bacterial species and genera (Boucher e*t al.,* 2009). There are more than 172 variations of *bla*CTX-M associated with multiple infection outbreaks in community hospitals or hospitals (Dhillon and Clark, 2018). Studies from various countries indicate that once the CTX-M β-lactamase predominates with the TEM gene, it leads to the penetration within a certain geographical region (Bonnet, 2004; Livermore *et al*., 2013). Girlich *et al.* (2012) demonstrates that some of the CTX-M enzymes may contribute to conferral of antibiotic resistance to carbapenem.

1.2.7.2. Outer Membrane permeability and Efflux Pumps

Gram-negative bacteria generally possess an outer membrane that preventing the entry of foreign substances into the bacterial cell (Pagès *et al*., 2008). The outer membrane is made up of proteins defined as porins proteins that allow the hydrophilic pathways to gain certain compounds and nutrients, like antibiotics (Armand-Lefevre *et al.,* 2003). *K. pneumonia* produces two

main of porin proteins, outer membrane protein C (OmpC) or outer membrane protein F (OmpF). Alterations in porins contribute to the effect of the inflow of drugs and efflux during the movement through the membrane (He *et al.,* 2015). When there is an interaction between the antibiotic and the intracellular target molecules, that helps the description of the intrinsic mechanism of antibiotic resistance in bacteria is eventually given (Kumarsamy *et al.,* 2010). Carbapenem resistance initially noted in *K. pneumoniae* when overexpressed by the chromosome *bla*AmpC gene that produced intrinsic cephalosporins as well as modified porins OmpC and/ or OmpF (Paltansing *et al.,* 2013). This resistance prevails when plasmid-encoded expression AmpC association with reduced cell membrane permeability (Shin *et al.,* 2012).

Other carbapenem resistance mechanism in *K. pneumonia* is efflux pumps act synergistically with a reduction in the permeability of the membrane. This mechanism is protein-based structure were which reduces undesirable substances such as antibiotics and thus reduces the concentrations of this substance within the bacterial cell (Pitout *et al.,* 2005). Efflux pump resistant mechanism is the most significant type of antibiotic resistance in order that the efflux pump can remove several antibiotics such as β-lactams, fluoroquinolones, aminoglycosides, and chloramphenicol. MDR *K. pneumoniae* isolated during antibiotic therapy are associated with the elevated expression for AcrAB or AcrAB-like pumps characterized that as a significant mechanism of antibiotic resistance (Coque *et al.,* 2010).

1.2.7.3. AmpC β-Lactamase Production

AmpC expression in many *Enterobacteriaceae* is low but inducible in reply to exposure to β-lactam. AmpC-type enzymes are generally resistant to cephalosporins, but lower efficacy toward carbapenems (Poirel *et al.,* 2011).

Nevertheless, carbapenems resistance noted when AmpC enzymes combine with reduced carbapenems cell penetration due to a loss of porin protein (Qin *et al.,* 2008). Overproduction of AmpC β-lactamases combinations occurring when associated with ESBLs and modified membrane channel this leads to increasing carbapenem resistance of *K. pneumoniae* clinical isolates (Neyestanaki *et al.,* 2014).

AmpC β-lactamase has been found either chromosomal mediated or plasmid-mediated in *K. pneumoniae* or other genera of *Enterobacteriaceae* (Denton, 2007). Either mutation or plasmid encoding responsible for AmpC hyper production in *K. pneumoniae,* which is usually resistant to most monobactams as well as cephalosporins but is not repressed by β-lactamase inhibitors (Rfin *et al.,* 2008). Plasmid-mediated AmpC may harbor additional genes of resistance for antibiotics other than β-lactams (Rodriguez-Martinez *et al.*, 2012; Zhao and Hu, 2013).

Plasmid-borne AmpC β-lactamases are divided into five families according to their amino acids sequence (Rodriguez-Martinez *et al.,* 2012). A cephamycin- resistant strain of *K. pneumoniae* that produced plasmid-mediated AmpC β-lactamase (CMY-1), was first recorded in Korea in 1989 (Wassef *et al.,* 2014). These enzymes typically make the bacteria resistant to penicillins, combinations of penicillin/β-lactamase inhibitors, cephalosporins including cephamycins and aztreonam (Paltansing *et al.*, 2013).

1.2.7.4. Carbapenemase Production

Carbapenemases have lately appeared as a subset of β-lactamase enzymes observed producing by pathogenic *Enterobacteriaceae* in different regions of the world (Poirel *et al.,* 2010). Carbapenem antimicrobials have often regarded the last resort to the therapy of intricate MDR or XDR infections occasioned by Gram-negative bacteria, and therefore the existence of carbapenemase threatens the usefulness as a curative choice

(LaBombardi, 2007). Carbapenemases converse resistance to β-lactam antibiotics and are generally noticed in transportable genetic components that enable resistance mechanisms to be transferred from one strain to another. However, the bacteria that produce carbapenemases may also spread from one patient to another (Jones-Dias *et al.,* 2014). The presence of carbapenemase gene combinations with ESBL and other antimicrobials resistance genes carried on mobile genetic components is a significant problem, when it is found in *Enterobacteriaceae* and therefore leads to the increased resistance acquisition to new classes of antibiotics (Zimmerman *et al.,* 2013). The uncontrollable transfer of mobile genetic elements intra- and inter- species have also provided to the evolution of bacteria that are resistant to nearly all antimicrobials (Gazin *et al.,* 2012).

The evolution of the carbapenemases genes in *K. pneumoniae*, therefore of considerable interest because enzyme diversity is emerging rapidly and thus, these pathogens often cause problems with recognition, therapy, and response (Nordmann *et al.,* 2012). Carbapenemases classified in Ambler classes either A, B or D according to molecular classes of β-lactamases. we prime mechanisms are accountable for the creation of carbapenem resistant *K. pneumoniae*, firstly mechanism suggests the procuration of the carbapenemase genes which express enzymes that degrade carbapenem, while secondly needs reducing in antimicrobial intake by lack of porin expression correlated with overexpression of β-lactamases with a limited affinity to carbapenem antibiotics (Livermore *et al.,* 2012).

1.2.7.4.1. Class A Carbapenemases

Class A carbapenemases seem to be the most varied and commonly distributed class, where most of the clinically significant β-lactamases belong to this class. This class is sensitive to cephalosporins, but does not

have any activity against carbapenems, aztreonam as well as penicillins (Bedenić *et al.,* 2014).

KPCs are the most clinically important of the plasmid-coded class A carbapenemases (Cabral *et al.,* 2012). The KPC gene was first described in the United States and presently internationally recognize. Its
variant of the KPC-2 was distinguished in the *K. pneumoniae* in the Eastern United States in 1996 (Cannatelli *et al*., 2014). There are present of the *bla*KPC gene have 17 variations was spared globally (Perez and Van Duin, 2013). The emergence of MDR β-lactam isolates of the harbor *bla*KPC gene is worrying because this gene will be combined with other carbapenem resistance genes, as well as many non-β-lactam antibiotics genes, thus will finally limit efficient therapeutic regimens (Nordmann *et al*., 2009).

K. pneumoniae isolates producing KPC is now widely recognized with nosocomial pathogens and systemic diseases. In a few years, *bla*KPC gene producer is distribute internationally and reported in Greece, China, Italy, and the North and South American countries, where they deemed endemic (Mataseje *et al.,* 2012; Munoz-Price *et al.,* 2013; Chan *et al.,* 2014). The high distribution of the *bla*KPC gene may be due in part to its carriage on the plasmid (Poirel *et al.,* 2013). Another carbapenemase type related to this class described as GES. GES was recognized in different clinical *K. pneumoniae* strains in various hospitals in Cape Town and South Africa (Brink *et al.,* 2012).

1.2.7.4.2. Class B (Metallo-β-Lactamases, MBLs)

The class B MBLs has higher clinical importance globally and can be hydrolytic effectively against penicillins, cephalosporins, and carbapenems but is not inhibited clavulanate, sulbactam, or tazobactam (Neyestanaki *et al.,* 2014). The hydrolytic activity count on the combination of the β-lactams ring with a cofactor (Zn2+) ions in the active site and therefore, this

compound inhibited by EDTA (Nucleo *et al.,* 2013). The first MBL has been recognized in opportunistic and environmental bacteria like *Salmonella maltophilia*, *Bacillus cereus,* and *Aeromonas* spp., where MBL genes are encoded in these species, both intrinsically and chromosomally. Since the 1990s, *Enterobacteriaceae* has reported a dramatic rise in the acquisition or transfer of MBL genes (Yang and Bush, 1996). The most common MBL families include the VIM (Verona-integron-encoded MBL), NDM (New-Delhei MBL), IMP (imipenem), SPM (Sao-Paulo MBL), SIM (Seoul imipenemase) and GIM (German imipenemase) enzymes. Genes that encode of MBL enzymes are predominantly found in the multitude of integron structures and combine into gene cassettes (Carmeli *et al.,* 2010; Calhau *et al.,* 2014*).*

The blaIMP is the most common family of MBL genes in the world. The IMP was found in *Acinetobacter* spp., *Pseudomonas* spp as well as Enterobacteriaceae (Shanthi and Balagurunathan, 2014). Up to 33 varieties of carbapenemases of the IMP type were detected have distributed throughout the world (Braun *et al*., 2018).

The blaVIM is a type of MBL genes. The VIM-1 variant was first recognized in *P. aeruginosa* obtained from a patient in Verona/ Italy and showed predominantly integrons associated MBLs and, sometime subsequently, VIM-2 occurred from P. aeruginosa isolates in France (Gupta et al., 2014). The VIM is detected in K. pneumoniae in France, also reported in *E. coli* in Greece (Kassis-Chikhani *et al.,* 2006). The VIM enzymes eventually consisted up more than 50 variants (Lam *et al*., 2018). There are some similarities between the VIM and IMP families where have the ability to hydrolyze all β-lactam antibiotics unless monobactams and sensitivity to all β-lactam inhibitors. Besides, both genes can begin nosocomial infections and may be responsible for the enhanced mortality rate (Marsik and Nambiar, 2011).

1.2.7.4.3. Class D OXA-Type-β-lactamases

The oxacillinase (OXA) enzymes are serine-β-lactamases belonging to class D that is weakly inhibited by EDTA and/or clavulanic acid. The OXA enzymes are of reduced activity against carbapenems antibiotics (Ahn *et al.*, 2015). These enzymes fundamentally found in non-fermented species like *A. baumannii*, *P. aeruginosa* and occasionally in *Enterobacteriaceae* isolates in several countries, including Candia, Denmark and the United Kingdom (Elufisan *et al*., 2012; Stalder *et al*., 2012). The OXA β-lactamase was first identified in MDR *A. baumannii* strains in 1985 in Edinburgh, Scotland (Paton *et al.,* 1993). OXA carbapenemases are considered to be of major concern due to their capability to mutate quickly and expand their range of activity (Carrer *et al.,* 2010). Research by Mathers *et al.* (2013) confirmed the widespread occurrence of Class D in the *Enterobacteriaceae*, causing global concern and major public health concerns.

OXA-48 is one of the few members of this class to have a significant activity of carbapenem-hydrolyzing. In 2003 in Turkey, the first organism to produce OXA-48 recognized in *K. pneumoniae* isolate (Livermore, 2012). This OXA-48-producing organism emerged from nosocomial infections in Turkey and was widespread in North Africa and Southern Europe (Lee *et al.*, 2016). Organisms producing OXA-48 like *E. coli* and *Enterobacter cloacae* revealed from through nosocomial transmission among patients in Russia, Spain, and Italy (Aktas *et al.,* 2012). A point mutant variant of OXA-48, OXA-181, has been reported have similar carbapenemase activity in isolates from Indian or Asian. OXA-48/OXA-181 are idiosyncratic as they are weakly hydrolyzing to carbapenems but have wide-spectrum toward cephalosporins like ceftazidime and aztreonam (Queenan and Bush, 2007; Castanheira *et al*., 2011).

The OXA-24 non-nosocomial type found in *Acinetobacter* spp. OXA-23 is also a non-nosocomial type that spreads worldwide and is more prevalent in the USA and Europe (Evan and Amyes, 2014).

1.2.8. Cause of nonsusceptibility (NS) to third generation cephalosporin

Infection with 3GCREB frequently results in inappropriate antimicrobial therapy and may compromise treatment outcome. While the impact of inappropriate therapy on mortality remains a controversial issue, it is significantly associated with increased length of stay and hospital costs (Frakking *et al.,* 2013). 3GCREB colonization of the gut is the reservoir for infections with these organisms (Woerther *et al.*, 2013).

Colonization rates differ greatly between countries and continents, with a lower prevalence in Europe and much higher rates in Africa or South-East Asia, reaching up to 69.3% in healthy volunteers in Thailand (Woerther *et al.,* 2013; Frakking *et al.,* 2013; Seiffert *et al.,* 2013). Documented risk factors for colonization with 3GC-REB are hospitalization within the previous 12 months, prior antimicrobial treatment, travel to regions with 3GCREB endemicity (e.g. India and South-East Asia) or eating pork (Seiffert *et al.,* 2013; Leistner *et al.,*2013).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Study design, Patients, and Samples:

2.1.1 Study design

This cross-sectional study was achieved to investigate the incidence of monomicrobial *Enterobacteriaceae* that are resistant to 3GC among hospitalized patients with bacteremia and study their antimicrobial susceptibility patterns.

2.1.2 Ethical Approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal approval before sample was taken. The study protocol and the patients consent forms were reviewed and approved by General Directorate of the medical city and the committee on publication ethics at college of Medicine, University of Babylon, Iraq, under the reference No. BMS 0338/016.

2.1.3. Patients and Samples:

This study included 266 patients (aged <1 to 80 years) suffering from blood stream infections. These patients were admitted to three hospitals and one teaching center (Baghdad teaching hospital, Ghazi Hariri Hospital, Peadiatric Teaching Hospital, National center of teaching Laboratories) in Baghdad city during the period from November 2021 to March 2022. These patients were admitted to different hospital wards, in addition to samples taken from blood stream for private clinics during this period.

Two-hundred sixty six blood specimens were generally collected from blood. These samples were collected under the help of advisory staff to avoid any possible contamination. Each sample was immediately inoculated in the blood bottle and incubated aerobically for 24 hours at 37°C. After appearance of growth in bottles, these growths immediately inoculated on blood agar MacConkey agar and EMB agar plate, Then incubated aerobically for 24 hours at 37° C.

Criteria used to categorize isolates as contaminants versus blood stream infection were the following:

1. Fever or signs of sepsis at the time of blood culture;
2. Isolation of the same potential skin contaminant from two or more blood cultures drawn on separate occasions within a 48-hour period and isolated from a patient with an intravascular access device inserted at least 48 hours before and physician institutes appropriate antimicrobial therapy.
3. Absence of any other possible site of infection.

2.2. Materials

2.2.1. Instrument and Equipments

The instrument and equipment used in this study with manufacturing companies and countries of origin were listed in (Table 2-1).

Table (2-1): The instruments and equipment used in the study

Type of equipment	Company (Origin)
Autoclave	HicCZave- Hirayama (Japan)
Bench centrifuge	Hettich (Germany)
Compound light microscope	Olympus (Japan)
Deep freezer	GFL (Germany)
Digital camera	Sony (Japan)
Distillator (Water distiller)	GFL
Electric oven	Memmert (Germany)
Electrophoresis unit	Labner (Taiwan)
Gel documentation system	Optima (Germany)
Gradient PCR	Biometra (Germany)
Incubator	Memmert
Magnetic stirrer	Labtech (Germany)

Micropipette set (1-1000µl)	Eppendorf (Germany)
Millipore filter (0.22 µm)	Difco (USA)
Nano drop	Thermo (USA)
Petridish (15 and 9 cm)	China
Sensitive balance	Memmert
Shaker water bath	Memmert
Standard loop 0.01 ml	Himedia (India)
UV spectrophotometer	Shimadzu (Japan)
Visible spectrophotometer	Apel (USA)
VITEK 2 system	BioMerieux (France)
Vortex	Thermolyne (USA)

2.2.2. Biological and Chemical Materials

The biological and chemical materials used in this study are listed in (Table 2-2).

Table (2-2): The biological and chemical materials used in the study

Materials	Company (origin)
Agarose	Promega (USA)
Absolute ethanol	BDH (England)
DNA Loading Buffer	Promega (USA)
Ethanol (96%)	BDH (U.K)
Ethidium bromide	Sigma (USA)
Ethyenediamine tetra-acetic acid(EDTA)	BDH (U.K)
Glycerol (C3H8O3)	Fluka (Switzerland)
Gram stain	Himedia (India)
Hydrochloric acid (HCI)	BDH
Isopropanol	Mast Diagnostic (India)
Kovac's reagent	Himedia (India)

McFarland standard tubes (0.5)	Biomerieux (France)
Methyl red	BDH (U.K)
Nuclease free water	Ambion (USA)
Peptone	Himedia (India)
Phenol	Scharlau, Sigma (USA)
Potassium hydroxide (KOH)	BDH(U.K)
Sodium chloride (NaCI)	BDH (U.K)
Sodium dodecyl sulfate (SDS)	AppliChem (Germany)
Sodium hydroxide (NaOH)	BDH (U.K)
Tetramethylp-phenyl diamine dihydrochloride	BDH (U.K)
Tris-(hydroxymethyl) methylamine	BDH (U.K)
Tris-borate-EDTA buffer (TBE buffer)	Promega, Bioworld(USA)
Tris-EDTA (TE) buffer molecular grad	Promega (USA)
Yeast extract	Bioworld (India)
α-naphthol	BDH (U.K)

2.2.3. Culture Media

The culture media that used in this study are listed in (Table 2-3).

Table (2-3): The different culture medium used in the study

Medium	Company (origin)
Blood agar base	Himedia (India)
Brain heart infusion agar	Mast Diagnostic (UK)
Brain heart infusion broth	Mast Diagnostic (UK)
MacConkey agar	Biolife (Italy)
Eosin methylen blue agar	Himedia (India)
MR-VP broth	Oxoid (UK)
Muller Hinton agar	Cyperss (Bilugim)

Nutrient agar and broth	Biolife (Italy)
Peptone water	Biolife (Italy)
Simmons citrate agar	Mast Diagnostic
Triple sugar iron agar	Biolife (Italy)
Tryptic soy broth	Biolife (Italy)
Trypton	Oxoid (UK)

2.2.4. Antibiotic Discs

Twelve types of antibiotic discs belonging to six classes of the antibiotics applied in this study are listed in the (Table 2-4).

Table (2-4): Types of Antibiotic discs, provided from Bioanalyzed Company (Turkey).

No	Antibiotic class	Antibiotic	Abbrevation	Concent.
1	Penicillins	Ampicilin	AM	25 (µg)
2	Cephalosporins	Ceftriaxone	CRO	10 (µg)
		Cefotaxime	CTX	30 (µg)
		Cefuroxime	CXM	30(µg)
		Cefepime	FEP	10 (µg)
		Ceftazidime	CAZ	30 (µg)
3	Carbapenems	Meropenem	MEM	10 (µg)
		Imipenem	IPM	10 (µg)
4	β-lactam-β-lactamase Inhibitor	Ampicillin/Sulbactam	AMS	20 (µg)
5	Quinolones	Ciprofloxacin	CIP	10 (µg)
6	Aminoglycosides	Amikacin	AK	30 (µg)
		Gentamicin	CN	10 (µg)

2.2.5. Kits

Types of kits that are used in the this study are listed in the (Table 2-5).

Table (2-5): Types of kits used in the study

Kit type	Manufacturer (Origin)
AST-GN-N222	BioMerieux (France)
VITEK-2 system	
Genomic-DNA Extraction	The Presto TM Mini gDNA (Geneaid Biotech Ltd / Taiwan)

2.2.6. Master Mix and Molecular Weight DNAMarker

Table (2-6): Materials used in PCR techniques in this study

Material	Description	Company	Country
PCR Green Master Mix	2X *Taq* DNA polymerase supplied in a reaction buffer (pH 8.5),400µM dATP, 400 µM dGTP, 400 µM dCTP,400 µM dTTP, and 3mM MgCl2	Promega	USA

Table 2.7. Primers used in the study

Target gene	Primer name	Primer sequence (5´-3´)	Molec. weight	Ref.
***bla*$_{TEM}$**	TEM – F	ATGAGTATTCAACATTTCCG	867	Rasheed *et al*., 1997
	TEM-R	CTGACAGTTACCAATGCTTA		
***bla*$_{SHV}$**	SHV-F	GGGTTATTCTTATTTGTCGC	930	Rasheed *et al*., 1997
	SHV-R	TTAGCGTTGCCAGTGGTC		
***bla*$_{OXA}$**	OXA-F	GGCACCAGATTCAACTTTCAAG	564	Dallenne *et al*., 2010
	OXA-R	GACCCCAAGTTTCCTGTAAGTG		
***bla*$_{AmpC}$**	AmpC-F	CCC CGC TTA TAG AGC AAC AA	634	Shahid *et al.*, 2009
	AmpC-R	TCA ATG GTC GAC TTC ACA CC		
***bla*$_{CTX-M}$**	CTX-	TCAAGCCTGCCGATGGT	561	

	M-F			Enwuru *et al*., 2013, Madhavan and Jayalakshmi 2016
	CTX-M-R	TGATTCTCGCCGATCTG		

2.3. Methods

2.3.1. Preparation of Reagents and Buffers

The following solutions and reagents were used in this study; those that require sterilization were autoclaved at 121°C for15-20 minutes. The pH of the solution was adjusted using 1N NaOH and/or 1N HCl.

2.3.1.1. Preparation of Reagents

The following reagents were prepared as described by (Forbes *et al*.,2007) and (MacFaddin, 2000).

2.3.1.1.1 Catalase Reagent

Hydrogen peroxide, H_2O_2 (3% vol/vol) was prepared and refrigerated in a dark bottle. This reagent was used to detect the presence of the catalase enzyme produced by bacteria.

2.3.1.1.2 Voges-Proskauer Reagents

The reagents were prepared fresh as follows:

Reagent A: 5 gm of α-naphthol in 100 of absolute ethanol (5% wt/vol)

Reagent B: 40 gm of KOH in 100 ml of DW (40% wt/vol)

These reagents were used in Voges-Proskauer test for detection of acetyl-methyl-carbinol (acetoin) from glucose fermentation.

2.3.1.2. Preparation of Buffers and Solutions

2.3.1.2.1. Solutions used in DNA Extraction

The following solutions were prepared as described by (Cheng and Jiang, 2006) with some modifications.

2.3.1.2.1.1.Tris-EDTA (TE) Buffer

It was prepared by adding 10 mM Tris-HCl and 1 mM EDTA to 800 ml ofDW. The pH was adjusted to eight. The buffer completed to one litter by DW, sterilized by autoclave, and stored at 4°C until used.

2.3.1.2.1.2 Salt-Tris-EDTA (STE) Buffer

This buffer was prepared by adding 100 mM NaCl, 10 mM Tris-HCl and 1 mM EDTA to 800 ml DW. The pH was adjusted to eight. The buffer completed to one litter by DW, sterilized by autoclave, and stored at 4°C until used.

2.3.1.2.3. Solution used in gel electrophoresis

The solutions were prepared as described by (Green and Sambrook, 2012) as follows:

2.3.1.2.3.1 Solution of Tris-Borate-EDTA (TBE) Buffer

Tris-borate-EDTA buffer was used at concentration of 1X (1:10 dilution of the concentration stock). The stock solution diluted by DW and stored at room temperature.

2.3.1.2.3.2 Ethidium Bromide Solution

A five μg/ml stock solution was prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of DW and kept in dark reagent bottle.

2.3.4. Preparation of Culture Media

All the culture meida were prepared according to the manudacture instructions.

2.3.4.1. Eosine methylen blue agar (EMB)

EMB Agar is used for differentiation of gram negative bacteria. Procedure Dissolve 36 grams in 1 litter of distilled water. Stir the suspension until it is

uniform. Heating of the medium for dissolving. Autoclave at 15 lbs pressure (121°C) for 15 minutes for sterile. Cool the medium to 45-50°C and shake it to oxidize the methylene blue and suspend the flocculent precipitate. (If EMB Agar is inoculated on the same day, no autoclave sterilization is required.

2.3.5. Identification of bacterial isolates

2.3.5.1. Gram stain

Bacterial isolates were examined for Gram stainability, shape and arrangement was observed using a light microscope (Forbes *et al.*,2007).

2.3.5.2. Colony morphology

All isolates were identified primary according to general cultural characteristic (color, shape, texture and size) of colony onto MacConky agar, Blood agar, and EMB agar after incubated overnight at 37 C°. Other characteristics were observed like lactose fermentation and blood hemolysis.

2.3.5.3. Biochemical Tests

With the following traditional tests bacterial isolates were identified biochemically.

2.3.5.3.1 Indole Production Test

For this test, conventional tube method was used. Fresh colonies of microorganism to be tested were inoculated in peptone water tube and incubate overnight to 48 hours at 37°C. Then, a few drops of Kovacs reagent was added to the broth culture. The tube did not shake and the result was considered positive if a pink colored ring appeared after the addition of reagent (MacFaddin, 2000).

2.3.5.3.2 Citrate Utilization Test

The fresh isolate colonies from the culture plate taken with a straight wire loop, inoculated into the Simmon's citrate slant, and incubated at 37˚C for 48-72 hours. The color change from green to blue and the growth of colonies on the streak line was indicated a positive test.

2.3.5.3.3 Voges-Proskauer Test

Methyl red/Voges-Proskauer broth was inoculated with a young bacterial culture and was incubated at 37˚C for 48 hours. Two drops of 40% KOH solution followed by 6 drops of 5% solution of α-naphthol were added to tube. A positive test was represented by the development of a pink color 15 minutes after the addition of the reagents (MacFaddin, 2000).

2.3.5.3.4 Urease Production Test

Colonies of isolated bacteria from an overnight culture were streaked onto the surface of a urea agar slant. The tube was incubated at 37˚C and was read after 24 hours, and every day for 6 days. The test was positive if a purple pink color appeared (MacFaddin, 2000).

2.3.5.3.5 Oxidase Test

On a nonporous surface such as glass plate or Petri dish, a strip of filter paper (Whatman No. 1) was soaked with a few drops of freshly made 1% of oxidase reagent, and then the overnight growth colony on nutrient agar was picked up with the help of a sterile wooden stick and was applied over the filter paper. Deep purple-color in 10 seconds was taken as positive test (MacFaddin, 2000).

2.3.5.3.6 Catalase Test

With a sterile inoculating loop or wooden applicator stick, the center a colony derived from an overnight culture plate picked and placed on a clean,

glass slide. A drop of catalase reagent (3%) was place onto the smear. The rapid elaboration of oxygen as evidenced by bubbling indicated as a positive result (MacFaddin, 2000).

2.3.5.3.7 Triple Sugar Iron Agar Test (TSI)

With a straight inoculation needle, an isolated overnight growth colony from the culture plate was taken and stabbed through the center of the medium to the bottom of the tube and then streaked the surface of the agar slant in a zigzag manner. The slant was incubated at 37˚C for 24 hours. The expected results including as follow. An acid-acid (yellow slant-yellow butt) reaction indicated the fermentation of dextrose, lactose, and/or sucrose. An alkaline-acid (red slant-yellow butt) reaction indicated of dextrose fermentation only. An alkaline-alkaline (red slant, red butt) reaction indicated absence of carbohydrate fermentation results; blackening of the medium, designated as the presence of H2S; and bubbles or cracks in the agar, indicated the production of gas (MacFaddin, 2000).

2.3.5.3.8. Growth on EMB medium

Each isolates which grown on Maconkey agar and apperaed as lactose fermented were re-streaked again on eosin methylen blue (EMB) aiming to look for metalic shiny green colony which is a diagnostic features of E. coli bacteria.

2.3.6. Identication using Vitek-2 system

Bacterial isolates were identified at species level by using VITEK® 2 system with Identification- Gram Negative Bacteria (ID-GNB) cards according to the manufacturer's instructions. The software provided with the VITEK® 2 Compact system includes analysis and data-management programs as an identification system depends on the biochemical reactions between the bacterial isolates suspended in their solutions and the media in the VITEK-2 Identification Cards.

The bacterial isolates inoculated at 37°C on MacConky agar plates and after incubated overnight taken a single colony then suspended. The turbidity measurement for suspension bacterial to match the McFarland (0.5) standard in 0.45% sodium chloride, then the (Gram Negative Vitek 2 ID card), the bacterial suspension tubes were loaded manually into the Vitek-2 system, the software also prepared according to (BioMerieux, France) the manufacturer's instructions (Ling *et al.*, 2003).

Vitek2 compact system composed mainly of two parts: Instrument and computer. the instrument consists of five parts:

a. Keypad.

b. Fill door in which the sample was transport from khan tube into the card by transfer tube through 70 seconds.

c. Load door, in this part the transfer tube was cut from the card and loading the latter into the incubator during 3-5 min.

d. User access door, in this part all changes which occur as a result of Bacterial growth were measured to give the end result.

Waste door, in this part a collection of cards was discarded at the end of process.

2.3.7. Preservation and Maintenance of Bacterial Isolates

The bacterial isolates were preserved on nutrient agar slant at 4˚C. The isolates were maintained monthly by culturing on new culture media. For long-term maintenance, an overnight culture of the bacteria to be stored was prepared on blood agar plate. By using a sterile loop and aseptic technique, several colonies were transferred into nutrient broth tube supplemented with 15% glycerol, resulting in a dense suspension. The broth was shook well and let sit at room temperature for approximately 30 minutes before placing into long-term freezer storage. Then, the broth was stored at -70˚C. At this temperature, the culture can be stored indefinitely (Mahon *et al*., 2007).

2.3.7.1 Subculture of Preserved and Frozen Stock Cultures

Frozen stock cultures were sub-cultured on fresh blood agar plates, and then incubated in aerobic conditions at 37˚C for 24 hours (Mahon *et al*., 2007).

2.3.8. Antibiotic Susceptibility testing

2.3.8.1. Determination of Antibiotic sensitivity test by disc diffussion test

All identified bacterial isolates were subjected to antibiotic susceptibility testing against 12 different antibiotics. All antibiotics used for this test are listed in (Table 2.4). The susceptibility was determined by the Kirby-Bauer disk diffusion test (DDT) according to CLSI, (2022) guidelines.

Fresh 3-5 well-isolated colonies of same appearance were inoculated in tube containing 2 ml of nutrient broth and were kept for approximately 2-3 hours to be enriched. The inoculum density was adjusted to a 0.5 McFarland standard (approximately 1.5 x 10^8 cfu/ml, corresponding to 0.1 optical densities at 600 nm) with sterile normal saline. This suspension was used within 15 minutes of preparation. A sterile cotton swab was dipped into the adjusted suspension and then the swab was rotated and pressed firmly several times against the side of the tube above the level of the suspension to remove excess fluid. The swab was used to inoculate the dried surface of the Mueller-Hinton agar plates by streaking over the entire agar surface, this process was repetitive three more times.

To ensure an even spreading of inoculum, the plate was rotated approximately 60° each time. The edge of the agar was then swabbed as a final step of streaking, and the surface of agar was left for 5 minutes to allow any excess surface moisture to be absorbed before applying the antibiotic disks. Then antibiotic disks were placed onto the surface of the inoculated Mueller Hinton agar plate by using sterile forceps, which evenly distributed in the inculcated plate. A maximum of 6 antibiotic disks were placed circular on a 100-mm

inoculated plates and 13 disks were placed on a 150-mm inoculated plate. Each disk was gently pressed down with the forceps to ensure its contact with the agar surface. The forceps was sterilized by immersing in alcohol and burning it. The inverted plates were incubated at 37 °C for 18 hours under aerobic conditions. Following incubation, the diameter of each zone of the inhibition was measured in millimeters by using a caliper on the underside of the plate. According to the CLSI (2021) guidelines, the results were interpreted as sensitive (S), intermediate (I), or resistance (R).

2.2.8.2. Determination of Antibiotic sensitivity (AST) test by Vitek

- The isolated bacteria suspension is measured to be diagnosed by the turbidity device Vetik 2 (Densichek), which the turbidity must equal (0.5) approximately 1.5×10^8 CFU/ml. An aliquout of 145 µL was transferred. The first tube to the second for the antibiotic susceptibility test, The two test tube containing bacterial suspension was placed into a cassette the device cut off the transport tube then transferred into the incubator card to incubate at 37°C, and the result was reading and printed diagnostic report for each card existing within the reader with antibiotic susceptibility test as (Table 2-7) according to company instruction (biomerieux).

2.3.8.3.Detection of antibiotic susceptibility patterns

Multidrug resistant (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antibiotic classes. Extensively drug resistant (XDR) was defined as non-susceptibility to at least one agent in all but one or two antibiotic classes. Pan drug resistant (PDR) was defined as non-susceptible to all-antibiotic classes (Magiorakos *et al.*, 2012).

2.3.9. Molecular Analysis Techniques

2.3.9.1. DNA Extraction

2.3.9.1.1. Presto™ Mini gDNA Bacteria Kit for DNA extraction

The Presto TM Mini gDNA Bacteria Kit (Geneaid Biotech Ltd / Taiwan) were used. The absolute ethanol was added to Washed Buffer then mixed by shaking it for a few seconds. Checked the box on the bottle. Be sure and close the bottle tightly after each use to avoid ethanol evaporation, Lysozyme were stored at -20ºC for extended periods and dd H_2O were added to Proteinase K then vortex to ensure Proteinase K is completely dissolved. Checked the box on the bottle. Once it is dissolved completely, centrifuge for a few seconds to spin down the mixture. For extended periods, the ddH_2O and Proteinase K mixture should be stored at 4ºC. Use only fresh ddH_2O as ambient CO_2 can quickly cause acidification.

Bacterial DNA extraction performed according to the Chang and Jiany method (2006). The bacterial culture was grown in five ml LB broth and, then one ml of the culture transferred to Eppendorf tubes. These tubes centrifuged for 3 min at 8000 rpm and the supernatant was discarded without touching the pellet. Genomic DNA extracted according to ABC DNA Isolation Kit. The amount and integrity of DNA were determined using the spectrophotometer (Nanodrop).

2.3.9.2. Molecular method for the detection resistance genes by PCR

2.3.9.2.1. Making the Primers preparation

The DNA primers listed in the (Table 2-7) were prepared depended on the manufacturer's instructions by dissolving the lyophilized primer product with TE buffer grade after a short spin down. The stock solution was diluted with nuclease - free PCR water with each primer in the ratio 1:10 to make a working solution. The final picomoles depended on each primer's procedure.

2.3.9.2.2. PCR reaction mix preparation

Aseptically prepared of the PCR reaction mix by using Taq Ready master mix Kit according to the manufacturer's instructions for a final reaction volume of 25 µl with 5 µl of DNA extract as shown in (Table 2-8).

Table (2-8): Total volume of PCR reaction mix

Reagent	Volume per PCR reaction
PCR master mix (QIA GEN) buffer, 2X	12.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
DNA template	5 µl
Nuclease free water	5 µl
Total volume	25 µl

2.3.9.10. PCR thermocycling Conditions

Polymerase chain reaction assays was performed at a reaction volume of 25 µl. Depending on their reference procedure as in (Table 2-9), the PCR amplification conditions performed with a thermal cycler were specific to each single primer set.

Table (2-9): Thermocycling conditions for primers used in the present study

Genes	Initial denaturation	Cycling condition			Final extension	Cycles
		Denaturation	Annealing	Extension		
bla$_{TEM}$	94/1 min	94/1 min	58/1 min	72/1 min	72/10 min	30
bla$_{SHV}$	94/1min	94/1min	56/1 min	72/1 min	72/10 min	30
bla$_{OXA}$	94/10 min	94/40 sec	60/40 sec	72/1 min	72/5 min	30
bla$_{AmpC}$	95/15min	94/1min	58/2min	72/3min	72/7min	35
bla$_{CTX-M}$	94/4min	94/30sec	63/1min	72/1min	72/5min	35

2.3.9.11. Agarose Gel Preparation

All gel electrophoresis requirements were carried out using the method described by (Green and Samboroak ,2012). Agarose gel has been made in 100

ml (1X) TBE buffer by adding 1.5 gm of agarose powder. The solution was boiled until it was clear, then cooled to 50° C, and added 0.5 µg / ml of ethidium bromide to the agarose solution. The agarose flowed into an earlier balanced gel tray leaving until it was cooled and solid. The comb gently removed from the tray. The wells made by comb used to load DNA product. The agarose gel wells were loaded with five microliters of amplified PCR product. A 5 µl DNA ladder was used to compare the size of the molecular standard. The gel tray in the electrophoresis chamber was set and the liquid was connected to the (IX) TBE buffer until the gel layer was covered. The electrical current was carried out at 70 volts for 1.5-2 hours. Finally, Biometra gel documentation system using for visualization of the PCR products. Based on the ladder, positive results were observed when the DNA band base pairs of the sample are equal to the target size of the DNA product.

3.2.9. 12. Statistical Analysis

The Chi-square test was used to determine any significant differences in resistance or prevalence where appropriate. Differences were considered significant at $p < 0.05$.

Chapter Three

Results and Discussion

Chapter Three: Results and Discussion

3.1. Clinical specimens and bacterial isolations

A total of 266 blood samples were collected from different patients from both sexes suffering from bacteremia admitted to different hospitals of medical city in Baghdad City during the period from November 2021 to March 2022. Blood samples were collected from 226 inpatients and 40 outpatients. The patients were 152 (57.1%) females and 114 (42.8%) males.

The results found that only 46 (17.2%) blood samples wrere positive culture, of these cultures, 25 (54.3%) were from females and 20 (44.4%) from males. The male to female ratio were 1:1.25 and the mean age of pateints (n=45) was (23.64±12.8) years as illustrated in (Table 3-1).

After observing the cultural and morphological characteristics of bacterial isolates and performing the traditional biochemical tests, and out of 46 (97.8.%) positive blood samples, 45 samples (16.9%) were belonged to Gram negative bacterial isolates and one sample (2.1%) was identified as fungal growth. However, no bacterial isolates belonged to Gram positive were recovered during the study. Also, no mixed infections were found among bacterial isolates recovred from positive blood cultures.

The identication of the Gram negative bacterial isolates revealed that they belonged to *E.coli* were 25 (55.5%) and isolates *K. pneumoniea*e 20 (44.4%). The identification of all *E.coli* and *K. pneumoniae* isolates was confirmed by Vitek-2 automated identification system. However, no other Enterobacteriaceae species were recovered (Table 3-1).

These results revealed that there were monomicrobial enterobacteriaceae bacteremia (mEB) infection among patients with bloodstream infections.

Several authors worldwide investigated the prevalence of Enterobacteriaceae among hospitalized patients with bacteremia (Lin, *et al*., 2021; Lee *et al*., 2014; Chang *et al*., 2020; Chiu *et al*., 2015; Yusef *et al*., 2017; Saleem *et al*., 2013; Naqid *et al*., 2020).

Lin and his colleagues (2021) found that *E.coli* and *K. pneumoniae* isolates were the two major causative species of Enterobacteriaceae among hospitalized patients with bacteremia in southern Taiwan. However, other Enterobacteriaceae species were recovered with low rate of infection such as *Proteus mirablis*, *Provedentia stuartii*, and *Citobacter koseri*. These findings were in agreement with the investigation by Lee et al., (2014), where these two species caused 62.8% of bacteremia cases. Also, Chang and his colleagues (2020) found these two species were the major causes of bacteremia, but in lesser percentage (34.8%), while the study by Chiu et al., (2015) revealed higher rate (58.1%). A study of Duhok City in Kurdistan Region of Iraq by Naqid et al., (2020) found that *K. pneumoniae* was the most prevalent species.

Choi and his colleagues (2021) compared the antibiotic resistance of pathogens causing community-acquired infections (CAIs), healthcare-associated infections (HCAIs), and hospital-acquired infections (HAIs) among 146 patients in Korea, and they found that the prevalence of Enterobacteriaceae pathogens showing third-generation cephalosporins resistance was 8.3%, 50.0%, and 60.0% in the CAIs, HCAIs, and HAIs groups, respectively. The prevalence of extended-spectrum beta-lactamase-positive pathogens was 6.3%, 47.3%, and 55.0% in the CAIs, HCAIs, and HAIs group, respectively, with no significant difference between the HCAIs and HAIs groups. Resistance patterns of the HCAIs group more closely resembled those of the HAIs group than those of the CAIs group.

Although Gram-positive and *Staphylococcus* spp. are the most predominant causative agents among hospitalized patients with bacteremia (Al-Charrakh and Obayes, 2014, Al-Sultany and Al-Charrakh, 2020, Taylor and Unakal, 2022). The results of the present study revealed that no bacterial isolates belonged to *Staphylococcus* spp. were recovered. This may be due to the patients had previously taken antibiotics or they were under antibiotic therapy during samples collection.

3.2. Distribution the age groups with the type of infections

The results found that most cases of enterobacteriacae infections were 13(28.9%) out of 45 cases occured among age groups (1-19) years while the frequencies and percentage of enterobacteriaceae infections were decreased among the patients at the sixth to seventh decade of age 3 (12.0%) and 1 (5.0%) respectively (Figure 3-1).

Regarding *E. coli* infections the results found that most cases (32.0%) were among age group (1-19) years out of 25 cases, while most cases (25%) of *K. pneumonieae* infections were distributed among most the age groups of 20 cases, statisticaly these differences were non-significant (P value= 0.7).

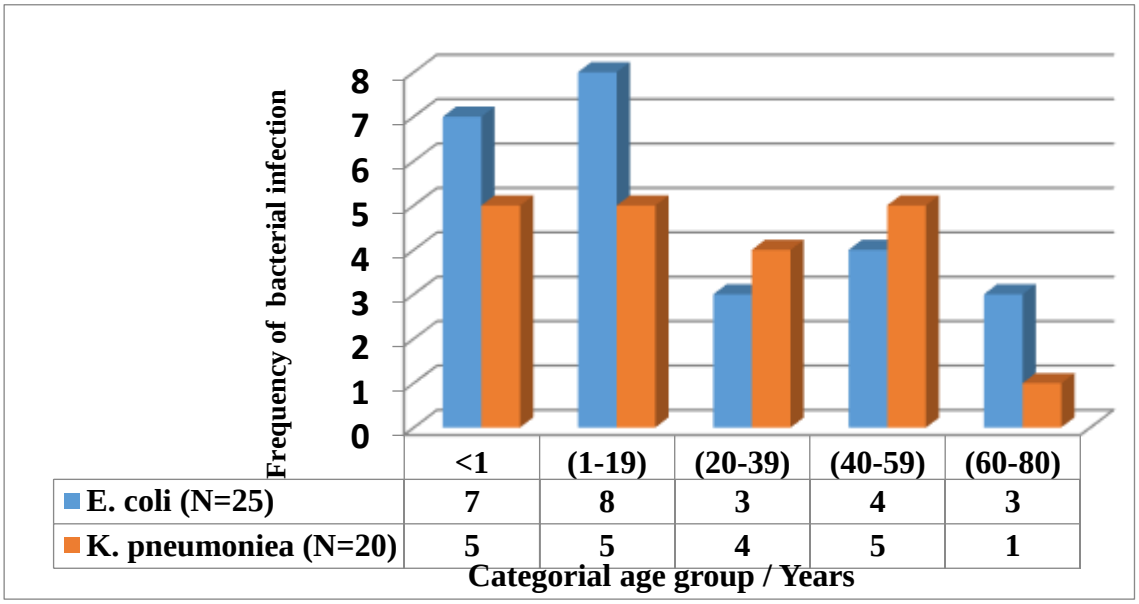


Figure 3-1: Distribution the type of bacterial infectios according to categorial age groups.

3.3. Distribution the type of infection according to sex

Table-2 showed that 13 (52.0%) out of 25 patients were infected with *E. coli* among femlae group versus 12 (48.0%) were recorded among male group, while the most patients infected with *K. pneumonieae* infections were recorded among female group 12 (60.0%) versus 8 (40.0%) among male group, statisticaly these differences were non-significant (P-value = 0.5). These result

indicated that there was a slight predomenance of females (55.6%) than males (44.4%).This finding was compatible with the results obtained by several authors worldwide (Chiu *et al*., 2015; Naqid *et al*., 2020).

Table 3-1: Distribution the type of bacterial infections according to sex

Sex		Study Groups		Total	P-value
		E. coli	***K. pneumoniae***		
Male	N (%)	12 (48%)	8 (40.0%)	20 (44.4%)	P-value=0.5 (N.S)
Female	N (%)	13 (52.0%)	12 (60.0%)	25 (55.6%)	
Total	N (%)	25 (100.0%)	20 (100.0%)	45 (100.0%)	

N.S; non significant

These results were compatible with results of Lin *et al.,* (2021) who documented that the female were the predominated groups infected with monomicrobial Enterobacteriaceae bacteremia (mEB) which accounting (53%) of all patients versus 47 for male groups, but the results were incompatible with the results of Lin *et al.* (2021) (regarding the predominance of infected age group) who revealed that the mean age of 499 cases of adults infected with mEB included were 74.5 years. Naqid et al., (2020) from Kurdistan region of Iraq found that the female patients were more affected by *K. pneumoniae* isolated from clinical specimens.

Regarding the types of monomicrobial Enterobacteriaceae bacteremia (mEB), the results of the current study were compatible with results of (Lin *et al*., 2021), who found that the *E. coli* were the major causative species of mEB which accounting (62%) more than *K. pneumoniae* which accounting (21%).

The results of this study were compatible with results obtained by (Lee *et al.*, 2014) who found that 1208 cases of community-onset bacteremia were identified, of which 551 (45.6%) and 206 (17.1%) were caused by *E. coli* and *K. pneumoniae* respectively. They also revealed that 548 patients with *E. coli* (75%)

bacteremia, and 183 patients with *K. pneumoniae* (25%) bacteremia were recorded which compatible with results of this study.

The results of this study were also compatible with results obtained by Chiu *et al*. (2013) who found that 518 adults with community-onset bacteremia were eligible with slight predominance of female cases of bactermia (262 patients, 50.6%). Out of a total of 565 bacteremic isolates, *E. coli* (228 isolates, 40.4%) and *K. pneumoniae* (100, 17.7%) were the major microorganisms.

Chang *et al*., (2020**)** studied the bacteremia due to enterobacteriaceae over a 5-year period. They found that isolates *E. coli* and *K. pneumoniae* together accounted for 34.8% from a total 15,665 blood culture. They also revealed that 90% of clinicaly significant isoaltes were community-acquired bacteremia.

Sepsis remains a major cause of morbidity and mortality in preterm infants. There has been a significant increase in neonatal sepsis caused by multi-drug resistant organisms in the past decade (Saleem *et al*., 2013,Yusef *et al*., 2017).

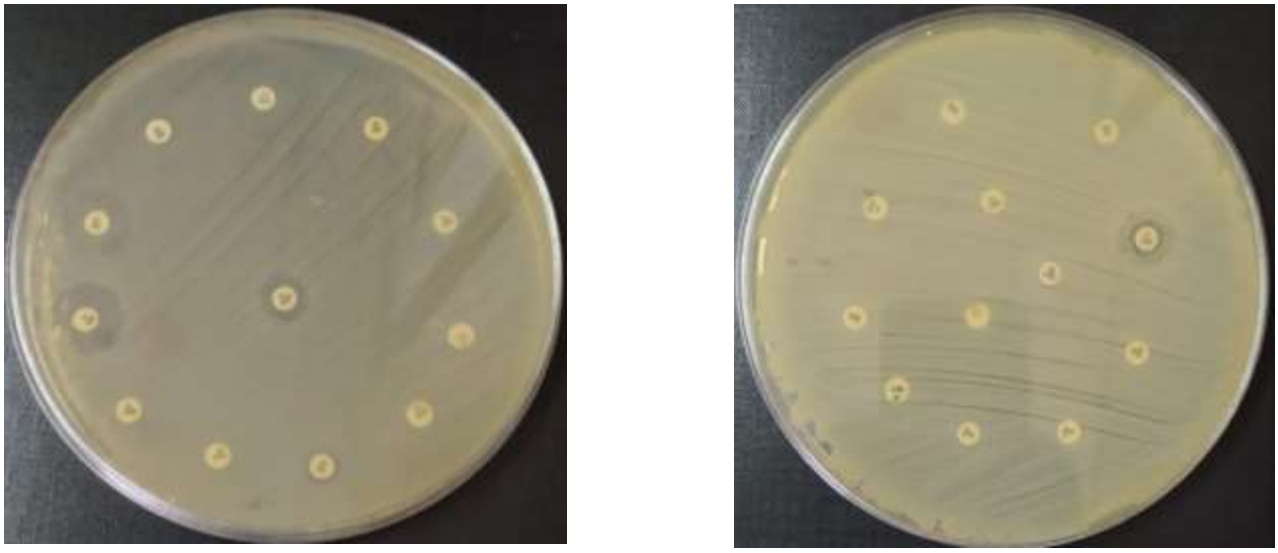
Infections with multi-drug resistant gram-negative organisms, especially Enterobacteriaceae, are of concern in preterm infants. Neonatal sepsis caused by these pathogens is increasing and there are limited choices available for treatment. Infections with multi-drug resistant Enterobacteriaceae (MRDE) are associated with poor outcome and high case fatality rates, especially in low and middle income countries (Folgori *et al*., 2017).

The results of the present study revealed that the age group ≤ 1 year had bacteremia due to *E.coli* (28%) more than *K. pneumoniae* (25%) from total bacterial cases (n=45) This result was incompatible with results of Ballot *et al*., (2019) who found that the most common isolates which cause sepsis of neonate was *K. pneumoniae,* followed by *Enterobacter cloacae,* and *E. coli*. Naqid *et al*. (2020) revealed that *K. pneumoniae* was the second causes of bactermia isolated from both sexes from total study cases 130 from differen clincal samples including blood samples.

Augustine *et al.* (2017) studied the hospitalized adults with bloodstream *Enterobacteriaceae* and they found most bloodstream infections (60%) were were due to *E. coli.*

3.4. Antibiotic susceptibility patterns of the bacterial isolates

The antibiotic susceptibility (AST) of *E. coli* (N =25) and *K. pneumoniae* (N=20) isolates was perfomed by disk diffusion test (DDT) using 12 antibiotics. The results of AST were interpreted based on CLSI guidelines (2021) (Figure 3-2). The results of the present study showed that both *E.coli* and *K. pneumoniae* isolates were phenotypicaly resistance to Ampicillin with 80% and 90% respectively.



(a) **(b)**

Figure 3-2: Antibiotic susceptibility profiles of *K. pneumoniae* (a), and *E. coli* (b) isolates recovred from bloodstream infections using DDT.

The results of the presnt study also found the both *E.coli* and *K. pneumoniae* isolates were resistant to ceftriaxone (CRO), Cefotaxime (CTX), and cefepime (FEP) with resistance rates 84%, 90%, 96%,100%, 80%, and 90% respectively, while both *E.coli* and *K. pneumoniae* isolates were absolutely resistance to ceftazidime (CTXM) antibiotics with 100%, 100% respectively. Of

note, both *E.coli* and *K. pneumoniae* isolates exhibited low resistance rate against the ciprofloxacine (CIP) with 60% and 75% respectively. Also *E.coli* isolates exhibited low resistance rates against ceftazidime (CAZ) with 76% rates versus 95% resistance rated exhibited by *K. pneumoniae* isolates. In contrast the carbapenem antibiotic meropenem (MEM) were still the most lower resistance rate agaisnt *E.coli* with 12% versus 70 % against a *K. pneumoniae* isolates as arranged in (Table 3-3) and (Figure 3-3).

Table 3-2: Antibiotic susceptibility profiles of ***E.coli*** (n=25) and ***K. pneumoniae*** (n=20) isolates by DDT

Antibiotic group	Antibiotic	Suscept. status	Bacterial isolates		Total	P-value
			E. coli	*K. pneumoniae*		
Penicillins	**AM**	R	20 (80.0%)	18 (90.0%)	38 (84.4%)	0.5 (N.S)
		S	4 (16.0%)	2 (10.0%)	6 (13.3%)	
		I	1 (4.0%)	0 (0.0%)	1 (2.2%)	
Ciphalosporins	**CRO**	R	21 (84.0%)	18 (90.0%)	39 (86.7%)	0.8(N.S)
		S	4 (16.0%)	2 (10.0%	6 (13.3%)	
		I	-	-	-	
	CTX	R	24 (96.0%)	20 (100.0%)	44 (97.8%)	0.2(N.S)
		S	-	-	-	
		I	1(4.0%)	0 (0.0%)	1 (2.2%)	
	CXM	R	25 (100.0%)	20 (100.0%)	45 (100.0%)	0.5 (N.S)
		S	-	-	-	
		I	-	-	-	
	FEP	R	20 (80.0%)	18 (90.0%)	38 (84.4%)	0.3(N.S)
		S	5 (20.0%)	2 (10.0%)	7 (15.6%)	
		I	-	-	-	
	CAZ	R	19 (76.0%)	19 (95.0%)	38 (84.4%)	0.07(N.S)
		S	2 (8.0%)	1 (5.0%)	3 (6.7%)	
		I	4 (16.0%)	0 (0.0%	4 (8.9%)	
Carbapenems	**MEM**	R	3 (12.0%)	14 (70.0%)	17 (37.8%)	0.001 (H.S)
		S	22 (88.0%)	6 (30.0%)	28 (62.2%)	
		I	-	-	-	
	IPM	R	3(12.0%	11 (55.5%)	14 (31.1%)	0.001 (H.S)
		S	22 (88.0%)	7 (35.0%)	29 (64.4%)	
		I	0 (0.0%)	2 (10.0%)	2 (4.4%)	
β-lactam/β-lactamase inhibitors	**AMS**	R	8 (32.0%)	13 (65.0%)	21 (46.7%)	0.04 (S)
		S	10 (40.0%)	6 (30.0%	16 (35.6%)	
		I	7 (28.0%)	1 (5.0%)	8 (17.8%)	
Quinolones	**CIP**	R	15 (60.0%)	15 (75.0%)	30 (66.7%)	0.07 (N.S)
		S	6 (24.0%)	5 (25.0%)	11 (24.4%)	
		I	4 (16.0%)	0 (0.0%)	4 (8.9%)	
Aminoglycosid	**AK**	R	3 (12.0%)	11 (55.0%)	14 (31.1%)	

es		S	18 (72.0%)	8 (40.0%)	26 (57.8%)	0.006 (H.S)
		I	4 (16.0%)	1 (5.0%)	5 (11.1%)	
	CN	R	10 (40.0%)	5 (25.0%)	15 (33.3%)	0.3 (N.S)
		S	15 (60.0%)	14 (70.0%)	29 (64.4%)	
		I	0 (0.0%)	1 (5.0%)	1 (2.2%)	

Abbreviations: AM: Ampicillin; CRO: ceftriaxone; CTX: cefotaxime; CXM: cefuroxime; FEP: cefepime; CAZ: ceftazidime; MEM: meropenem; IPM: imipenem; AMS: ampicillin-sulbactam; CIP: ciprofoloxacin; AK: amikacin; CN: gentamicin.

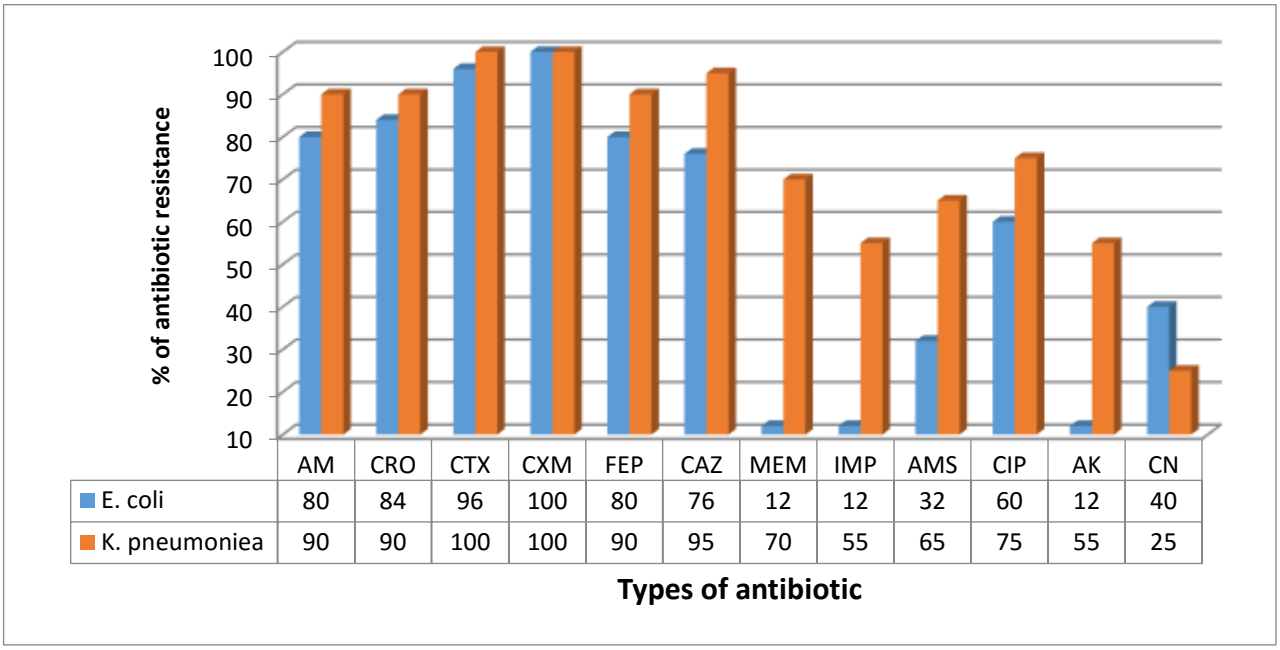


Figure 3-3: Antibiotic Resitance profiles of *E.coli* and *K. pneumoniae* isolates.
Abbreviations: AM: Ampicillin; CRO: ceftriaxone; CTX: cefotxaime; CXM: cefuroxime; FEP: cefepime; CAZ: ceftazidime; MEM: meropenem; IPM: imipenem; AMS: ampicillin-sulbactam; CIP: ciprofoloxacin; AK: amikacin; CN: gentamicin.

As the criteria proposed by Magiorakos *et al.*,(2012), for a multiple antibiotic resistant organism, current study showed that all *E. coli* and *K. pneumoniae* isolates exhibited multiple antibiotic resistant (resistant to at least one antibiotic in three or more of the 10 antimicrobial classes tested in this study. Based on this criterion, multi-resistant isolates were classified as MDR, possible

XDR or possible PDR as arranged in (Table 3-4).

The results of (Table 3-4) showed that multi-drug resistance patterns of monomicrobial enterobacteriaceae bacteremia (mEB) isolates were as follows: out of 45 isolates, 6 isolates (13.3 %) were PDR, 9 (20%) were XDR, and 30 isolates (66.6%) were MDR.

Results also found that out of 25 *E. coli* isolates recovred from patients with bacteremia, 3 isolates (12%) were PDR and 22 (88%) isolates were MDR. However, no XDR patterns were found amomg *E. coli* isolates.

Regarding *K. pneumoniae* isolates, out of 20 isolates, 9 (45%) were XDR, 3 isolates (15%) were PDR, and 8 isolates (40%) were MDR. However, no isolates from both *E. coli* and *K. pneumoniae* were found to be sensitive (susceptibe) to all antibiotics tested.

Regarding the antibiotic resistance patterns among mEB isolates, the results revealed that *K. pneumoniae* isolates were more resistant to the antibiotics than *E.coli*, because *K. pneumoniae* have XDR resistance pattern (45%) while isolates of *E. coli* did not have this type of resistance. In addition to that, the isolates of *E. coli* were the highest in possession of MDR (88%) compared to in *K. pneumoniae* isolates (40%), which indicates the low efficiency of these isolates in the antibiotic resistance.

Parajuli *et al*., (2017) concuded that out of 739 *E coli* isolates, (64.9%) were multidrug resistant (MDR) and (5%) were extensively drug resistant (XDR), while Jain *et al*., (2021) revealed that (98%) of *E. coli* isolates were MDR.

Lee *et al*., 2021 revealed that there was no PDR pattern among *E.coli* isolates recovered from patients with bacteremia. They also revealed that there was no XDR pattern among *K. pneumoniae* isolates recovered from the same patients.

Table 3-3: Multi drug resistance patterns of monomicrobial enterobacteriaceae bacteremia (mEB) *E.coli and K. pneumoniae* isolates.

% of mEB isolates	**Type of multidrug resistance pattern (N,%)**		
	MDR	**PDR**	**XDR**
***K. pneumonia* (n=20)**	8 (40%)	3 (15%)	9 (45%)
***E.coli* (n=25)**	22 (88%)	3 (12%)	0

Table 3-4 Antibiotic susceptibility pattern (ASP) and multidrug resisatnce patterns of mono-microbial enterobacteriaceae (mEB) *E.coli* (N=25) & *K.pneumoniae* (N=20) isolates

No	Name	AM	AMS	CN	AK	IPM	CRO	CTX	CXM	FEP	CiP	CAZ	MEM	ASP*
1	*E.coli-1*	S	I	S	S	S	R	R	R	R	R	R	S	**MDR**
2	*E.coli-2*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
3	*E.coli-3*	R	R	R	I	S	R	R	R	R	R	R	S	**MDR**
4	*E.coli-4*	R	R	R	I	S	R	R	R	R	I	R	S	**MDR**
5	*E.coli-5*	R	R	R	I	S	R	R	R	R	I	R	S	**MDR**
6	*E.coli-6*	R	I	R	S	S	R	R	R	R	R	R	S	**MDR**
7	*E.coli-7*	R	S	S	S	S	R	R	R	R	S	R	S	**MDR**
8	*E.coli-8*	R	R	R	S	S	R	R	R	R	R	I	S	**MDR**
9	*E.coli-9*	R	S	S	S	S	R	R	R	R	R	R	S	**MDR**
10	*E.coli-10*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
11	*E.coli-11*	R	R	S	I	S	R	R	R	R	R	R	S	**MDR**
12	*E.coli-12*	S	S	S	S	S	S	R	R	S	S	S	S	**MDR**
13	*E.coli-13*	S	S	R	S	S	R	R	R	R	R	R	S	**MDR**
14	*E.coli-14*	I	S	S	S	S	R	R	R	R	S	R	S	**MDR**
15	*E.coli-15*	R	S	S	S	S	S	R	R	S	R	R	S	**MDR**
16	*E.coli-16*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
17	*E.coli-17*	R	I	S	S	S	S	I	R	S	S	S	S	**MDR**
18	*E.coli-18*	R	I	S	S	S	R	R	R	R	R	R	S	**MDR**
19	*E.coli-19*	R	S	S	S	S	S	R	R	S	R	R	S	**MDR**
20	*E.coli-20*	S	I	S	S	S	R	R	R	R	R	R	S	**MDR**
21	*E.coli-21*	R	S	S	S	S	R	R	R	S	I	I	S	**MDR**
22	*E.coli-22*	R	I	S	S	S	R	R	R	R	R	R	S	**MDR**
23	*E.coli-23*	R	S	R	S	S	R	R	R	R	I	R	S	**MDR**
24	*E.coli-24*	R	I	S	S	S	R	R	R	R	S	I	S	**MDR**
25	*E.coli-25*	R	S	S	S	S	R	R	R	R	I	I	S	**MDR**
26	*K.P-1*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
27	*K.P-2*	S	S	S	S	S	R	R	R	R	S	R	S	**MDR**
28	*K.P-3*	R	R	S	R	R	R	R	R	R	R	R	R	**XDR**
29	*K.P-4*	R	R	I	R	R	R	R	R	R	R	R	R	**XDR**
30	*K.P-5*	R	S	R	R	R	R	R	R	R	R	R	R	**XDR**
31	*K.P-6*	R	S	S	S	S	R	R	R	R	S	R	S	**MDR**
32	*K.P-7*	R	R	S	I	R	R	R	R	R	R	R	R	**XDR**
33	*K.P-8*	R	R	S	R	R	R	R	R	R	R	R	R	**XDR**
34	*K.P-9*	R	R	S	R	I	R	R	R	R	R	R	R	**XDR**
35	*K.P-10*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
36	*K.P-11*	R	R	S	R	R	R	R	R	R	R	R	R	**XDR**
37	*K.P-12*	R	R	S	R	R	R	R	R	R	R	R	R	**XDR**
38	*K.P-13*	R	R	R	R	R	R	R	R	R	R	R	R	**PDR**
39	*K.P-14*	R	R	S	R	R	R	R	R	s	R	R	R	**XDR**
40	*K.P-15*	S	S	S	S	S	R	R	R	S	S	R	S	**MDR**
41	*K.P-16*	R	I	S	S	S	S	R	R	S	S	S	S	**MDR**
42	*K.P-17*	R	R	S	S	I	R	R	R	R	R	R	R	**MDR**
43	*K.P-18*	R	S	S	S	S	S	R	R	R	R	R	S	**MDR**
44	*K.P-19*	R	R	R	S	S	R	R	R	R	R	s	S	**MDR**
45	*K.P-20*	R	S	s	S	S	R	R	R	R	R	R	R	**MDR**
Total (45)		**MDR (30); XDR (9), PDR (6)**												

Abbreviations: AM: Ampicillin; CRO: ceftriaxone; CTX: cefotaime; CXM: cefuroxime; FEP: cefepime; CAZ: ceftazidime; MEM: meropenem; IPM: imipenem; AMS: ampicillin-sulbactam; CIP: ciprofoloxacin; AK: amikacin; CN: gentamicin.

Table 3-5: Antibiotic resistance status of monomicrobial enterobacteriaceae bacteremia (mEB) *E.coli* (N=25) and *K. pneumoniae* (N=20) isolates

% of mEB isolates	AM	CRO	CTX	CXM	FEP	CAZ	MEM	IPM	AMS	CIP	AK	CN
% of Resistant *K. pneumoniae*	90	90	100	100	90	95	70	55	65	75	55	25
% of Resistant of *E.coli*	80	84	96	100	80	76	12	12	32	60	12	40
Total % of Resistance	85	87	98	100	85	85.5	41	33.5	48.5	67.5	33.5	32.5

Abbreviations: AM: Ampicillin; CRO: ceftriaxone; CTX: cefotaxime; CXM: cefuroxime; FEP: cefepime; CAZ: ceftazidime; MEM: meropenem; IPM: imipenem; AMS: ampicillin-sulbactam; CIP: ciprofoloxacin; AK: amikacin; CN: gentamicin.

The results of Table 3-5 showed the total antibiotic resistance status of the monomicrobial enterobacteriaceae bacteremia (mEB) isolates. The mEB isolates showed high rate of resistance especially for 3rd and 4th generation cephalosporins and penicillins including ampicillin, ceftriaxone, cefotaxime, cefuroxime, ceftazidime and cefepime) which indicates that the resistance is due to production of ESBL enzymes.

On the other hand, the mEB isolates showed susceptibility to meropenem, imipenem, ampicillin-sulbactam, ciprofoloxacin, amikacin, and gentamicin (Table 3-5).

The results also revealed that the total antibiotic resistance rates among *E. coli* and *K. pneumoniae* isolates recovered from patients with monomicrobial enterobacteriaceae bacteremia were 80%,84%,96%,100%,80% and 85%, 90, 100%,100%,90% 95% against AM, CRO, CTX, CXM, FEP, and CAZ respectively, However, these antibiotic resistance rates were completely different towards the antibiotics MEM, IPM, AMS, CIP, AK, and CN as *K. pneumoniae* isolates were the most resistant (Table 3-5).

Lin *et al.,* (2021) found that patients infected by 3GC-NS monomicrobial Enterobacteriaceae bacteremia (mEB) isolates had a higher mortality rate than those by 3GC-susceptible isolates (21% vs 11%, P=0.005).

Choi *et al*. (2021) compared the antibiotic resistance of pathogens causing community-acquired infections (CAIs), healthcare-associated infections (HCAIs), and hospital-acquired infections (HAIs) among 146 patients in Korea, and they found that the prevalence of pathogens showing piperacillin-tazobactam resistance was (8.3%, 7.7%, 15.0%) in the CAIs, HCAIs, and HAIs groups, respectively. The prevalence of ESBL-positive pathogens was (6.3%, 47.3%, and 55.0%) in the CAIs, HCAIs, and HAIs group, respectively, with no significant difference between the HCAIs and HAIs groups. Resistance patterns of the HCAIs group more closely resembled those of the HAIs group than those of the CAIs group.

3.5. Molecular detection of ESBL genes among mEB isolates

Only 24 out of 45 mEB isolates were subjected to detect the presence of five commone types of ESBLs genes including *bla*TEM, *bla*SHV, *blaOXA*, *bla*CTX-M and AmpC. Amplified PCR-products for the five genes among both mEB *E. coli* and *K. pneumoniae* exhibited different molecular weights (867, 930, 564, 561, and 634 respectively) bp respectively, as illustrated in (Figures 3-5, 3-6, 3-7, 3-8, 3-9, and 3-10).

Genotypically, 92.8%, 78.5%,85.7%, 92.8% and 85.7% of *K. pneumoniae* (N=14) isolates barborourd *bla*TEM, *bla*SHV, *blaOXA*, blaCTX-M and Amp resistance genes respectively, while ESBL producing *E.coli* (N=10) isolates were harbouring *bla*TEM, *bla*SHV,*blaOXA*, blaCTX-M and Amp with less prevalence rate except the SHV genes which were more prevalent among *E.coli* than *K. pneumoniae* with 90%, 90%, 90%, 90% and 70% resistance rates respectively as shown in (Table 3-6, Table 3-7, and Figure 3-4).

The prevalence of ESBLs genes was approximately similar among mEB isolates except for *AmpC* gene which was predominant among *K. pneumoniae* (85.7%) than *E. coli* (70%). The prevalence of *OXA*, *TEM*, and *CTX-M* in *E. coli* isolates were 80, 90, and 90%, repectively; while for *K. pneumoniae,* 85.7, 92.8,

and 92.8%, respectively; which means these genes were also more predominant in the latter species.

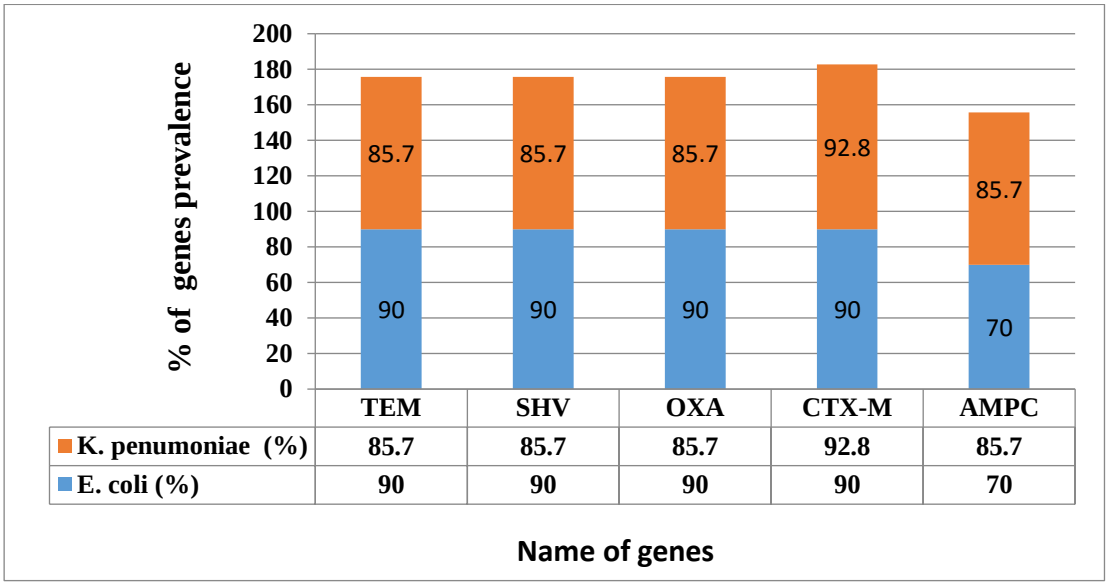


Figure 3-4: Prevalence of types of ESBLs genes among both *E.coli* and *K. pneumoniae* isolates

Table 3-6: Prevalence of antibiotic resistance genes of *E. coli* (N=10)

Isolate No	*TEM*		*SHV*		*OXA*		*CTX-M*		*AmpC*	
	+ve	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**
1	+ve		+ve	-	+ve		**+ve**		**+ve**	
2	+ve		+ve	-	+ve		**+ve**			-ve
3	+ve		+ve	-	+ve		**+ve**			-ve
4	+ve		+ve	-	+ve		**+ve**			-ve
5	+ve		+ve	-	+ve		**+ve**		**+ve**	
6	+ve		+ve	-	+ve		**+ve**		**+ve**	
7		-ve		-ve	-+ve		**+ve**		**+ve**	
8	+ve		+ve	-	-	-ve	-	-ve	**+ve**	
9	+ve		+ve	-	+ve		**+ve**		**+ve**	
10	+ve		+ve	-	+ve		**+ve**		**+ve**	
Total	9/10	1/10	9/10	1/10	9/10	1/10	9/10	1/10	7/10	3/10
%	90	10	90	10	90	10	90	10	70	30

Table 3-7: Prevalence of antibiotic resistance gene of *K. penumoniae* (N=14)

Isolate No.	*TEM*		*SHV*		*OXA*		*CTX-M*		*AmpC*	
	+ve	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**
11	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
12	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-ve
13	+ve	-	+ve	-	+ve	-	+ve	-	-	-ve
14	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
15	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
16	+ve	-	-	-ve	-		+ve	-	+ve	-
17	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
18	-	-ve	-	-ve	-	-ve	+ve	-	+ve	-
19	+ve	-	+ve	-	+ve	-ve	-	-ve	+ve	-
20	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
21	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
22	-	-ve	+ve	-	+ve	-	+ve	-	+ve	-
23	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
24	+ve	-	+ve	-	+ve	-	+ve		+ve	-
Total	13/14	1/14	11/14	3/14	12/14	2/14	13/14	1/14	12/14	2/14
%	85.7	14.3	85.7	14.3	85.7	14.3	92.8	7.2	85.7	14.3

*bla*TEM

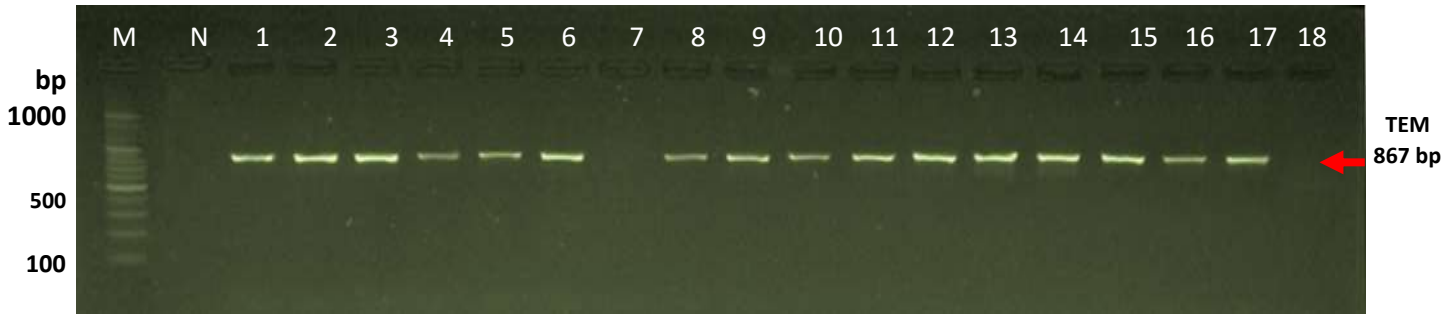


Figure 3-5. a. Agarose gel electrophoresis of PCR products of **TEM** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 1 to 10 represents *E.coli* isolates,11 to 24 represents *K. pneumonia*e isolates

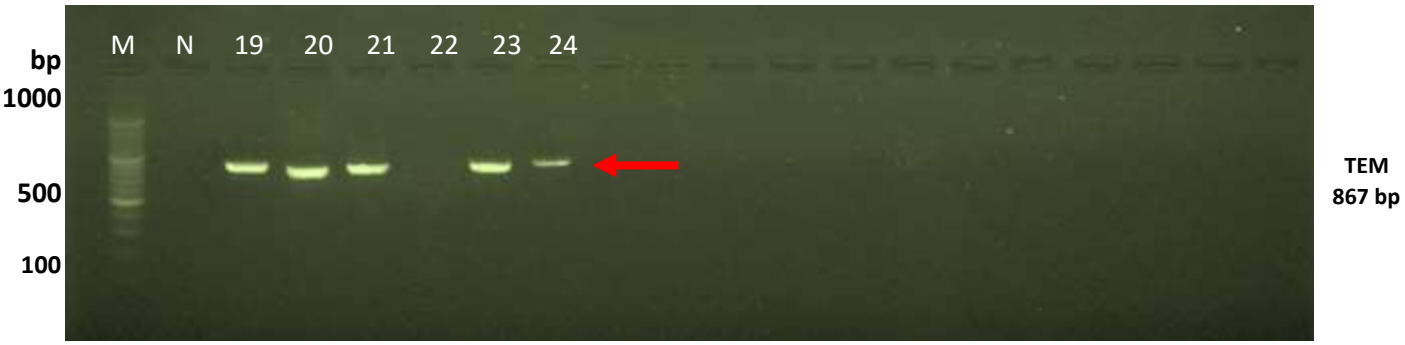


Figure 3- 5: b. Agarose gel electrophoresis of PCR products of **TEM** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 19 to 24 represents *K. pneumoniae* isolates.

*bla*SHV

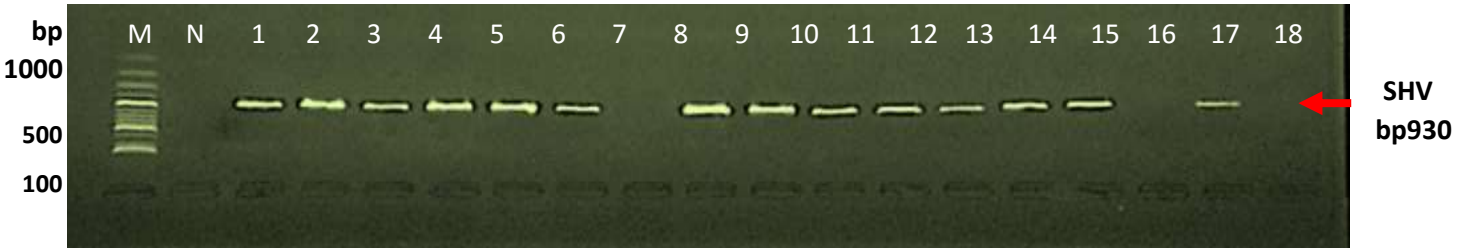


Figure 3-6a. Agarose gel electrophoresis of PCR products of **SHV** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 1 to 10 represents *E.coli* isolates,11 to 24 represents *K. pneumoniae* isolates

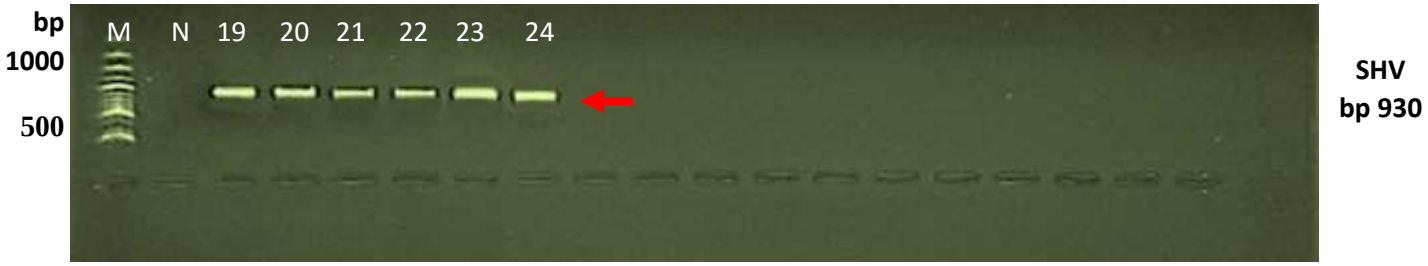


Figure 3-6.b. Agarose gel electrophoresis of PCR products of **SHV** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 19 to 24 represents *K. pneumoniae* isolates.

***bla*OXA**

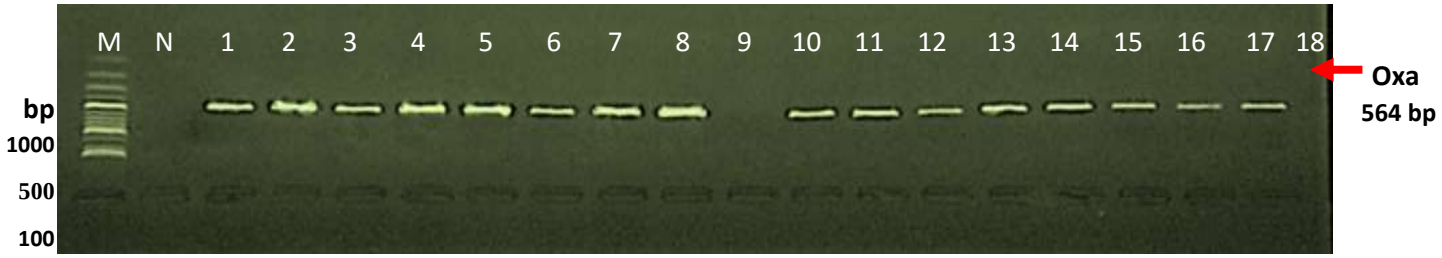


Figure 3-7.a. Agarose gel electrophoresis of PCR products of **OXA** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 1 to 10 represents *E.coli* isolates,11 to 24 represents *K. pneumoniae* isolates

Figure 3-7b. Agarose gel electrophoresis of PCR products of **OXA** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 19 to 24 represents *K. pneumonia* isolates.

*bla*CTX-M

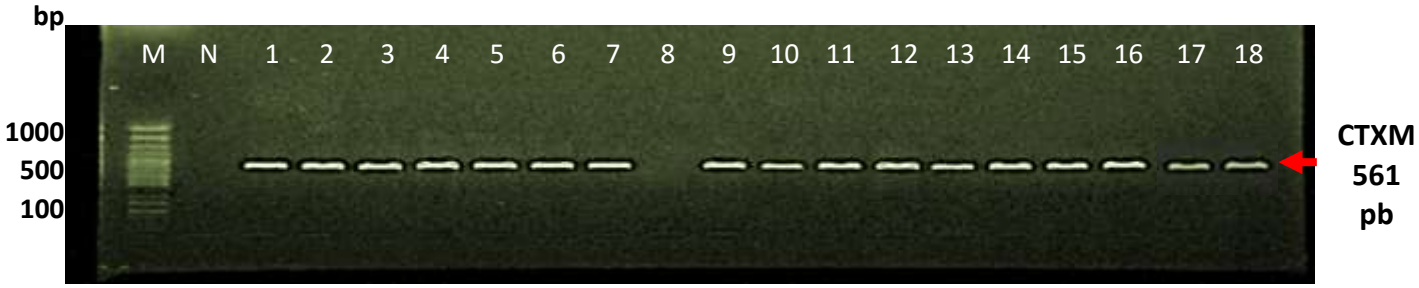


Figure 3- 8. a. Agarose gel electrophoresis of PCR products of **CTX-M** gene among *E.coli* and *K. pneumonia* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 1 to 10 represents *E.coli* isolates,11 to 24 represents *K. pneumoniae*

Figure 3-8.b. Agarose gel electrophoresis of PCR products of **CTX-M** gene among ***E.coli*** and ***K. pneumonia*** isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 19 to 24 represents *K. pneumoniae* isolates.

***bla*Ampc**

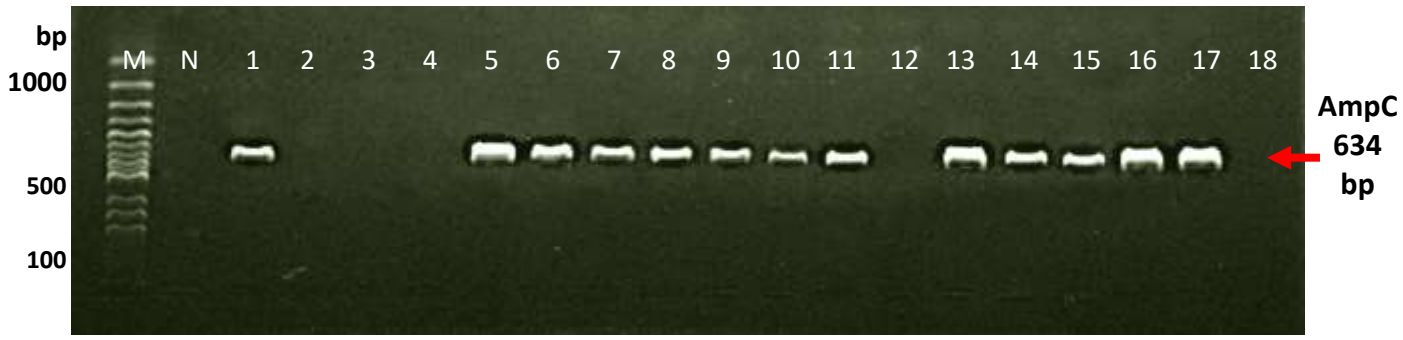


Figure 3-9.a. Agarose gel electrophoresis of PCR products of **AmpC** genes among ***E.coli*** and ***K. pneumonia*** isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 1 to 10 represents *E.coli* isolates,11 to 24 represents *K. pneumoniae*

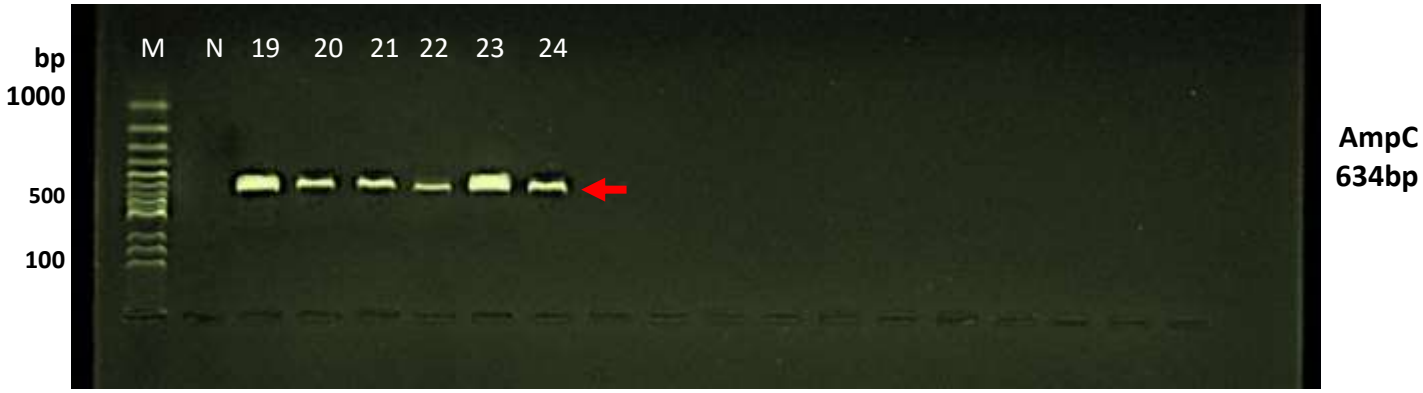


Figure 3-9.b. Agarose gel electrophoresis of PCR products of **AmpC** gene among *E.coli and K. pneumoniae* isolates recovered from patients with bloodstream infections. M=DNA molecular marker size (1000 bp), 19 to 24 represents *K. pneumonia* isolates.

Augustine *et al.*, (2017) studied the hospitalized adults with bloodstream *Enterobacteriaceae* (BSI) infection. They revealed that among 910 patients with *Enterobacteriaceae* BSI, 42 (4.6%) had ESBL bloodstream isolates. Most

ESBL- producing bloodstream *Enterobacteriaceae* isolates were community onset (79%), and 25 (60%) of them were due to *E. coli*.

A study by Dirar *et al*., (2020) found that *bla*TEM and *bla*CTX-M genotypes are the most prevalent genes in ESBLs-producing Enterobactriaceae.

Zaniani *et al*., (2012) concluded that the frequency of SHV among the ESBLs producing *E.coli* and *K. pneumoniae* isolates was 14.4%, while Doosti *et al*., (2015) found that 8.37% of *K. pneumoniae* isolates were positive for SHV. A study by Kiratisin and his colleagues (2008) concluded that *bla*OXA was encoded by ESBL-producing *E. coli* and *K. pneumoniae* for 8.1% and 11.8%, respectively.

A study in Bangladesh by Jain and his colleagues (2021) found the PCR analysis using 25 selected isolates showed the predominance of ESBL genes blaOXA (48%) and blaCTX-M-15 (32%). The AmpC gene was detected in 68% of the isolates.

Singh and his colleagues found that conjugation assays of plasmid DNA indicated that blaTEM, and blaCTX-M-15 were plasmid-mediated and possibly transmit between genera through horizontal gene transfer (HGT), and thus the prevalence of these genes are high among *E. coli* isolates.

A survey conducted by Ramakrishnan and his colleagues (2022) concluded that mong the dmoninat resistance genes were them *bla*TEM (n = 14), followed by *bla*CTX-M-15 (n = 11) of β-lactamase genes and AmpC was (n = 12).

Ruppé *et al.* (2015) reported that ESBL- and carbapenemase-encoding plasmids frequently bear resistance determinants for other antimicrobial classes, including aminoglycosides (aminoglycoside-modifying enzymes or 16S rRNA

methylases) and fluoroquinolones (Qnr, AAC (6′)-Ib-cr or efflux pumps), a key feature that fosters the spread of multidrug resistance in *Enterobacteriaceae*.

Kpoda and his colleagues (2018) studied ESBL-encoding genes among 187 ESBL producing Enterobacteriaceae strains. They found that the prevalence of ESBL-producing strains with *bla*TEM, *bla*SHV and *bla*CTX-M genes were 26.2% (49/187), 5.9% (11/187) and 40.1% (75/187) respectively. The association of ESBL encoding genes with health centers was statistically significant (p= 0.0209). Approximately 39.6% of *E. coli* harbored CTX-M and *Klebsiella* spp. 5.9%.

Kiratisin *et al*. (2008) conducted a molecular detection and characterization of ESBL-related bla genes, including blaTEM, blaSHV, blaCTX-M, blaVEB, blaOXA, blaPER, and blaGES, among 362 isolates of ESBL-producing *E. coli* (n = 235) and ESBL-producing *K. pneumoniae* (n = 127) collected from patients with health Care-Associated Infection in Thailand. They found that a total of 87.3% of ESBL-producing E. coli and ESBL-producing K. pneumonia carried several *bla* genes. The prevalence of blaCTX-M was strikingly high: 99.6% for ESBL-producing *E. coli*. Up to 77.0% and 71.7% of ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae*, respectively, carried blaTEM. ESBL-producing *K. pneumoniae* carried blaSHV at 87.4% but only at 3.8% for ESBL-producing *E. coli*. bla genes encoding OXA-10 were found in both ESBL-producing *E. coli* (8.1%) and ESBL-producing *K. pneumoniae* (11.8%).

Conclusions and Recommendations

Conclusions

1- The female were the predominated group infected with monomicrobial Enterobacteriaceae bacteremia (mEB) versus for male group in present study.

2- Most of (mEB) isolates were among age group (1-19) years of hospitalized patients with blood stream infections.

3- *E.coli* and *K. pneumoniae* isolates were the two major causative species of Enterobacteriaceae among hospitalized patients with bacteremia. However, no other Enterobacteriaceae species were recovered.

4- The mEB isolates have shown great resistance to third generation Cephalosporins and penicllins.

5- ESBL producing isolates were harboring *blaTEM, blaSHV, blaOXA, blaCTX-M* and *AmpC* with less prevalence rate except for *AmpC* gene which was predominant among *K. pneumoniae* than *E. coli*.

6- The dissemination of TEM, SHV and CTX-M genes in ESBL producing (mEB) isolates and there continuous spread of these bacteria poses great public health risk in Iraq.

Recommendations:

- Efforts to rapid identification of antimicrobial resistance profiles of 3GC-NS *enterobacteriaceae* bacteremia in adults should be incorporated into antimicrobial stewardship programs to achieve favorable outcomes.
- Although they were active in combating 3GC-NS entero-bacteriaceae bacteremia isolates in adults, 3rd and 4th GC should to be used wisely.
- Increased monitoring and regulation of antibiotic use (particularly third and fourth generation cephalosporins) is imperative in Iraq.
- Study the third generation cephalosporins resistance in bacteremia due Gram positive bacterial infections.
- Study the Metallo-β-lactamases (MBLs) among 3GC-NS bacteremia non-sugar fermentative Gram negative bacteria.

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Appendices (I)

Vitek (1) Reports:

المركز الوطني للمختبرات التعليمية

Laboratory Report

bioMérieux Customer:
System #:
Patient Name: 0 ,كمال الدين
Isolate: 181-1 (Approved)

Printed by: Labadmin
Patient ID: 181

Card Type: GN Bar Code: 2411602103142114 Testing Instrument: 0000148FFC51 (9517)
Card Type: AST-N204 Bar Code: 5741726403175561 Testing Instrument: 0000148FFC51 (9517)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 2607734673564010
Organism Quantity:

Selected Organism: Klebsiella pneumoniae ssp pneumoniae

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411602103	**Expires:**	Apr 16, 2022 13:00 CDT
	Status:	Final	**Analysis Time:**	4.08 hours	**Completed:**	Oct 29, 2021 15:18 CDT
Organism Origin	VITEK 2					
Selected Organism	99% Probability **Klebsiella pneumoniae ssp pneumoniae**					
	Bionumber: 2607734673564010			Confidence: Excellent identification		
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Susceptibility Information	**Card:**	AST-N204	**Lot Number:**	5741726403	**Expires:**	Aug 18, 2022 13:00 CDT
	Status:	Final	**Analysis Time:**	8.72 hours	**Completed:**	Oct 29, 2021 19:56 CDT

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Meropenem	>= 16	R
Ampicillin	>= 32	R	Amikacin	>= 64	R
Amoxicillin/Clavulanic Acid	>= 32	R	Gentamicin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Cefotaxime	>= 64	R	Norfloxacin	>= 16	R
Ceftazidime	>= 64	R	Fosfomycin	>= 256	R
Cefepime	>= 64	R	Nitrofurantoin	>= 512	R
Ertapenem	>= 8	R	Trimethoprim/ Sulfamethoxazole	80	R
Imipenem	>= 16	R			

AES Findings:	**Last Modified:** May 31, 2021 11:09 CDT **Parameter Set:** Global CLSI-based +Phenotypic 2019	
Confidence Level:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	CARBAPENEMASE (+ OR - ESBL),IMPERMEABILITY CARBA (+ESBL OR +HL AmpC)
	AMINOGLYCOSIDES	RESISTANT GEN TOB NET AMI

Installed VITEK 2 Systems Version: 9.02
MIC Interpretation Guideline: Global CLSI-based 2019
AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019
AES Parameter Last Modified: May 31, 2021 11:09 CDT

المركز الوطني للمختبرات التعليمية

bioMérieux Customer:

Laboratory Report

System #:

Printed by: Labadmin

Patient Name: 53 فيصل تركي خالف

Patient ID: 8BB

Isolate: 8BB-1 (Approved)

Card Type: GN Bar Code: 2411602103141232 Testing Instrument: 0000148FFC51 (9517)

Card Type: AST-N222 Bar Code: 6221572403308409 Testing Instrument: 0000148FFC51 (9517)

Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0405610570526610

Organism Quantity:

Selected Organism: Escherichia coli

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411602103	**Expires:**	Apr 16, 2022 13:00 CDT
	Status:	Final	**Analysis Time:**	4.80 hours	**Completed:**	Nov 3, 2021 14:25 CDT
Organism Origin	VITEK 2					
Selected Organism	96% Probability **Escherichia coli**					
	Bionumber: 0405610570526610				Confidence: Excellent identification	
Analysis Organisms and Tests to Separate:						
Analysis Messages: The following antibiotic(s) are not claimed: Rifampicin,						
Contraindicating Typical Biopattern(s) Escherichia coli dTAG(22),PHOS(81),						

Susceptibility Information	**Card:**	AST-N222	**Lot Number:**	6221572403	**Expires:**	Mar 17, 2022 13:00 CDT
	Status:	Final	**Analysis Time:**	7.73 hours	**Completed:**	Nov 3, 2021 17:20 CDT

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Ampicillin/Sulbactam			Amikacin	16	*I
Ticarcillin	>= 128	R	Gentamicin	<= 1	S
Ticarcillin/Clavulanic Acid	64	*R	Tobramycin	>= 16	R
Piperacillin	>= 128	R	Ciprofloxacin	>= 4	R
Piperacillin/Tazobactam	8	*R	Pefloxacin		
Ceftazidime	16	R	Minocycline	<= 1	S
Cefepime	8	S	Colistin		
Aztreonam	>= 64	R	Rifampicin		
Imipenem	<= 0.25	S	Trimethoprim/ Sulfamethoxazole	>= 320	R
Meropenem	<= 0.25	S			

*= AES modified **= User modified

Installed VITEK 2 Systems Version: 9.02

MIC Interpretation Guideline: Global CLSI-based 2019

AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019

AES Parameter Last Modified: May 31, 2021 11:09 CDT

bioMérieux Customer:
System #:
Patient Name: 53 فيصل تركي خلف
Isolate: 8BB-1 (Approved)

Laboratory Report

Printed by: Labadmin
Patient ID: 8BB

Card Type: GN Bar Code: 2411602103141232 Testing Instrument: 0000148FFC51 (9517)
Card Type: AST-N222 Bar Code: 6221572403308409 Testing Instrument: 0000148FFC51 (9517)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0405610570526610
Organism Quantity:

Selected Organism: Escherichia coli

AES Findings:	**Last Modified:** May 31, 2021 11:09 CDT **Parameter Set:** Global CLSI-based +Phenotypic 2019	
Confidence Level:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	EXTENDED SPECTRUM BETA-LACTAMASE,HL CEPHALOSPORINASE (AmpC)
	AMINOGLYCOSIDES	RESISTANT TOB NET AMI (AAC(6'))

المركز الوطني للمختبرات التعليمية

Laboratory Report

bioMérieux Customer:
System #:
Patient Name: تبارك جمال, -RCU
Isolate: 84BL-1 (Approved)
Card Type: GN Bar Code: 2411602103141320 Testing Instrument: 0000148FFC51 (9517)
Card Type: AST-N204 Bar Code: 5741813503730418 Testing Instrument: 0000148FFC51 (9517)
Setup Technologist: Laboratory Administrator(Labadmin)

Printed by: Labadmin
Patient ID: 84BL

Bionumber: 2405610440524610
Organism Quantity: **Selected Organism: Escherichia coli**

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411602103	**Expires:**	Apr 16, 2022 13:00 CDT
	Status:	Final	**Analysis Time:**	4.78 hours	**Completed:**	Dec 14, 2021 16:55 CST
Organism Origin	VITEK 2					
Selected Organism	95% Probability **Escherichia coli**					
	Bionumber: 2405610440524610			Confidence: Very good identification		
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Escherichia coli	ADO(9),PHOS(81),					

Susceptibility Information	**Card:**	AST-N204	**Lot Number:**	5741813503	**Expires:**	Nov 13, 2022 12:00 CST
	Status:	Final	**Analysis Time:**	5.52 hours	**Completed:**	Dec 14, 2021 17:38 CST

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Meropenem	>= 16	R
Ampicillin	>= 32	R	Amikacin	>= 64	R
Amoxicillin/Clavulanic Acid	>= 32	R	Gentamicin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Cefotaxime	>= 64	R	Norfloxacin	>= 16	R
Ceftazidime	>= 64	R	Fosfomycin	<= 16	S
Cefepime	>= 64	R	Nitrofurantoin	64	I
Ertapenem	>= 8	R	Trimethoprim/ Sulfamethoxazole	>= 320	R
Imipenem	>= 16	R			

AES Findings:	**Last Modified:** May 31, 2021 11:09 CDT **Parameter Set:** Global CLSI-based +Phenotypic 2019	
Confidence Level:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	CARBAPENEMASE (+ OR - ESBL),IMPERMEABILITY CARBA (+ESBL OR +HL AmpC)
	AMINOGLYCOSIDES	RESISTANT GEN TOB NET AMI

Installed VITEK 2 Systems Version: 9.02
MIC Interpretation Guideline: Global CLSI-based 2019
AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019
AES Parameter Last Modified: May 31, 2021 11:09 CDT

الخلاصة

تم جمع 266 عينة دم من مرضى من كلا الجنسين يعانون من تجرثم الدم حيث تم إدخالهم إلى مستشفيات مختلفة في المدينة الطبية في مدينة بغداد. ووجدت النتائج أن 46 (17.3٪) عينة دم فقط تحتوي على نمو زرعي، 25 (55.6٪) من الإناث و 20 (44.4٪) من الذكور.

بعد ملاحظة الخصائص المظهرية للعزلات البكتيرية وإجراء الاختبارات البيوكيميائية التقليدية تم عزل 45 (16.9٪) عزلة بكتيرية منها 25 عزلة (55.6٪) بكتريا قولونية و 20 (44.4٪) عزلة *K. pneumonieae*. بعد ذلك، تم تأكيد التشخيص بواسطة نظام فايتيك الآلي Vitek-2. أظهرت هذه النتائج وجود عدوى بكتريا معوية أحادية الميكروبية (mEB) بين مرضى التهابات مجرى الدم.

أظهرت النتائج أن معظم حالات تجرثم الدم (28.9٪) من أصل 45 حالة حدثت بين مرضى الفئة العمرية (1-19 سنة) بينما انخفضت نسبة الإصابة بالبكتيريا بين المرضى في العقد السادس إلى الثامن من العمر.

تم إجراء اختبار الحساسية لمضادات الميكروبات لكل من العزلات باستخدام كل من نظام Vitek-2 الآلي وطريقة انتشار القرص في الاكار، وشملت مضادات الامبسلين، السفترياكزون، السيفوتاكزيم، السيفيروكزيم، السيفيبيم، السفتازيديم، الميروبينيم، امبسلين-سلباكتام، الاميكاسين، السبروفلوكساسين والجنتاميسين.

أظهرت النتائج أن نسبة مقاومة بكتيريا *E.coli* كانت 80٪ للأمبيسيلين، بينما أظهرت عزلات الكليبسيلا الرئوية مقاومة بنسبة 90٪.. بالنسبة للسيفالوسبورينات، كانت جميع العزلات مقاومة بنسبة 92.8% لـ CTX-M ، بينما كان هناك تفاوت في مقاومتها لمركبات β-lactam الأخرى. بالنسبة للكاربابينيمات ، كانت عزلات الإشريكية القولونية شديدة التأثر بالميروبينيم والإيميبينيم (12٪) ، بينما أظهرت عزلات الكليبسيلا الرئوية حساسية متوسطة للمضادات الحيوية المذكورة أعلاه ، 70٪ و 55٪ على التوالي. بالنسبة لمضادات للأمينوكلايكوسيدات (أميكاسين ، جنتاميسين) أظهر كلا النوعين من العزلات حساسية عالية تجاه هذه المضادات الحيوية. بالنسبة للسيبروفلوكساسين، أظهرت عزلات K. الرئوية مقاومة 75٪ ، بينما كانت الإشريكية القولونية 60%

فيما يتعلق بالدراسة الجزيئية، تم إخضاع 24 عزلة فقط من أصل 45 عزلة بكتيرية معزولة من مرضى التهابات مجرى الدم لغرض الكشف عن وجود خمسة أنواع من جينات البتالاكتاميز واسعة الطيف ESBLs وهي blaTEM و blaSHV و blaOXA و blaCTX-M و ampC.

الخلاصة

أظهرت نواتج تفاعل السلسلة المتبلور بين عزلات كل من *E. coli* و *K. pneumoniae* أوزانًا جزيئية مختلفة لجينات البتالاكتاميز واسعة الطيف على التوالي.

كان انتشار جينات ESBLs متشابهًا تقريبًا بين عزلات mEB باستثناء جين CTX-M الذي كان أكثر انتشارًا بين *E. coli* من *K. pneumoniae* ، بينما كان جين AmpC أكثر انتشارًا بين *K. pneumoniae* منه في عزلات الاشريشيا القولون.

كانت نسبة انتشار جينات OXA و TEM و CTX-M في عزلات الإشريشية القولونية 80% و 90% و 90٪ على التوالي. بينما كان معدل هذه الجينات أكثر انتشارًا في عزلات الكليبسيلا الرئوية مقارنة بالبكتريا المعوية.

وزارة التعليم العالي والبحث العلمي
جامعة بـابـــل / كـلية الطـــب
فرع الاحياء المجهرية الطبية

دراسة عدم حساسية الجيل الثالث من السيفالوسبورينات بين المرضى المصابين بتجرثم الدم بفعل العائلة المعوية-أحادية المايكروبات

رسالة مقدمة إلى
مجلس كلية الطب / جامعة بابل
وهي جزء من متطلبات نيل درجة الماجستير في
علوم الاحياء المجهرية الطبية
من قبل

جواد حميد حسن ظاهر
(2012- بكالوريوس أحياء مجهرية ــ كلية مدينة العلم الجامعة)

بأشـــراف

الاستاذ الدكتور
علاء هاني حسن الجراخ

الاستاذ المساعد الدكتور
عباس عبدالمعيد مصطفى

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