ISSN: 1343-4292
Volume 140, Issue 02, May, 2022

Prevalence of Extended-spectrum and Metallo Beta-Lactamases in some Gram-negative bacteria

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ABSTRACT— The rapid increase in the production of MBL and EXBL among the members of *Enterobacteriaceae*, primarily *Klebsiella pneumoniae* (*K. pneumoniae*), *E. coli*, and *Pseudomonas aeruginosa* (*P. aeruginosa*) that represent the most frequent human infection causes, is a severe global issue related to the public health. Because there are few options of the treatment for infections resulting from MBL and ESBL-producing bacteria, treating these infections can be complicated, and treatment failure is common. Regular drug resistance surveillance amongst clinical isolates will assist to determine true severity of the situation and, as a result, establish the appropriate policy for reducing the prevalence of drug resistance among bacteria. Furthermore, understanding local antimicrobial susceptibility patterns will aid in starting timely adequate preliminary treatment [1].

KEYWORDS: beta- lactamases, ESBL, MBLs, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*

1. INTRODUCTION

1.1 Overview of Proteus mirabilis

Proteus spp. might result in infections of the respiratory tract, urinary tract, otitis media, wounds, and burns, as well as bacteremia, newborn meningoencephalitis, osteomyelitis, and empyema in the community and in hospitals [2]. Urinary tract infections (UTIs) are caused by *P. mirabilis* in people who have structural or functional problems in their urinary tract. They frequently develop cystitis, bladder and kidney stones, acute pyelonephritis, and catheter obstruction as a result of stone encrustation. *P. mirabilis* is most known in the medical community for its capacity to create stones in the kidneys and bladder, along with crystalline bio-films on outer surface as well as in indwelling urinary catheter lumen [3], [4]. Due to the fact that they lack a chromosomally encoded AmpC cephalosporinase, *P. mirabilis* is frequently vulnerable to ß-lactams. Yet, such species were found to have a diverse range of acquired b-lactamases, like the extended- and broad-spectrum ß-lactamases, as well as AmpC enzymes. ESBL producing P mirabilis had resulted in the increase of the different geographical places; the most common are the CTX-M enzymes and the TEM-type enzymes, with a few PER- and VEB-types being less common [5].

1.2 Overview of Pseudomonas aeruginosa

Extreme *P. aeruginosa* infections are frequently nosocomial infections, and they're almost always linked to weakened host defenses, like severe burns, neutropenia, or cystic fibrosis. With regard to clinical practice, it results in various infections, including UTIs, lung infections, endocarditis (resulting from intravenous drug use), corneal ulceration (resulting from wearing contact lenses), and pneumonia (resulting from using ventilator and endotracheal tube) [6], [7]. As a result of the low permeability regarding its outer membrane, constitutive expression of different efflux pumps, along with the production of antimicrobial

inactivation enzymes, *P. aeruginosa* has often been specified as a complicated target for antimicrobial chemotherapy; additionally, *P. aeruginosa* forms biofilm, which is considered as a sessile bacterial growth related to many human infections [8- 10]. Antibiotic resistance mediated by plasmids is widespread in *P. aeruginosa* and plays an important role in bacterial multidrug resistance. The generation of MBLs and ESBLs is a major issue in clinical isolates of *P. aeruginosa*, because organisms which produce MBL or ESBL are linked to increased morbidity and mortality. With the increased creation of ESBL in hospitals, the issue of MBL production is becoming more of a problem; both MBLs and EXBLs genes were observed to transfer from *P. aeruginosa* to various *Enterobacteriaceae* members [11], [12].

1.3 Overview of Klebsiella pneumoniae

It's a Gram-negative encapsulated bacterium is located on the mammals' mucosal surfaces and in environment (water, soil, and so on). *K. pneumoniae* can be defined as one of the opportunistic pathogens causing a wide variety of the illnesses and is more and more resistant to antimicrobial agents. Approximately one-third of all Gram-negative infections, like UTIs, pneumonia, cystitis, surgical wound infections, septicemia, and endocarditis, are caused by this organism. Pyogenic liver abscesses, endogenous endophthalmitis and necrotizing pneumonia are other symptoms. Infections produced by this bacterium are frequently related to prolonged hospitalization, high fatality rates, and significant costs [13]. *K. pneumoniae* have become index species for the plasmids-encoding (ESBLs) plasmids giving expanded-spectrum cephalosporin resistance in the 1980s. Initially, SHV and TEM type ESBLs coexisted on the plasmids with components encoding aminoglycoside, tetracycline, and trimethoprim-sulfamethoxazole resistance. The cefotaximase-M (CTX-M) family of ESBLs emerged in the 1990s, and they are now the most common ESBLs in the *K. pneumoniae*.

As a result of the widespread usage of carbapenems, multidrug-resistant, carbapenemase-producing *K. pneumoniae* strains emerged and spread quickly. The *K. pneumoniae* carbapenemases (KPC) (KPC2 to KPC13), metallo-β-lactamases (Verona integronencoded metallo-β-lactamase, VIM; imipenemase, IMP; and New Delhi metallo-β-lactamase, NDM types), and oxacillinase (OXA)-type enzymes are the most common carbapenemas (mainly OXA-48). MDR transmissible plasmids which give resistance to several antimicrobial agents frequently have carbapenemase-encoding genes [14].

1.4 ß- lactamases enzymes

The most significant resistance mechanism is the hydrolysis of ß-lactam ring by ß-lactamases (cephalosporinases and penicilinases), which has developed in many microorganisms for overcoming the inhibitory action of ß-lactam antibiotics. The following mechanisms were proposed for explaining bacterial resistance to β-lactam antimicrobial agents: Changes in target site, Changes in access to the target site, and Production of β-lactamases enzymes [15], [16]. PBPs evolved into ß-lactamases, which were able to bind ß-lactam antibiotics, create an acyl enzyme molecule, and after that deacylate and hydrolyze the ß-lactam ring. A few bacteria carry ß-lactamase genes on their chromosomes; the spread of ß–lactamase genes among bacteria has led to spread of resistant organisms, culminating in the emergence of clinically significant adverse effects. The first classification of ß-lactamases was established by Richmond and Jack in 1970, based on their susceptibility to inhibitors, hydrolytic spectrum, and if they are encoded via the plasmids or chromosome [17]. There are several different varieties of the beta-lactamase types, and numerous different classification methods were suggested; β-lactamases were classified according to their inhibitor susceptibility, hydrolytic spectrum, and whether they are encoded via the plasmids or chromosome (4).

There are three main groups of β-lactamases enzymes [18], [19]:

1) Class A, ESBL. They are plasmid-mediated enzymes that hydrolyze penicillins and extended cephalosporins of the 1st, 2nd, 3rd, and 4th generations (ceftriaxone, cefotaxime, ceftazidime) monobactam (aztreonam), yet not cephomycin.
2) Class B, they are carbenem hydrolyzing enzymes:
a) Serin- β-lactamases
b) Metalo-β-lactamases contain metal ion, like zinc.
3) Class C, cephalosporinase (AmpC). They are plasmid mediated, have the ability to hydrolyze cephomycin (cefotaten and cefoxitin), along with oxyimino-cephalosporins (ceftazidime and cefotaxime) and monobactams (aztreonam).

1.5 Production of Extended spectrum beta lactamases

Bush suggested a substantial redesign in 1989, which was updated in 1995. β-lactamases were categorized in the revised Bush system according to their substrate preference, which included oxacillin, penicillin, cephaloridine, carbenicillin, expanded-spectrum cephalosporins, and imipenem, in addition to their resistance to clavulanate inhibition [6]. Depending on amino acid sequencing, Ambler developed a classification in 1980 that implies four unique molecular classes: A, B, C, and D. Because of its phylogenetic links and simplicity among the enzymes, Ambler's molecular classification is more universally accepted compared to Bush's phenotypic classification [20], [21].

Jung Hune Lee and colleagues suggested a more inclusive definition of ESBLs in 2010. In addition, The Ambler Class A ESBLs have been referred to as the ESBLs, class C enzymes with the extended spectrum as cESBLs, and class D extended-spectrum variant of the OXA enzymes as dESBLs [10]. Antimicrobial use results in the selection of bacteria that produce ESBLs, and ESBL genes are frequently found on self-transmissible or mobilizable broad-host range plasmid types. Invasive infections were produced by a significant incidence of ESBLs in *P. mirabilis*. Peniclillins, oxyimino-thiazolyl cephalosporins (including 3rd and 4th generation cephalosporins), early cephalosporins, and monobactams are hydrolyzed by ESBLs, yet carbapenems and cephamycins are not. Suicide inhibitors like tazobactam, clavulanic acid, and sulbactam inhibit them [22].

1.6 Production of Metallo- beta lactamases

MBLs were once widespread in *P. aeruginosa* and *Acinetobacter* spp., yet have lately become increasingly common among *Enterobacteriaceae* members [23]. Carbapenems, wide-spectrum antibiotics with exceptional stability against the beta-lactamases, particularly ESBLs, were a major relief to clinicians. Such relief, yet, has been short-lived, as infections caused by carbapenemase-producing *Enterobacteriaceae* appeared shortly. Carbapenemases hydrolyze not only carbapenems, yet any hydrolysable beta-lactams as well, and have resistance to beta-lactamase inhibitors. In addition, carbapenemases are divided into two classes based on the hydrolytic processes present at the active site. The first group (A, C, & D) uses serine at active site for implementing its hydrolytic activity; the 2nd group (metallo β-lactamases) utilizes at least one zinc atom at active site for the facilitation of the hydrolysis. Metallo-ß-lactamase genes are transported on mobile genetic elements, allowing resistance genes to spread quickly [24]. With the exception of azetreonam and monobactames, MBL can easily hydrolyze all β-lactams. Metal chelators, like ethylenediamine tetra acetic acid (EDTA), block such enzymes because they need divalent cations, mainly zinc ions, as metal cofactors. Many nations have observed increases in the frequency of carbapenem resistance caused by acquired MBLs such as VIM and IMP. There are approximately nine main types of acquired MBL. Cabapenem-hydrolyzing enzymes are divided into four classes depending on molecular studies: A, B, C, and D. MBLs are classified as part of ambler group B, which is separated into 3 subclasses: BI, BII, and BIII. The VIM and IMP forms of acquired MBLs are the most common and

pervasive. This enzyme has a broad substrate spectrum, since it can hydrolyze penicillins, carbapenems, and cephalosporins, yet it cannot hydrolyze aztreonam [25].

The goal of this work was to look for ESBLs and Metallo-lactamases in Gram negative bacteria which are accountable for a variety of dangerous infections, as well as determining antimicrobial susceptibility patterns of isolates producing MBL and EXBL. This could result in a vision that can help limit the spread of bacteria which have a higher resistance to anti-microbial agents and control the infections that they cause.

2. Materials and Methods

Between Nov. 2018 and Feb. 2020, 28 isolates of *P. aeruginosa*, 22 clinical isolates of P. *mirabilis*, and 23 isolates of *K. pneumoniae* have been isolated from 73 clinical specimens, including wounds, burns, and UTI specimens, from patients at various hospitals in Baghdad, Iraq. After that, all of the specimens were transported to the laboratory, where they were isolated and identified with the use of sterile equipment and media. MacConkey, Blood, XLD, Citrmide, and Milk agars were used to streak the samples. All of the plates have been incubated aerobically for 24 hrs at a temperature of 37, and the generated isolates were identified using the following criteria: [26]. The results have been confirmed with the use of Vitek 2 identification technique, which relies on a series of biochemical tests performed simultaneously using Vitek cards; the results were digitized and recorded by Vitek software after the bacterial solution was placed into the Vitek cards.

2.1 Antimicrobial activity

- Antimicrobial susceptibility test

The antimicrobial susceptibility of 23 antimicrobial agents against Gram negative bacteria has been specified using Kirby-Bauer approach, as recommended by CLSI (2011) (27). Through preparing serial dilutions of 18 hours, a sterile cotton swab has been submerged in bacterial suspension that has been standardized to meet the turbidity of 0.50 McFarland turbidity standard (1.5x108CFU/ml). Following comparing it to the 0.50 McFarland turbidity standard, the 3rd dilution of the Brain Heart Infusion culture of the tested bacterium has been utilized. The bacterial suspension was dispersed across the surface of Mueller Hinton agar plates in 4 directions, and the plates have been left to dry for 10 minutes. The disks regarding the antimicrobial agents were after that placed on the surface of agar with sterile forceps and strongly pressed for ensuring that they made contact with the agar. Furthermore, the plates have been incubated at a temperature of 37oC for 18-24 hrs. According to the Clinical Laboratories Standards Institute, inhibition zones generated around antimicrobial dicks have been evaluated with the use of a metric ruler in millimeters (CLSI, 2011). By comparing the isolate to typical inhibition zones, it was determined if it has been susceptible or resistant to specific anti-microbial agents.

- Detection of Extended Spectrum β-Lactamases (ESBLs)

The Double-disk synergy test (DDST) approach was used for detecting isolates expressing Extended Spectrum β-Lactamases [1], [28], [29].

1) Serial dilutions of 18hrs. Brain Heart Infusion culture of the tested bacteria were prepared, a sterile cotton swab was submerged into the third dilution of bacterial suspension which has been standardized for the purpose of matching a 0.50 McFarland standard of turbidity (1.5x108CFU/ml)

2) The bacterial suspension was spread into four directions on Mueller Hinton agar plates' surface, the plates were left for 10 min. to dry.

3) Amoxicillin/ Clavulanic acid (30µg) disc has been placed in middle of the Mueller Hinton agar plate, after that, Ceftazidime, Cefotaxime and Aztronam discs have been arranged around Amoxicillin/ Clavulanic acid (30µg) disc within 2cm-3cm distance. Plates have been inverted and incubated at a

ISSN: 1343-4292
Volume 140, Issue 02, May, 2022

temperature of 37oC for 18hrs-24hrs. After the incubation, the activity of the synergism between central disk and any surrounding anti-microbial disc has been noted to detect Extended Spectrum beta-lactamase producing isolates.

- Detection of Metallo β-Lactamases (MBLs)

Double-disk synergy test approach has been utilized in order to detect metallo- β-Lactamases producing isolates [30], [31]

1) A sterilized cotton swab has been submerged into the bacterial suspension that has been standardized for the matching of 0.50 McFarland turbidity standard ($1.5x10^8$CFU/ml) turbidity through the preparation of the serial dilutions of 18hrs. The Brain Heart Infusion culture of the tested bacteria, and the 3rd dilution has been utilized after the comparison with 0.50 McFarland turbidity standard.

2) The bacterial suspension was dispersed across the surface of Mueller Hinton agar plates in 4 directions, and the plates were left to dry for 10 minutes.

3) An Imipenem (10 µg) and Imipenem - EDTA discs were pressed on plates within 2cm-3cm distance. The plates have been inverted and incubated at 37 degrees Celsius for 18hrs-24hrs. Synergism activity between Imipenem-EDTA and Imipenem disk was seen after incubation for detecting Metalo-lactamases producing isolates.

3. Results and discussion

3.1 Isolation and Identification

From patients attending to different Hospitals in Baghdad through the period from Nov. 2018 to Feb. 2020, 73 clinical isolates from burns (17 isolates, 23.3 %) wounds (22 isolates, 30.1 %) and urine (34 isolates, 46.5 %) were collected. As in figure (1).

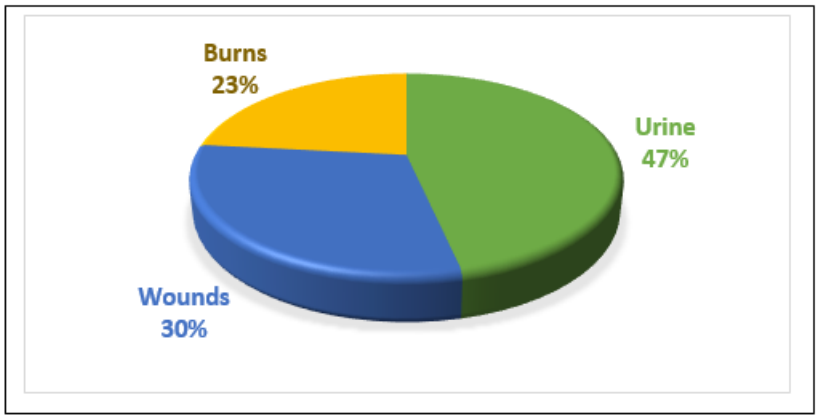


Figure (1): results of isolated bacteria

After culturing on XLD agar and MacConkey agar, which are selective and differential medium utilized for isolating and identifying *Pseudomonas*, *Proteus*, and *Klebsiella* spp. from other *Enterobacteriaceae* species as a primary identification depending on the most widespread characters like: non-lactose fermenter isolates that appeared as convex, pale, smooth and circular colonies, with a special fish-like After that, all of the isolates were sub-cultured on blood agar. The swarming behavior was used to identify *Proteus* spp., which had no hemolytic activity on blood agar media [27]. For isolation and primary identification of *Pseudomonas spp*.; presence of non – lactose fermenter isolates on MacConkey agar in general, then sub – cultured the result on citramide agar as a specific selective media for growth *Pseudomonas* only, finally sub-cultured the isolates on milk media as differential media to notice the ability of isolates to produce

pigments as further morphological detection for *Pseudomonas* [32- 34]; while *Klebsiella spp.* were identified by lactose fermenter large mucoid colonies on MacConkey agar and Yellow, surrounded by yellow zones, opaque, mucoid colonies on XLD agar.

Vitek 2 identification system assay has been utilized for confirming the identification of collected isolates; results of Vitek 2 identification system assay identified 22 isolates as *P. mirabilis*, 28 isolates as *P. aeruginosa* and 23 isolates as *K. pneumoniae*, as in table (1).

Table (1): Result of isolation according to the type of specimens

Species of bacteria	specimen	No. of isolates	Percentage of isolation %
Proteus mirabilis	Urine	12	54.5 %
	Wound	8	36.3
	Burn	2	9 %
Pseudomonas aeruginosa	Urine	10	35.7 %
	Wound	7	25 %
	Burn	11	39.2 %
Klebsiella pneumoniae	Urine	18	78 %
	Wound	5	21.7 %
	Burn	0	0 %

3.2 Susceptibility of P. mirabilis towards antimicrobial agents

The investigation of identified isolates susceptibility to 4 antimicrobial agents; including: Ceftazidime, Cefotaxime, Meropenem, and Imipenem, was performed through the use of Kirby-Bauer method. The results were showed in (figure 2).

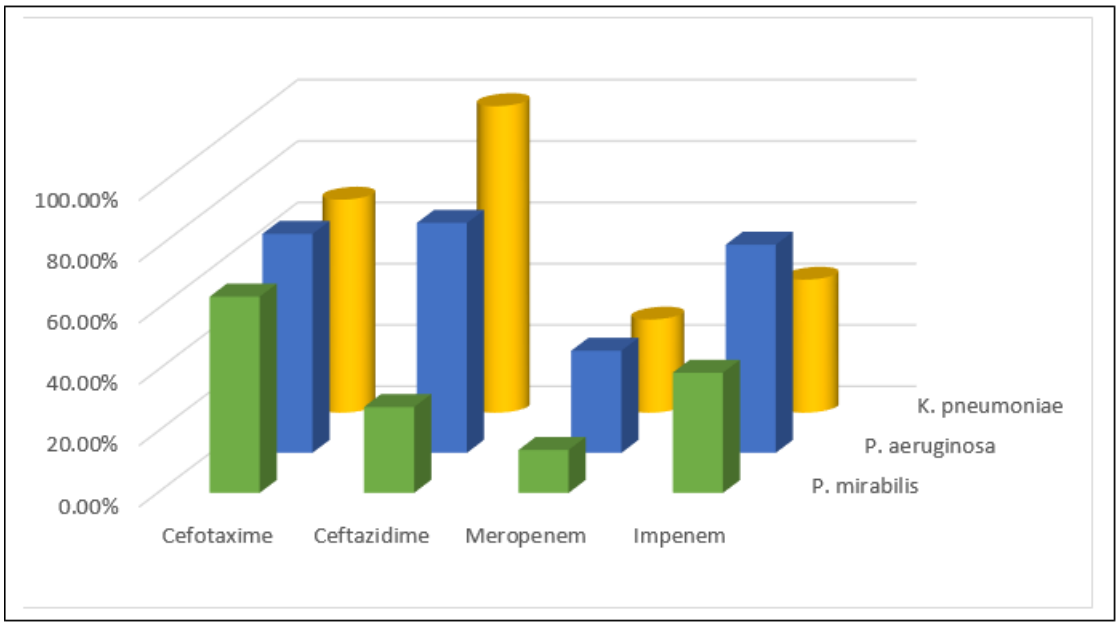


Figure (2): Susceptibility of tested isolates to antimicrobial agents

As in the figure (2) *P. mirabilis* isolates were high resistant towards cefotaxime (64%) in compression to other antimicrobial agents, while they showed moderate resistant towards ceftazidime (28%). These results were differed from what [35] had mentioned, that *P. mirabilis* resistance to cefotaxime was 15.7% but it was close to ceftazidime (26.4%). The results of this study indicate increasing resistance of *P. mirabilis* towards third generation of cephlaosporins which are drug of choice to treat *Proteus spp.* infections due to increasing randomly wide usage of these antimicrobial agent to treat such infections. On other hand the tested isolates result of Impineme were close to the previous (39.2%) which it disagreed with [35] who mentioned that *P. mirabilis* isolates were sensitive to imipenem; while the results showed low resistant towards meropenem (14%).

The results of *P. aeruginosa* showed that 21 (75%) isolates have been resistant to ceftazidime, which was approach to a local study [36] that mentioned the percentage of resistant was (69.2%), but it was disagree with another local study [37] that showed 30% resistance to the same antimicrobial agent. Also, the results were the less than [38], who recorded that the resistant to ceftazidime was (94.77%) & (92%) respectively.

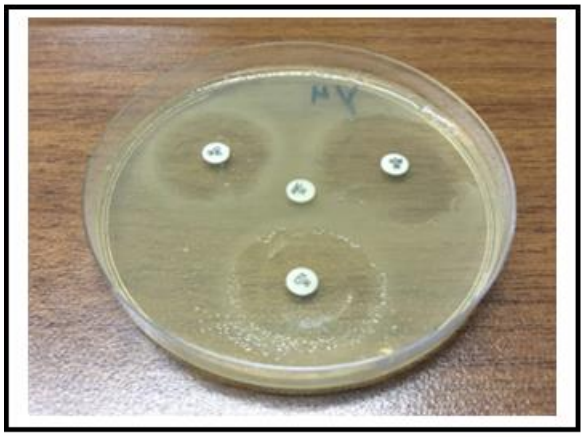
The isolates showed (71.4%) resistant to cefotaxime; which approach with [39] who reported that the resistant to cefotaxime was (76.3%). The isolates resistant to Imipenem was (67.9%), this result was disagreeing with [36], [37], [39], who mentioned that the percentage was (55.3%), (28.2%) and (zero %) respectively. Tested *P. aeruginosa* indicate resistant toward Meropenem (33.3%).

The results confirmed the increasing of cefotaxime and ceftazidime resistant producing bacteria epidemiology across wide regions around the world due to the random and high antimicrobial agents' usage that leads to increasing resistant towards antimicrobial agents' usage with passing time. The importance of these two antimicrobial agents belong to the fact that they belong to the third generation of cephalosporins, which theoretically have wide activity against Gram negative bacterial infection [40]. While resistant to imipenem which is a drug of choice to β-lactamases producing *Pseudomonas* was moderate between all mentioned results.

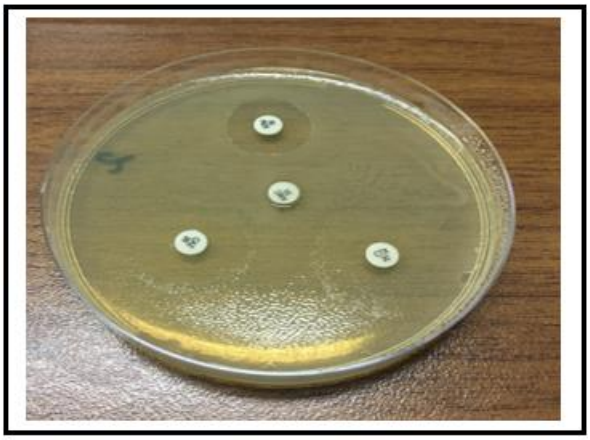
K. pneumoniae tested isolates were all resistant towards cftazidime (100%) which indicate significant resistant; while they showed moderate resistant towards cefotaxime (69.5%). This result disagrees with [30]. That mentioned low resistant to ceftazidime (38.9%) but it was more approximate with result of cefotaxime (50%). Resistant towards Impineme and meropenem was approximately close (43.4%) and (30.4%) respectively. Testes isolates were more resistant than what [30] indicated.

3.3 Production of EXBL in tested isolates

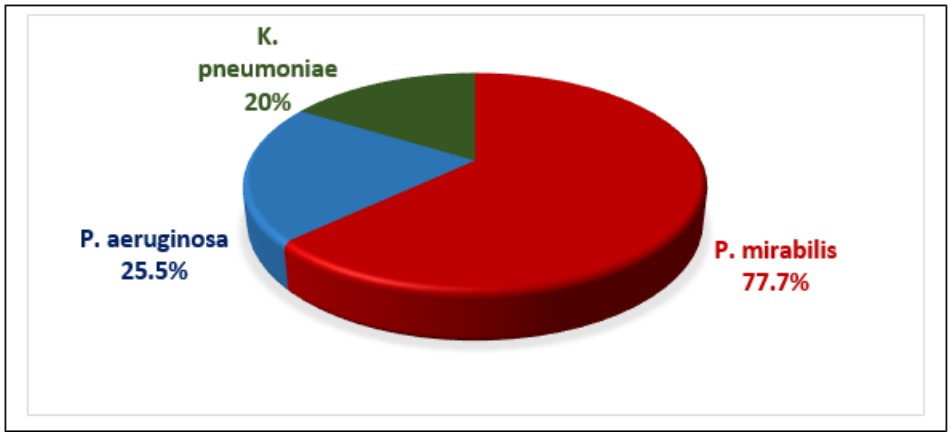
Detection of Extended Spectrum β-Lactamases producing isolates that resistant to cefotaxime was carry out by using DDST approach, by determining the increasing of inhibition zone against amoxicillin/ clavulanic acid, cefotaxime, ceftazidime and aztronam, and compared with same antimicrobial agents lacking clavulanic acid by the same method (figure 3,4). The results indicate that 14 (77.7 %) of 18 *P. mirabilis* isolates resistant to cefotaxime were ESBL producers; *P. aeruginosa* producing EXBLs was 8 (25.5%) isolates, while *K. pneumoniae* showed 4 (20%) of 20 isolates resistant to cefotaxime were ESBL producers (figure 5).



(Fig.3): ESBL producing tested bacteria



(Fig.4): Non-ESBL producing tested bacteria



(Fig.5): ESBL producing tested bacteria percentage

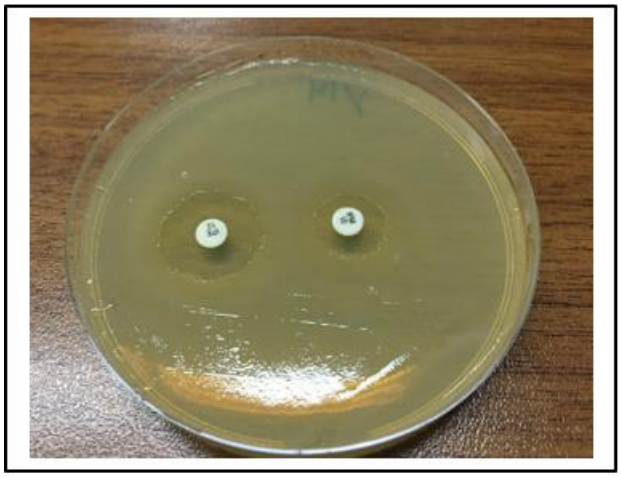
The results of this study showed that EXBL β-lacatmase are produced higher in *P. mirabilis* than *P. aeruginosa* and *K. pneumoniae* sequentially, which showed approximate results. It is confirmed with most studies results which indicate high EXBL producers are *Proteus spp.* and this enzyme could be found in other bacteria specially in Gram negative bacteria because of the ability of genetic elements especially plasmids with different sizes that are carrying ESBLs β-lacatmase genes of transferring, cloning and conjugation.

Treatment of ESBL-producing Enterobacteriaceae strains has become a main challenge in both community and hospitalized patients because of the main risk factors for infection or colonization with ESBL-producing organisms such as prolonged hospitalization, long-term antibiotic exposure, high rates of 3rd-generation cephalosporin utilization, and invasive procedures [41].

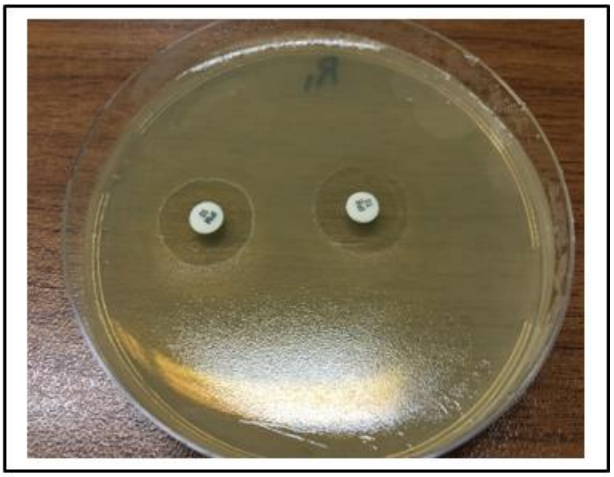
3.4 Production of MBL in tested isolates

The capability of isolates for resisting imipenem which produce MBL enzymes (a type of β-lactamases) was found using the double disk synergy approach, which increased the inhibition zone in the EDTA-Impenem combination disk by more than 7 mm when put to comparison with Imipenem disk alone (figure 6,7).

ISSN: 1343-4292
Volume 140, Issue 02, May, 2022

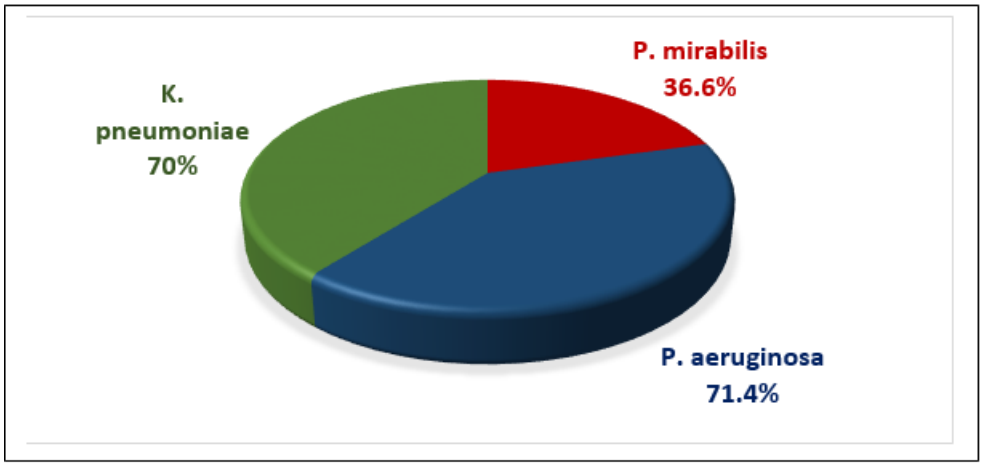


(Fig.6): MBL producing tested bacteria



(Fig.7): non-MBL producing tested bacteria

According to this method; it was found that out of 11 (50%) *P. mirabilis* isolates resistant to imipenem, 4 isolates (36.3%) were MBL producers; 15 isolates (71.4%) were MBL producers out of 21 (75%) *P. aeruginosa* isolates resistant to imipenem; while Out of 10 (43.4%) *Klebsiella* isolates resistant to imipenem, 7 isolates (70%) were MBL producers (figure 8).



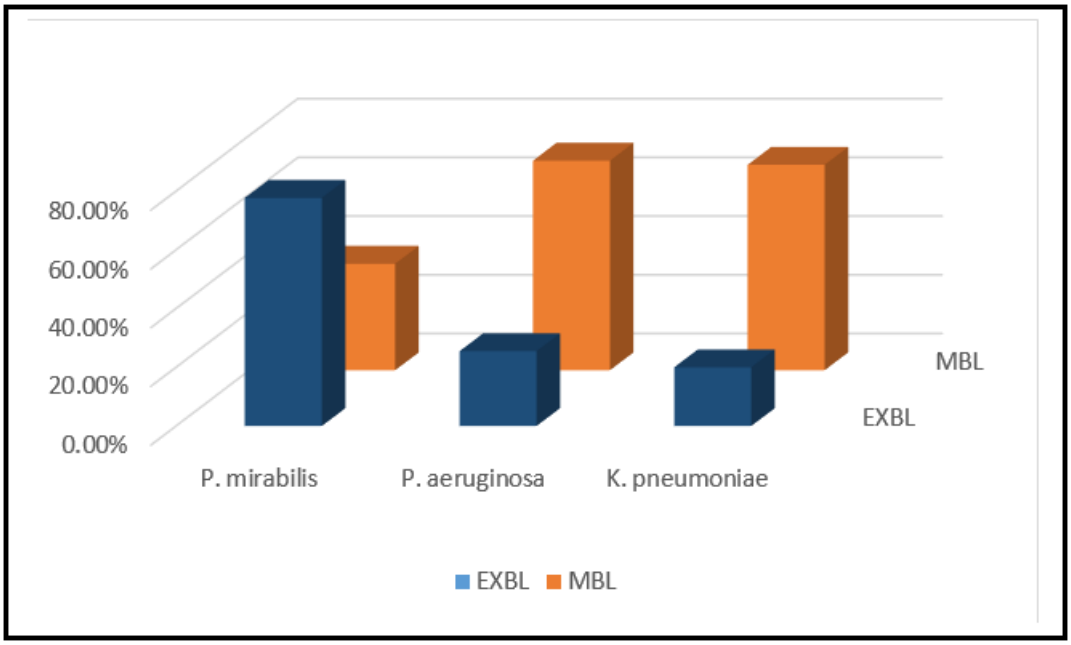
(Fig.8): MBL producing tested bacteria percentage

Results indicate that *P. aeruginoas* produce MBL β-lacatmase significantly in comparison with *K. pneumonie* and *P.mirabilis.* which agree with the present data. In general, presence of phenotype detection of MBLs is rare with *Proteous spp.,* but found higher in *Klebsiella spp.;* this may be to low resistant of these bacteria to carapenems, such as: imipenem and meropenem, or due to the ability of *Proteus* to harbor ESBLs gene instead of MBLs genes, but a phenotype detection results of this study showed the possibility of such organisms to carry MBL genes, which could be a threat to infection control attempts and might lead to bad clinical outcomes in the case when carbapenems are utilized to treat those with severe infections; such a hypothesis requires additional analysis, since early detection is critical for preventing the consequences of such worrying resistance mechanism.

Finally, the (figure 9) showed the percentage of the main types of ß-lactamases enzymes in *P. mirabilis*, *P. aeruginosa* and *K. pneumoniae* that tested in this study, which displayed that the ESBLs (77.7%) were the most dominant type of enzymes produced by *P. mirabilis*; which forms the main concern faced the

treatment of *Proteus* infections; in addition to the presence of other types of enzymes; such as: MBLs (36.3%).

At the same time; *P. aeruginosa* (71.4%) and *K. pneumoniae* (70%) indicate that MBLs were the most dominant enzymes in tested isolates in addition to produce EXBLs (25.5%) and (20%) for *P. aeruginosa* and *K. pneumoniae* respectively.



(Fig.9): distribution of beta-lactamases enzymes in tested isolates

These results indicate the increased resistant to other types of antimicrobial agents that can be alternative drug of choice, which make the treatment of such infections failed with ordinary choices of antimicrobial agents because of growing resistant towards antimicrobial agent and this need to aware the physician with these informations to find another combination of drugs and determine the right way to use these drugs to prevent such a problem in the future.

Better patient outcomes with regard to avoiding incorrect therapy are possible advantages, as is the failure to detect the kind of ß-lactamase producers, which can result in inappropriate antimicrobial treatment and higher mortality. This is concerning and necessitates immediate intervention, both therapeutically and in terms of infection management.

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