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Detection and molecular characterization of Metallo beta-lactamases producing *Pseudomonas aeruginosa* isolated from burn patients

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Abstract

Due to drug resistance, *P. aeruginosa* becomes more contagious that able to cause more severe infection. Those patients with acute burn existing an immunosuppressive situation and, therefore, advanced defenselessness to diseases through nosocomial pathogens along with high mortality. The purpose of the research was isolation and identification of *P. aeruginosa* from burn samples and determined its antibiotic sensitivity pattern and then molecular identification of MBL genes using PCR. A total of 200 clinical samples were collected from burn patients of Allied Hospital Faisalabad. *P. aeruginosa* was isolated on cetrimide agar, and identification was made by gram staining and biochemical tests. All isolates checked the antibiotic sensitivity pattern. Phenotypic screening of Metallo Beta Lactamases (MBL) producing *P. aeruginosa* and genotypic detection of MBL genes were determined. Out of 200 samples, 46.5% were positive. All isolates exhibited high resistance against multiple antibiotics except colistin, polymyxin, and aztreonam. All of 7 strains were detected PCR positive for the *OprL gene* using specific primers. 35.48% isolates have shown MBLs production in the combined disk diffusion method. Phenotypically detected ten isolates positive for MBLs encoded genes as followed by blaNDM 5(50%) blaVIM 3(30%) blaIMP 2(20%) and blaSIM, blaDIM, blaSPM, blaGIM, and blaAIM were not detected. MBLs producing isolates such as *Pseudomonas aeruginosa* are a distressing threat in health care institutes and can be control through proper following the isolation and strict detection methods that will really helpfully in reduce serious infections and death rate among the hospitalized patients.

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Introduction

Nosocomial infections affect patients in hospitals or other health care facility. The leading nosocomial pathogens are *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Clostridium difficile*, *E. coli,* and *Acinetobacter spp*. They cause Urinary tract infections (UTIs), burn infections, respiratory infection, and bacteremia (Doyle *et al*., 2011). In Developed countries, the total prevalence of hospital-acquired diseases varies between 5.1 to 11.6 %, and in developing countries, it is 5.7 to 19.1 %. The maximum public bacterial reason to cause nosocomial infections 17–26% of burn wound infections is *P. aeruginosa* (Saaiq M *et al*., 2015).

In 1850 Se´dillot detected *P. aeruginosa* for the first time on surgical wound bandages. In 1860 Firdos done the pigment's obstruction, and Luke originate relationship between blue-green pigment and some rod-shaped organisms in 1862 (Lister *et al*., 2009). *P. aeruginosa* has various virulence factors classified as cell-associated factors and secretory virulence factors. Flagellum pilus and non-pilus adhesions and LPS (lipopolysaccharide) are cell-associated virulence factors, while exo-enzymes includes, protease, pyocyanin, hemolysins, exotoxin A elastase, phospholipase, siderophores, rhamnolipids are recognized as secretory virulence factors. (Church *et al*., 2006).

According to the National Center for Injury Prevention and Controls statistics, information from the United States displays that about 2 million fires are testified that consequence annually in 1.2 million injuries through burns (National Center for Injury Prevention and Control, 2002).

Roughly 100,000 of these reported cases need hospitalization, as well as about 5000 serious patients, expire annually from burn-associated difficulties (Eriksen *et al*., 2005).

The persistence frequency for severe burn patients has enhanced significantly in the previous years via developments in intensive care organizations in specific burn hubs. Moreover, in the middle of patients with severe burns finished over 40% of the (EBSA) entire body surface area, 75% of the losses are presently linked to sepsis from severe wound infection or other complications related infectious (Nanvazadeh *et al*., 2013). Serious infection may lead to bacteremia, in burn patients by Gram-negative bacteria that have been stated to be accompanying with predicted mortality 50% for patients with bacteremia compared with those deprived of having it. Burns is only the extreme public and distressing kind of serious physical injury (trauma) which is triggered by severe thermal damage to soft and skin tissue.

Metallo-beta-lactamases (MBLs) producing *Pseudomonas aeruginosa* are categorized into different types which are based on different amino acid sequences including AIM, DIM, FIM, GIM, IMP, KHM, NAM, SBM, SIM, SPM, TMBs, and VIMs (Pollini *et al*., 2013).The most common categories of MBLs encoded genes identified in family Enterobacteriaceae particularly in *Pseudomonas aeruginosa* include the IMP and VIM, along with the evolving NDM group (Walsh *et al*., 2005).

Commonly, the resistance of carbapenem detected for MBL-producing strains varies, with high-frequency mortality linked with MBL production, which varies from 18% to 67%. The purpose of this research was isolation and identification of *Pseudomonas aeruginosa* from clinical samples, to check antibiotic sensitivity pattern by Kirby-Bauer test (Disk diffusion method) and then molecular identification of MBL genes using PCR.

Materials and methods

Sample collection

200 clinical samples were collected from patients in the burn ward by using sterilized swabs from Allied hospitals Faisalabad. Then samples were delivered to the postgraduate research laboratory Institute of Microbiology University of Agriculture Faisalabad as soon as possible through Amie's Transport Medium Agar.

Isolation and identification of P. aeruginosa

P. aeruginosa was isolated through culturing on Pseudomonas Cetrimide agar and selective medium *Pseudomonas* agar by using the streak plate method. Standard procedures checked morphological characterization such as shape, Gram reaction, colony morphology, motility. Different biochemical tests were performed according to standard procedures, like Citrate, Catalase, Oxidase, Methyl Red, and Indole. To check the motility of bacteria, wet preparations were used. Motility testing supports the detection of *P. aeruginosa* (Jensen *et al.*, 2001).

Antibiotic sensitivity testing

Antibiotic sensitivity pattern was checked of all *P. aeruginosa* isolates according to the CLSI guidelines. Nutrient broth was used to grow *P. aeruginosa* isolates and turbidity was adjusted to 0.5 McFarland standards. Mueller Hinton agar plates were used for antibiotic sensitivity testing.

The Mueller Hinton agar plates were taken, and a suspension containing *Pseudomonas aeruginosa* was spread. Anti-biotic disks were placed as ceftriaxone (30-µg), amikacin (30-µg) ciprofloxacin (5-µg). Piperacillin (100-µg), imipenem (100-µg), meropenem (10-µg), nalidixic acid (30-µg), norfloxacin (10-µg), tetracycline (30-µg), ceftazidime (30-µg), ceftriaxone (30-µg), cefotaxime (30-µg), aztreonam (30-µg), gentamicin (100-µg). Incubation was given to plates for 24 hours at 37˚C. Then comparison was done between zone of inhibitions and CLSI scale after measuring the zone of inhibition (Beige *et al.*, 2015).

DNA extraction (Phenol and chloroform Method)

This practice is most frequently used for purifying DNA from any samples. First of all, DNA was extracted through a phenol, chloroform, and isoamyl alcohol mixture to eliminate protein impurities, and precipitated by using ethanol 100%. Afterward, the precipitation step, ethanol 70% was used to wash DNA by eradicating small organic molecules and salts, and for more experimentation suspended in the buffer. Although this is an inexpensive and proficient method (Chomczynski and Sacchi, 2006).

PCR (Polymerase chain reaction)

Master Mix 250 unit kit (Qiagen) and Master Cycler Gradient PCR were used to amplifying the DNA. Primers listed in (Table 1) along with base-pair size (Matthijs *et al.*, 2013).

Phenotypic screening of MBL producing Pseudomonas aeruginosa

Combine disk diffusion Test (CDDT)

Taken samples of wound swab from patients admitted in tertiary care hospital Faisalabad. MBL detection methods EDTA impregnated with imipenem, meropenem, and clavulanic acid and without EDTA Imipenem, meropenem and clavulanic acid were followed in combined disk diffusion test (CDDT) to detect MBL producers. Standard distance and EDTA Concentration in CDDT, it was seen that between 1cm, 1.5cm, 2cm, 2.5cm and 3cm distances, 1.5cm distance gives a clear outcome. For CDDT, 10µl of 0.5 M EDTA solutions provided a clear extended zone of inhibition.

It is indicated that there was MBL producer isolates *Pseudomonas aeruginosa* occurs (Yong *et al.*, 2012). If inhibition zone diameter ≥7 mm between of the antibiotic EDTA combined disk and the only disk was considered to be a positive for the existence of MBLs (Khosravi *et al.*, 2012).

Genotypic detection of MBL genes

Pseudomonas aeruginosa which were isolated and showed resistance against imipenem and meropenem, tested for MBL production. These are metallo-β-Lactamase class B genes encoded for the (blaDIM, blaNDM, blaAIM, blaIMP, blaVIM, blaSPM, blaGIM, and blaSIM) were used to determine from genomic DNA via gene specific primers by PCR amplification. Phenotypically confirmed MBL producing isolates of *P. aeruginosa* were tested for genes encoding (blaDIM, blaSIM, blaNDM, blaIMP, blaVIM, blaSPM, blaGIM, and blaAIM) by PCR using the primers listed in (Table 2) along with base pair size (Fallah *et al.*, 2013).

PCR (Polymerase chain reaction)

The amplification was done by using a Master cycler Gradient PCR (Eppendroff) and Master Mix 250-unit kit (Qiagen).

Agarose gel electrophoresis

For purity and quality of Genomic DNA was checked by gel electrophoresis. For this purpose agarose gel 0.8 %(w/v) was prepared in buffer 0.5X TAE (pH 8.0) (Fallah, F *et al.*, 2013) and after that added an Ethidium bromide about to 10 µl. Now about to 5µl DNA sample along with gel 1µl loading stain was mixed thoroughly and loaded in gel. The gel was run in Electrophoresis for 30 minutes at 80V after that DNA was visualized in the gel.

Results

The occurrence of Pseudomonas aeruginosa

Overall, 200 clinical samples were collected from the Allied Hospital Faisalabad. Out of two hundred samples, 93 (46.5%) were positive and 107 (53.5%) were negative for the existence of *Pseudomonas aeruginosa* (Fig. 1).

Colony morphology and microscopic characteristics

Pseudomonas aeruginosa was observed on *Pseudomonas* agar that shows smooth round colonies with the production of bluish-green pigment diffusing into the agar and fruity aroma (Fig. 2).

Gram-negative short rods that presented positive motility tests underneath the microscope were observed. Results of biochemical tests were shown that oxidase, along with catalase and citrate, were positive, but methyl red and indole tests were negative.

After 24 hours of incubation on triple sugar iron agar classic metallic shine with not any color change was examine that shown no sugars fermentation in existing medium without any acid and alkali production.

Table 1. List of primer sequences.

Gene type	Primer sequences5´-sequence-3´	Reference	Base pair
OprI	F ATGAACAACGTTCTGAAATTC TCTGCT R CTTGCGGCTGGCTTTTTCCAG	[12]	249
OprL	F ATGGAAATGCTGAAATTCGGC R CTTCTTCAGCTCGACGCGACG	[12]	504

Antibiotic susceptibility testing

All ninety-three (93) isolates of *Pseudomonas aeruginosa* were tested for antibiotic susceptibility against some common anti-pseudomonas antibiotics. All isolates exhibited high resistance against multiple antibiotics except colistin, polymyxin and aztreonam.

The antibiotic susceptibility testing showed high susceptibility against polymyxin B and colistin with (89.25%) and (83.88%), respectively.

The highest resistance was observed against ceftriaxone (86.02%), levofloxacin (84.74%), imipenem (81.72%), meropenem (76.34%), amikacin (73.15%), gentamicin (70.96%), ciprofloxacin (69.89) % and aztreonam (68.61%) (Table 3).

Molecular (PCR) detection of Pseudomonas aeruginosa

All of seven (7) morphologically confirmed isolates of *Pseudomonas aeruginosa* were detected PCR positive for OprL gene of *Pseudomonas aeruginosa* by using specific primers as shown in (Fig. 3).

Phenotypic detection of MBLs in Pseudomonas aeruginosa

Thirty-three (33) (35.48%) out ninety-three (93) (46.5%) isolates of *Pseudomonas aeruginosa* have shown MBLs production in the combined disk diffusion method.

Table 2. MBL genes with primer sequences.

Gene type	Primer sequences-5´-sequence-3´	Reference	Base pair
blaSIM	F 5-TACAAGGGATTCGGCATCG-3 R 5-TAATGGCCTGTTCCCATGTG-3	[29]	570
blaDIM	F 5-GCTTGTCTTCGCTTGCTAACG-3 R 5-CGTTCGGCTGGATTGATTTG -3	[29]	699
blaNDM	F 5-GGTTTGGCGATCTGGTTTTC-3 R 5 - CGGAATGGCTCATCACGATC-3	[29]	621
blaIMP	F 5-GAA GGY GTT TAT GTT CAT AC-3 R 5- GTA MGT TTC AAG AGT GAT GC-3	[29]	587
blaVIM	F 5-GTT TGG TCG CAT ATC GCA AC-3 R 5-AAT GCG CAG CAC CAG GAT AG-3	[29]	382
BlaSPM	F 5-AAAATCTGGGTACGCAAACG-3 R 5- ACATTATCCGCTGGAACAGG-	[29]	271
BlaGIM	F 5-TCGACACACCTTGGTCTGAA-3 R 5-AACTTCCAACTTTGCCATGC-3	[29]	477
blaAIM	F 5-CTGAAGGTGTACGGAAACAC-3 R 5-GTTCGGCCACCTCGAATTG-3	[13]	322

The zone of inhibitions was observed ≥7 mm increase in diameter for imipenem, meropenem, and clavulanic acid combined with EDTA, meropenem and compared with without EDTA disks.

Genotypic detection of MBLs genes (blaDIM, blaSIM, blaNDM, blaIMP, blaVIM, blaSPM, blaGIM, and blaAIM) genes in Pseudomonas aeruginosa

Table 3. Antimicrobial resistance pattern of *Pseudomonas aeruginosa*.

Antimicrobial agents (ug)	Susceptible isolates (%)	Resistant isolates (%)
imipenem (10 µg)	17 (18.28%)	76 (81.72%)
ciprofloxacin (5 µg)	28 (30.11%)	65 (69.89%)
Meropenem (10 µg)	22 (23.66%)	71 (76.34%)
norfloxacin (10 µg)	25 (26.88%)	68 (73.11%)
ceftazidime (30 µg)	33 (35.49%)	60 (64.51%)
ceftriaxone (30 µg)	13 (13.98%)	80 (86.02%)
Cefotaxime (30 µg)	20 (21.50%)	73 (78.50%)
aztreonam (30 µg)	29 (31.19%)	64 (68.81%)
gentamicin (10 µg)	27 (29.04%)	66 (70.96%)
Amikacin (30 µg)	68 (73.15%)	25 (26.88%)
Piperacillin-tazobactam(100 µg)	65 (69.89%)	28 (30.10%)
Colistin (10 µg)	78 (83.88%)	15 (16.12%)
Polymyxin B(100 µg)	83 (89.25%)	10 (10.75%)
Piperacillin (30 µg)	35 (37.64%)	58 (62.36%)
Cefepime (30 µg)	31 (33.34%)	62 (66.66%)
Levofloxacin (5 µg)	14 (15.26)	79 (84.74%)

All fifty-three (93) isolates were resistant to meropenem and imipenem. The MBLs genes encoding for the following (blaDIM, blaSIM, blaNDM, blaIMP, blaVIM, blaSPM, blaGIM, and blaAIM) were confirmed from DNA of *P. aeruginosa* via genes specific primers through multiplex PCR as shown in table 3. From total of thirty-three (33) that were phenotypically detected ten (10) (30.30%) isolates of *P. aeruginosa* positive for MBLs encoded genes as followed by blaNDM 5(50%) blaVIM 3(30%) blaIMP 2(20%)and blaSIM, blaDIM, blaSPM, blaGIM, and blaAIM were not detected. As shown in table 4.

Table 4. Percentage positive result of metalo β lactamase enzymes.

Gene	Percentage result
blaDIM	0
blaSIM	0
blaNDM	50%
blaIMP	20%
blaVIM	30%
blaSPM	0
blaGIM	0
blaAIM	0

Discussion

In our study, the prevalence rate of *Pseudomonas aeruginosa* is (46.5%) out of 200 clinical samples. The prevalence rate of MBL producing Pseudomonas *aeruginosa* was 33 (35.48%) out of 93 isolates. After that, all ninety-three (93) isolates of *P. aeruginosa* were confirmed through biochemical tests such as (oxidase, catalase, citrate, triple sugar iron, indole and methyl red test). Similar studies were carried out by different regions of the world for more and fundamental confirmation of the isolates (Kaushik *et al.*, 2001).

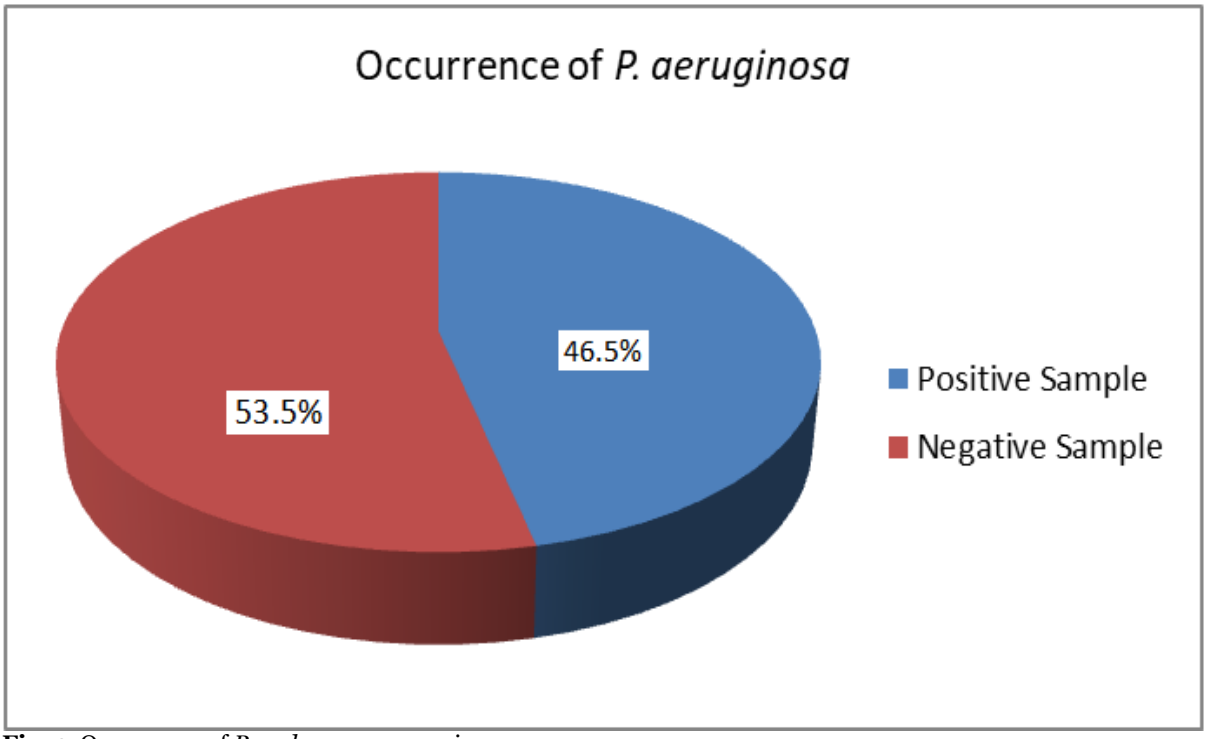

Fig. 1. Occurrence of *Pseudomonas aeruginosa*.

Multiple studies reported that various classes of antibiotics are used as anti-pseudomonas drugs (Livermore *et al.*, 2001; Wroblewska *et al.*, 2006). In our study, the antibiotic susceptibility testing showed high susceptibility against polymyxin B and colistin with (89.25%) and (83.88%) respectively.

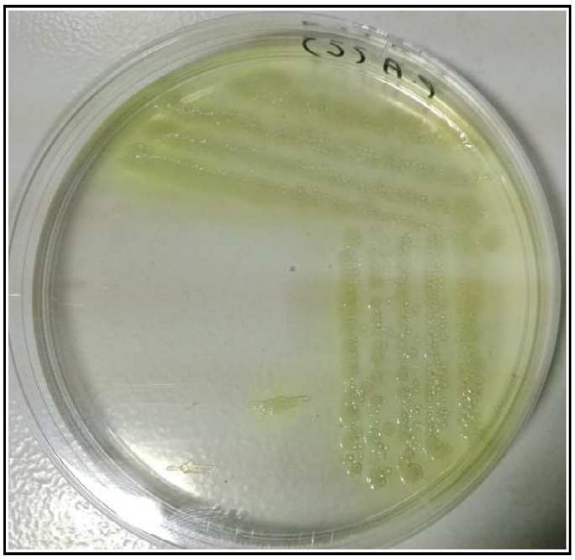

Fig. 2. Colony morphology of *Pseudomonas aeruginosa* on cetrimide agar.

The highest resistance was observed against ceftriaxone (86.02%), levofloxacin (84.74%), imipenem (81.72%), meropenem (76.34%), amikacin (73.15%), gentamicin (70.96%), ciprofloxacin (69.89) % and aztreonam (68.61%) as shown in table 3. Very similar resistance rates were observed in Pakistan by Zubair *et al.*, 2018, ceftriaxone (82.98%), imipenem (78.73%), meropenem (75%), gentamicin (72.72%), ciprofloxacin and aztreonam (69.23%) and Ceftazidime (65.95%). Haque and Salam (2010) detected (100%) resistance to ceftriaxone and (80%) resistance to aztreonam, ciprofloxacin, ceftazidime and gentamicin. Kamaria *et al.*, (2016) observed high resistance against pseudomonas aeruginosa (93.24%) to Gentamycin, (89.18%) Ciprofloxacin, (85.13%) Amikacin, 83.78%) Aztreonem, (81.08%) Ceftazidime, (72.97%) Piperacillin and (0%) resistance against Colistin and Polymyxin B.

The zone of inhibitions was observed ≥7 mm increase in diameter for imipenem, meropenem, and clavulanic acid combined with EDTA, meropenem and compared with without EDTA disks.

In our study, in the Combined disk diffusion method (CDDT), thirty- three (33) (35.48%) out of ninety-three (93) Pseudomonas aeruginosa were MBLs producing. Picao *et al.*, (2008) proved a total of 46 genetically unrelated MBL producing Pseudomonas aeruginosa, by a combined disk diffusion method. Overall, 46 isolates were confirmed by a combined disk diffusion test (CDDT). In the next study Owlia *et al.* (2008) an emerging threat in hospital isolates of Pseudomonas aeruginosa that were Metallo-beta-lactamases (MBLs) resistant. By using the combined disk diffusion test (CDDT) phenotypic method, it was detected that MBLs producing P. aeruginosa isolated from burned patients. These P. aeruginosa were used for the detection of MBLs production (53.2%) isolates were found MBL positive (30.06%). Peshattiwar *et al.*, (2011) commenced to identify the Metallo β lactamases (MBL) in isolates of *Pseudomonas aeruginosa*. The Imipenem spotted the existence of MBL enzyme – EDTA Double combined Disk Diffusion test (CDDT). The result showed that 10 (7.8%) isolates out of 126 were MBL producers.

In the our conducted study from total of thirty-three (33) (35.48%) that were phenotypically detected Ten (10) (30.30%) isolates of *P. aeruginosa* positive for MBLs encoded genes as followed by blaNDM 5(50%) blaVIM 3(30%) blaIMP 2(20%)and blaSIM, blaDIM, BlaSPM, BlaGIM, and blaAIM were not detected as shown in table 4. On the other hand, multiple similar studies were conducted worldwide. In other comparable study isolated the blaNDM-1 (Walsh, 2005). In another study also detected blaSIM of MBLs from *P. aeruginosa*. Poirel *et al.*, (2011) reported blaIMP, blaVIM, blaSPM, blaNDM, from *Pseudomonas aeruginosa*. Furthermore, it is included carbapenemase resistant genes blaSIM, blaDIM. Saaiq *et al.*, (2015) were detected blaIMP, blaVIM, blaSPM, blaSIM, blaGIM 16(76.1%) isolates. Shanthi *et al.*, (2014) were reported about NDM-1 New Delhi Metallo Beta-lactamase gene from *P. aeruginosa* isolates. Four isolates of *P. aeruginosa* were positive for NDM-I that further confirmed through PCR. Irfan *et al.*, (2008) conducted a study on metallo-*β*-lactamases (MBL).

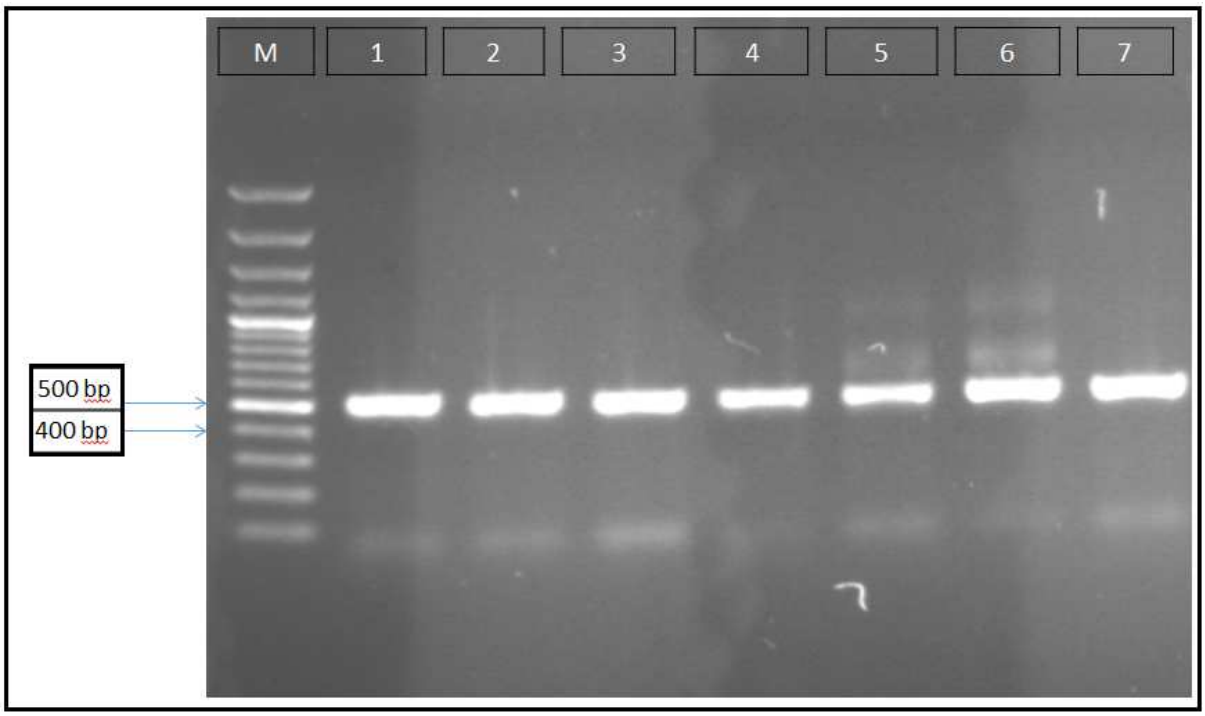

Fig. 3. PCR amplification of OprL gene using *P. aeruginosa* specific primers.

From MBLs Initially reported genes in Egypt were blaIMP-1, blaNDM. Unfortunately, MBLs genes of blaDIM, blaNDM, blaVIM, was not noticed in all isolates. Poirel *et al.*, (2011) were also conducted the same work and detected several carbapenemase resistant genes (blaDIM, blaSIM, blaNDM, blaIMP, blaVIM, blaSPM, blaGIM, and blaAIM) from different clinical isolates.

Conclusion

In conclusion, *P. aeruginosa* is the most prevalent organism found in the burn patients and the resistant rate was high in hospital admitted patients as compared to the community. This organism was found resistant to imipenem and menepenem which are mostly used antibiotics in burn patients. There is a need of more effective antibiotics and other ways for the treatment of burn patients. Otherwise, it will become an emerging threat in patients.

It was shown in this research work MBLs producing isolates such as *Pseudomonas aeruginosa* are a distressing threat in health care institutes. It can be controlled through proper isolation and strict detection methods that will reduce serious infections and death rates among hospitalized patients.

Ethical approval

Any of the authors have conducted no studies with humans and animals for this article.

Conflict of interest

The authors affirm that they have no divergence of interest.

Informed consent

Informed permission was carried out from all the authors.

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