ORIGINAL ARTICLE

Molecular Detection of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* Clinical Isolates in Khartoum State, Sudan

NAJEM ALDIN M. OSMAN[1*], AMMAR M. HASHIM[2], NADIR ABUZEID[1], HISHAM ALTAYB[3], MUATAZ MOHMED ELDIRDERY[4], ALI SULEIMAN ELWAKEEL[5] ALI MOHAMED ELHASSAN ELERAGI[6]
[1]*Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, Omdurman Islamic University, Sudan*
[2]*Department of Microbiology, Sudan Academy of Science, Sudan*
[1]*Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, Omdurman Islamic University, Sudan*
[3]*Department of Microbiology, College of Medical Laboratory Sciences, Sudan University for Science and Technology*
[4]*Department of Microbiology, Tropical Medicine Research Institute, National Center for Research, Sudan*
[5]*Research Board member, Faculty of Medical Laboratory Sciences, Omdurman Islamic University, Sudan*
[6]*Department of Microbiology, Faculty of Medicine, University of Bisha, KSA*
Correspondence to Dr. Najem Aldin M Osman, Email: najemosman@hotmail.com, Cell: 00249 - 912636573

ABSTRACT

Background: Antibiotics resistant including carbapenems among pseudomonas species is one of the critical health problems which lead to limited treatment options. Therefore, precise identification and antibiotic testing is necessary for effectual treatment.
Aim: To determine Metallo-β-Lactamase (*bla*$_{VIM}$ and *bla*$_{IMP}$**)** producing pseudomonas species among different Pseudomonal infections.
Methods: Standard microbiological methods, antibiotics susceptibility tests and PCR were performed to identify Metallo-β-Lactamase producing pseudomonas species
Results: A total of 40 Pseudomonas isolates were recovered. *Pseudomonas fluorescence* was found to be 85% (n = 34) while *Pseudomonas aeruginosa* was 15% (n = 6). All *Ps. aeruginosa* isolates were IMP and VIM positive (100%), while just nine (22%) isolates of *Ps. fluorescence* were bearing IMP and eight (20%) were VIM positive.
Conclusion: Our finding revealed a high frequency of MBLs producing Pseudomonas species where *Ps. fluorescens* was more frequently isolated.
Keywords: Carbapenems, *Psaeruginosa*, *Ps. fluorescence,* IMP, VIM.

INTRODUCTION

Pseudomonas species is part of the normal microbial flora of the human. It is adaptable to a variety of physical conditions and is genetically resistant to several antibiotics[1]. Metallo-β-Lactamase (MBLs**)** enzymes including *bla*$_{VIM}$ and *bla*$_{IMP}$, is the agents responsible for hydrolyzing β-Lactam antibiotics[2]. and thus resistance to carbapenems including Meropenem (MEM) and Imipenem (IPM) which considered the most potent and the last choice drugs to treat pseudomonas infections[3]. The *bla*$_{VIM}$ and *bla*$_{IMP}$ are mobile genes that can be transmitted through plasmids, integrons or transposons[4]. *Pseudomonas aeruginosa* as human-related species is one of leading causes of healthcare-associated infections giving rise to a different range of opportunistic infections[5]. Its infections are associated with mortality due to treatment failure as it is bearing MBLs[6]. Moreover *Ps. aeruginosa* is identified as a multidrug resistant (MDR) since it is strongly resist routinely used antibiotics[7,8,9]. Although *Pseudomonas fluorescence* is less clinically significant and not commonly isolated as human pathogen but it is likely to be widely spread in hospitals environment and maybe occasionally isolated from hospitalized patients[10]. *Ps. fluorescence* was reported to cause nosocomial infections even in a higher incidence rates than *Ps. aeruginosa*[11] . It has been reported for hemolytic activity, which induces cytotoxic and pro-inflammatory responses in intestinal cells[12]. On the other hand carbapenemase genes were detected in it and was considered as a reservoir of resistance determinants[10]. For useful and appropriate treatment, MBLs producing Pseudomonas species should be detected to prevent their spread and subsequent complications.

The current study was undertaken to detect bla$_{VIM}$ and bla$_{IMP}$ genes among Pseudomonas isolates from a hundred hospitalized and non-hospitalized patients in Khartoum State, Sudan.

METHODS

The current study was conducted in Khartoum State, Sudan. One major hospital was taken as representative for each city (Khartoum, Omdurman and Khartoum North). The subjects were patients suffering from any suspected pyogenic bacterial infection as diagnosed by a physician. Scientific and ethical approval of the study was obtained from Scientific and ethical approval of the study was obtained from Tropical Medicine Research Institute ethical committee (Sudan) where the research was conducted, and the verbal or the written consent was obtained from all patients.
Specimens: Clinical specimens from different sites of infection including ear, wound, urine and diabetic foot ulcer were collected and directly transported to the laboratory.
Bacterial identification:: Isolation and identification of bacterial isolates was done using standard bacteriological procedures as described by Cowan and Steel [13] and Cheesbrough [14].

Antibiotic susceptibility testing: Muller-Hinton agar was used to perform Kirby-Bauer susceptibility testing to different antibiotics according to CLSI guidelines (2015) [15].

Molecular detection of Metallo β-Lactamase gene

DNA extraction: DNA extraction was carried out using modified bacterial DNA extraction protocol as described by Reischl *et*, al [16].

PCR amplification: Metallo ß-lactamase specific primers for *bla* $_{IMP}$ and *bla* $_{VIM}$ were used[17] The primers were manufactured by (Eurofins MWG Operon, Germany) the primers were as follows: (Table 1)

Single PCR amplifications were performed in 25µl reaction mixture (Maxime PCR PreMix Kit i-Taq 5µl) (INTRON Biotechnology, South Korea), containing 3µl of bacterial DNA template, 1µl of 5 pmol/µl from each primer and the mixture was completed with 15 µl of sterile distilled water in a 0.2 Eppendorf (PCR) tube. Amplifications were performed by using the thermal cycler as follows: initial denaturation step at 95 °C for 5 minutes; 30 cycles of denaturation at 94 °C for 30 seconds, (annealing at 55 °C for 30 seconds for bla_{IMP}, 63 °C for 30 seconds for bla_{VIM}), extension at 72 °C for 1 minute, followed by a final extension step at 72 °C for further 10 minutes.

RESULTS

Bacteriological result: A total of forty *Pseudomonas* species were isolated from different clinical specimens. Among them, six isolates were *Ps.aeruginosa (*15%) while thirty-four isolates were *Ps. fluorescens* (85%) as shown in table 2.

Distribution of *Pseudomonas* isolates according to the site of infection: Among the collected clinical specimens throughout this study, ear infection was the most observed site of infection (n=19) while wounds were the least ones (n=2) (Table 3).

Distribution of *Pseudomonas* isolates according to the patient's hospitalization status

Most *Pseudomonas* isolates were recovered from hospitalized patient (n = 33) while the outpatients yielded only seven isolates (Table 4).

Table 1: Primer sequences and Amplicon sizes used in this study[27].

Primer	Sequence (5'-3' direction)	Amplicon size
bla $_{IMP}$ F	TCGTTTGAAGAAGTTAACGG	568
bla $_{IMP}$ R	ATGTAAGTTTCAAGAGTGATGC	
bla $_{VIM}$ F	GGTGTTTGGTCGCATATCGCAA	502
bla $_{VIM}$ R	ATTCAGCCAGATCGGCATCGGC	

Antibiotic susceptibility: Resistance against Gentamicin (57.5%) was found to be the most abundant followed by Meropenem (47.5**%**), Imipenem (45%), Ciprofloxacin (37.5%) and Cefotaxime (32.5%) (Table 5,6)

Molecular detection of Carbapenem resistance genes: The study of *Pseudomonas* isolates showed that two genes were detected concerning carbapenem resistance genes. IMP gene was detected in 15 (37.5%), while VIM gene was detected in 14 (35%) isolates (Table 7, Fig.1,2)

Table 2. Frequency and percentage of isolated *Pseudomonas* species from different clinical specimens.

Pseudomonas species	Frequency	%age
Ps. fluorescens	34	85
Ps. aeruginosa	6	15
Total	40	100

Table 3. Frequency and percentage of Pseudomonas isolates according to the infection site

Site infection	Frequency	%age
Ear infections	19	47.5
Urine tract infections	14	35
Diabetic foot ulcer	5	12.5
Wounds	2	5
Total	40	100

Table 4: Frequency and percentage of Pseudomonas isolates according to the patient's status

Patients status	Frequency	%age
Hospitalized	33	82.5
Outpatient	7	17.5
Total	40	100

able 5: Antibiotic Susceptibility pattern of isolated *Pseudomonas* species

Antibiotic	Sensitive	Intermediate	Resistant
Gentamicin	15(37.5%)	2(5%)	23(57.5%)
Ciprofloxacin	15(37.5%)	10(25%)	15(37.5%)
Cefotaxime	20 (50%)	7(17.5%)	13(32.5%)
Imipenem	16 (40%)	6(15%)	18(45%)
Meropenem	16 (40%)	5(12.5%)	19(47.5%)

Table 6. The Resistant pattern of isolated *Ps. aeruginosa* and *Ps .fluorescens*

Antibiotic	Resistant	Ps aeruginosa	Ps fluorescens
Gentamicin	23(57.5%)	6	17
Ciprofloxacin	15(37.5%)	5	10
Cefotaxime	13(32.5%)	4	9
Imipenem	18(45%)	6	12
Meropenem	19(47.5%)	6	13

Table 7. Molecular detection of VIM \ IMP genes among *Pseudomonas* clinical isolates

Type of isolate	Ps aeruginosa	Ps Flourescens	Total
IMP+ve	6(15%)	9(22%)	15(37.5%)
IMP –ve	0	25(62.5%)	25(62.5%)
VIM+ve	6(15%)	8(20%)	14(65%)
VIM –ve	0	26(65%)	26(65%)

Fig.1: Molecular detection of *bla* $_{IMP}$ gene among *Ps. aeruginosa* and *Ps. flourescens* analyzed in agarose gel.
M: 100 bp molecular ladder; lane 17: positive control; lane 18: negative control; lanes 1, 6, 8, 13 and 15: typical IMP positive isolates (amplicon size 568 bp); lanes 2, 3, 4, 5, 7, 9,10,11,12,14 and 16: IMP negative isolates.

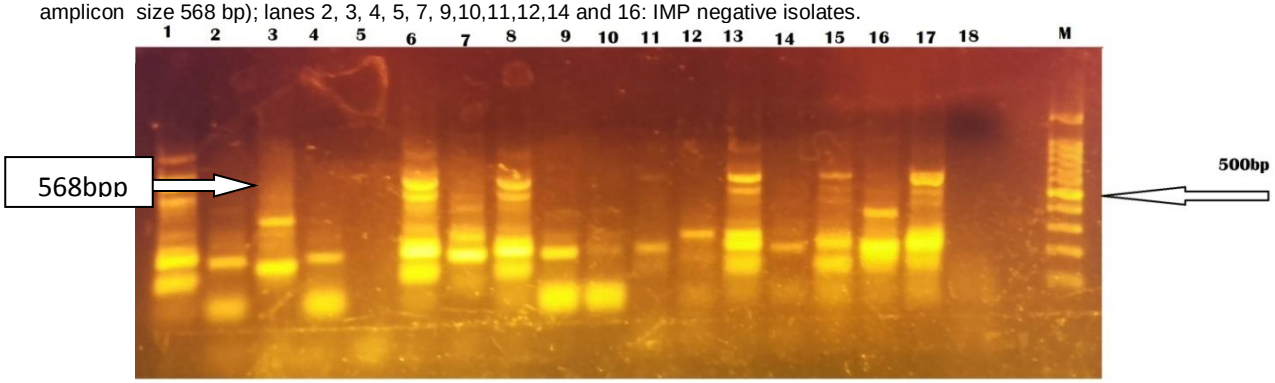
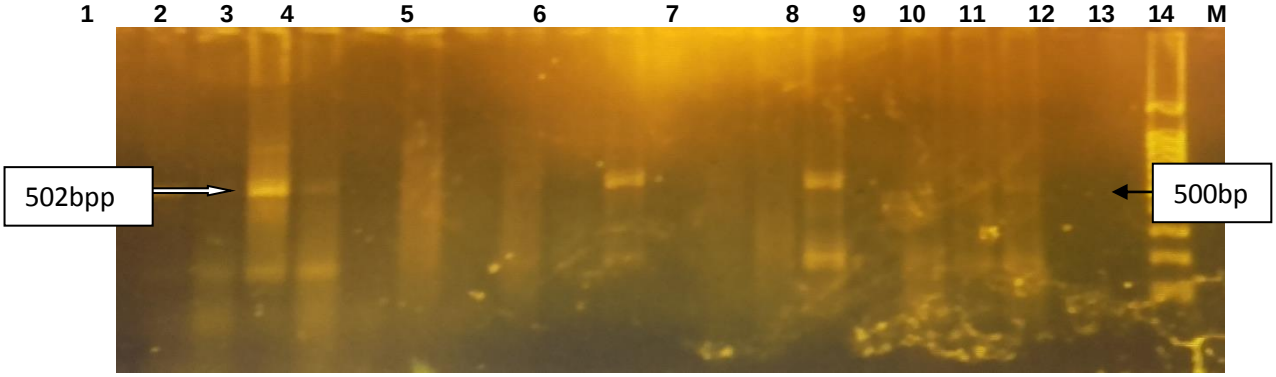


Fig. 2: Molecular detection of bla $_{VIM}$ gene among Ps. aeruginosa and Ps fluorescens analyzed in agarose gel.
M: 100 bp molecular ladder; lane 1: positive control; lanes 3, 4, 7, 10: typical VIM positive isolates (amplicon size 502 bp); lanes 2, 5, 6, 8, 9, 11,12, 13 and 14: VIM negative isolates



DISCUSSION

Pseudomonas is one of the main opportunistic pathogens in the hospital environment. It imposes a great challenge to treat its infections due to its natural resistance against drugs and the rapid changes in the pattern of resistance [18]. In the current study and among forty recovered *Pseudomonas* clinical isolates, 85% were *Ps. fluorescens* and 15% were *Ps. aeruginosa*. Although *Ps. fluorescens* is an unusual cause of disease in humans and usually affects immune-compromised patients, some previous studies showed that it was the commonly isolated from bloodstream infections after exposure to contaminated heparinized saline flush[19], in addition it was also isolated from three successive blood cultures from patients with septicemia[20]. Because of the significant numbers of isolated *Ps. fluorescens* from various clinical specimens, the consideration that it is of no clinical importance is questionable. Recent reports on clinical strains of *Ps. fluorescens* have caused serious health problems[12,21-23] which may require some alternative treatment approach. In the present investigation the ear is the most common site of infection (47.5%), followed by urinary tract (35%) and wounds (5%), while in Nigeria it was reported that the high to low frequency rate of *Pseudomonas* was as follows: wound (51.5%); ear (16%); and urine (13.5%)[24]. This was not in line with this study in terms of sites order, but in general these sites in addition to blood are the most sites infected by Pseudomonas species. This study reports a high frequency of isolated pseudomonas from hospitalized patients rather than outpatients, which emphasize the existence of Pseudomonas in hospital environments [20]. Moreover; among 40 clinical Pseudomonas species isolates, multiple-resistance phenotypes were observed, where 45% of *Pseudomonas* isolates were found resistant to Imipenem, this finding is in accordance with Moradian *et al*, who found that the resistance to Imipenem was 53.7%[25]. The resistant of *Pseudomonas* isolates to the Meropenem was 47.5%. This finding is not comparable with that reported by Gad GFe and Ashour [26], who found the resistance to Meropenem was 68%. This study indicated that all phenotypically Carbapenem-resistant *Pseudomonas aeruginosa* is bearing both *bla*$_{VIM}$ and *bla*$_{IMP}$ (100%), this frequency is a higher than in the previous study carried out in Sudan by Satir *et al*[27]. On the other hand, 9 (22%) of *Ps. fluorescens* were positive for *bla*$_{IMP}$ whereas 8 (20%) were *bla*$_{VIM}$ positive. Similar study in Singapore reported *bla*$_{IMP}$ positive *Ps. fluorescens* recovered from hospitalized patients[10].

CONCLUSION

The present study reported a high frequency of MBLs producing Pseudomonas species where *Ps. fluorescens* was more frequently isolated, therefore alarming us not to neglect it as an opportunistic pathogen. It is recommended that, precise identification protocols should be adopted and good infection control practices should be applied for

optimal management and to decrease further dissemination of such resistant strains.

Competing interests: No competing interests were disclosed.

Acknowledgement: Authors wish to thanks Ms Hanifa Mohammed, Mawada Abbakar, Noora Mahmoud and Ohood Mohammed for their technical assistance.

REFERENCES

1. Deplano A, Denis, O., Poirel, L., Hocquet, D., Nonhoff, C., Byl, B., Nordmann, P., Vincent, J. L. and Struelens, M. J. . Molecular characterization of an epidemic clone of pan antibiotic-resistant *Pseudomonas aeruginosa*. *Journal of clinical microbiology.* 2005; 43(3): 1198-1204.
2. Slama T. Clinical review: balancing the therapeutic, safety, and economic issues underlying effective antipseudomonal carbapenem use. *Crit Care.* 2008; 12(5):233.
3. 3. Valenza G JB, Elias J, Claus H, Oetterlein A, Engelhardt K, Turnwald D, Frosch M, Abele-Horn M Schoen C First survey of Metallo-β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in a German university hospital. *Antimicrob Agents Chemother* 2010; 54: 3493-3497.
4. Livermore D. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare. *Clinical Infectious Disease.* 2002; 34:634-40
5. Poole K. "Efflux-mediated multiresistance in Gram-negative bacteria". *Clin. Microbiol. Infect.* 2004; 10 (1): 12–26.
6. Cornaglia G GH, Rossolini GM. . Metallo-β-lactamases: a last frontier for β-lactams. *Lancet Infectious Diseases.* 2011; 11:381-93
7. G.Giske C, Monnet DL, O.Cars, Y.Carmeli. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrobial agents and chemotherapy.* Mar 2008; 52 (3): 813-821.
8. AP Magiorakos AS, RB Carey , Y Carmeli , ME Falagas , CG Giske . Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection.* 2012; 18: 268-8.
9. Paphitou N. Antimicrobial resistance: Action to combat the rising microbial challenges. *International journal of antimicrobial agents.* 2013; 42 Suppl: S25-8.
10. Koh Tse Hsien GCYW, and Li-Hwei Sng. IMP-1 and a Novel Metallo--Lactamase, VIM-6, in Fluorescent Pseudomonads Isolated in Singapore *Antimicrobial agents and chemotherapy.* 2004; 48: 6.
11. MG Farquhar PG. Junctional complexes in various epithelia. *J Cell Biol.* 1963; 17: 375-412.
12. A Madi OL, HM Blottière , M Guyard-Nicodeme, KLe Roux. The clinical *Pseudomonas fluorescens* MFN1032 strain exerts a cytotoxic effect on epithelial intestinal cells and induces Interleukin-8 via the AP-1 signaling pathway. *BMC Microbiol.* 2010; 10: 215.
13. ST Cowan KS, GI Barrow , RKA Feltham Cowan and Steel's manual for the identification of medical bacteria. 3rd ed. Cambridge ; New York: ; . . Cambridge University Press, 1993.
14. Cheesbrough M. District laboratory practice in tropical countries. 2nd ed. Cambridge ; New York: Cambridge University Press; 2005.
15. CLSI. performance standards for antimicrobial disk diffusion test. USA: clinical and laboratory standard institute. 2015; Contract No: M2- M9.
16. Udo Reischl H-Jrl, Michaela Metz, Birgit Leppmeier, Norbert Lehn. Rapid Identification of Methicillin-Resistant *Staphylococcus aureus* and Simultaneous Species Confirmation Using Real-Time Fluorescence. *PCR journal of clinical Microbiology.*2000; 38: 6.
17. Zong Z, Lu X, Valenzuela JK, Partridge SR, Iredell J. An outbreak of carbapenem-resistant Acinetobacter baumannii producing OXA-23 carbapenemase in western China. *International journal of antimicrobial agents.* 2008; 31 (1): 50-54.
18. Kohler T, Epp SF, Curty LK, Pechere JC. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *Journal of bacteriology.* 1999;181(20): 6300-6305.
19. Gershman MD KJ, Noble –waqng J, Kim C, Gullian J, Kacica M, Jensen B,Pascoe N, Salman L, Michile J, Willkins M, Schoomaker – Bopp D, Clyton J, Andrio M, Srinivasan A. Multistate outbreak of *Pseudomonas fluorescens* in blood stream infection after exposure to contaminated heparinized saline flush prepared by a compounding pharmacy. *Clinical infectious diseases*. (2008) 47: 11.
20. Sutter, VL. Identification of Pseudomonas Species Isolated from Hospital Environment and Human Sources. 1968; *Appl. Microbiol*. 16:1532-1538.
21. L Picot SA, A Merieau , P Lerouxb , L Cazina *Pseudomonas fluorescens* as a potential pathogen: adherence to nerve cells. . *Microb & Infect* 2001; 3: 985-995.
22. A Chapalain GR, O Lesouhaitier ,A Merieau , C Gruffaz . . Comparative study of 7 fluorescent pseudomonad clinical isolates. *Canad J Microbiol* 2007; 54: 19-27.
23. V Wong KL, B Baddal , JTurton ,T Boswell. Spread of *Pseudomonas fluorescens* due to contaminated drinking water in a bone marrow transplant unit. *J Clin Microbiol* 2011; 49: 2093-2096.
24. Zubair KO, Iregbu KC. Resistance Pattern and Detection of Metallo-beta-lactamase Genes in Clinical Isolates of *Pseudomonas aeruginosa* in a Central Nigeria Tertiary Hospital. *Niger J Clin Pract* 2018; 21:176-82
25. Moradian Fatemeh kouchaksaraei EFS, Zahra Molana,Masomeh Moradian. Molecular Detection of Integrons Genes and Pattern of Antibiotic Resistance in *Pseudomonas aeruginosa* Strains Isolated from Intensive Care Unit, Shahid Beheshti Hospital, North of Iran. . *Iranian Journal of Molecular C Microbiology.* 2012; 1: 4.
26. Gad GFe-D, R. A.Ashour, H. M .Antimicrobial susceptibility profile of *Pseudomonas aeruginosa* isolates in Egypt. Urol 2008; 180 (1):176-81.
27. Salma B Satir AIE, Musa A Ali, Abdel Rahim M El Hussein, Isam M Elkhidir and Khalid A Enan. Detection of Carbepenem resistance genes among selected Gram Negative bacteria isolated from patients in-Khartoum State, Sudan. *Clin Microbiol*. 2016; 5(6): 26