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Molecular Analysis of Metallo Beta Lactamase in Multi Drug Resistant Pseudomonas Aeruginosa among the Clinical Isolates

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Abstract: ***Background: One of the major clinical problems regarding pseudomonas aeruginosa is attributed to the production of metallo-betalactamase (MBL) enzymes. This group of enzymes are a member of beta-lactamases which constitute Ambler class B that hydrolyze - carbapenems. This study was carried out to find out the predominant resistance mechanisms among MDRPA and the prevalence of corresponding resistance genes. Materials and Methods: MDRPA isolates collected from various clinical samples for a period of one year from March 2015 to February 2016 were included to detect the predominant mechanism of resistance using phenotypic and molecular methods. Molecular characterization of all these isolates was done by polymerase chain reaction (PCR) for the presence of blaVIM-2, blaIMP-1, blaOXA-23, and blaNDM-1 genes with specific primers. Results: Among 120 MDRPA isolates 70 (58.33%) were MBL producers. Molecular characterization studied by PCR showed 15(12.5%) of Vim2 gene and only 2 (1.66%) of IMP 1 gene. None of the 120 MDRPA have produced OXA 23 and NDM gene in our study. Conclusion: The prevalence of MBLs has been increasing worldwide, particularly among P. aeruginosa, leading to severe limitations in the therapeutic options for the management. Thus, proper resistance screening measures and appropriate antibiotic policy can be strictly adopted by all the healthcare facility providers to overcome these superbugs.***

Keywords: Pseudomonas aeruginosa ,metallo β-lactamase, genes, blaVIM-2, PCR

1. Introduction

Pseudomonas aeruginosa is one of the most importantpathogens causingnosocomial infections, it is naturally resistant to many antimicrobial agents. It has a distinctivecapacity to become resistant to many available antimicrobial agents via multiple mechanisms(1).In the past decade, acquired multidrug resistance, relating to selective antibiotic pressure, has emerged in several countries; and in some cases, infections caused by multidrug resistant P. aeruginosa have been untreatable (2). Carbapenems, including meropenem and imipenem, are the most effective antibiotic against this organism isolated from patients. However, resistance to carbapenems has emerged by different mechanisms such as impermeability to drug due to loss of OprDporin, the up-regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms or production of metallo-beta-lactamases (MBLs) that hydrolyze all carbapenems (3,4). As carbapenems are the potent antimicrobial weapon against multi drug resistant P. aeruginosa (MDRPA), this bacterium has developed resistance even against this group of drugs by producing MBLs (carbapenemase)(5). Imipenem and meropenem among carbapenems have gained increased therapeutic access in many medical centers against MDRPA. However, as this pathogen has gained already resistance even to these available drugs, identification of nosocomial strains capable of producing MBL has aroused more interest and importance in the recent years(6).

Carbapenemases are Class B MBLs; IMP, VIM or Class D oxacillinases (*OXA* 23-*OXA* 27) (carbapenem-hydrolyzing Class D β -lactamases or Class A clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). Class A β -lactamases with activity again carbapenems, are uncommon and divided into five groups (GES, IMI, KPC, NMC-A and SME). ESBLs and carbapenemases are typically encoded by plasmid or transposon-borne genes, often on integron, Which are genetic elements capable of capturing and subsequently mobilizing resistance genes, although some β -lactamase genes are associated with novel mobile insertion sequences termed insertion sequence common region elements (6).Acquired MBLs includes the VIM and IMP enzymes, of which there are numerous variants of the original VIM-1 and IMP-1 MBLs as well as the SPM-1, GIM-1, NDM-1, AIM-1 and SIM-1 enzymes(7,8). The VIM and IMP enzymes are by far the most common MBLs found in carbapenem-resistant bacteria, including carbapenem-resistant *P. aeruginosa*(9). Thus, this study was conducted to know the prevalence of MBL producing multidrug resistant *P. aeruginosa* and the molecular characterization of prevalent genes present in them in order to improve the therapeutic options and to decrease the morbidity and mortality.

2. Materials and Methods

The present study was carried out on *P. aeruginosa* obtained from various clinical samples from clinical laboratories during the period of March 2015 to February 2016. One hundred and twenty MDR P. aeruginosa isolates were obtained from 400 clinical samples The samples from which the strains were isolated include blood, Pus, Urine, Broncho alveolar lavage (BAL) and endotracheal (ET) aspirates and tissues. All the samples were processed for isolation and antibiotic sensitivityby Kirby Bauer method. All clinical isolates of *P. aeruginosa* which were found to be multidrug resistant (total of 120 MDRPA*)* were included in

this study for further characterization. Repeated isolates from the same patients were excluded.

Detection of MBL producing P. aeruginosa was performed by the **Imipenem- EDTA combined disc test (CDT):** The CDT was performed as described by Yong et al. (10) and Molecular characterization for these isolates were done by polymerase chain reaction (PCR) for the presence of *VIM* 2, *IMP* 1, *OXA* 23 and *NDM* 1 genes. ATCC *P. aeruginosa* 27853 was used as a control strain for all the procedures.

Polymerase chain reaction amplification of resistance genes

The genomic DNA was extracted from all the 120 MDRPA isolates followed by agarose gel electrophoresis. The isolated DNA was used as a template for amplification of specific genes described below. PCR amplification was done with specific gene primers and checked for the presence of the corresponding gene responsible for MBL production such as $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$, $bla_{\text{OXA-23}}$, and $bla_{\text{NDM-1}}$ genes. The PCR amplification was performed with Eppendorf Master cycler.

Amplification of the resistant genes were carried out with the following reaction mixture composition (10 µl): DNA template (50 ng), 1 µl each of deoxynucleotide triphosphates (2.5 µM), Taq buffer (10ul), forward and reverse primers (2.5 µM) and 1 U of Taq DNA polymerase (Merck Biosciences, Darmstadt, Germany) (Table-1). All PCR amplifications were performed using thermal cycler (Veriti Thermal cycler, Applied Biosystems, USA.) using the following conditions for 30 cycles: 94 °C for 5 min, 94 °C for 45 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s. The PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide, and the amplicons were purified using HiPura PCR product purification kit (Himedia, Mumbai, India).

Table 1: List of primers used

Primer name	Sequence (5' to 3')	Amplicon size (bp)
bla VIM-2	Forward- ATGTTCAAACTTTTTGAGTAGTAAG Reverse - CTACTAACGACTGAGCG	801
bla IMP	Forward - CTACCGCAGCAGAGTCTTTGC Reverse - GAACAACCAGTTTTGCCTTACC	640
Bla OXA-23	Forward - GATGTGTCATAGTATTCGTCGT Reverse - TCACAACAACTAAAAGCACTGT	1058
blaNDM-1	Forward - GGTTTGGCGATCTGGTTTTC Reverse - CGGAATGGCTCATCACGATC	621

Statistical Analysis

All the data were entered in Microsoft Excel sheet and the results were analyzed by SPSS software. (IBM, USA)

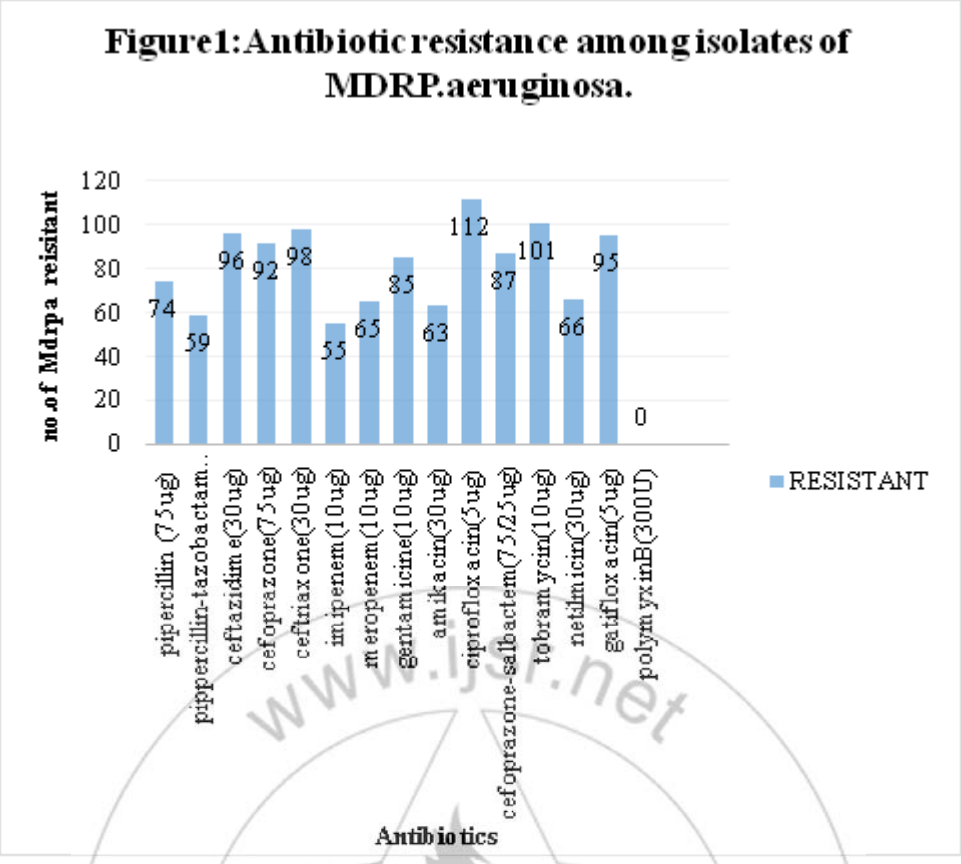
3. Results

A total of 120 MDRPA, carbapenemase resistance was documented among 54.16% (65) towardsmeropenem and 45.83% (55) towards imipenem, respectively. All these 120 isolates showed 100% susceptibility towardspolymxinB by Kirby Bauer disc diffusion method.Antibiotic resistance among isolates of MDRP.aeruginosa reflected in **(Table 2, Fig-1).** The minimum inhibitory concentration (MIC) of meropenem for resistant strains ranged from 8 µg/ml to >64 µg/ml. (Break point MIC-≤4 µg/ml to ≤16 µg/ml.

Amongst these 120 MDR *P. aeruginosa*, Seventy isolates (58.33%) were found to be MBL producers.Fifteen (12.5%) of MDRPA showed presence of *Vim* 2 gene and only 2(1.66%) was positive for *IMP* 1 gene. None of the 120 MDRPA have produced *OXA* 23 and *NDM* gene in our study.

Table 2: Antibiotic resistance among isolates of MDRP.aeruginosa

Antibiotics	*Resistant*	*Sensitive*	*Intermediate*
piperacillin (75ug)	74 (61.66%)	46 (38.33%)	0 (0%)
piperacillin-tazobactam (100/10ug)	59 (49.1%)	54 (45%)	7 (5.83%)
ceftazidime(30ug)	96 (80%)	15 (12.5%)	9 (7.5%)
cefoperazone(75ug)	92 (76.66%)	9 (7.5%)	19 (15.83%)
ceftriaxone(30ug)	98 (81.66%)	9 (7.5%)	13 (10.83%)
imipenem(10ug)	55 (45.83%)	65 (54.16%)	0 (0%)
meropenem(10ug)	65 (54.16%)	54 (45%)	1 (0.8%)
gentamicin(10ug)	85 (70.83%)	35 (29.16%)	0 (0%)
amikacin(30ug)	63 (52.5%)	44 (36.66%)	13 (10.83%)
ciprofloxacin(5ug)	112 (93.33%)	4 (3.3%)	4 (3.3%)
cefoperazone-sulbactam(75/25ug)	87 (72.5%)	11 (9.16%)	22 (18.33%)
tobramycin(10ug)	101 (84.16%)	19 (15.83%)	0 (0%)
netilmicin(30ug)	66 (55%)	54 (45%)	0 (0%)
gatifloxacin(5ug)	95 (79.16%)	18 (15%)	7 (5.83%)
PolymyxinB(300U)	00 (0%)	120 (100%)	0 (0%)



4. Discussion

The rapid spread of MBLs among major gram-negative pathogens, particularly P. aeruginosa, is an emerging threat and a matter of concern worldwide (11, 12). These organisms are resistant to almost all commonly available antibiotics with limited treatment options. Thus, this study was conducted to know the prevalence of MBL producing multidrug resistant *P. aeruginosa* and the molecular characterization of prevalent genes present in them in order to improve the therapeutic options and to decrease the morbidity and mortality.

In the present study, MDRPA isolates showed the resistance to carbapenems like meropenem (54.16%) and imipenem (45.83%), which were found to be the precious weapon against MDRPA infections and this is an alarming sign. All the isolates showed 100% sensitive to polymyxin B. In2014, a study by Samira et al 2014 reported , imipenem and meropenem resistance was observed to be fifty five isolates (45.83%) and sixty five isolates (54.16%) respectively which is more likely case in our study (13). In 2008, a study by Alis, kan*et al.* with 1071 MDRPA, reported resistance to imipenem (22.5%) and meropenem (31%)(14).Deepak *et al.* during 2009 to 2010 with 193 *P. aeruginosa* reported resistance to imipenem (3.7%), which is less compared with the present study(15).Minimum Inhibitory concentration of meropenem ranged from 8 µg/ml to >64 µg/ml, which is comparable to other studies. About 63.33% of these MDRPA isolates showed higher MIC to meropenem. In 2006, Shashikala *et al.*carbapenem resistant *P. aeruginosa* had reported MIC ranging from 8 µg/ml to 64 µg/ml(16). A study by Fernɑndez*et al.in 2010.* higher MIC of 128 µg/L for meropenem got documented (17).All these resistance ranging pattern is mostly directly dependent on various factors, which mainly includes the antibiotic policy in practice in the respective healthcare setups.

As MBLs production is the major mechanism of resistance among MDRPA, A study by Jayakumar *et al in 2007.* reported 54.5% MBL producers(18). Morten et al in 2001 have reported 47% of MBL producers in P.aeruginosa(19). Upadhyay*et al in 2010.* reported 46.6% of MBL production among MDRPA strains (20). Poirel L et al in 2000 reported 36% of MBL producers among the MDRPA (21).Navaneeth*et al in2002.* reported 12% MBL production in *P. aeruginosa*(22).Another recent study by Varaiya*et al in2008.* showed 20.8% of MDRPA were found to be MBL producers (23).In comparisonwith our present study, we reported a high prevalence of 58.33% of MBL producers. The Remaining MDRPA strains may be harborin some other resistance mechanism like, ESBL production, AmpC production, biofilm formation or through various virulence factors.

In *P. aeruginosa* number of different β-lactamases has been described including MBL, ESBL and *OXA* production. This present study investigated the predominant β-latamase coding genes such as, *VIM*-2, *IMP*-1, *OXA*-23 and *NDM*-1 through PCR. Among MBL producing isolates in our study, the presence of *VIM*-2 gene is predominant when compared with *IMP*-1 MBL gene. Surprisingly, none of our isolates were positive for *OXA*-23 and *NDM* genes. The presence of *VIM*-2 gene appears to be more prevalent in our study.

In 2007, the first case of MBL gene bla_{VIM-2} was reported in a strain isolated in India. This VIM-2, which is present on integron had its ancestral Class I integron documented in

United States and Russia. (24,25,26) This Class I integron having 3' conserved sequence have arisen from an ancestral integron predating the formation of 3' conserved, which was found in United States and Russia. The present study documented occurrence of *bla*$_{VIM-2}$among its collection, wherein 15 isolates of its collection were positive for *bla*$_{VIM2}$. Interestingly, the DNA sequence analysis of all our VIM-2 showed 100% identity with the sequence of global genotypes retrieved from the GenBank public database. This finding suggests successful global dissemination of VIM-2 resistant gene that is of great concern. *VIM*-2 gene was found to be more prevalent among MDRPA in our study as revealed by PCR method.

Fortunately in our study we encountered very less prevalence of resistance genes among our *P. aeruginosa* isolates when compared to rest of the world, wherein, high incidence of MBL have been reported. From the results obtained through our investigation, it can be concluded that VIM-2 gene was the most frequently isolated β-lactamase gene among the *P. aeruginosa* . The sequencing results further confirmed, there is less variance among our β-lactamase genes when compared to global genotypes.

MDRPA infections are likely to affect critically ill patients who require prolonged hospitalization. Infections with MDRPA are also associated with adverse clinical outcome. Strict isolation of patients infected with MDRPA and judicial use of antibiotics should be emphasized in order to prevent the spread of MDRPA infections. Further, more clinical studies are needed to identify risk factors for MDRPA development and to determine the economic impact of these infections, as well as to determine the most efficacious antimicrobial regimens and duration of therapy to maximize the outcome of MDRPA infections.

5. Conclusion

The present study gives the alarming sign toward the high prevalence of carbapenem resistant nonfermenting pathogens. Thus, this calls for stringent preventive measures, which includes strict infection control practices and judicious use of antibiotics with implementation of antibiotic policy. These kind of important measures might overcome the challenge of high mortality posed by MDRPA and other nonfermenting bacterial pathogens.

6. Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Hocquet D, Berthelot P, Roussel-Delvallez M, Favre R, Jeannot K, Bajolet O etal. Pseudomonas aeruginosa may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. Antimicrob Agents Chemother 2007; 51:3531–6.

[2] Makedou KG, Tsiakiri EP, Bisiklis AG, Chatzidimitriou M, Halvantzis AA, Ntoutsou K, et al. Changes in antibiotic resistance of the most common Gram-negative bacteria isolated in intensive care units. J Hosp Infect. 2005; 60: 245 – 248.

[3] KOHLER T., MICHEA-HAMZEHPOUR M., EPP SF., PECHERE J.C. (1999). Carbapenem activities against Pseudomonas aeruginosa: respective contributions of OprD and efflux systems. Antimicrob. Agents. Chemother. 43, 424-427.

[4] LIVERMORE D.M. (1992). Interplay of impermeability and chromosomal beta-lactamase activity in imipenemresistant Pseudomonas aeruginosa. Antimicrob. Agents. Chemother. 36, 2046-2048.

[5] Soraya S. Andradel, Ronald N.Jones, Ana C. Gales and HelioS.Sader. Increasing prevalence of antimicrobial resistance among *Pseudomonas aeruginosa* isolates in Latin American medical centers: 5 year report of the SENTRY Antimicrobial Surveillance Program (1997-2001). J AntimicrobChemother 2003;52:140-1.

[6] Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-lactamases: The quiet before the storm? ClinMicrobiol Rev 2005;18:306-25.

[7] Zavascki AP, Gaspareto PB, Martins AF, Gonçalves AL, Barth AL. Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing SPM-1 metallo-{beta}-lactamase in a teaching hospital in Southern Brazil. J AntimicrobChemother 2005;56:1148-51.

[8] Gales AC, Menezes LC, Silbert S, Sader HS. Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-β-lactamase. J AntimicrobChemother 2003;52:699-702.

[9] Cornaglia G, Akova M, Amicosante G, Cantón R, Cauda R, Docquier JD, *et al.*Metallo-beta-lactamases as emerging resistance determinants in Gram-negative pathogens: Open issues. Int J Antimicrob Agents 2007;29:380-8.

[10] Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J ClinMicrobiol 2002; 40:3798-801.

[11] Nam Hee R, Jung Sook H, Dong Seok J, Jae Ryong K. Prevalence of Metallo-β-lactamases in Pseudomonas aeruginosa and Acinetobacter baumannii. Korean J ClinMicrobiol. 2010;13(4):169-72.

[12] Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. Journal of clinical microbiology. 2004;42(5):2074-9.

[13] Samira Aghamiri, NourAmirmozafari, JalilFallahMehrabadi, BabakFouladtan, and Hossein SamadiKafil. Antibiotic Resistance Pattern and Evaluation of Metallo-Beta Lactamase Genes Including bla-IMP and bla-VIM Types in Pseudomonas aeruginosa Isolated from Patients in Tehran Hospitals. Hindawi Publishing Corporation ISRN Microbiology 2014; 10.1155-941507.

[14] Aliþkan H, Colakoðlu S, Turunç T, Demiroðlu YZ, Erdoðan F, Akin S, *et al*. Four years of monitoring of antibiotic sensitivity rates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains isolated from patients in intensive care unit and inpatient clinics. MikrobiyolBul 2008;42:321-9.

[15] Deepak A, Neerja J, Rajiv K, Romit . Emerging Antibiotic resistance in *Pseudomonas* - A challenge. Int J Pharm PharmSci 2011;3:82. Kindly provide author initial.
[16] Shashikala KR, Srinivasan S, Devi S. Emerging resistance to carbapenem in hospital acquired Pseudomonas infection: A cause of concern. Indian Pharmacol 2006;38:287-8.
[17] Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in Pseudomonas aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother 2010;54:3372-82.
[18] Jayakumar S, Appalaraju B. Prevalence of multi and pan drug resistant Pseudomonas aeruginosa with respect to ESBL and MBL in a tertiary care hospital. Indian J Pathol Microbiol2007;50:922-5.
[19] Morten Hentzer,L Gail M. Teitzel, Grant J. Balzer, Arne Heydorn, L SørenMolin,L Michael Givskov,L , Matthew R. Parsek.Alginate Overproduction Affects Pseudomonas aeruginosa. Biofilm Structure and Function .Journal of Bacteriology. 2001; 5395–5401.
[20] Upadhyay S, Sen MR, Bhattacharjee A. Presence of different beta-lactamase classes among clinical isolates of *Pseudomonas aeruginosa* expressing AmpC beta-lactamase enzyme. J Infect Dev Ctries 2010;4:239-42.
[21] Poirel L, Naas T, Nicholas D, Collet L, Bellais S, Cavallo JD, et al. Characterization of VIM-2, a Carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid-and integron-born gene from a Pseudomonas aeruginosa clinical isolate in France. Antimicrob Agents Chemotherapy. 2000; 44:891-97.
[22] Navaneeth BV, Sridaran D, Sahay D, Belwadi MR. A preliminary study on metallo-beta-lactamase producing *Pseudomonas aeruginosa* in hospitalized patients. Indian J Med Res 2002;116:264-7.
[23] Varaiya A, Kulkarni N, Kulkarni M, Bhalekar P, Dogra J. Incidence of metallo beta lactamase producing *Pseudomonas aeruginosa* in ICU patients. Indian J Med Res 2008;127:398-402.
[24] Sader HS, Reis AO, Silbert S, Gales AC. IMPs, VIMs and SPMs: The diversity of metallo-beta-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. ClinMicrobiol Infect 2005;11:73-6.
[25] Freshteh S, Mohammad RS, Hanieh N. Molecular identification of ESBL genes blaGES-1, blaVEB-1, blaCTx-M, blaOXA-1, blaOXA-4, blaOXA-10 and blaPER-1 in *Pseudomonas aeruginosa* strains isolated from burns patients by PCR, RFLP and sequencing techniques. Internal J Biol Life Sci 2010;6:138-41.
[26] Pitout JD, Chow BL, Gregson DB, Laupland KB, Elsayed S, Church DL. Molecular epidemiology of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* in the Calgary Health Region: Emergence of VIM-2-producing isolates. J ClinMicrobiol 2007;45:294-8.

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