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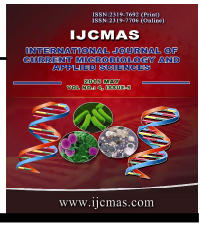


Original Research Article

Detection of Metallo-βeta-Lactamase Enzyme among Isolated *Pseudomonas aeruginosa* From Nosocomial Infected Patients at Suez Canal University Hospital

Nermine N. El-Maraghy[1]*, Gehan Saddik El-Hadidy[1], Mahmoud Kamel Mansour[1] and Moshira Mohammed El-Saeiyed[2]

[1]Microbiology & Immunology Department, Faculty of Medicine, Suez Canal University; Ismailia, Egypt
[2]Resident of Medical Microbiology, El-Tal -Kebeer Hospital, Ismailia, Egypt

**Corresponding author*

Keywords

Metallo-β-lactamase, *Pseudomonas aeruginosa*

A B S T R A C T

Pseudomonas aeruginosa, a non-fermentative Gram- negative bacillus, which can survive in hospital environment, as the wards encourage its growth. Multidrug-resistant *Pseudomonas aeruginosa* accounts for about 10%–20% of nosocomial infections at the in intensive care unit. Also, it is defined as the resistance to one or more of anti-microbial agents in three or more anti-pseudomonal anti-microbial classes; beta-lactam antibiotics (penicillins, cephalosporins, carbapenems & monobactam); aminoglycosides and fluoroquinolones. Enzyme production is the major mechanism of acquiring resistance towards β -lactam antibiotics among clinical isolates of *P. aeruginosa* that is usually referred to as β – lactamases. To determine the prevalence of Metallo- β – lactamases among multidrug-resistant *Pseudomonas aeruginosa* isolates from different clinical specimens of patients admitted at Suez Canal University hospital. This study was carried out at Suez Canal University Hospital; Ismailia, Egypt from December 2012 to February 2014 over 65 patients acquiring *Pseudomonas aeruginosa* nosocomial infections. Modified Hodge Test, disk synergy test and double disk test were performed for the phenotypic detection of the carbapenemase and metalo- β -lactamase enzymes production. We found that 33 isolates were MDR with 60.6% of these isolates were carbapenemase production. And the highest percentage of Metallo-β-lactamase production was in the Intensive Care Unit (40%) with the resistance to Imipenem & Meropenem by 56% & 66.7% respectively. Therefore, restricting the use of carbapenems and routine detection of Metallo- β – lactamases enzyme in all nosocomial infected cases could solve the problem of multidrug- resistant *Pseudomonas aeruginosa.*

Introduction

Pseudomonas aeruginosa (P. aeruginosa), a non-fermentative Gram- negative bacillus that can survive in hospital environment; as the wards encourage its growth (Akova *et al.,* 2012). It accounts for about 10% -20% of nosocomial infections at the intensive care unit (ICU) as bacteremia, sepsis, pneumonia of cystic fibrotic lung, urinary tract infections (UTI), burn and wound infection are the causes (Carmeli *et al.,* 2002).

Multidrug-resistant (MDR) *P. aeruginosa* is the resistance to one or more of anti-microbial agents in three or more anti-pseudomonal anti-microbial classes; beta-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactam); aminoglycosides and fluoroquinolones (Murray *et al.,* 2010).

Multidrug-resistant *P. aeruginosa* is a major problem due to genetic resistant inherence to different drug classes with an increasing in the ability of acquiring resistance to all effective antimicrobial drugs due to the enzymatic and mutational mechanisms (Gad *et al.,* 2007).

Because of the low permeability of *P. aeruginosa* outer membrane, the constitutive expression of various efflux pumps with wide substrate specificity [5], the naturally occurring chromosomal AmpC- β -lactamase also known as cephalosporinase, it provide resistance to beta –lactam antibiotics (Livermore, 2000).

Enzyme production is the major mechanism of acquiring resistance towards β -lactam antibiotics among clinical isolates of *P. aeruginosa* that is usually referred to as β –lactamases and that act by destroying the amide bond of the β -lactam ring, so that why the destroyed products lack of antibacterial activity and become ineffective (Nordmann and Pand, 2002).

Carbapenem antibiotics were originally obtained from thienamycin that is a naturally derived product of Streptomyces (very similar to the penicillins), but the sulfur atom has been replaced with a carbon atom on position number one, and the unsaturation happened, hence the name of the carbapenems (Tomás *et al.,* 2010).

Carbapenems are a class of β-lactam antibiotics with a broad spectrum of antibacterial activity that acts as one of the last resort antibiotics used for the treatment of *P. aeruginosa.* But, recently the production of carbapenemase enzyme raised an alarm over the spread of drug resistance among clinical isolates of *P. aeruginosa*. A nightmare scenario start to appear due to worldwide spread of the genetic resistance to carbapenem, in spite of lack of antibiotics used to combat bacteria resistant to carbapenems due to their structure that renders them highly resistant to most β-lactamases (Nordmann *et al.,* 2012).

Moreover, carbapenemases are group of extended spectrum beta-lactamase (ESBLs) that have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems. Carbapenemases are members of the molecular class A, B, and D β-lactamases. Class A and D enzymes have a serine-based hydrolytic mechanism, while class B enzymes are metallo-β-lactamases (MBLs) that contain zinc at the active site (Murphy *et al.,* 2003).

Several literature announced the increase prevalence of carbapenem resistance mediated by acquiring MBLs including, IMP (Imipeneme active metallo-β-lactamases) and VIM (Verona Integron –encoding Metallo β lactamase) but VIM is reported worldwide (Rossolini, 2005).

So far, in Egypt, a study was conducted by Gad and his colleagues at Menoufia University Hospital, they found that MDR *P. aeruginosa* isolates were 52% and ESBLS producers were 45.6% (Gad *et al.,* 2007).

However, little information is available concerning the distribution of MBLs producing isolates in our country, so we aimed in this study to determine the prevalence of MBLs among MDR *P. aeruginosa* isolates from different clinical specimens of patients admitted at Suez Canal University hospital.

Patients and Methods

Patients

This study is a cross-sectional descriptive study, carried out at SCU (Suez Canal University) Hospital in Ismailia during the period from December 2012 to February 2014 over 65 patients acquiring infections 48 hours after their admission regardless their age or sex after obtaining written consents from all patients. With an exclusion of patients having signs or symptoms of infection before 48 hours after admission and those receiving antibiotics 48 hours prior to the collection of specimens.

Methods

Various clinical specimens (Urine, sputum, blood and pus) were collected under aseptic conditions and inoculated onto Pseudomonas Isolation Agar Medium (selective media for *P. aeruginosa*) that appeared as greenish colonies after 18 hours of incubation and turned blue to blue-green after 24–48 hours incubation, with diffusion of the pigment into the medium with positive and negative QC (Quality control) strains" *P. aeruginosa* 27853" &" *Escherichia coli* 25922" respectively were used (Isenberg and Garcia, 2004) and oxidase and catalase tests were performed.

Then, *P. aeruginosa* isolates were tested for antibiotic susceptibility pattern by using disc diffusion method of Kirby Bauer according to the Clinical and Laboratory Standard Institute (CLSI, 2012).

In order to define MDR *P. aeruginosa* the following antibiotics were used different antibiotics. For β lactam antibiotics: Aminopenicillins: "ampicillin10 μg" Urido penicillins: "Piperacillin 100 μg", Pipracilline-Tazobactame100/10 μg", Carboxy penicillins: "Ticarcillin 75 μg", Ticarcilline-clavulanic acid 75/10 μg, Cephalosporines: "Cefpiem 30 μg", Monobactams: "Azetronam 30 μg" and Carbapenems: "Merobenem 10 μg" & Imipenem 10 μg (Oxoid, England). For Aminoglycosides: Gentamicin 10 μg (Oxoid, England) was used and for Fluoroquinolones: Ciprofloxcacin 5μg (Oxoid, England) was used,

MDR *P. aeruginosa* was detected as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (Murray *et al.*, 2010).

For the phenotypic detection of the carbapenemase and metalo- β -lactamase enzymes were done by using Modified Hodge Test (MHT) and disk synergy test and double disk test respectively.

MHT is characterized by the cloverleaf-like indentation after using indicator strain (*E.coli* ATCC 25922) (Lee *et al.*, 2003).

For Imipenem- EDTA Combination disk synergy test (CDT-IPM) was considered positive when the growth-inhibitory zone diameter around the Imipenem disc with EDTA increase ≥7 mm compared with the growth-inhibitory zone diameter around the disc containing Imipenem alone.

And Imipenem-EDTA Double disk synergy test (DDST-IPM) was considered positive if there was a zone of inhibition between imipenem and EDTA disk (Yang and Livermore, 1990).

Result and Discussion

The study was conducted at SCU hospital on 210 admitted patients. *P. aeruginosa* were isolated from the oncology department and from pus specimen with 32.3% and 36.9% respectively as shown figure 1 & 2. Table 1 showed the sensitivity profile of the specimens for the tested antibiotics. Thirty –three isolates out of the 65 specimens were MDR as shown in table 2.

According to MHT, 53.8% of the isolated *P. aeruginosa* were carbapenemase enzyme producer, with MDR strains in 60.6% of enzyme production as presented in table 3.

Figure 3 showed the frequency distribution of *P. aeruginosa* isolates according to the Metallo-β-

lactamase enzyme production by using DDST&CDT.

Figure 4 showed that among the 33 MDR *P. aeruginosa* patients, 60.6% of resistance caused by carbapenemase enzyme was detected by MHT & Metallo-β-lactamase test was 70%detected by CDST.

Table 4 showed the relationship between the Metallo-β-lactamase production and the resistance to Imipenem & Meropenem by 56% & 66.7% respectively as well as different types of antibiotics as presented in figure 5.

Finally, table 5 showed that the highest percentage of Metallo-β-lactamase enzyme production was present in the ICU (40%),with (35.3%) in blood specimen.

MDR resistant gram-negative bacilli have been increasingly reported globally as a major cause of nosocomial infections(Garau *et al.*,2004). *Pseudomonas aeruginosa* is one of the most gram-negative bacilli causing nosocomial infections. The problem arise from the genetic inherently resistant to many drug classes and the ability in acquiring the resistance to all effective antimicrobial drugs. Nowadays, MDR *P. aeruginosa* is emerged in Egypt at several hospitals.

This study conducted over 210 patients with different nosocomial infection. *P. aeruginosa* strains were isolated with 30% and they were responsible for the nosocomial infections in Suez Canal University hospital.

In comparison to Mahmoud and his colleagues and Gad and his colleagues, the former detected that *P. aeruginosa* strains were responsible for 19% of nosocomial infections in El Menoufia University Hospital, while the later detected it was responsible for 18% of nosocomial infections in three hospitals in El Minia, Egypt. The present study is higher than these two due to difference in sample size and the prevalence of the other types of infection included in these researches.

Concerning the antibiotic resistance, most of isolated *P. aeruginosa* strains in this study were resistant to carbapenem with 18.5% and 38.5% resistance to meropenem and imipenem respectively. In Egypt nearer results were reported by Zafer where *P. aeruginosa* strains were isolated from Kasr El-Aini Hospital and National Cancer Institute, Cairo University hospitals, and Mahmoud and his collegues showed that 33.3% of the isolated *P. aeruginosa* strains were resistant to imipenem.

Carbapenem resistance become a major problem facing the world and requires prefect surveillance system for the detection of new cases of resistance and MDR bacteria. This rate of carbapenem resistance reflects great limiting the treatment options in our country. This can be explained by the increase in the consumption of different antimicrobial agents in the last decade leading to a selective pressure on certain types antibiotics used to treat *P. aeruginosa* and consequently the bacteria modified the resistant mechanisms. This high rate of resistance was recorded all over the world.

In the Middle East, imipenem resistant *P. aeruginosa* was recorded in Saudi Arabia with resistance rate of 38.57% in 2011 (Mahmoud *et al.*, 2013).

Among thirty three European countries participating in the European Antimicrobial Resistance Surveillance System in 2007, six countries reported carbapenem resistance rates were more than 25% among *P. aeruginosa* isolates; with the highest rate reported from Greece (51%) (Souli *et al.*, 2008).

MDR *P. aeruginosa* phenotype is defined as resistant to one anti-microbial agent in three or more anti-pseudomonal anti-microbial classes (carbapenems, fluoroquinolones, penicillins, cephalosporins and aminoglycosides) (Murray *et al.*, 2010).

This study reported that 50.8% of the specimens were MDR. Our results are in convenient with Gad and Mahmoud who reported that MDR *P. aeruginosa* were 52% of all isolated nosocomial infection specimens. The situation in other countries was completely different, as in Iran, Zahra reported that MDR *P. aeruginosa* was

30%. In Turkey, Unan, it was 60%. In Europe, a study conducted over a 10years from1990 to 1999 the hospital prevalence of MDR *P. aeruginosa* increased from 8% to 17% and this result increased to 20.1% by 2010 (Tam, 2010). In America**,** the prevalence rates were close to 15% in 2001 and 2002 (Liakopoulos, 2013).

According to the previous results there was increase in the prevalence of MDR *P. aeruginosa* in Africa and Asia rather than Europe and America where their prevalence rate is increasing, this might be due to perfect surveillance system and regular recording the early cases of MDR *P. aeruginosa* in these developing countries that enable them to attack the problem.

Our research detected that 60.6% of MDR strains were carbapenemase enzyme producer, until now no recommended test by CLSI for detection of carbapenemase enzyme in *P. aeruginosa* but many studies reported that Re-Modified Hodge Test was the best test for detection of carbapenemase enzyme in *P. aeruginosa* (Lee, 2003). Our results were higher than Liakopoulos who reported that carbapenemase produced in 28% of isolated MDR *P. aeruginosa* in Greece, this change in the results might be due to change in the sample size and the types of the specimens.

Our study detected that MBL produced in 36.9% of all isolated *P. aeruginosa*, that didn't match with Zafer who detected that MBL-produced only at 27%of isolated *P. aeruginosa* strains. However higher results were reported by several authors (El-Kholy *et al.*, 2005; Franklin *et al.*, 2009) who reported that MBLs enzyme was produced in (62%), (50%) and (53.4%) of isolated *P. aeruginosa* strains respectively.

In India MBL production were detected in different percentage by Khakhkhar where MBL produced in 20.8%, 46.6%, 11.11% of isolated *P. aeruginosa* respectively. In Iran, higher results were reported by Masoumeh that MBL produced in 80% of isolated *P. aeruginosa*. This could be explained according to their antibiotics profile and infection control policies used in these areas.

Our results matched with El-Kholy in Egypt and Noyal in Indian**,** who reported that MBLs was a major type of carbapenemase enzyme in the *P. aeruginosa* strians but in lower rate 50%.The differences in the reported values might be due to the difference in geographical regions, difference in kind of infections, the enormous usage of antibiotics, or difference in antibiotic therapy regimens in the selective hospital in this study than those in other studies.

In our study, we used (DDST) and (CDST) on one plate to increase the accuracy of the test. And the MBL enzyme was detected in 30.7% by DDST and 36.9% by CDST from all the isolated *P. aeruginosa* strains. El-Kholy revealed that CDT was much accurate test for detection of MBL in *P. aeruginosa* as well as Yan. In India, Singh reported that EDTA containing CDST and DDST had the same sensitivity and specificity by 100%. In Brazil, Franco detected that CDST and DDST are equal in sensitivity and specificity. But in contrast, Yalda in Malaysia reported that sensitivity of the both tests were equal with higher specificity of DDST (96.6%) than the specificity of CDST (43.1%).

This difference of sensitivity and specificity of CDST and DDST in different studies require several researches to standardize the phenotypic method recommended for the detection of MBL producers with standardizing all factors of study.

In our study, we detected that highest percentage of MBL production was presented in the ICU (40%), the oncology department (38.1%), surgery ward, and the burn unit (27.3%) for both, the urology ward (20%) and lastly the neonatal ICU (14.3%). In contrast to Mehul who reported highest percentage of MBL in surgical ward was 75% followed by medical ward patient suffering from pleural effusion. This difference of results might be due to change of types of infection included in the researches.

Our study, reported that MBL detected mostly from blood specimens 35.3% followed by pus specimens 33.3%. In contrast to Khakhkhar who detected that MBL enzyme producing isolates were high in pus followed by urine and respiratory secretion. While, Shashikala reported

that 20.7% MBL producing *P. aeruginosa* isolates were from respiratory specimen. This difference might be due to the small representing number of respiratory specimens involved in our study and this might had underestimated the frequency of such isolated in their respiratory specimens.

Among the MBL positive *P. aeruginosa* strains, Aztreonam was the most active drug, but instead of MBL enzyme has no effect on monobactam (aztreonam) 17.9% of the MBL-producing isolates were not susceptible to aztreonam, suggesting a possible association with other resistance mechanisms or enzymes. Our results match with Murray in Brazil, Golshani in Iran and Zafer in Egypt, who demonstrated that a large proportion of MBL genes are associated with one or more aminoglycoside or beta-lactam resistant genes, partially explaining MDR cases.

In relation to carbapeneme resistance, MBL produced only in 66.7% and 56% of resistant cases to meropenem and imipenem respectively and the remaining cases of resistance were caused by other causes such as efflux systems, decreased outer membrane permeability, or production of *Ampc* enzymes. Our results did not match with Murray in Brazil where MBL produced in 30.4% of imipenem-resistant *P. aeruginosa*, and Aghamiri who detected that 70% of imipenem-resistant *P. aeruginosa* were MBLs producers. Lower result reported by FranclinIn USA who detected that 30% of MBL-carrying isolates, were found to be susceptible to imipenem and 3.9% of MBL MBL-carrying isolates were from Meropenem sensitive strains.

The presence of MBL among Meropenem and Imipenem sensitive strains indicates that there might be a hidden MBL among isolated strains which cannot be diagnosed by phenotypic tests, leading to the dissemination of these MBL genes in the hospital silently among patients even within normal health workers whom act as carriers for MBL genes in future. Other causes are due to the fact that in this study, Meropenem and Imipenem were selected to be tested against isolated bacteria as an example of carbapenem agent, but there were other generations such as etrapenem and doripenem which were not used and resistance might be detected in these cases of MBL positive strains.

Therefore, the reliable detection of the MBL-producing strains is essential for the optimal treatment of infected patients and to control the nosocomial spread of resistance.

MBL producing MDR *P. aeruginosa* represent a prevalent problem in SCU Hospital. The unjustified use of carbapenems can be considered as a major cause of such problem. Multidrug resistance, difficulty in detection and treatment and increase mortality represent the major problems encountered with MBL. Therefore, restricting the use of carbapenems and routine detection of MBL enzyme in all nosocomial infected cases especially those resistant to carbapenems, along with implementation of infection control measures, are the most effective means of controlling and decrease the spread of MBL producing isolates.

Recommendations

From the previous results, in case of MBL producing MDR *P. aeruginosa*, we recommend a combinational treatment of carbapenems and aminoglycosides and or fluoroquinalones. Lastly, CDT could be used as gold standard for detecting MBL producing MDR *P. aeruginosa* but further researches are still in need for the approval of this result.

Table.1 Frequency distribution of the studied patients infected with *P. aeruginosa* causing nosocomial infection according to the antibiotic sensitivity of the specimens (N=65)

Variable		Sensitive		Resistant	
		No.	%	No.	%
Antibiotic sensitivity	Ampicillin	0	0%	65	100%
	Pipracillin	0	0%	61	93.8%
	Ciprofloxacin	32	49.2%	31	47.7%
	Cifepime	20	30.8%	41	63.1%
	Aztronam	31	47.7%	28	43.1%
	Gentamicin	45	69.2%	20	30.8%
	Meropenem	41	63.1%	12	18.5%
	Imipenem	29	44.6%	25	38.5%
	Pipracillin-tazobactam	20	30.7%	23	35.3%
	Ticarcillin-clavulanic acid	10	15.3%	33	50.7%

Table.2 Frequency distribution of the studied patients infected with *P. aeruginosa* causing nosocomial infection according to the presence of multi-drug resistance among the specimens (N=65)

Variable		Number	Percentage
MDR	Present	33	50.8%
	Absent	32	49.2%

Table.3 Frequency distribution of the studied patients according to the presence of carbapenemase enzyme production among *P. aeruginosa* which were detected according to Modified Hodge Test (N=65)

Variable	Positive Carbapenemase production		Negative Carbapenemase production	
	No.	%	No.	%
***Total Isolated P. aeruginosa* (n=65)(*)**	35	53.8%	30	46.2%
MDR strains (n=33)(*)	20	60.6%	13	39.4%

(*) P-value<0.01, statistically significant.

Table.4 Frequency distribution of the studied patients infected with *P. aeruginosa* causing nosocomial infection according to the relationship between Metallo-β-lactamase enzyme production detected by CDST and the resistance to Imipenem & Meropenem (N=65)

Variable		Metallo-β-lactamase production	
		No.	%
Imipenem resistance(*)	Sensitive (n=29)	5	17.2%
	Resistant (n=25)	14	56%
	Intermediate (n=11)	5	45.4%
Meropenem resistance(*)	Sensitive n=41)	8	19.5%
	Resistant n=12)	8	66.7%
	Intermediate (n=12)	8	66.7%

(*) P-value<0.01, statistically significant.

Table.5 Frequency distribution of the studied patients according to the relation of Metallo-β-lactamase enzyme production detected by CDST and the source & the type of the specimens (N=65)

Variable		Metallo-β-lactamase production			
		Positive		Negative	
		No.	%	No.	%
Department (*)	Surgery (n=11)	4	27.3%	7	72.7%
	Burn unit (n=11)	4	27.3%	7	72.7%
	Oncology (n=21)	9	38.1%	12	61.9%
	ICU (n=10)	4	40%	6	60%
	Urology (n=5)	2	20%	3	80%
	NICU (n=7)	1	14.3%	6	85.7%
Specimen(*)	Urine (n=8)	2	12.5%	6	87.5%
	Pus (n=24)	10	33.3%	14	66.7%
	Blood (n=17)	6	35.3%	11	64.7%
	Sputum (n=16)	6	31.3%	10	68.8%

(*) P-value<0.01, statistically significant.

Figure.1 The distribution of the studied patients infected with *P. aeruginosa* according to different departments from which specimens were collected

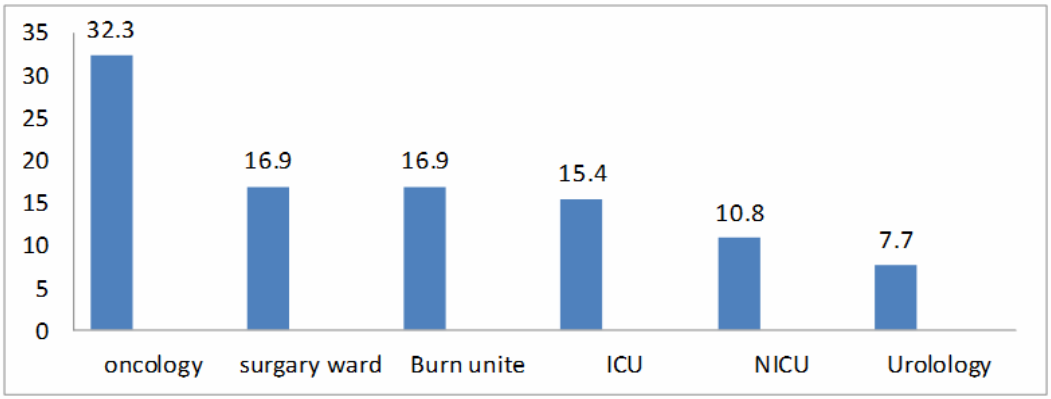


Figure.2 The distribution of the studied patients infected with *P. aeruginosa* according to the types of collected specimens

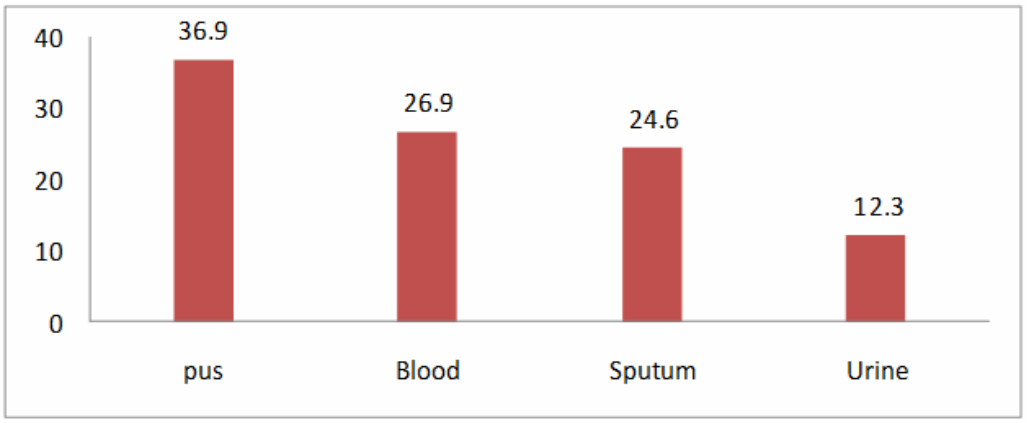


Figure.3 Frequency distribution of isolated *P. aeruginosa* according to the presence of Metallo-β-lactamase enzyme production (using DDST and CDST) (N=65)

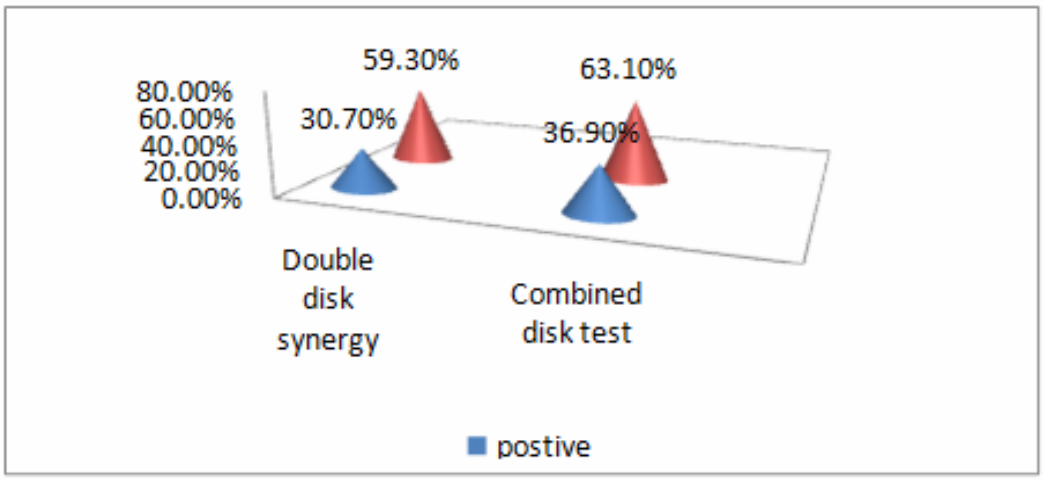


Figure.4 Frequency distribution of the studied patients according to the relation between carbapenemase and Metallo-β-lactamase production among the multidrug resistant *P. aeruginosa* (N=33)

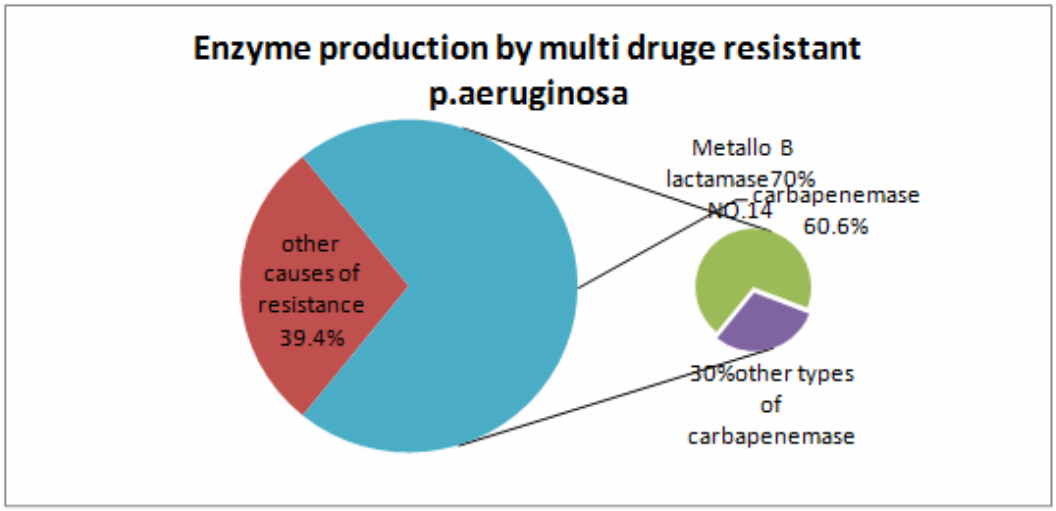
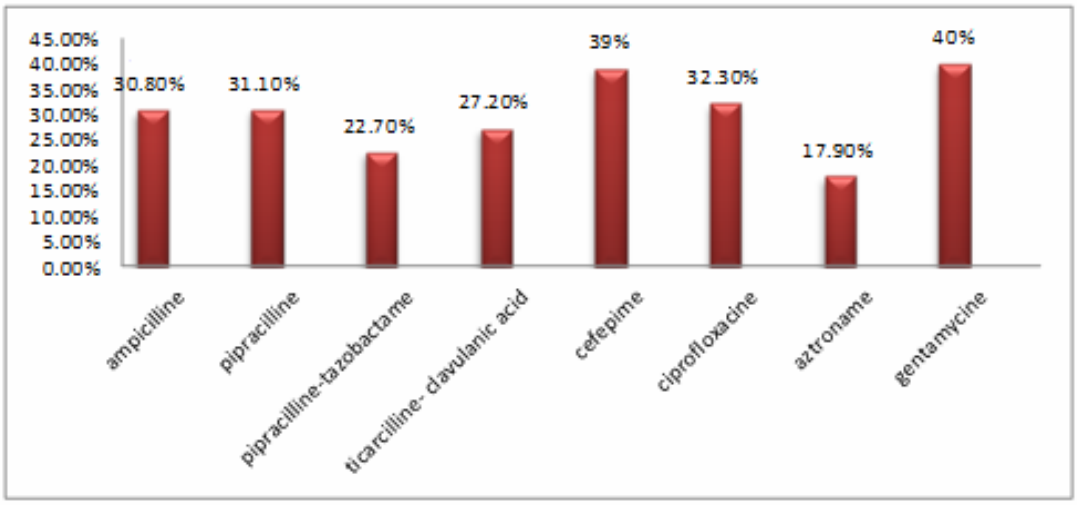


Figure.5 The relationship between the production of Metallo-β-lactamase enzyme and the resistance towards different types antibiotics



(*) P-value<0.01, statistically significant.

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