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Molecular detection of Metallo-beta lactamase producing *Pseudomonas aeruginosa* isolated from different sites of infection

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Abstract

In The present study, included the collection of (100) samples from different clinical sites. Clinical samples were collected from patients who were visit and admitted All-Hilla teaching hospital at the period from November (2017) to February (2018). Cultural, biochemical and VITEK$_2$ system were used for identification, and depending on the VITEK$_2$ system (VITEK-$_2$ GN Kit), revealed that twenty one (21) *Pseudomonas aeruginosa* isolates were recovered, The percentage conformational identification of *P. aeruginosa* was performed using VITEK$_2$ system of (21) *P. aeruginosa* was (99%). Nine (42.8%) samples were isolated from burns, 5(23.8%) samples from wound, 3(14.2%) from urine, 2(9.5%) from ear swab, and 1(4.7%) sample was isolated from both blood and sputum. The antibiotics discs diffusion test was done by using (7) different clinically important antibiotics. This study showed that most of *P. aeruginosa* isolates were highly resistant against the antibiotics used particularly the β-lactams, such as Amoxicillin 21(100%), Cefixime 20(95.2%), Piperacillin 19(90.4%), Ceftriaxone 18(85.7%), Cefotaxime 17(81%) and 16(76.1%) isolates were resistant to (carbapenems) both Meropenem and Imipenem antibiotics. All *P. aeruginosa* isolates were further investigated for the presence of chromosomal or plasmid mediated Metallo-β-lactamases. The presence of MBL genes that include *bla*IMP, *bla*VIM, *bla*SPM, *bla*SIM, *bla*GIM were detected by conventional PCR technique. These genomic *bla*IMP, *bla*VIM, *bla*SPM, *bla*SIM, *bla*GIM were detected in 16(76.1%) isolates. These results achieved using specific genes primers.

Keywords: *Pseudomonas aeruginosa*, Metallo-β-lactamases, *bla*IMP, *bla*VIM, *bla*SPM, *bla*SIM, *bla*GIM, PCR

INTRODUCTION:

Pathogenic members of these genera include *P. aeruginosa*, *P. oryzihabitans*, and *P. plecoglossicida*. *P. aeruginosa* founds in hospital environments and is a common problem in this environment since it is the second most common causative agent of nosocomial infections. This pathogenesis may in part be due to the metabolites secreted by *P. aeruginosa*. The bacterium possesses a wide range of secretion systems, which secrets numerous proteins relevant to the pathogenesis of clinical strains (1). The symptoms of such infections are generalized inflammation and septic. If such infection occurs in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment, causing cross-infections in hospitals and clinics (2). *P. aeruginosa* has become an important agent of infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen extracted from patients who have been hospitalized longer than 1 week. It is a frequent cause of nosocomial infections such as urinary tract infections (UTIs), pneumonia, and bacteremia (3). *P. aeruginosa* is an opportunistic pathogen. It rarely causes disease in healthy persons. In most cases of infection, the integrity of a physical barrier to infection (eg. mucous membrane, skin) is lost or an underlying immune deficiency (eg, neutropenia, immunosuppression) is present (4). Antibiotics are traditionally defined as natural compounds, produced by microorganisms, with selective antibacterial activity that does not have any strong effects on human cells. Their mechanism of action is either through killing the bacteria (bactericidal effect) or by inhibiting bacterial growth (bacteriostatic effect). With the advent of synthetic antibacterial drugs the term antimicrobial agents was initially used to include both synthetic and natural compounds, but as the concept of antibiotics had already become so well established this term took over and is now generally used to include all antibacterial agents (5). *P. aeruginosa* is a very relevant opportunistic pathogen. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility. This low susceptibility is count to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (e.g., mexAB, mexXY etc.) and the low permeability of the bacterial cellular envelopes (6). *P. aeruginosa* producing MBLs were first reported from Japan in (1991), and since then has been described from various parts of the world including Asia, Europe, Australia, South America, and North America (7). MBLs belong to Ambler class B and have the ability to hydrolyze a wide variety of β-lactam agents, such as penicillins, cephalosporins and carbapenems, and consist of five groups of enzymes namely IMP, VIM, SPM, GIM and SIM. These enzymes require zinc for their catalytic activity and are inhibited by metal chelators such as EDTA and thiol-based compounds (8).

The present study is carried out to achieve the following objectives:

1. Isolation of *P. aeruginosa* from different sites of infection.
2. Identification of *P. aeruginosa* by microbial, biochemical and VITEK$_2$ system.
3. Phenotypic detection of some antibiotics.
4. Molecular detection for MBL antibiotic genes.

MATERIALS AND METHODS:

Patients and study design: Cross sectional study, this study included (100) patients (aged 5 year-70 years), the specimens were collected from different sits of infections burns, wounds, urine, seputum, ear swab, blood from patients who were visit All-Hilla teaching hospital laboratory in AL-Hilla city at the period from November (2017) to February (2018).

Ethical Approval: A valid consent was achieved from each patients before their inclusion in the study.

Identification of *P. aeruginosa*:

1. **Microscopic Properties**:
2. Gram's stain was used to examine the bacteria for studying the microscopic properties as initial identification of *P. aeruginosa* (9).
3. **Cultural Characteristics:** Morphological colonies characteristics were recorded on the specific media for primary identification of *P. aeruginosa* (10).
4. **Biochemical tests** of *P. aeruginosa* :were done by 1-2 colony tested for oxidase, catalase, Simmone Citrate, Kligler Iron agar and Indole tests and these entire tests positive (9).
5. **Definitive Identification by VITEK$_2$ – Compact:** using the identification of *Pseudomonas aeruginosa* was confirmed using of VITEK$_2$-Compact, which represent an advanced

colorimetric technology for bacterial identification; gram-negative (GN) card was used for this purpose for *Pseudomonas aeruginosa* Identification. All the following steps were done according to the manufactures instructions (Biomerieux, France). Three ml of normal saline was placed in plane test tube and inoculated with a lope full of isolated colony. The test tube was inserted into the Dens Check machine for standardization of the colony to McFarland standard solution ($1.5X10^{8}$cfu/ml). The standardized inoculum was placed into the cassette and a sample identification number entered into the computer software via barcode. The VITEK-$_2$ card type was then read from barcode placed on the card during manufacture and the card was thus connected to the sample identification port. The cassette was placed in the filter module, when the card were filled, transferred the cassette to the reader/ incubator module. All the subsequent steps were handled by the instruments, the instrument controls the incubation temperature, the optical reading of the cards and continuously monitors and transfers test data to the computer for analysis. When the test cycle was completed, the system automatically ejected the cards into a waste container.

Antibiotics Susceptibility Test: Disk Diffusion Test (DDT): It was performed by using a pure culture of previously identified bacterial organism. The inoculum to be used in this test was prepared by adding growth from (5) isolated colonies grown on blood agar plates to (5 ml) of nutrient broth; this culture was then incubated for (2) hours to produce a bacterial suspension of moderate turbidity that compared with turbidity of ready-made (0.5) McFarland tube standard. A sterile swab was used to obtain an inoculum from the standardized culture; this inoculum was then swabbed on Muller–Hinton agar plate. The antibiotic discs were placed on the surface of the medium at evenly spared intervals with flamed forceps, then incubated at (37ºC) for a full (18) hours before reading the results to identify cells expressing hetero-resistance. Antibiotics inhibition zones were measured using a transparence ruler. Zone size was compared to standard zones to determine the susceptibility of organism to each antibiotics (11).

DNA extraction form bacterial culture: Genomic DNA was extracted from the *Pseudomonas aeruginosa* isolates according to instruction provided by manufacturer using Genomic DNA purification kit supplemented by (Geneaid, USA). The viewed using UV-trans illuminator**.** Bacterial cells (in the sediment) was collected from swab samples that diluted in phosphate buffer solution and transferred to a (1.5ml) micro centrifuge tube then centrifuged in high speed centrifuge at (14000-16000) rpm for (1) minute then the supernatant discarded. Add (20µl) of proteinase k (make soured H_2O was added). Incubate at (60ºC) for (10) minutes and inverted every (3) minutes through incubation periods. GB buffer (200µl) were added to each tube and mixed by shaking vigorously for (5) seconds. Then the tubes were incubated in water bath at (60ºC) for (10) minutes and inverted every (3) minutes through incubation periods.Meanwhile, Elution buffer (200µl per sample) was pre-heated at 60°C (to be used in step 9 DNA Elution). Absolute ethanol (200µl) were added to the clear lysate and immediately mixed by shaking vigorously, and then precipitates broke it up by pipetting. A GD column was placed in a (2ml) collection tube and transferred all of the mixture (including any precipitate) to the GD column. Then centrifuged at (14,000-16,000) rpm for (2) minutes. In addition, the (2ml) collection tube containing the flow-through were discarded and placed the GD column in a new (2ml) collection tube. W1 buffer (400µl) were added to the GD column, then centrifuge at (14,000-16,000) rpm for (30) seconds. The flow-through was discarded and placed the GD column back in the (2ml) collection tube. Wash Buffer (ethanol was added) (600µl) were added to the GD column. Then centrifuged at (14,000-16,000) rpm for 30 seconds. The flow-through was discarded and placed the GD column back in the (2ml) collection tube. All the tubes were centrifuged again for (3) minutes at 14,000-16,000 rpm to dry the column matrix. The dried GD column was transferred to a clean (1.5ml) micro centrifuge tube and (150µl) of pre-heated elution buffer were added to the center of the column matrix. The tubes were let stand for at least (3) minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at (14,000-16,000) rpm for (30) seconds to elute the purified DNA. The extracted DNA was stored at – (16°C).

Plasmid DNA Extraction: DNA preparation from bacterial cells was performed by salting out method (12) with some modification as following: Bacterial cell of (50ml) culture were precipitated by centrifugation (1000 rpm for 10 minutes). Rewashed (3) times in TE buffer, and then the pellet was suspended in (5ml) TE buffer. A volume of (600 µl) of (25%) SDS was added, mixed by inversion to the cell suspension and incubated for (5) minutes at (55oC). About (2ml) of (5M) NaCl solution was added to the lysate, mixed thoroughly by inversion and let to be cooled to (37oC).Then (5ml) of (phenol: chloroform: isoamylalcohol) (25:24:1 v/v) was added to the lysate and mixed by inversion for (30) minutes at (25ºC) and the spun by centrifuge (4500) rpm for (10) minutes. The aqueous phase was transferred to a fresh tube, which contain the nucleic acid then isopropanol (0.6 volume) was added to the extract and mixed by inversion, after (3) minutes DNA spooled on to a sealed pasture pipette. The DNA rinsed in (5ml) of (70%) ethanol, air dried, and dissolved in (300µl) TE buffer, and then DNA extract was kept at (-20ºC) until use.

RESULTS AND DISCUSSION:

Isolation and Identification of *Pseudomonas aeruginosa*: The present study included the collection of (100) samples from different clinical sites. Clinical samples were collected from patients who were visit All-Hilla teaching hospital laboratory in AL-Hilla city at the period from November (2017) to February (2018). Cultural, biochemical and VITEK$_2$ system were used for identification, and depending on the VITEK$_2$ system revealed that twenty one (21) *Pseudomonas aeruginosa* isolates were recovered. These results were shown in Table (1). These samples were recovered from patients their age ranged from (5-70) years and the males were more infected than females as shown in Figure (1). The Table (1) indicates that *P. aeruginosa* were isolated mainly from burn samples (42.8%), followed by wound (23.8%) and urine (14.2%), while ear swabs (9.5%), blood and seputum (4.7%). These results are in agreement with study done by Latif in Iraq who showed that *P. aeruginosa* were most common (44.4%) in burn infections, followed by (38.1%) otitis media, (16.6%) wounds and (6.6%) urinary tract infections, while *P. aeruginosa* cannot be isolated from eye infections (13). However, these results are different from the results obtained by (14) who found that most of *P. aeruginosa* isolates (80.6%) were recovered from burns. In northeastern Nigeria which found that significant proportion of isolates were recovered from wounds (39.6%), followed by ear (otitis media) (30.2%), and UTI (7.5%) (15). Al-Mamori, (16) who found that most isolates were obtained from burns (8.55%), wounds (3.95%), ear swabs (3.30%) and (1.97%) isolates from each urine and blood in Hilla city. Al-Derzi, (17) in the North of Iraq (Mosul and Duhok) revealed that, the most common *P. aeruginosa* isolates comes from purulent specimens collected from skin wounds and burns (44.4%) followed by

isolates from UTI (31.8%) and ear discharge specimens (12.4%). Also, the study of (18) in Kurdistan region of Iraq found highest percentage of *P. aeruginosa* was obtained from burn samples (%10.9) whereas the lowest percentage were obtained from otitis samples (%1.81). There are differences in the percentage of infections between our results and others, and the reasons for these variations in all studies may be due to the percentage of distribution of isolates, which varied according to the place of clinical samples collection, environmental factors, nutrition requirements and virulence factors (19). To initiate infection, *P. aeruginosa* usually requires a substantial break in the skin, which considered the first-line of defenses against microbes. Such a break can result from breach or bypass of normal cutaneous or mucosal barriers (e.g., trauma, surgery, serious burns). *P. aeruginosa* associated burn infections was common (20). *P. aeruginosa* is a common cause of infections in burns, established through colonization of the burn wound by the patient's own normal flora or from the environment. Patients with burns infected with *P. aeruginosa* have an increased mortality rate and longer hospital stays compared to non-infected patients. They also have an increased number of surgical procedures and higher associated antibiotic costs (21). *P. aeruginosa* isolates (21 isolates) were identified using traditional morphological and biochemical diagnostic tests according to the methods of (9). The results were show in Table (2). *P. aeruginosa* are gram-negative bacilli and the colonies on nutrient agar are approximately (2mm) in diameter with bluish green coloration and irregular, feathered edges (22). The conformational identification of *P. aeruginosa* was performed using $VITEK_2$ system ($VITEK-_2$ GN Kit). The percentage of (21) *P. aeruginosa* was (99 %).

Antibiotic Susceptibility Testing (AST): The antibiotic disc diffusion test was done using (7) different clinically important antibiotics This study showed that most of *P. aeruginosa* isolates were highly resistant against the antibiotics used particularly the β-lactams, such as Amoxicillin 21(100%), Cefixime 20(95.2%), Piperacillin 19(90.4%), Ceftriaxone 18(85.7%), Cefotaxime 17(81%), Meropenem 16(76.1%) and Imipenem 16(76.1%). The results were shown in shown in Table (3) and Figure (2). The high resistance level of the bacteria against these β-lactam antibiotics since most of resistant *P. aeruginosa* isolates were recovered from burned hospitalized patients, while the hospital environment is a focus of contamination with the resistant bacteria for the most using antimicrobial drugs. This result agreed with results of a local study conducted by (14) who found that the resistance level of *P. aeruginosa* against β-lactam antibiotic was increased. Ceftriaxone is a third generation cephalosporin used frequently for the treatment of infections caused by gram-negative bacilli. This study showed that the resistance rate against this antibiotic was (85.7%) and this result agreed to the result of (14) who found that the resistance of *P. aeruginosa* against ceftriaxone was (96%). However, these results disagreed with the results obtained by (23) who found that the percentage of resistance to Ceftriaxone was (34.6%), also disagreement with the result obtained by (24) regarding to Ceftriaxone as he found that the percentage of resistance was (55.9%). In present study most isolates also appear to have resistance (81%) to Cefotaxime, and the result in this study is in agreement with the result obtained by (25) that found (95%) of isolates were resistant to Cefotaxime. The resistances of *Ps. aeruginosa* isolates to Cephalosporin may be due to the synthesis of β-lactamase as well as loss of PBP by mutation confer resistance to this antibiotic. This study showed that resistance rate against Cefixime was (95.2%) this result similar to the results obtained by (26) who showed all *P. aeruginosa* isolates resist Cefixime in the percent (100%); whereas (27) showed *P. aeruginosa* isolates resist Cefixime in the percent (80%). *P. aeruginosa* characterized by inherent resistances to a wide variety of antimicrobials. Its intrinsic resistance to many antimicrobial agents and its ability to develop multidrug resistance and mutational acquired resistance to antibiotics through chromosomal mutations imposes a serious therapeutic problem (28, 29 and 30). In present study, all isolates also appear to have resistance (100%) to Amoxicillin the result in this study is in agreement with the result obtained by (31) who found that (91%) of the isolates of *P. aeruginosa* are resistant to Amoxicillin. Also, agreed the result obtained by (32) found that all isolates of *P. aeruginosa* are resistant to Penicillin and Amoxicillin. This study showed that resistance rate against piperacillin was (90.4%), this result similar to the results obtained by (14) who found that the resistance to the antibiotic was (88%). The *P. aeruginosa* naturally resistant against penicillins such as piperacillin (33). AmpC cephalosporinase is characteristically chromosomally encoded in *P. aeruginosa*. Stably depressed mutants that hyperactive produce the AmpCβ-lactamase may lead to resistance to ticarcillin, piperacillin, and third-generation cephalosporins (34). The higher resistance level of *P. aeruginosa* against the cephalosporins may be due to increase usage of these antibiotics and the bad usage of these antibiotics help the bacteria to develop several resistance mechanisms one of them is the production of β-lactamase enzymes that destroy these antibiotics (35). This study showed that *P. aeruginosa* isolates have (76.1%) against the carbapenems (imipenem and meropenem). Carbapenem consumption was found to be significantly associated with carbapenem resistance rate (36). However the resistance level of *P. aeruginosa* against carbapenems in this study disagreed with the result of (23) who found that the resistance rate for imipenem and Meropenem was (3.85%), (7.7%) respectively and agree with the result of (24) who found that all *P. aeruginosa* isolates were susceptible to imipenem (100%). In various studies around the world, varying resistance (2-60%) has been seen toward imipenem (37). (38) showed that about (56%) of *P. aeruginosa* isolates were susceptible to imipenem. Out of these, the resistant mechanism because of Carbapenem hydrolyzing enzymes is commonest and has gained much importance because of their horizontal spread to other Gram-negative bacteria by plasmid-mediated transfer (39). Some investigators have recommended that double-antibiotic therapy must be administered, in accordance with local susceptibility pattern, for skin and soft tissue infections (40, 41).

Table (1): Distribution of *Pseudomonas aeruginosa* isolates among clinical samples

Sources of isolates	No. of *P. aeruginosa* isolates	Isolates %
Burns	**9**	42.8%
Wound	**5**	23.8%
Urine	**3**	14.2%
Ear swab	**2**	9.5%
Blood	**1**	4.7%
Sputum	**1**	4.7%
Total number	**21**	100%

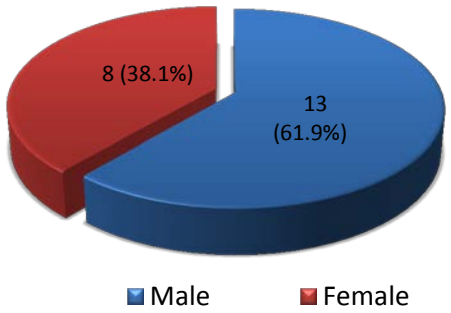

Figure (1): Distribution of infected patients according to gender

Table (2): Biochemical tests for Identification of *Pseudomonas aeruginosa*

Test	Gram stain	Indole	MR	VP	Citrate	Oxidase	Motility	Catalase	TSI Slant/ butt H2S Gas
Results	Gram -ve	-	+	-	+	+	+	+	K/K - -

(+) positive; (-) negative; (Alk) alkaline; (A) acid; (TSI) triple sugar iron; (MR) methyl red; (VP) voges-proskauer

Table (3): Number and percentage of resistant isolates against tested antibiotics using disc diffusion method

No.	Antibiotic discs	No. and % of resistant isolates
1.	Amoxicillin	21(100%)
2.	Cefixime	20(95.2%)
3.	Piperacillin	19(90.4%)
4.	Ceftriaxone	18(85.7%)
5.	Cefotaxime	17(81%)
6.	Meropenem	16(76.1%)
7.	Imipenem	16(76.1%)

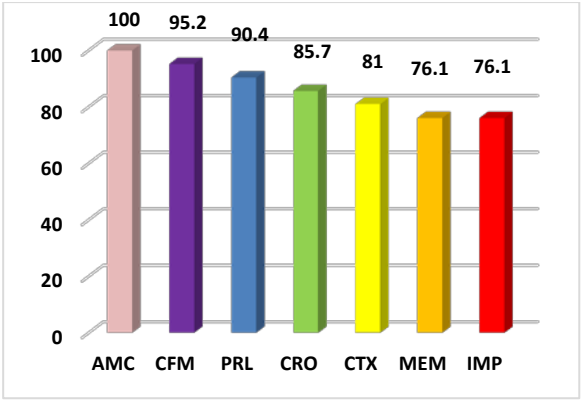

Figure (2): Percentage of resistant *P. aeruginosa* isolates against β-lactam antibiotics

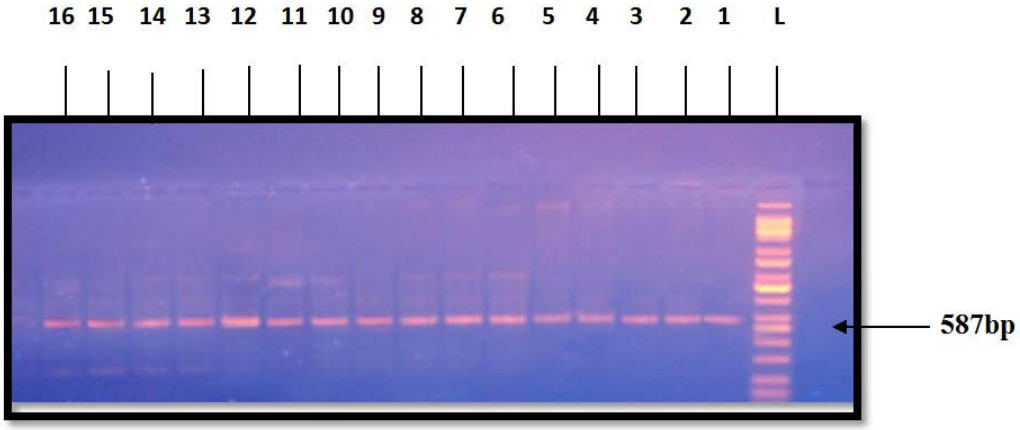

Figure (3): Ethidium bromide stained agarose gel showing PCR amplification products with *bla* IMP gene (587bp) primers for *P. aeruginosa* extracted DNA
L: ladder, (1, 2, 3,……16) samples of *P. aeruginosa* extracted DNA

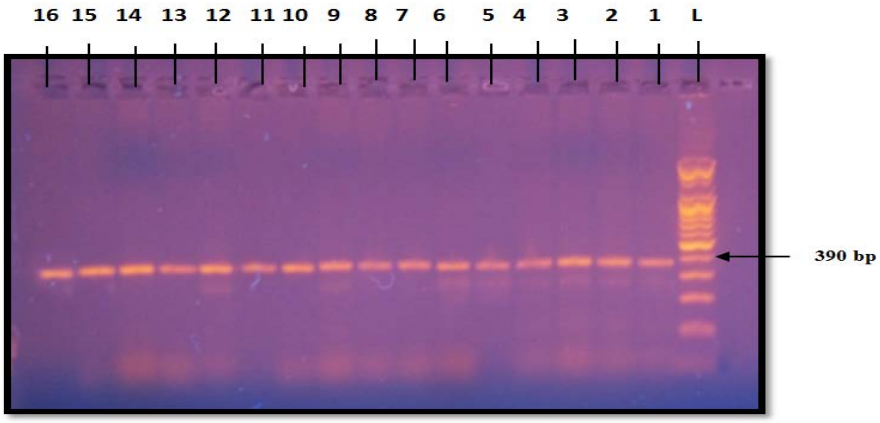

Figure (4): Ethidium bromide stained agarose gel showing PCR amplification products with *bla*VIM gene (390 bp) primers for *P. aeruginosa* extracted DNA
L: ladder, (1, 2, 3,……16) samples of *P. aeruginosa* extracted DNA

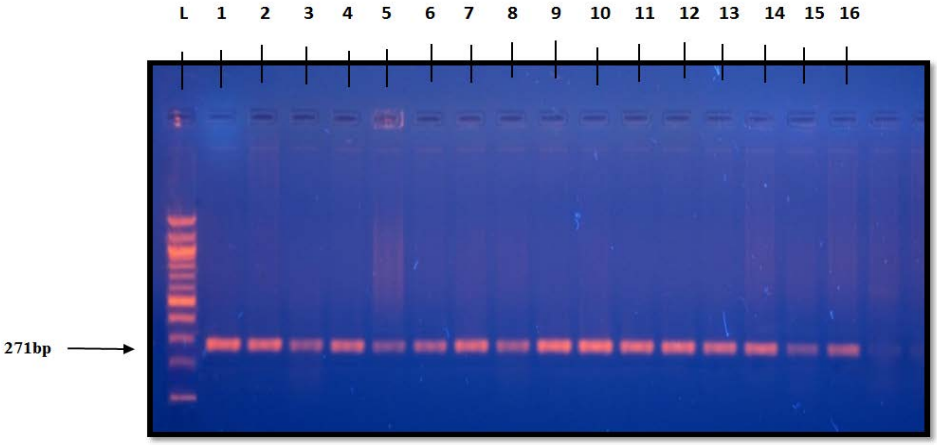

Figure (5): Ethidium bromide stained agarose gel showing PCR amplification products with *bla* SPM gene (271bp) primers for *P. aeruginosa* extracted DNA
L: ladder, (1, 2, 3,……16) samples of *P. aeruginosa* extracted DN

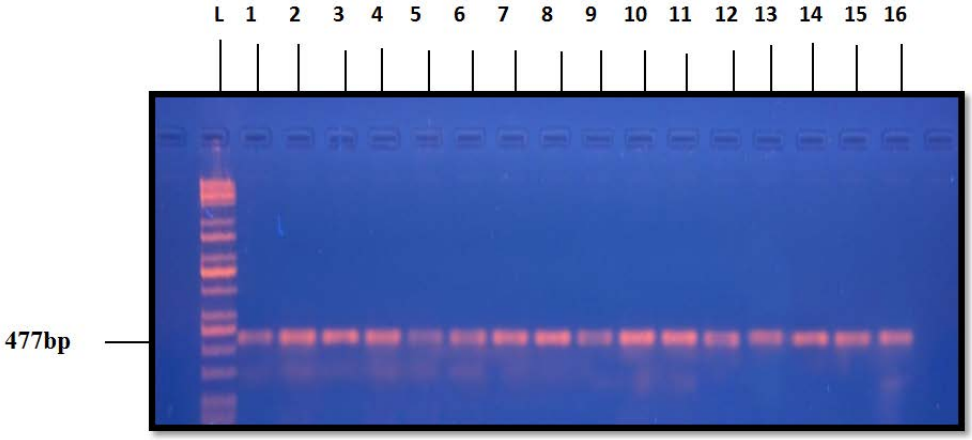

Figure (6): Ethidium bromide stained agarose gel showing PCR amplification products with *blaGIM* gene (477bp) primers for *P. aeruginosa* extracted DNA.
L: ladder, (1, 2, 3,……16) samples of *P. aeruginosa* extracted DNA

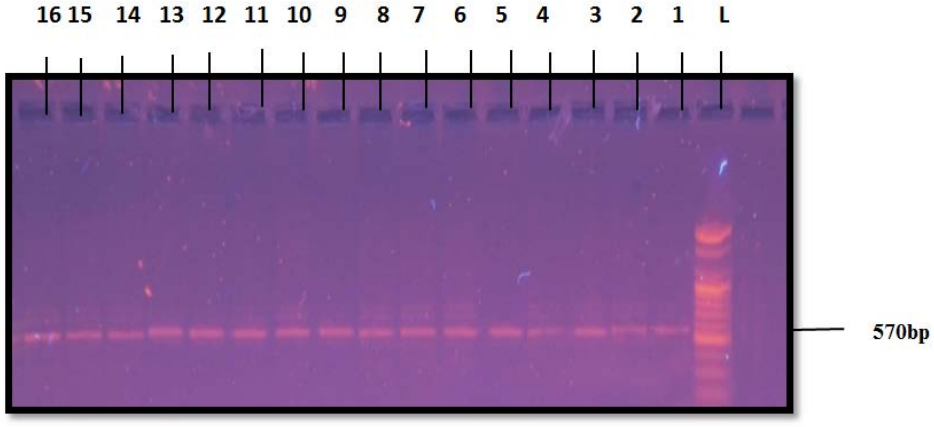

Figure (7): Ethidium bromide stained agarose gel showing PCR amplification products with *bla*SIM gene (570bp) primers for *P. aeruginosa* extracted DNA
L: ladder, (1, 2, 3,……16) samples of *P. aeruginosa* extracted DNA

Molecular detection of metallo-β-lactamase genes among *Pseudomonas aeruginosa* Isolates: All *Pseudomonas aeruginosa* isolates were further investigated for the presence of chromosomal or plasmid mediated Metallo-β-lactamases. The presence of MBL genes that include *bla*IMP, *bla*VIM, *bla*SPM, *bla*SIM, *bla*GIM was detected by conventional PCR technique. Metallo-β-lactamase (MBL) producing Gram-negative bacteria are an increasing public health problem worldwide because of their resistance to all β-lactams except aztreonam (42). MBL genes are often located in mobile or mobilizable genetic elements (plasmids, insertion sequences and integrons), which can contribute to the acquisition of new resistance mechanisms, and may increase the

plasticity of the *P. aeruginosa* genome and improve the environmental adaptation of *P. aeruginosa*. MBLs are also commonly associated with multidrug- resistant epidemic high-risk clones (43, 44). The most common transferable MBL families include the IMP, VIM, SPM, GIM, and SIM-type enzymes, which have been detected primarily in *P. aeruginosa* but were also found in other gram-negative bacteria, including non-fermenters and members of the family enterobacteriaceae (45). The most common and widespread acquired MBLs are those of the IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known. Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antibiotic class (46). Members of SPM, GIM, and SIM are restricted to certain geographical regions (45).

***bla*IMP gene:** The genomic *bla*IMP was detected in 16(76.1%) isolates. These results achieved using specific blaIMP gene primers. The results were shown in Figure (3). The IMP enzymes were originally detected in Asia, but later appeared in Europe, Americas and in Australia (47). The genes encoding MBLs are in nearly all cases located on class 1 integrons, although class 3 integrons harbouring IMP-type enzymes have been reported. Integrons are genetic elements consisting of two conserved regions (5'CS and 3'CS), as well as a variable region where gene cassettes encoding resistance determinants can be inserted (48). Earlier studies showed that *bla*IMP-1 was found on conjugative plasmids. Repeated attempts to detect *bla*IMP-1 on plasmids using a conjugation experiment were unsuccessful. Rather, fragments of genomic DNA separated by PFGE hybridized with a gene probe for *bla*IMP-1, suggesting a chromosomal location. However, we cannot exclude the possible carriage of the *bla*IMP-1 determinant by a large plasmid that may be strongly associated with the chromosome (49). Moreover, observation obtained by PCR analyses suggested that several metallo-β-lactamase gene-positive *P. aeruginosa* isolates also had the integrase gene and/or the aac (6')-Ib gene. From these findings, it is possible that the *bla*IMP-like genes of P. *aeruginosa* have been trans located into various plasmids or chromosomes with the help of an integron or similar element and disseminated among the *P. aeruginosa* and other Gram-negative bacteria (50).

***bla*VIM gene:** In this study pointed out that 16(76.1%) *bla*VIM gene was detected in a these isolates. These results achieved using specific *bla*IMP gene primers. The results were shown in Figure (4). VIM β-lactamases are classified into three subgroups, VIM-1(VIM-1, -4, -5, -11A), VIM-2 (VIM-2, -3, -6, -8, -9, -10, -11B) and VIM-7. The VIM enzymes were first reported in Europe. However, shortly thereafter emerged on the other continents (47). The *bla*VIM determinants have rapidly established a condition of high-level endemicity in Europe. Even the large outbreak reported in Greece was caused by a single clone and was apparently confined to the hospital wards (51). As found for *bla*IMP genes, the *bla*VIM-1 gene was also integrated as a gene cassette into a class 1 integron (52). Worldwide, VIM-2 is the dominant MBL gene associated with nosocomial outbreaks due to MBL producing *P. aeruginosa* (53). The MBLs have been identified from clinical isolates worldwide with increasing frequency over the past few years, and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections (54). The occurrence of an MBL-positive isolate in a hospital setting poses a therapeutic problem, as well as a serious concern for infection control management. The accurate identification and reporting of MBL-producing *P. aeruginosa* will aid in infection control practitioners and preventing the spread of these multidrug-resistant isolates (55).

***bla*SPM gene:** The *bla*SPM-1 gene was isolated from Sao Paulo, Brazil in (1997). On comparison with the sequence of other MBLs, maximum identity was seen with IMP1 enzyme (56). Gene encoding SPM-1 Metallo-β-lactamase was commonly found on the plasmid of several members of *P. aeruginosa*. However, it was widely disseminated among Brazilian hospitals (57). In this study pointed out that 16(76.1%) *bla*SPM-1 gene was detected in all isolates. These results achieved using specific *bla*IMP gene primers as shown in Figure (5). Sao Paulo Metallo-β-lactamase (SPM-1)was the most prevalent Brazilian Metallo-β-lactamase, as this enzyme was originally described in Sao Paulo, and its gene was detected in plasmids and not carried out by integrons, allowing a more effective horizontal dissemination (57). SPM-1 clonal dissemination is present in distinct Brazilian regions but is still restricted to Brazil. Recently, the SPM-1 producing isolate in Europe was described, but the patient had previously been in a Brazilian hospital for medical assistance (58). In a previous study, (59) applied scheme for determining the epidemiology of colistin-only-sensitive (COS) Brazilian. *P. aeruginosa* clone harboring *bla*SPM-1, and it was demonstrated that this clone had already been circulating outside Brazil before the recent isolate identification in Europe. The gene encoding SPM-1 enzyme, like both IMP and VIM, has been shown to be associated with a mobile genetic element and thus, may have originated in another bacterium (54).

***bla*GIM gene**: In this study, 16(76.1%) *bla*GIM gene was detected in all isolates. These results achieved using specific *bla*IMP gene primers as shown in Figure (6). GIM-1 (German imipenemase) is a carbapenemase that was first identified in five clonally related *Pseudomonas aeruginosa* clinical isolates recovered in North Rhine-Westfalia, Germany, In (2004) (60). The original report came from a hospital in Du¨ sseldorf, Germany, and most reports of GIM-1 have been from locations within a distance of 40 km. (60, 61). Five of GIM-1 producing *P. aeruginosa* isolates were then reported in the same region in (2012) (61). These isolates were shown to possess a novel class B β-lactamase designated, as GIM-1. The isolates were susceptible to polymyxin only. The amino acid sequence of GIM-1 displayed maximum identity with IMP-6, IMP-1 and IMP-4 isolates. Like majority of MBL genes, blaGIM-1 was also found on class 1 integron that is carried on a small plasmid of 45 kb (60). The *bla*GIM-1 gene was found to be located on plasmids, however, their transferability (either by conjugation or by transformation) has not been demonstrated. Whereas other MBL genes such as *bla*IMP, *bla*VIM, and *bla*NDM have been found in either *P. aeruginosa* or enterobacteriaceae, the occurrence of *bla*GIM-1 had only been described in *P. aeruginosa* and then recently in a single *Serratia marcescens* isolate (61).

***bla*SIM gene:** In this study, *bla*SIM gene was detected, and it was found that 16(76.1%) isolates have *bla*SIM gene. These results achieved using specific *bla*SIM gene primers. The results were shown in Figure (7). The *bla*SIM-1 gene was first discovered in *Acinetobacter pittii* from a Korean hospital in (2003), (62), and then found in two additional *Acinetobacter* species *Acinetobacter bereziniae* and *Acinetobacter nosocomialis* in Korea (63, 64). SIM-1 is a rarely detected member of the MBLs, and displays > (64%) amino acid sequence identity with IMPs, which are its closest relatives (62). These *bla*SIM-1 genes from Korea are mostly located on ~280-kb plasmids and on chromosomes on a few rare occasions (65). Class B metallo-β-lactamases (MBLs), such as IMPs, NDMs and SIM-1, are capable of hydrolysing almost all β-lactams including carbapenems but not monobactams, and they can be inactivated by metal chelators such as EDTA but not by sulbactam and tazobactam that are effective against class A serine β-lactamases (66). Transferable MBL genes have been extensively found in *Pseudomonas*, *Acinetobacter* and enterobacteriaceae, resulting in the global spread of broad-spectrum β-lactam resistance (67).

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