ORIGINAL ARTICLE

Detection of Extended Spectrum β-lactamases and Metallo β-lactamases in *Pseudomonas Aeruginosa* isolated from Burns

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ABSTRACT

Introduction: *P. aeruginosa* is one of the complex targets for antimicrobial chemotherapy. Also, it is intrinsically resistant to several antibiotics. It produces β-lactamases enzymes that are responsible for the widespread β-lactam antimicrobial resistance. There are three major groups of β-lactamase enzymes, MBLs and ESBLs forming *Pseudomonas* is a major issue for the treatment of burns victims. **Methods:** A total of 28 clinical isolates related to *P. aeruginosa* have been obtained from the burns specimens from patients attending to AL-Imam hospital/Baghdad-Iraq, through the period from October 2015 to March 2016. Also, all isolates have been recognized as *P. aeruginosa* via utilizing bacteriological assay and confirmed by Vitek 2. In addition, the susceptibility regarding *P. aeruginosa* isolates towards many antibiotics is identified detected. **Results:** it was found that the susceptibility regarding *P. aeruginosa* isolates towards ceftazidime and cefotaxime respectively is (75%) and (71.4%), while *P. aeruginosa* isolates' susceptibility towards imipenem was (67.9%). Extended-spectrum β-lactamases producing *Pseudomonas* was (30 %) while metallo β-lactamases producing *P. aeruginosa* was (78.9 %) by double-disk synergy test, in general, the percentage of *P. aeruginosa* producing ESBL and MBL was (11.1%). Production of EXBLs and MBLs was determined to be plasmid-mediated that could be eliminated by using UV light as a curing agent. **Conclusion:** The importance of MBL and ESBL forming *P. aeruginosa* as evidence of increasing resistance to the antimicrobial agent; especially penicillins and cephalosporins as a drug of choice, also it was noticed that *P. aeruginosa* have the ability to produce MBLs more than ESBL; and these enzymes producing genes are harbored on a plasmid that can be affected by curing chemical agent.

Keywords: Beta-lactamases, ESBL, MBLs, Pseudomonas aeruginosa, Curing plasmid.

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INTRODUCTION

P. aeruginosa is considered a free-living bacteria, majorly identified in the water and soil. It is a Gram-negative bacillus which is considered an important opportunistic pathogen for humans, animals, and plants (1,2,3). An important characteristic of *P. aeruginosa* is its capability to grow at high temperatures (40-41C) and its inability to grow at low temperatures (4C) (4).

Often, dangerous *P. aeruginosa* infections are considered nosocomial infections; fairly related to compromised host defenses, like severe burns, neutropenia, or cystic fibrosis. It has been found to cause a variety of infections in clinical practice /besides chronic cystic fibrosis, lung infections, urinary tract infections, corneal ulceration (caused by wearing contact lenses), endocarditis (caused by intravenous drug usage), and pneumonia (caused by the usage of the ventilator and endotracheal tube) (5,6). Burns is providing an adequate site for bacterial multiplications and have been a more resistant and rich infection source compared to surgical wounds, majorly due to the large area and long patient stay duration in hospitals (7). Also, infections were the main causes of mortality and morbidity in hospitalized burns patients; it is recently evaluated that 75% of mortalities after burns injuries were associated with infections instead of osmotic shock and hypopovolemia (2). The 2 major pathogens accountable for burn wound infections were *P. aeruginosa* and *S. aureus* (8). Infections are spread easily via bed sheets, contact with visitors and burn unit workers, bedsheets, clothes, dressing materials, and other equipment utilized for patient care, poor hygiene rules, decreases host defense, and unsuitable antibiotics use were the major significant causes for nosocomial

infection in burns unit (9).

P. aeruginosa is one of the complex targets for antimicrobial chemotherapies (10); it has intrinsic resistance to many antibiotics as a result of the low permeability regarding its outer membrane, the constitutive expression of many efflux pumps as well as the production of antimicrobial inactivation enzymes; also, *P. aeruginosa* is considered as one of the biofilm's forms which was a sessile bacterial growth related to many humans' infections (11, 12).

Along with the intrinsic resistance of *P. aeruginosa*, it is also producing β-lactamases enzymes accountable for the widespread β-lactam antimicrobial resistance. These enzymes hydrolyze the amide bond attached to β-lactam ring.(13,14).

There are three major groups of β-lactamases enzymes (A, B & C) (15, 16). Class A, ESBL. They are plasmid mediated enzymes, hydrolyze penicillins, extended cephalosporins; first, second, third, and 4th (cefotaxime, ceftriaxone, ceftazidime) monobactam (aztreonam) but cannot hydrolyze cephomycin). Class B they are carbenem hydrolyzing enzymes: Serin-β-lactamases & Metalo-β-lactamases contain metal ion, like zinc.

Finally, Class C, cephalosporinase (AmpC) are plasmid mediated with the ability to hydrolyze cephomycin (cefoxitin and cefotaten) as well as oxyimino-cephalosporins (cefotaxime and ceftazidime) and monobactams (aztreonam).

ESBLs & MBLs producing *Pseudomonas* pose a major problem for treating burns victims in addition to being resistant to other drugs classes, like cotrimoxazole, aminoglycoside, tetracyclins, and fluoroquinolones (1, 16); therefore, the carbapenem group regarding antimicrobial agents is of high importance in managing the hospital acquired Gram-negative bacterial infections due to their various stabilities and activities to hydrolysis through the majority of β-lactamases, involving ESBLs. In addition, the nosocomial outbreak related to carbapenem- resistance *P. aeruginosa* because of metallo-β-lactamases production (MBL) was indicated from many regions (17). Also, MBLs were class-B enzymes that hydrolyze carbapenem and were encoded through genes like the VIM, IMP, and so on. They were indicated as enzymes requiring divalent cations, typically zinc, as metal cofactors for their enzymatic activity. Recently, MBL genes were spread from *P. aeruginosa* to other Enterobacteriacae. Their nonstop spread is one of the main therapeutic challenges (18).

Plasmid-mediated antibiotic resistance is considered to be common in *P. aeruginosa*, and it is involved in bacterial multi-drug resistance. Also, MBLs and ESBLs production are one of the considerable problems in the clinical isolates regarding *P. aeruginosa*, while the organisms producing MBL or ESBL were also associated with the high morbidity and mortality. The problem of MBL production was increased with the increase in the production of ESBL in hospitals; MBLs and EXBLs genes were indicated to spread from *P. aeruginosa* to a few members regarding Enterobacteriaceae (19, 20). Therefore, this work aims to determine the incidence of MBL and ESBL between P. aeruginosa isolates from the specimens of burns, for helping in the formation of effective antimicrobial agents control strategy for preventing the outbreak regarding antimicrobial agents' resistant isolates.

MATERIALS AND METHODS

Samples

Between Oct 2015 to Mar 2016, 43 clinical specimens regarding burns have been collected from the patients in Al-Imam hospital/Baghdad- Iraq; then, all the specimens were transferred to the laboratory by using transport media for the identification and isolation of *Pseudomonas* via utilizing sterile equipment and media.

Isolation and Identification of Pseudomonas isolates

The specimens have been streaked on Mac Conkey, citrmide, and milk agar. All the plates have been aerobically incubated for 24 hrs at a temperature of 37 Celsius; following incubation, the produced isolates have been identified based on (21). Along with the morphological features on culture media; like non-lactose fermented growth on Mac Conkey agar, bacteria growth on citramide agar, and forming pigements on the milk agar, the results have been verified via the use of Vitek2 identification system depending on serial of bio-chemical tests that have been accomplished together via utilizing Vitek cards; the results are computerized as well as recorded through Vitek software following bacterial suspension loaded in Vitek cards.

Antimicrobial activity

a) Antimicrobial susceptibility test

Kirby-Bauer's approach is made based on (21) for carrying out the antimicrobial susceptibility test for sixteen antimicrobial agents.

Inhibition zones created around the antimicrobial dicks were evaluated via utilizing a metric ruler in mm, based on Clinical Lab Standards Institute (CLSI, 2011). Also, the isolate is interpreted as intermediate, susceptible, or resistant to specific antimicrobial agents through comparison to the standard inhibition zones.

b) Detection of Extended Spectrum β-Lactamases (ESBL)

The double-disk synergy test approach is utilized for detecting the ESBLs producing isolates (15, 16, 22, 23). For the purpose of examining the isolates capability for producing EXBLs, Amoxicillin/ Clavulanic acid (30μg)

disc has been placed in the middle of cultured Mueller Hinton agar plate with bacterial suspension that has been standardized for matching turbidity regarding 0.5 McFarland turbidity standard (1.5 x 108 CFU/ml), after that the discs of Ceftazidime, Cefotaxime, and Aztronam are arranged around Amoxicillin/ Clavulanic acid (30µg) disc within a distance between 2 and 3 cm. In addition, the plates are inverted and subjected to incubation at a temperature of 37°C for a period between 18 and 24 hrs. Following incubation, the synergism activities between central disk and any surrounding antimicrobial disc detects the ESBLs producing isolates.

c) Detection of Metallo β-Lactamases (MBLs)

The double-disk synergy test approach is utilized for detecting the MBLs producing isolates (23, 24).

The surface related to Mueller Hinton agar plates is spread into 4 directions through bacterial suspension that has been standardized for matching the 0.50 Mc Farland turbidity standard (1.50 x108CFU/ml); also, the plates are left to dry for 10 mins, after that, Imipenem (10µg) and Imipenem (10µg) + EDTA (which it prepared through dissolving 186.10g Na-EDTA in 1,000ml of DW, pH is adjusted to 8.0 through NaOH; also, the mix is sterilized through auto-claving, after that, 10 µl is added on imipenem disc); discs are added on plates within a distance of 2cm-3cm. After incubation of the plates have been at 37oC for 18hrs-24hrs., synergism activity between Imipenem disk and Imipenem+ EDTA was noted to detect the Metalo β-lactamases producing isolates.

Bacterial plasmid curing

In order to examine the existence of ESBLs & MBLs producing genes on a plasmid or chromosomal DNA in studied isolates; curing plasmid of two isolates of P. aeruginosa is performed (one was ESBLs producing isolates and the other was MBLs producing isolates) by exposure of the selected isolates to UV light, as a curing agent at wavelength 260 nm for ten minutes. Plasmid DNA of two tested isolates was extracted by using (pureyeild TM plasmid miniprep system, Promega, USA); the pattern and number of plasmids were examined by preformed gel electrophoresis (0.8% agarose).

RESULTS

Out of 43 specimens of burns, 28 isolates were identified as *P. aeruginosa* according to the bacteriological detection. The colonies have been recognized based on the observing characters, as follows: the presence of non – lactose fermenter isolates on MacConkey agar as a selective and differential media for identification and differentiation of *Enterobactereacae* in general, then subcultured 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*

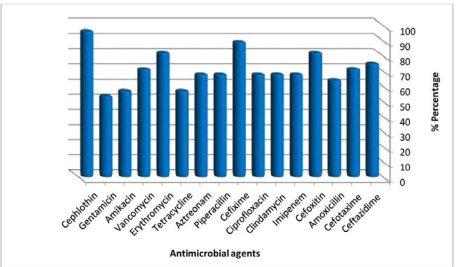

Figure 1: Susceptibility of P. aeruginosa isolates to the antimicrobial agents. The susceptibility of 28 *P. aeruginosa* isolates to 16 antimicrobial agents has been researched with the use of the Kirby-Bauer approach.

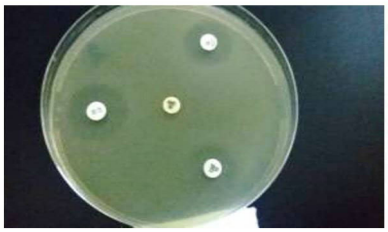

Figure 2: Non ESBL producing *P.aeruginosa*. has been detected by the double disk synergy method, a Clavulanic acid / Amoxicillin (30µg) disc has been put in the middle of the cultured Mueller Hinton agar plate with the bacterial suspension that has been standardized for the purpose of matching the 0.50 Mc Farland turbidity standard (1.50 x 108CFU/ml), after that, Aztronam, Cefotaxime, Ceftazidime discs have been arranged around Clavulanic acid / Amoxicillin (30µg) disc in a distance of 2cm-3cm. Following the incubation, the activity of the synergism between central disk and any surrounding anti-microbial disc has been noted for the detection of Extended Spectrum beta-lactamase producing isolate types.

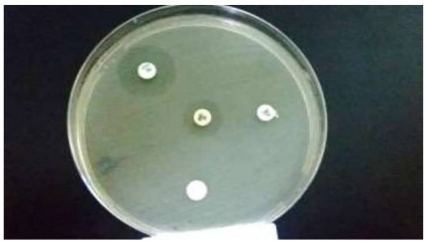

Figure 3: ESBL producing *P.aeruginosa* has been found by the double disk synergy method, a Clavulanic acid / Amoxicillin (30µg) disc has been put in middle of the cultured Mueller Hinton agar plate with the bacterial suspension that has been standardized for the purpose of matching the 0.50 Mc Farland turbidity standard (1.50 x 108CFU/ml), after that, Aztronam, Cefotaxime, Ceftazidime discs have been arranged around Clavulanic acid / Amoxicillin (30µg) disc in a distance of 2cm-3cm. Following the incubation, the activity of the synergism between central disk and any surrounding anti-microbial disc has been noted for the detection of Extended Spectrum beta-lactamase producing isolate types.

(25, 26, 27).

Vitek 2 identification system assay is utilized for identifying the collected isolates as *P. aeruginosa.*

The susceptibility regarding 28 *P. aeruginosa* isolates to 16 antimicrobial agents is examined via utilizing the Kirby-Bauer method (Figure 1).

The results showed that 21 (75%) isolates of *P. aeruginosa*

have been resistant to ceftazidime, (71.4%) resistant to cefotaxime, and the isolates resistant to imipenem was (67.9%).

All *P. aeruginosa* isolates in the presented work showed multi-drug resistance (100%); They have been divided to 3 groups, every one of the groups including five antimicrobial agents.

The ability of *P. aeruginosa* isolates resistant to cefotaxime because of producing ESBLs; a type of β-lactamases has been detected by double-disk synergy method, (figure 2, 3) by determining the increasing of inhibition zone against certain β-lactam the results showed that 6 (30 %) isolates were ESBL producers. The ability of isolates to resist imipenem that produces MBL enzymes, a type of β-lactamases, was detected by the imipenem-EDTA combined disk method (figure 4, 5), 15 (78.9 %) isolates were MBL producers. The most highly EXBLs & MBLs producing isolates were selected for plasmid curing. The results showed that the two isolates have two plasmids; The results of curing showed that one of them is almost eliminated after exposure to UV light as a curing agent at wavelength 260 nm for 10 minutes (figure 7), which was capable of suppressing development and spread of EXBLs & MBLs producing enzymes. The tested isolates lost the ability to produce Я- lactamases by showing negative results for phenotypic detection after curing, leading to consider that the EXBLs & MBLs harboring gene is located on plasmid DNA of local *P. aeruginosa.*

DISCUSSION

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 increased resistance towards antimicrobial agents' usage with as the time passes. The importance of these two antimicrobial agents belongs to the fact that they belong to the third generation of cephalosporins, which

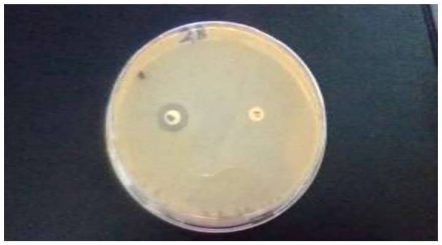

Figure 4:Non MBL producing *P.aeruginosa*. the approach of the double-disk synergy testing has been utilized for the detection. Mueller Hinton agar plate surfaces have been spread by bacterial suspension that has been standardized for the purpose of matching the 0.50 Mc Farland standard of turbidity (1.50 x 108CFU/ml), an Imipenem (10µg) and Imipenem (10µg) + EDTA discs have been added on plates within 2-3 cm distance. Following the incubation, the activity of the synergism between the Imipenem disk and Imipenem+ EDTA was noted to detect the Metalo β-lactamases generating isolates.

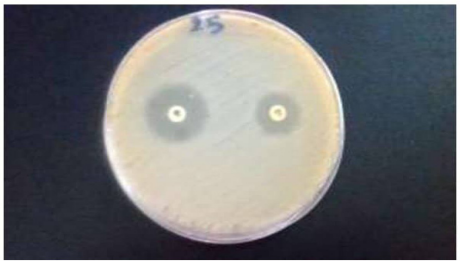

Figure 5:MBL producing P.aeruginosa. the approach of the double-disk synergy testing has been utilized for the detection. Mueller Hinton agar plate surfaces have been spread by bacterial suspension that has been standardized for the purpose of matching the 0.50 Mc Farland standard of turbidity (1.50 x 108CFU/ml), an Imipenem (10µg) and Imipenem (10µg) + EDTA discs have been added on plates within 2-3 cm distance. Following the incubation, the activity of the synergism between the Imipenem disk and Imipenem+ EDTA was noted to detect the Metalo β-lactamases generating isolates.

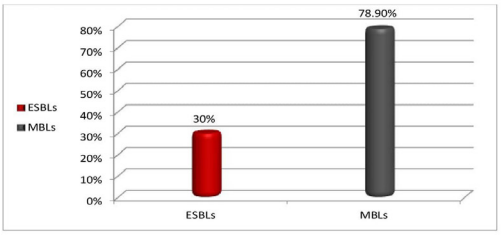

Figure 6: Percentage of ESBLs&MBLs Production in *P.aeruginosa* isolation.

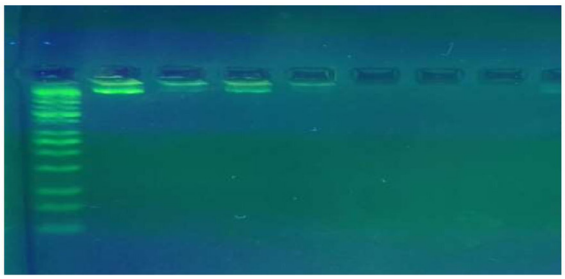

Figure7: pattern of plasmid was examined by preformed gel electrophoresis (0.8% agarose).
a- ESBLs producing *P. aeruginosa* before curing
b- ESBLs producing *P. aeruginosa* after curing
c- MBLs producing *P. aeruginosa* before curing
d- MBLs producing *P. aeruginosa* after curing

theoretically have wide activity against Gram negative bacterial infection. (14). While resistance to imipenem which is a drug of choice to β-lactamases producing *Pseudomonas* was moderate between all mentioned results.

In general, all antimicrobial agent results showed approximate results because of the capability of *P. aeruginosa* for resisting many chemotherapeutic agents. Cephlotine and Cefixime showed a high percentage of resistance, while the isolates show low resistance to gentamycin.

The phenomenon of multi-drug-resistant (MDR) (present of more than one antimicrobial agent resistant to the bacteria) is a serious problem facing the possibility of

treating bacterial infections and increasing mortality.

The capability regarding *P. aeruginosa* isolates resistance to cefotaxime because of producing ESBLs, a type of β-lactamases, is detected via the double-disk synergy method (figure 2, 3). The main risk factors for infection or colonization with the ESBL producing organisms were prolonged hospital stay, long-term antibiotic exposures, high rates of 3rd cephalosporin utilization, and invasive processes. Also, treatment of ESBL producing strains regarding Enterobacteriaceae was one of the main problems in the community and hospitalized patients (7).

The result of a high percentage of isolates that expressed MBLs might be because of the high rate of the antimicrobial agents' utilization, as well as capability of genetic elements, especially plasmids with different sizes that are carrying MBL β-lactamase genes of transferring, cloning, and conjugation.

The ability of isolates to resist imipenem that produces MBL enzymes, a type of β-lactamases, has been detected via the imipenem-EDTA combined disk method (figure 4, 5).

MBL can be defined as a group of carbapenem hydrolyzing β-lactamase. Also, MBLs were inhibited in-vitro through $FeCl_3$, EDTA, $CuCl_3$ and thiol compounds such as 2 mercaptopropionic acid, 2 mercaptoethanal, and sodium mercapto acetic acid, yet no via β-lactamase inhibitors such as sulbactam, tazobactam or Clavulanic acid. In addition, the detections of the production of the MBL in the MDR organisms from burn infections has massive therapeutic consequences since the options of the treatment for these isolates were aztreonam or possibly toxic polymyxin B and colistin. In this work, not every Gram-negative bacteria are tested for the production of the MBL; just these gram-negative bacilli that have resistance to the imipenem are screened for the production of the MBL (7).

The production of MBLs in *P. aeruginosa* is higher than their production of ESBLs (figure 6). This is because of the genetic structure and mobile genetic elements, such as a plasmid, that transfer the genes responsible for this type of resistance to antimicrobial agents. The results of curing showed that one of them is almost eliminated after exposure to UV light as a curing agent at wavelength 260 nm for 10 minutes (figure 7), which was capable of suppressing the development and spread of EXBLs & MBLs producing enzymes. The tested isolates lost the ability to produce Я- lactamases by showing negative results for phenotypic detection after curing, leading to consider that the EXBLs & MBLs harboring gene is located on plasmid DNA of local *P. aeruginosa.*

CONCLUSION

The conclusions of the present study are referred to the importance of ESBL and MBL producing *P. aeruginosa* as evidence of increasing resistance to the antimicrobial agent; especially penicillins and cephalosporins as a drug of choice, also it was noticed that *P. aeruiginosa* have the ability to produce MBLs more than ESBL; and these enzymes producing genes are harbored on a plasmid that can be affected by curing chemical agent.

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