行政院國家科學委員會專題研究計畫　成果報告

carbapenem 抗藥性綠膿桿菌之分子流行病學調查及其產生金屬性 beta-環環環酶環行環研究

計畫類別： 個別型計畫
計畫編號： NSC93-2314-B-002-084-
執行期間： 93 年 08 月 01 日至 94 年 07 月 31 日
執行環位： 國立臺灣大學醫學院內科

計畫主持人： 王振泰
共同主持人： 環望徽，張上淳

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 94 年 10 月 14 日

計畫中文摘要。（五百字以內）

綠膿桿菌（*Pseudomonas aeruginosa*）是革蘭氏陰性桿菌之一。其臨床上的重要性在於本身的高抗藥性特質，以及是院內感染的重要致病菌之一。在美國的院內感染監控系統中，綠膿桿菌是引起院內感染的第二常見致病菌；而在台大醫院長達 20 年的院內感染監控追蹤調查中，綠膿桿菌一直是前三名之一的重要致病菌。由於綠膿桿菌天生即對許多抗生素具抗藥性，所以當其造成臨床上的感染時，可以選用治療的抗生素也就有限。而 carbapenem 類的抗生素，是臨床上治療綠膿桿菌及其它許多抗藥性細菌的主要抗生素之一；唯近年來許多國家（日本，義大利，法國，英國，美國等）發現有越來越多的綠膿桿菌菌株對 carbapenem 類的抗生素產生抗藥性，對臨床治療造成莫大的衝擊。而台大醫院 2002 內造成院內感染的 357 株綠膿桿菌中，已有 13%對 carbapenem 是具有抗藥性的。這對臨床治療綠膿桿菌的影響不可謂不大。

綠膿桿菌對 carbapenem 產生抗藥性的機轉，早年的研究發現主要是因為細胞外膜的 OprD 減少、multidrug efflux pump、細胞通透性減低與β-環單醯酶（β-lactamase）的交互作用等。但最近的文獻報告指出，某些綠膿桿菌菌株因獲得了金屬性 -環單醯酶（metallo-β-lactamase）而產生了 carbapenem 抗藥性。而此種抗藥性因為是由 integron 所攜帶，所以可以在不同的細菌細胞間傳遞，而進一步造成抗藥性的散播。因之，此種因獲得金屬性β-環單醯酶而產生的 carbapenem 抗藥性，愈來愈受到微生物學界與醫學界的注意。本研究即針對台大醫院、國家衛生研究院 TSAR 研究中所收集的 carbapenem 抗藥性綠膿桿菌進行實驗，研究其抗藥性機轉是否為金屬性β-環單醯酶所引起；並探討這些菌株的分子流行病學。研究結果顯示，台灣地區的 carbapenem 抗藥性綠膿桿菌仍屬於 polyclonal，沒有 clonal spread 的現象；而 carbapenem 抗藥性綠膿桿菌產生金屬性β-環單醯酶的比率為 18%。

關鍵詞：綠膿桿菌，carbapenem 抗藥性，金屬性β-環單醯酶，分子流行病學

計畫英文摘要。（五百字以內）

Pseudomonas aeruginosa is a Gram-negative bacillus. It is intrinsically resistant to many antimicrobial agents and one of the leading pathogens causing nosocomial infections. According to the data from National Nosocomial Infection Surveillance of USA, *P. aeruginosa* is the second leading pathogen of nosocomial infection. In the longitudinal surveillance of nosocomial infection during a period of 20 years at National Taiwan University Hospital (NTUH), *P. aeruginosa* is always one of the leading three nosocomial pathogens at NTUH. Given the fact of intrinsically multi-drug resistance of *P. aeruginosa*, number of effective antibiotics to treat infections caused by *P. aeruginosa* is limited. Carbapenem is one of the major antibiotics to treat *P. aeruginosa*. However, more and more reports about emergence of carbapenem-resistant *P. aeruginosa* in Japan, Italy, France, United Kingdom, America, and so on, have been noted. This will further limit the choice of effective antibiotics for *P. aeruginosa*.

The carbapenem resistance mechanism in *P. aeruginosa* is previously reported to be associated with loss of OprD, existence of multidrug efflux pump, and interplay between impermeability and chromosome-encoded β-lactamase. However, recent reports from Japan, Italy, France, and United Kingdom demonstrate that this resistance can be mediated by acquirement of a foreign metallo-β-lactamase, which is encoded by an integron-borne gene element. The resistant gene can be transferred between different bacteria and will lead to further spread of carbapenem resistance. Therefore, integron-borne gene mediated carbapenem resistance, due to the production of metallo-β-lactamase, has become a growing problem for microbiology and clinical medicine. The present study investigated the moleculary epidemiology and prevalence of metallo-β-lactamase producing in carbapenem resistant *P. aeruginosa* collected at NTUH and TSAR study conducted by National Health

Research Institute. We found that the isolates of carbapenem-resistant *P. aeruginosa* remained polyclonal at NTUH and in Taiwan. The prevalence of carbapenamase-production among carbapenem-resistant *P. aeruginosa* was 18%.

Key words: *Pseudomonas aeruginosa*, carbapenem resistance, metallo-beta-lactamase, pulsed field gel electrophoresis

研究計畫之前言、目的與文獻探討

Pseudomonas aeruginosa is a Gram-negative bacillus [1, 2]. It is intrinsically resistant to many antimicrobial agents and one of the leading pathogens causing nosocomial infections [1-3]. According to the data from National Nosocomial Infection Surveillance of USA, *P. aeruginosa* is the second leading pathogen of nosocomial infection [3]. In the longitudinal surveillance of nosocomial infection during a 20-year period at National Taiwan University Hospital (NTUH), *P. aeruginosa* is always one of the three leading nosocomial pathogens at NTUH [4, 5]. Given the fact of intrinsically multi-drug resistance of *P. aeruginosa*, number of effective antibiotics to treat infections caused by *P. aeruginosa* is limited.

Carbapenem, a potent β-lactam resistant to hydrolysis by most β-lactamases, have become the drugs of choice for *P. aeruginosa* infections and have retained better activity than other antimicrobials [6]. However, more and more reports about emergence of carbapenem-resistant *P. aeruginosa* have been noted [7-16]. This will further limit the choice of effective antibiotics for *P. aeruginosa*.

The carbapenem resistance mechanism in *P. aeruginosa* is previously reported to be associated with loss of OprD, existence of multidrug efflux pump, and interplay between impermeability and chromosome-encoded β-lactamase [7-11, 17]. But most recent reports describe β-lactamase-, carbapenemase-, mediated resistance [12-16, 18-20]. These carbapenemases are metallo-β-lactamases belonging to molecular class B, which is capable of hydrolyzing both imipenem and most β-lactam antibiotics, and can confer resistance to these agents in pathogenic bacteria [21, 22].

The metallo-β-lactamses found in *P. aeruginosa* are classified into two types, IMP and VIM type, and the IMP-1, VIM-1, VIM-2 carbapenemase are the most common ones [12-16, 18-20]. The genes coding for these carbapenemases are located in various integrons, which can be transferred between different bacteria [14, 16, 23,

24]. The transferability of these resistant genes leads to the possibility of further spread of carbapenem resistance between different bacteria. Therefore, metallo-β-lactamase producing *P. aeruginosa* has become a growing problem in microbiology, clinical medicine, and infection control.

P. aeruginosa causes 357 episodes of nosocomial infection at NTUH in 2002. Among the 357 isolates of *P. aeruginosa*, 13% of them are resistant to carbapenem. It is important to realize the prevalence of production of metallo-β-lactamses among carbapenem-resistant *P. aeruginosa* in Taiwan.

研究方法

Bacterial Isolates

Clinical isolates of *P. aeruginosa* stored at Laboratory of National Taiwan University Hospital (NTUH) during 1999-2003 were screened by antimicrobial susceptibility determined by disc diffusion method first [25]. Those that demonstrate carbapenem resistance were enrolled to undergo further microbiologic studies. No duplicate isolate from the same patient and no strains from a single outbreak will be included. Strains of carbapenem-resistant *P. aeruginosa* at NHRI collected from 22 other hospitals located in different regions in Taiwan between 1998 and 2000 were also enrolled. It is estimated that there will be 150 isolates that are carbapenem-resistant. The further microbiologic studies included antimicrobial susceptibilities (minimal inhibitory concentrations) determined by agar dilution method [26], molecular epidemiology by pulsed field gel electrophoresis, assay of the activities of metallo-β-lactamase, and production of metallo-β-lactamase determined by polymerase chain reaction (PCR).

Antimicrobial Agents

The antimicrobial agents were supplied by individual pharmaceutical companies as standard reference powder for laboratory use. The following antimicrobials were tested: amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, and ceftazidime (GalxoSmithKline Ltd.); ampicillin/sulbactam (Pfizer Ltd.); cefpirome (Aventis Ltd.); piperacillin (Wyeth-Ayerst Ltd.); aztreonam and cefepime (Bristol-Myers Squibb Ltd.); imipenem (Merck Sharp & Dohme Ltd.); meropenem (Sumitomo Ltd.).

Susceptibility Test

The minimum inhibitory concentrations (MICs) of each antimicrobial agent for the tested bacterial isolates were determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS) of the USA [26]. Inocula of 10^4 colony-forming units (CFU) of aerobic bacteria was inoculated onto the Mueller-Hinton agar plates containing a series two-fold dilution of tested antimicrobial agents with the Steers' replicator. Following inoculation, the agar plates were incubated at 35℃ in 5% CO_2 for 18-20 h, the MIC will be read as the lowest concentration of the antimicrobial agents that completely inhibited the growth of bacteria. The concentration of antimicrobial agents were tested for all bacteria ranged from 0.03 μg/mL to 256μg/mL. *P. aeruginosa* ATCC 27853 was used as internal control for each run of test. Carbapenem-non-susceptible strains will be defined as the MIC of the test strains = 8 (intermediate) and MIC ≧16 μg/mL (resistance).

Molecular Typing using Pulsed-field gel electrophoresis

All strains of carbapenem-resistant *P. aeruginosa* were enrolled to undergo pulsed-field gel electrophoresis, which was used to evaluate the epidemiological relatedness of those strains [27, 28]. Bacterial suspensions were prepared by scraping several bacterial colonies directly from overnight-incubated cultures on sheep blood agar. After washing in 1 mL PIV buffer (1 mol/L NaCl, 10 mmol/L Tris-Cl, pH8.0), the bacteria was resuspended in 0.5 mL PIV buffer and the suspension was adjusted to an optical density (OD_{620}) of 3.0. the bacterial suspensions were then mixed with an

equal volume of 1.6% low-melting agarose (Boehringer GmbH, Mannheim, Germany) in PIV buffer and allowed to solidify in plug molds. The bacteria was then lysed by incubation of the agarose plugs at 37°C for 4 hours with lysostaphin (50 μg/mL) (Sigma, St. Louis, MO, USA) and RNase (50 μg/mL) (Boehringer GmbH) in 1 mL EC buffer (6 mmol/L Tris, pH 8.0, 1 mol/L NaCl, 0.1 mol/L EDTA, pH 8.0, 0.2% sodium deoxycholate, 0.5% Sarkosyl). Next, the lysis buffer was replaced with 1 mL ESP buffer (0.5 mmol/L EDTA, pH 9.0, 1% Sarkosyl, 1 mg/mL proteinase K) and incubated overnight at 50°C. The agarose plugs was then washed three times with 10 mL of Tris EDTA (TE) buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 8.0) for 30 minutes at room temperature and transfer to a tube containing TE buffer and placed in a refrigerator at 4°C until use.

For restriction endonuclease digestion, approximately 1 to 1.5 mm of a plug was cut and incubated overnight with 250 uL of restriction buffer containing 20 U of SmaI (Biolab Laboratories, Beverly, MA, USE) at 25°C. After DNA digestion, the agarose plugs were incubated with 1 mL of TE buffer at 37°C for 1 hour. The plugs were then inserted into 1% agarose gels (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5x TBE buffer, and restriction fragments were separated using a contour-clamped homogeneous electric field system (CHEF-DRII; Bio-Rad Laboratories). Electrophoresis was performed at 200 V for 24 hours with pulse times of to 35 seconds at 4°C, and the gels were stained with ethidium bromide and photographed under ultraviolet light. Chromosomal DNA of *S. aureus* NCTC 8325 digested with *Sma*I was used as the molecular weight marker.

PCR amplification of metallo-β-lactamase genes

Method for PCR amplification and nucleotide sequencing

Template DNAs were prepared as described in previous report [28]. PCR amplification was performed in a 50-μl volume with the GeneAmp PCR system 9600. Reaction mixtures contained 1 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, 1 X reaction buffer containing 1.5 mM $MgCl_2$, 2.5 U of *Taq* polymerase, and approximately 25 ng of template DNA. After an initial denaturation step (2 minutes at 94℃), 30 cycles of amplification were performed, as follows: denature at 94℃ for 1 minute, annealing at 55℃ for 1 minute, and DNA extension at 72℃ for 15 minutes. After agarose gel electrophoresis, the ethidium bromide-stained PCR products will be visualized under UV light [18]. All PCR products were further sequenced using a 377 automated fluorescent DNA sequencing system (Perkin-Elmer, Foster City, Calif.). All results were repeated.

Primers using in PCR procedures

The following primers were used to detect the existence of bla_{IMP-1}, bla_{VIM-1}, and bla_{VIM-2} gene [14].

bla_{IMP-1}: 5'-CTACCGCAGCAGAGTCTTTGC-3'

5'-GAACAACCAGTTTTGCCTTACC-3'

bla_{VIM-1}: 5'-TCTACATGACCGCGTCTGTC-3'

5'-TGTGCTTTGACAACGTTCGC-3'

bla_{VIM-2}: 5'-ATGTTCAAACTTTTGAGTAGTAAG-3'

5’-CTACTCAACGACTGAGCG-3’

β-lactamase assays

β-lactamase activity in crude cell extracts were assayed spectophotometrically. Reactions were performed in 30 mM N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-NaOH buffer (pH 7.0) (AB) at 25℃ in total volume of 0.75 mL. Imipenem hydrolysis was monitored at 299 nm ($\Delta\varepsilon$ = -9,000 M^{-1} cm^{-1}) by using an initial substrate concentration of 150 μM. Inhibition of enzymatic activity by EDTA was assayed by measuring the residual carbapenemase activity after incubation of the crude extract for 20 minutes at 25℃ in the presence of 2 mM EDTA (EDTA was added to the crude extract from a 100 mM stock solution in AB). A control without EDTA was always run in parallel. Reactivation by Zn^{2+} alone on the enzymatic activity was assayed by measuring the carbapenemase activity after incubation of the EDTA-treated enzyme preparation for 20 minutes at 25℃ in the presence of 2 mM Zn^{2+} (Zn^{2+} will be directly added to the EDTA-treated extract as $ZnCl_2$, which was from a 100 mM stock solution in 10 mM HCL). Controls for the effect of Zn^{2+} alone on the enzymatic activity and on the substrate stability were also included. Crude cell extracts will be prepared as follows. Cells will be grown in Mueller-Hinton broth aerobically at 37℃ until the late exponential phase, collected by centrifugation, re-suspended in AB (1/10 of the original culture volume), and disrupted by sonication (six times for 15 seconds each time at 50W). The supernatant obtained after centrifugation at 10,000 X g for 10 minutes to remove the cell debris represents the crude extract. The protein concentration in the solution will be determined with a commercial kit (Bio-Rad protein assay; Bio-Rad, Richmond, Calif.), with bovine serum albumin used as a standard [23].

結果

A total of 111 isolates carbapenem-resistant *P. aeruginosa* were identified. The drug susceptibilities to various antimicrobial agents of these 111 isolates were listed in Table. Molecular typing using PFGE of these 111 isolates revealed they were polyclonal and no evidence of clonal spread (part of the PFGE result was demonstrated in Figure 1). Using PCR methods, 20 of these 111 isolates were found to bear the VIM2 (2 isolates), VIM3 (17 isolates), and VIM11 (1 isolates) gene (Figure 2). All 20 isolates demonstrated activity of metallo-β-lactamase.

Table

	AZT	CEF	CEP	CTZ	IMI	MEM	AM/C	PIP	TIC/C
MIC (μg/mL) range	0.25 ~ 128	1 ~ 64	1 ~ 128	8 ~ 128	64 ~ >128	4 ~ 128	128 ~ >128	2 ~ >128	8 ~ >128
MIC_{90}(μg/mL)	128	64	128	>128	>128	128	>128	>128	>128

Abbreviation: AZT, aztreonam; CEF, cefepime; CEP, cefpirome; CTZ, ceftazidime; IMI, imipenem; MEM, meropenem; AM/C, amoxicillin/clavulanate; PIP, piperacillin; TIC/C, ticarcillin/clavulanate.

Figure 1. Part of the PFGE result, which revealed that these carbapenem-resistant *P. aeruginosa* were polyclonal.

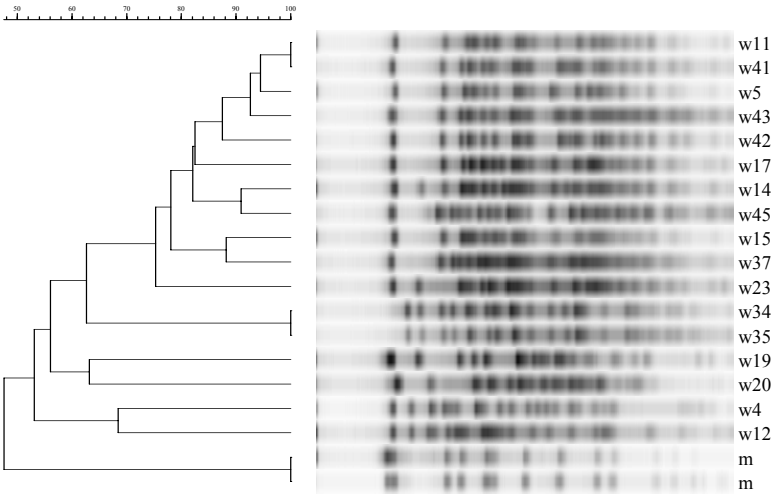
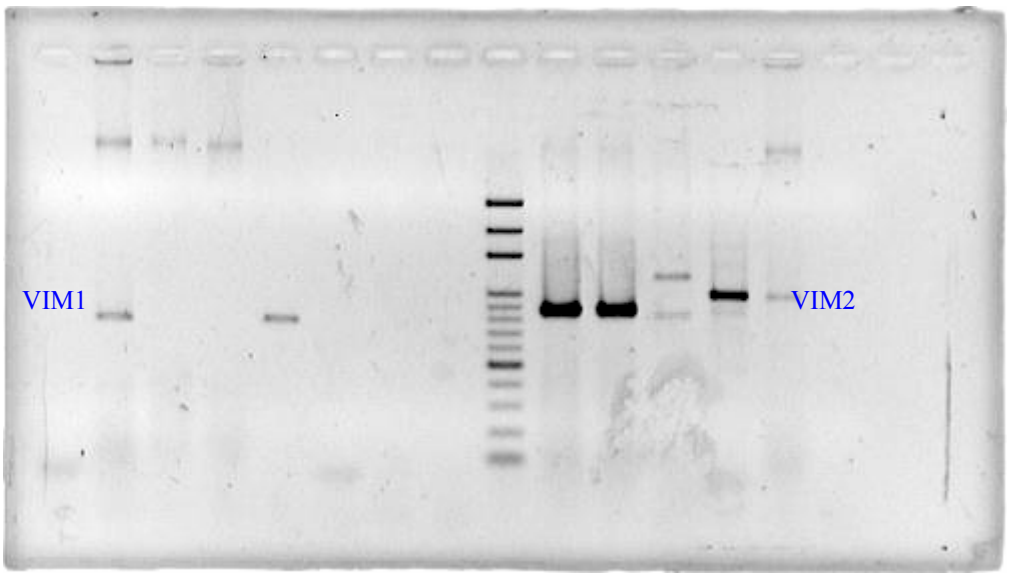


Figure 2. Result of gel electrophoresis of PCR products



結論

1. The prevalence rate of metallo-β-lactamase producing *P. aeruginosa* among carbapnem-resistant *P. aeruginosa* in Taiwan was around 18%, which was higher than the reports (5 ~ 7%) from foreign countries. This implied that the degree of spread of metallo-β-lactamase in *P. aeruginosa* was more severe in Taiwan, which might lead to further rapid increase of carbapnem resistance among *P. aeruginosa* in Taiwan.
2. VIM3 coding metallo-β-lactamase was predominant among metallo-β-lactamase producing *P. aeruginosa* in Taiwan, which was also different to reports from foreign countries (IMP1, VIM1, VIM2 predominant). The implication of this phenomenon was worthy of further investigation.
3. Among the isolates of carbapnem-resistant *P. aeruginosa*, cefepime is the most effective drugs (susceptible rate: 30%). However, it was still not so effective.

建議

Because the prevalence rate of metallo-β-lactamase producing *P. aeruginosa* in Taiwan was higher than in other countries, prevalence and mechanism of carbapenem resistance in *P. aeruginosa* needed continuous surveillance. If clonal spread of metallo-β-lactamase producing *P. aeruginosa* happens, it will leading to rapid increase of carbapenem resistance in *P. aeruginosa*.

References

1. Kiska DL, Gilligan PH: *Pseudomonas*. In: Murray PR, Baron EJO, Jorgensen J, Pfaller MA, Yolken RH, eds. Manual of Clinical Microbiology. 8th ed. Washington DC: ASM press, 2003: 719-728.
2. Pollack M: Pseudomonas aeruginosa. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 5th ed. Philadelphia: Churchill Livingstone, 2000: 2310-2335.
3. Centers for Disease Control and Prevention. National Nosocomial Infections Surveillance (NNIS) Report, October 1968 – April 1996. Am J Infect Control. 1996;24:380-388.
4. Chen ML, Chen YC, Pan HJ, Chang SC, Yang LS, Ho SW, Luh KT, Hsieh WC, Chuang CY. Secular trends in the etiology of nosocomial infection at a teaching hospital in Taiwan, 1981-1994. Chinese J Microbiol Immunol. 1995;28:230-217.
5. National Taiwan University Hospital. Nosocomial Infection Surveillance, 2002. Taipei: National Taiwan University Hospital, 2003.
6. Bush K, Jacoby GA, Medeiros A. A functional classification scheme for β -lactamase and its correlation with molecular structure. Antimicrob. Agents Chemother. 1995;39:1211-1233.
7. Kohler T, Michea-Hamzhpour M, Simone FE, Pechere JC. Carbapenem activities against *Pseudomonas aeruginosa*: respective contribution of OprD and efflux systems. Antimicrob Agents Chemother. 1999;43:424-427.
8. Kohler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechere JC. Characterization of Mex E-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol. 1997;23:345-354.
9. Livermore DM. Interplay of impermeability and chromosomal b-lactamase

activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1992;36:2046-2048.

10. Maseda H, Yoneyama H, Nakae T. Assignment of the substrate-selective suunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2000;44:658-664.
11. Ochs M, McCusker M, Bains M, Hancock R. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob Agents Chemother. 1999;43:1085-1090.
12. Senda K, Arakawa Y, Nadashima K, Hideo I, Ichiyama S, Shimokata K, Kato N, Ohta M. Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum β-lactams, including carbapenem. Antimicrob Agents Chemother. 1996;40:349-353.
13. Cardoso O, Sousa JC, Leitao R, Peixe L. Carbapenem-hydrolyzing β-lactamase from clinical isolates of *Pseudomonas aeruginosa* in Portugal. J Antimicrob Chemother. 1999;44:135.
14. Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo JD, Nordmann P. Characterization of VIM-2, a carbapenem-hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob Agents Chemother. 2000;44:891-897.
15. Tsakris A, Pournaras S, Woodford N, Palepou MFI, Babini GS, Douboyas J, Livermore DM. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J Clin Microbiol. 2000;38:1290-1292.
16. Livermore DM, Woodford N. Carbapenemases: a problem in waiting? Curr Opin Microbiol. 2000;3:489-495.
17. Pai H, Kim JW, Kim J, Lee JH, choe KW, Gotoh N. Carbapenem resistance

mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob Agents Chemother. 2001;45:480-484.

18. Senda K, Arakawa Y, Ichiyama S, Nakashima K, Ito H, Ohsuka S, Shimokata K, Kato N, Ohta M. PCR detection of metallo-β-lactamase gene (bla_{IMP}) in Gram-negative rods resistant to broad-spectrum β-lactams. J Clin Microbiol. 1996;34:2909-2913.
19. Hirakata Y, Izumikawa K, Yamaguchi T, Takemura H, Tanaka H, Yoshida R, Matshda J, Nadano M, Tomono K, Maesaki S, Kaku M, Yamada Y, Kamihira S, Kohno S. Rapid detection and evaluation of clinical characteristics of emerging multiple-drug-resistant Gram-negative rods carrying the metallo-β-lcatamase gene bla_{IMP}. Antimicrob Agents Chemother. 1998;42:2006-2011.
20. Laraki N, Galleni M, Thamm I, Riccio ML, Amicosante G, Frere JM, Rossolini GM. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related In5, which carries an unusual array of gene cassettes. Antimicrob Agents Chemother. 1999;43:890-901.
21. Livermore DM. Carbapenemase. J Antimicrob Chemother. 1992;29:609-616.
22. Rasmussen BA, Bush K. Carbapenem-hydrolyzing β-lactamases. Antimicrob Agents Chemother. 1997;41:223-232.
23. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, Rossolini GM. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother. 1999;43:1584-1590.
24. Arakawa Y, Murakami M, Suzuki K, Ito H, Wacharotayankun R, Ohsuka S, Kato N, Ohta M. A novel integron-like element carrying the metallo-β-lactamase gen bla_{IMP}. Antimicrob Agnets Chemother. 1995;39:1612-1615.
25. National Committee for Clinical Laboratory Standards. Performance Standards

for Antimicrobial Disk Diffusion Susceptibility Tests, -seventh ediдtion, Approved Standard. 2000; NCCLS documents, Wayne, Pennsylvania.

26. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. 2000; NCCLS documents, Wayne, Pennsylvania.
27. Thuong M, Arvaniti K, Ruimy R, de la Salmoniere P, Scanvic-Hameg A, Lucet JC, Regnier B. Epidemiology of *Pseudomonas aeruginosa* and risk factors for carriage acquisition in an intensive care unit. J Hosp Infect. 2003;53:274-282.
28. Engelhart S, Krizek L, Glasmacher A, Fischnaller E, Marklein G, Exner M. *Pseudomonas aeruginosa* outbreak in a haematology-oncology unit associated with contaminated surface cleaning equipment. J Hosp Infect. 2002;52:93-98.