T. Tada, T. Miyoshi-Akiyama, RK. Dahal, M.K Sah, H. Ohara, **T.** Kirikae, B.M. Pokhrel.	NDM-8 metallo-β-lactamase in a multidrug-resistant *Escherichia coli* strain isolated in Nepal.	Antimicrob. Agents. Chemother.	57(5)	2394-2396	2013
T. Tada, T. Miyoshi-Akiyama, Y. Kato, N. Ohmagari, N. Takeshita, N.V. Hung, D.M. Phuong, T.A. Thu, N.G. Binh, N.Q. Anh, T.T. Nga, P.H. Truong, P.T. Xuan, L.T. Thu, N.T. Son, T. Kirikae.	Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates in hospitals in Vietnam.	BMC Infect. Dis.	13(1)	251	2013
T. Tada, T. Miyoshi-Akiyama, K. Shimada, M. Shimima, T. Kirikae.	IMP-43 and IMP-44 metallo-β-lactamases with increased carbapenemase activities in multidrug-resistant *Pseudomonas aseurinosa*.	Antimicrob. Agents. Chemother.	57(9)	4427-4432	2013
T. Tada, T. Miyoshi-Akiyama, R.K. Dahal, S.K. Mishra, H. Ohara, K. Shimada, T. Kirikae, B.M. Pokhrel.	Dissemination of multidrug-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal.	Int. J. Antimicob. Agents.	42(4)	372-374	2013

Y. Hamada, K. Watanabe, T. Tada, K. Mezaki, S. Takeuchi, T. Shimizu, T. Kirikae, N. Ohmagari.	Three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* blood stream infection in Japan.	J. Infect. Chemother.	19(5)	956-958	2013
T. Tada, T. Miyoshi-Akiyama, R.K. Dahal, M.K. Sah, H. Ohara, K. Shimada, **T.** Kirikae, B.M. Pokhrel.	NDM-1 Metallo-beta-Lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal.	BMC Infect. Dis.	14(1)	56	2014
T. Tada, T. Miyoshi-Akiyama, K. Shimada, M. Shimojima, T. Kirikae.	Dissemination of 16S rRNA methylase ArmA-producing *Acinetobacter baumannii* and emergence of OXA-72 carbapenemase-coproducers in Japan.	Antimicrob. Agents. Chemother.	In press		
Kurushima J, Hayashi I, Sugai M, Tomita H.	Bacteriocin protein BacL1 of *Enterococcus faecalis* is a peptidoglycan D-isoglutamyl-L-lysine endopeptidase.	Journal of Biological Chemistry.	288	36915-36925	2103
Hirakawa H, Tomita H.	Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance.	Frontiers Microbiology.	4	114	2013

Matsumoto T, Hirayama Y, Hisamitsu Y, Fukumura M, Hirata A, Tanaka K, Kurokawa M, Tamura Y, Yoshida H, Suzuki K, Nagai T, Kawase I, Hoshino Y	Simultaneous and Longitudinal Comparison of Interferon Gamma Release Assays Among Health Care Workers in Japan	Mycobacterial Diseases	in press.		
Matsumoto T, Koshii Y, Sakane K, Murakawa T, HirayamaY, Yoshida H, Kurokawa M, Tamura Y, Nagai T, Kawase I	A novel approach to automated genotyping of Mycobacterium tuberculosis using a panel of 15 MIRU VNTRs.	Journal of microbiological methods	93	239-241	2013
Matsumoto T, Ogata H, Toyota E, Suzuki K, Saito T, Fujita A, Suetake T, Chikamatsu K, Mizuno K, Mitarai S	Clinical evaluation of a line probe assay kit for the identification of mycobacterium species and detection of drug-resistant mycobacterium tuberculosis	Kekkaku: [Tuberculosis]	88(3)	253-259	2013
Takaya A, Sato Y, Shoji K, Sone K, Yamamoto T.	Methylation of 23S rRNA nucleotide G748 by RlmA^{II} methyltransferase renders *Streptococcus pneumoniae* telithromycin-susceptible.	Antimicrob. Agents Chemother	57	3789-3796	2013
Morioka I, Takahashi N, Kitajima H; Committee for Infection Prevention and Vaccine Promotion of the Japan Society for Premature and Newborn Medicine.	Prevalence of MRSA colonization in Japanese neonatal care unit patients in 2011.	Pediatr Int.	2013 Oct 15. doi: 10.1111/ped.12232.		2013

別紙 5-1

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
松本智成	感染症の診断と治療、予防-最近の進歩- 4. IGRA による結核診断	渡辺彰	日本内科学会雑誌	日本内科学会	東京	2013	p2888-2899
松本智成	結核 －古くて新しい感染症－結核菌の分子疫学の展開		最新医学 68 巻 11 月	最新医学社	大阪	2013	p2496-2502
松本智成	多剤耐性結核の現状 呼　吸　32(8) p697-702　2013		呼吸 32(8)	一般社団法人 呼吸研究	東京	2013	697-702
山本友子	マクロライド耐性-肺炎球菌のもつマクロライド耐性機構を中心に-	富田治芳	化学療法の領域特集・細菌の進化から考える抗菌薬耐性	医薬ジャーナル社	東京	2013	44-54

Ⅳ. 研究成果の刊行物・別冊

J Infect Chemother xxx (2013) 1–4

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Case report

Chromobacterium violaceum nosocomial pneumonia in two Japanese patients at an intensive care unit

Hideharu Hagiya [a,*], Tomoko Murase [b], Masato Suzuki [c], Keigo Shibayama [c], Yumi Kokumai [d], Naoto Watanabe [b], Miyako Maki [b], Fumio Otsuka [a]

[a] Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan
[b] Department of Clinical Laboratory, Microbiology Division, Tsuyama Central Hospital, 1756 Kawasaki, Tsuyama, Okayama 708-0841, Japan
[c] Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan
[d] Department of Nursing, Microbiology Division, Tsuyama Central Hospital, 1756 Kawasaki, Tsuyama, Okayama 708-0841, Japan

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form
30 September 2013
Accepted 3 October 2013

Keywords:
Chromobacterium violaceum infection
Global warming
Nosocomial infection
Intensive care unit
Respiratory care device

ABSTRACT

Chromobacterium violaceum is sensitive to temperature and the infection is usually confined to tropical or subtropical regions. Since Japan has a warm climate, *C. violaceum* has been scarcely isolated from clinical specimens. With global warming, however, the geographical distribution of *C. violaceum* infection is likely to change. We report two cases of *C. violaceum* nosocomial pneumonia that occurred at an intensive care center in Japan. *C. violaceum* was first detected from a patient in the same center as a pathogenic organism of pneumonia. Later, the organism was isolated from sputum and a ventilator circuit tube of another patient in the center. The two patients were admitted to the center in nearby beds for several days. All of the pathogens were confirmed to be *C. violaceum* by the nucleic acid sequence of the 16S rRNA gene and were proven to be genetically identical organisms by pulsed field gel electrophoresis. Both patients were managed with well-humidified and heated oxygen using a venturi mask and ventilator to promote excretion of sputum. It was thought that the medical respiratory care devices that provide a humid and warm environment, an optimal condition for proliferation of *C. violaceum*, can contribute to *C. violaceum* infection in a hospital environment.

1. Introduction

Chromobacterium violaceum is a gram-negative, facultative anaerobic, oxidase-positive bacillus. The organism is ubiquitous in natural environments but is a rare pathogen in humans. Its growth is significantly affected by temperature, and infection has a predilection to tropical or subtropical regions (between latitudes 35°N and 35°S) [1]. Fewer than 200 cases of *C. violaceum* infection have been reported worldwide so far, and most cases have been seen in those geographically confined areas [2]. In the United States, a previous report showed that almost all cases occurred mainly in Florida, the southern most part of the country [3].

In a warm climate, *C. violaceum* has rarely been isolated from clinical specimens. To our knowledge, there has been only one reported case of *C. violaceum* infection in Japan to date [4]. According to that report, a 59-year-old man with a history of diabetes mellitus died of septic shock and multi-organ failure, which began from cellulitis in his right thigh. An autopsy revealed systemic dissemination of the organism. With global warming, however, the geographical distribution of *C. violaceum* infection is likely to change in the future [5]. Here, we report two cases of *C. violaceum* nosocomial pneumonia that occurred at an intensive care center in winter in Japan.

2. Case description

Following two cases occurred at an open-type combined medical/surgical intensive care center of Tsuyma Central Hospital (TCH; Okayama, Japan) where there are an 8-beded intensive care unit (ICU) and a 10-beded high care unit (HCU).

2.1. Case 1

In December 2012, a 66-year-old man with a past medical history of hypertension and chronic obstructive pulmonary disease was transferred to TCH due to a sudden onset of impaired

* Corresponding author. Tel.: +81 86 235 7342; fax: +81 86 235 7345.
E-mail address: e_dai_for_all@hotmail.com (H. Hagiya).

1341-321X/$ – see front matter © 2013, Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jiac.2013.10.001

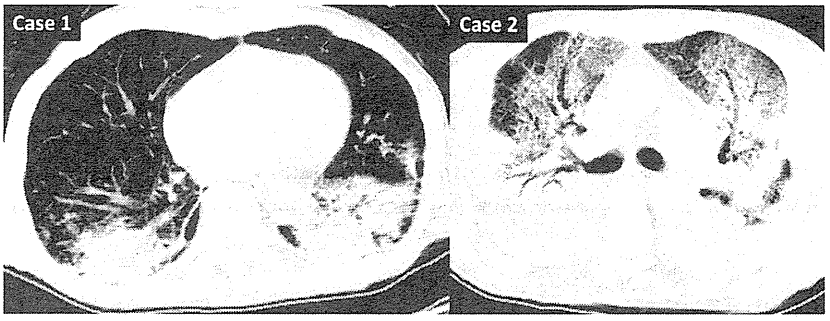

Fig. 1. Chest computed tomography findings. Case 1: Bilateral consolidation (day 10). Case 2: Bilateral consolidation and massive pleural effusion with ground glass opacity (day 31).

consciousness. He had no history of traveling abroad. A computed tomography (CT) scan of the head revealed subarachnoid hemorrhage (SAH), and an emergent clipping operation for a cerebral aneurysm was performed (day 1). After the operation, the patient was admitted to the ICU and extubation was successfully performed the next day. Beginning on day 8, well-humidified and heated highly concentrated oxygen was administered with a venturi mask for the purpose of promoting excretion of sputum. Subsequently, he had high fever and laboratory data on day 10 showed that inflammation was increasing (white blood cell [WBC], 9100/mm^3; C-reactive protein [CRP], 24.0 mg/dL) and a chest CT scan revealed bilateral consolidation (Fig. 1(A)). Gram staining of purulent sputum on day 11 (Miller & Jones classification: P3, Geckler classification: 5) showed many inflammatory cells phagocytozing Gram-negative rods, and bacterial culture revealed *C. violaceum* concurrently with *Neisseria* spp. and *Streptococcus* spp. (MicroscanWalkAway 40SI, NegCombo3.12J panel; SIEMENS, Tokyo, Japan). A full-body CT scan did not show any visceral organ abscesses, and serial blood cultures were all negative. Based on the diagnosis of *C. violaceum* pneumonia, administration of meropenem (1 g every 8 h) was initiated. The patient's respiratory condition improved after 2 weeks, and the patient was moved to a surgical ward. *C. violaceum* was isolated from his sputum five times throughout the course.

2.2. Case 2

An 80-year-old man who was administered 30 mg/day prednisolone for 1 month due to organizing pneumonia underwent gastric segmentectomy for gastric cancer at TCH 8 days after Case 1 had been admitted. He was managed at the HCU for a few hours after the operation and returned to a surgical ward. Approximately one week later, the patient developed aspiration pneumonia and was transferred to the ICU (day 17). Due to respiratory failure, he was intubated and required a tracheostomy on day 22. His respiratory state stabilized after that, and ventilatory support was discontinued. On day 26, he was transferred to the general surgical ward with a venturi mask to provide well-humidified and heated highly concentrated oxygen.

On day 31, his respiratory state deteriorated again and the patient was transferred again back to the ICU. Laboratory testing showed a high inflammatory state (WBC, 22,400/mm^3; CRP, 20.1 mg/dL), and a chest CT scan revealed bilateral consolidation and massive pleural effusion with ground glass opacity suggesting acute respiratory distress syndrome (Fig. 1(B)). Only *C. violaceum* was isolated from his purulent sputum (Miller & Jones classification: P1, Geckler classification: 4), and he was also diagnosed with *C. violaceum* pneumonia. Serial blood cultures were obtained but were negative for any organisms. Suspecting a carrier state of *C. violaceum* in the respiratory device, bacterial culture was performed using fluid inside the ventilator circuit tube; the result was positive for the organism. As in Case 1, meropenem (1 g every 8 h) was administered for 2 weeks and his respiratory condition improved.

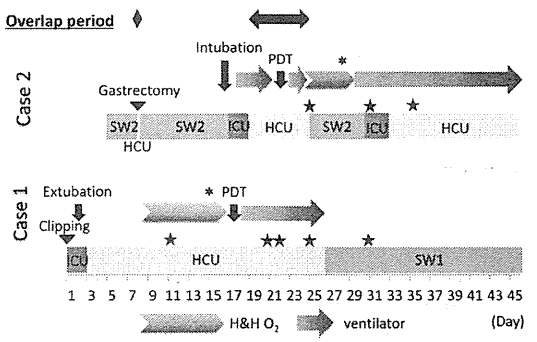

Fig. 2. Clinical courses of the two cases. ICU, intensive care unit: HCU, high care unit: SW, surgical ward. H&H O_2, humidified and heated highly concentrated oxygen. PDT, percutaneous dilatational tracheostomy. ★ indicates the day when *Chromobacterium violaceum* was isolated, and ∗ indicates the day of diagnosis of *C. violaceum* pneumonia. The day Case 1 was admitted to the ICU was set as day 1. During admission to the intensive care center, the two patients were simultaneously admitted to the HCU in close proximity but not right next to each other. H&H O_2 by using a venturi mask was given from day 8 in Case 1 and from day 25 in Case 2. Ventilator circuit tubes were also well-humidified through the course.

3. Clinical course

Time courses of the two cases are summarized in Fig. 2. The timing of hospital admission was different; however, the two patients were simultaneously admitted to the intensive care center, especially the HCU on day 8 (only a few hours) and from day 19 to

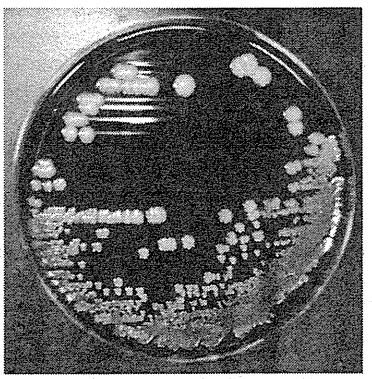

Fig. 3. Colonies of *Chromobacterium violaceum* from Case 1. The organism was incubated on sheep blood agar at 35 °C for 48 h, and only non-pigment colonies were seen. Colonies obtained from Case 2 also showed the same non-pigment appearance.

Please cite this article in press as: Hagiya H, et al., *Chromobacterium violaceum* nosocomial pneumonia in two Japanese patients at an intensive care unit, J Infect Chemother (2013), http://dx.doi.org/10.1016/j.jiac.2013.10.001

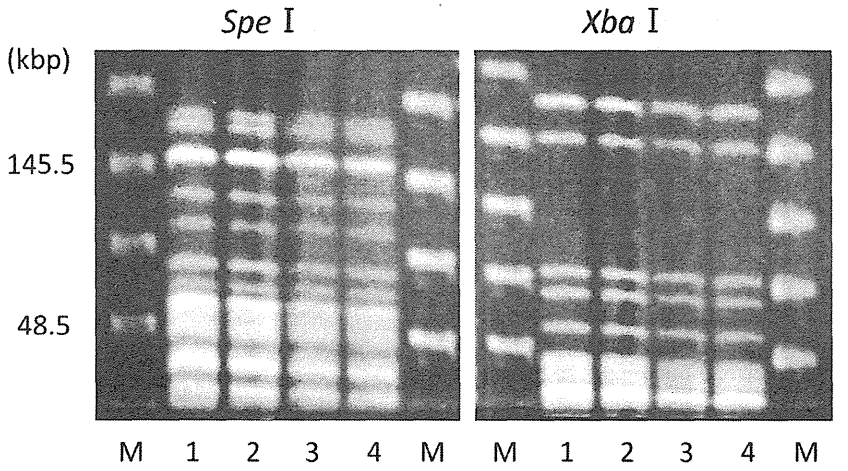

Fig. 4. Genotyping analysis by pulsed field gel electrophoresis (PFGE). Bacterial DNA was digested with *SpeI* and *XbaI* and was analyzed by PFGE. Lane M, CHEF DNA Size standards Lambda ladder marker (Bio-Rad). Lane 1, an isolate obtained from sputum in Case 1. Lanes 2 and 3, isolates obtained from sputum in Case 2. Lane 4, an isolate obtained from the ventilator circuit tube in Case 2. All 4 isolates showed the same PFGE band pattern.

25. They were admitted there closely but not right next to each other. Use of inhalants was unknown. Bronchoscopy was performed for both patients for the purpose of suctioning sputum and sampling respiratory specimens, which was also carried out for other ICU or HCU-admitted patients. Both patients were provided well-humidified and heated highly concentrated oxygen with the use of a venturi mask and ventilator during the course.

Screening cultures of their surrounding environment were performed. In total, 40 samples from independent places were obtained: 29 samples from the ICU, 9 samples from the HCU, and 2 samples from bronchoscopy. Frequent hand contact places, sinks, and medical tables were mainly targeted as sampling points. However, the results were all negative for *C. violaceum*, including routine screening cultures of respiratory specimens (twice a week) obtained from other patients in the ICU and HCU.

4. Bacterial analysis

All of the isolated organisms formed non-pigmented colonies (Fig. 3). The nucleic acid sequences of the organisms that were isolated from the sputum of both patients and from the ventilator circuit tube in Case 2 were examined by amplifying the partial 16S rRNA gene. The sequences were identical in all samples, and the organism was confirmed to have 99% homology with the published sequence of *C. violaceum* strain (GenBank Accession No.: HM449690) [6]. The sequence of our strain was registered at GenBank (Accession No.: AB851804). Moreover, all of the isolates were proven to be genetically identical by pulsed field gel electrophoresis (PFGE) using two different restriction enzymes: *SpeI* and *XbaI* (Fig. 4) [7].

The organism was considered to be sensitive to imipenem, meropenem, levofloxacin, ciprofloxacin, and sulfamethoxazole/trimethoprim but resistant to piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, gentamicin, and amikacin (Table 1).

5. Discussion

Yang et al. reviewed data for 106 patients with *C. violaceum* infection between 1952 and 2009 [5]. Of those 106 cases, 104 (98.1%) were community-acquired cases in tropical or subtropical regions, and only a few cases have been reported from outside the geographically confined area [8,9]. Tsuyama (Okayama, Japan) is located at latitude 35°4′N, which is slightly beyond the reported northern limit of previously reported infection. In addition, the present cases occurred in December, which is winter season in Japan, when the average temperature is less than 10 °C. Thus, our cases were geographically and epidemiologically uncommon. Moreover, although there are potential predisposing factors associated with *C. violaceum* infection include trauma and exposure to water or soil, the 2 cases presented herein were not related to these factors.

To date, there have been only 2 cases of healthcare-associated *C. violaceum* infection [10,11]. The reason for this can be attributed to its being a temperature-sensitive organism. Hospital environments are well controlled for patients to be comfortable but are different from the environments of subtropical and tropical areas. However, the inside of a well-humidified and heated tube of a respiratory care device closely resembles those environments. Case 1 was managed with a venturi mask in order to administer well-humidified and heated highly concentrated oxygen to promote excretion of sputum. We assume that the original *C. violaceum* had an optimal environment for proliferation inside the venturi mask tube. As a result, the organism grew aberrantly, causing pneumonia and subsequently infected the patient in Case 2. The results of PFGE confirmed that the organisms were genetically identical in the two cases. Thus, well-humidified and heated respiratory care devices can be considered to be a significant risk factor for *C. violaceum* infection in hospital environments.

Table 1
A result of antibiotics susceptibility testing of *C. violaceum*.

	MIC (μg/mL)	
	Case 1	Case 2
Piperacillin	>64	>64
Piperacillin/tazobactam	>64/4	64/4
Ceftazidime	>16	16
Cefepmie	>16	>16
Imipenem	4–8	4
Meropenem	≦1	≦1
Aztreonam	>16	>16
Gentamycin	>8	>8
Amikacin	>32	>32
Levofloxacin	≦0.5	≦0.5
Ciprofloxacin	≦0.25	≦0.25
Minocycline	4–8	4
Sulfamethoxazole/trimethoprim	≦2	≦2

MIC; minimum inhibitory concentration. Antimicrobial susceptibility testing was performed by using Microscan Walkaway (SIEMENS, Tokyo, Japan).

Please cite this article in press as: Hagiya H, et al., *Chromobacterium violaceum* nosocomial pneumonia in two Japanese patients at an intensive care unit, J Infect Chemother (2013), http://dx.doi.org/10.1016/j.jiac.2013.10.001

The original organism could have been carried by the patient in Case 1 or might have already existed in the hospital environment. However, *C. violaceum* has never been identified from a TCH, and we believe that the organism was brought into the ICU by the first patient.

Most strains of *C. violaceum* produce violacein, a pigment that provides a violet-dark color to the colonies. However, the isolates from our patients did not show such a characteristic color. A few cases of non-pigmented strains have already been reported [12]. The color can also be lost on subculture or due to the initial therapy [2]. The relationship between the epidemiology or severity of *C. violaceum* infection and positivity of the pigment color is unknown.

C. violaceum is usually sensitive to fluoroquinolones, chloramphenicol, tetracycline, trimethoprim/sulfamethoxazole, imipenem, and gentamicin [13]. Ciprofloxacin is the most active agent against *C. violaceum in vitro* [2], and cases with effective treatment have been reported [14]. Both of our patients were successfully with meropenem.

Though rare, *C. violaceum* infection can cause life-threatening sepsis with metastatic abscess, and the overall mortality rate has been reported to be 53% [5]. Superoxide dismutase and catalase, which protect the microorganism from phagocytosis, are considered to be virulent factors [15].

In summary, *C. violaceum* infection is still rare but may increase in non-tropical climates with further global climate change and with the development and wide prevalence of medical respiratory care devices. Since the organism can be multidrug-resistant and causes fatal sepsis with high mortality, the organism should be regarded as an emerging hospital-acquired pathogen in non-tropical climates.

Conflict of interest

The authors state that there are no conflicts of interest to report.

References

[1] Midani S, Rathore M. *Chromobacterium violaceum* infection. Southampt Med J 1998;91:464–6.

[2] Steinberg JP, Burd EM. Other gram-negative and gram-variable Bacilli. In: . Mandell GL, Bennett JE, Dolin R, editors. Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 7th ed., vol. 237. Churchill Livingstone Elsevier; 2009. pp. 3015–33.

[3] Ponte R, Jenkins SG. Fatal *Chromobacterium violaceum* infections associated with exposure to stagnant waters. Pediatr Infect Dis J 1992;11:583–6.

[4] Hiraoka N, Yoshioka K, Inoue K, Kawahito Y, Kasamatsu Y. *Chromobacterium violaceum* sepsis accompanied by bacteria-associated hemophagocytic syndrome in a Japanese man. Arch Intern Med 1999;159:1623–4.

[5] Yang CH, Li YH. *Chromobacterium violaceum* infection: a clinical review of an important but neglected infection. J Chin Med Assoc 2011;74:435–41.

[6] Lima-Bittencourt CI, Astolfi-Filho S, Chartone-Souza E, Santos FR, Nascimento AM. Analysis of *Chromobacterium* sp. natural isolates from different Brazilian ecosystems. BMC Microbiol 2007;7:58.

[7] Koburger JA, May SO. Isolation of *Chromobacterium* spp. From foods, soil, and water. Appl Environ Microbiol 1982;44:1463–5.

[8] Byamukama D, Farnleither AH, Kansiime F, Manafi M, Burtsher M, Mach RL. Contrasting occurrence of *Chromobacterium violaceum* in tropical drinking water springs of Uganda. J Water Health 2005;3:229–38.

[9] Bosch FJ, Badenhorst L, Le Roux JA, Louw JV. Successful treatment of *Chromobacterium violaceum* sepsis in South Africa. J Med Microbiol 2008;57:1293–5.

[10] Teoh AYB, Hui M, Ngo KY, Wong J, Lee KF, Lai PBS. Fatal septicemia from *Chromobacterium violaceum*: case reports and review of the literature. Hong Kong Med J 2006;12:228–31.

[11] Lee J, Kim JS, Nahm CH, Choi JW, Kim J, Pai SH, et al. Two cases of *Chromobacterium violaceum* infection after injury in a subtropical region. J Clin Microbiol 1999;37:2068–70.

[12] Aldridge KE, Valainis GT, Sanders CV. Comparison of the in vitro activity of ciprofloxacin and 24 other antimicrobial agents against clinical strains of *Chromobacterium violaceum*. Diagn Microbiol Infect Dis 1988;10:31–9.

[13] Moore CC, Lane JE, Stephens JL. Successful treatment of an infant with *Chromobacterium violaceum* sepsis. Clin Infect Dis 2001;32:E107–10.

[14] Miller DP, Blevins WT, Steele DB, Stowers MD. A comparative study of virulent and avirulent strains of *Chromobacterium violaceum*. Can J Microbiol 1988;34:249–55.

[15] Sabat AJ, Budimir A, Nashev D, Sá-Leão R, van Dijl JM, Laurent F, et al., ESCMID Study Group of Epidemiological Markers (ESGEM). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill 2013;18:20380.

Please cite this article in press as: Hagiya H, et al., *Chromobacterium violaceum* nosocomial pneumonia in two Japanese patients at an intensive care unit, J Infect Chemother (2013), http://dx.doi.org/10.1016/j.jiac.2013.10.001

Journal of Antimicrobial Chemotherapy Advance Access published December 18, 2013

Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother
doi:10.1093/jac/dkt498

A subclass B3 metallo-β-lactamase found in *Pseudomonas alcaligenes*

Masato Suzuki[1]*, Satowa Suzuki[1], Mari Matsui[1], Yoichi Hiraki[2], Fumio Kawano[3] and Keigo Shibayama[1]

[1]Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan; [2]Department of Pharmacy, National Hospital Organization Kumamoto Medical Center, 1-5 Ninomaru, Chuo-ku, Kumamoto 860-0008, Japan; [3]Department of Hematology, National Hospital Organization Kumamoto Medical Center, 1-5 Ninomaru, Chuo-ku, Kumamoto 860-0008, Japan

*Corresponding author. Tel: +81-42-561-0771; Fax: +81-42-561-7173; E-mail: suzuki-m@nih.go.jp

Keywords: nosocomial infections, drug resistance, bacterial genomics

Sir,

Metallo-β-lactamase (MBL) is an important resistance determinant among Gram-negative bacteria, and its clinical relevance is increasing.[1] Some MBL genes are carried on mobile gene elements that have spread among various clinically important bacterial species.[1] Here we report a case of a novel MBL-positive *Pseudomonas alcaligenes* strain, MRY13-0052, that caused a bloodstream infection in a medical institution in Japan.[2] *P. alcaligenes* is a Gram-negative aerobic bacillus belonging to the bacterial family Pseudomonadaceae, the members of which are common inhabitants of soil and water, and it is a rare opportunistic human pathogen.[3] However, little is known about the clinical importance of *P. alcaligenes*, mainly because of the difficulties in identifying and distinguishing this bacterium from closely related *Pseudomonas* species, such as *Pseudomonas aeruginosa*, *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes*, in medical settings. We report here our investigation of the draft genome sequence of *P. alcaligenes* strain MRY13-0052 and our finding that this strain contains a subclass B3 MBL,[4] PAM-1 (*P. alcaligenes* MBL-1), that can hydrolyse cephalosporins and carbapenems.

In 2012, *Pseudomonas* strain MRY13-0052 was recovered from a blood sample of a patient who was receiving therapy for Guillain–Barré syndrome. The patient had no recent history of travel abroad. Although the primary site of infection was unknown, the patient became afebrile soon after combination therapy with ceftazidime and clindamycin. The MRY13-0052 strain was identified as *P. mendocina* by the VITEK2 system (bioMérieux; 96% probability), but subsequently as *P. alcaligenes* based on 16S rRNA gene sequence analysis.[2] MRY13-0052 was resistant to penicillins, cephalosporins, aztreonam and fosfomycin, but susceptible to imipenem, meropenem, amikacin, fluoroquinolones, minocycline and trimethoprim/sulfamethoxazole, according to MICs determined using the VITEK2 system and the Etest (bioMérieux), applying the recommended breakpoints described by CLSI (2013).[5] The production of MBL was screened for using a disc containing sodium mercaptoacetic acid (SMA) (Eiken).[6] Apparent expansion of the growth inhibitory zone around the ceftazidime and meropenem discs was observed around the SMA disc following overnight incubation at 37°C, strongly suggesting that MRY13-0052 produces MBL. PCR tests to detect the bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{TMB} genes in MRY13-0052 were all negative; therefore, we analysed the whole-genome shotgun (WGS) sequence of MRY13-0052, obtained using the GS Junior system (Roche), to identify the responsible MBL gene (DDBJ/EMBL/GenBank accession number of the WGS project: BATO01000000).[2] BLAST-based similarity searches revealed that MRY13-0052 carries three class C β-lactamase genes and a novel subclass B3 MBL gene [which we named bla_{PAM-1} (DDBJ/EMBL/GenBank accession number of the gene: AB858498)] that might confer resistance to β-lactams.

The PCR product of the bla_{PAM-1} gene was ligated into pUCP19 (ATCC), a *Pseudomonas–Escherichia* shuttle vector, resulting in the PAM-1 expression vector pUCP19-blaPAM-1. *P. aeruginosa* strain PAO1 and *Escherichia coli* strain MC1061 were transformed with this vector, and transformants were selected on agar plates containing 20 mg/L piperacillin. Expression of the bla_{PAM-1} gene was driven by the *tac* promoter regardless of IPTG induction and confirmed by SMA disc-mediated expansion of the growth inhibitory zone around ceftazidime and meropenem discs. As shown in Table 1, bla_{PAM-1}-producing *P. aeruginosa* bacteria were more resistant to ceftazidime, imipenem, meropenem and doripenem than control bacteria harbouring the empty vector (MICs increased 32-fold, 2-fold, 6-fold and 21-fold, respectively), but were still as susceptible as control bacteria to aztreonam. Although bla_{PAM-1}-producing *E. coli* bacteria were slightly more resistant to ceftazidime and meropenem than control bacteria (MICs increased 4-fold and 1.4-fold), there was no apparent change in the susceptibility to aztreonam and other carbapenems. The differences in the contribution of the PAM-1 enzyme to cephalosporin and carbapenem resistance among *P. aeruginosa* and *E. coli* could reflect differences in expression levels, outer-membrane permeability and/or efflux systems in these hosts.[7]

The bla_{PAM-1} gene in *P. alcaligenes* strain MRY13-0052 is encoded in contig 73, which is part of the chromosome, and there is no transposable element, such as a transposon or integron, around the gene, suggesting that bla_{PAM-1} is an intrinsic species-specific MBL gene of *P. alcaligenes*. *Pseudomonas otitidis*, a *Pseudomonas* species that is associated with otic infections in humans,[8] also produces a resident MBL named POM-1 (*P. otitidis* MBL-1), which is active against carbapenems.[9] The PAM-1 protein exhibits close similarity to POM-1 (72.4% amino acid identity), suggesting that these enzymes have a common ancestor (Figure 1). PAM-1 and POM-1 are homologous with the L1 MBL of *Stenotrophomonas maltophilia* (63.3% and 62.1% identity,

Table 1. Antimicrobial susceptibility profiles of strains determined using the Etest

Strain	Antimicrobial agent MIC, mg/L				
	aztreonam	ceftazidime	imipenem	meropenem	doripenem
P. alcaligenes MRY13-0052	32	48	2	2	>32
P. aeruginosa PAO1/pUCP19	2	1	0.50	0.16	0.38
P. aeruginosa PAO1/pUCP19-blaPAM-1	2	32	1	1	8
E. coli MC1061/pUCP19	0.125	1	0.25	0.16	0.35
E. coli MC1061/pUCP19-blaPAM-1	0.125	4	0.25	0.23	0.35

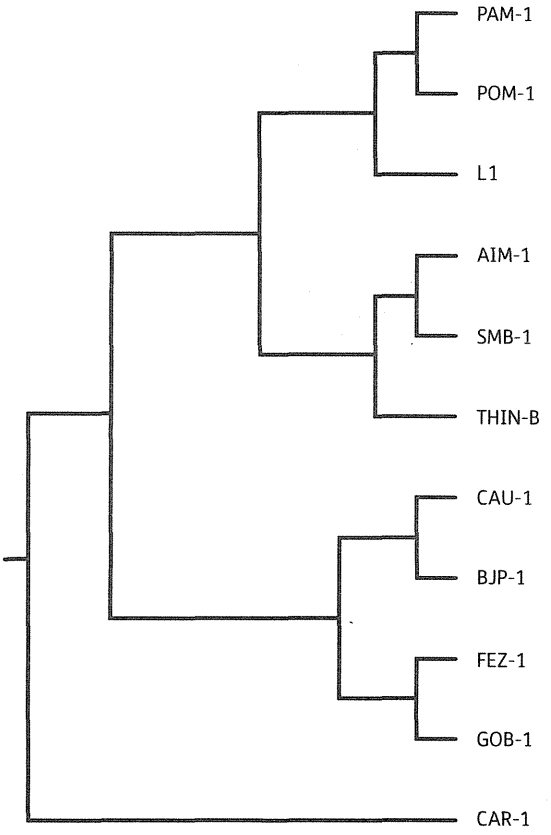

Figure 1. A rooted phylogenetic tree of subclass B3 MBL proteins, generated by ClustalW.

respectively, relative to the *S. maltophilia* strain IAM 1566 protein) (Figure 1). *S. maltophilia* is a Gram-negative bacterium found in a variety of environments, including soil, water and plants, and is therefore a potential reservoir of the MBL gene.[10] Similar to POM-1 and L1 MBLs, the ability of the PAM-1 enzyme to hydrolyse carbapenems might be relatively low; consequently, the PAM-1-positive MRY13-0052 strain was not categorized as carbapenem resistant. However, the combination of PAM-1-mediated β-lactam hydrolysis with genetic mutations that decrease outer-membrane permeability could confer high-level carbapenem resistance, leading to major concern for the treatment of *P. alcaligenes* infection.

Acknowledgements

We thank Kumiko Kai and Yoshie Taki for technical assistance. We are grateful to the participating medical institution for providing the strain and clinical information.

Funding

This work was supported by a Grant-in-Aid for Young Scientists (A) (25713017) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and H24-Shinkou-Ippan-010 from the Ministry of Health, Labour and Welfare, Japan.

Transparency declarations

None to declare.

References

1 Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamases: a last frontier for β-lactams? *Lancet Infect Dis* 2011; **11**: 381–93.

2 Suzuki M, Matsui M, Suzuki S *et al.* Genome sequence of a strain of the human pathogenic bacterium *Pseudomonas alcaligenes* that caused bloodstream infection. *Genome Announc* 2013; **1**: e00919-13.

3 Martino P, Micozzi A, Venditti M *et al.* Catheter-related right-sided endocarditis in bone marrow transplant recipients. *Rev Infect Dis* 1990; **12**: 250–7.

4 Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58.

5 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23*. CLSI, Wayne, PA, USA, 2013.

6 Shibata N, Doi Y, Yamane K *et al.* PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by Gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *J Clin Microbiol* 2003; **41**: 5407–13.

7 Miriagou V, Cornaglia G, Edelstein M *et al.* Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. *Clin Microbiol Infect* 2010; **16**: 112–22.

8 Clark LL, Dajcs JJ, McLean CH *et al.* *Pseudomonas otitidis* sp. nov., isolated from patients with otic infections. *Int J Syst Evol Microbiol* 2006; **56**: 709–14.

9 Thaller MC, Borgianni L, Di Lallo G *et al.* Metallo-β-lactamase production by *Pseudomonas otitidis*: a species-related trait. *Antimicrob Agents Chemother* 2011; **55**: 118–23.

10 Brooke JS. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 2012; **25**: 2–41.

Jpn. J. Infect. Dis., 67, 66-68, 2014

Laboratory and Epidemiology Communications

Evaluation of a Double-Disk Synergy Test with a Common Metallo-β-Lactamase Inhibitor, Mercaptoacetate, for Detecting NDM-1-Producing *Enterobacteriaceae* and *Acinetobacter baumannii*

Jun-ichi Wachino[1,2], Mari Matsui[1], Hoang Huy Tran[3], Masato Suzuki[1], Satowa Suzuki[1], and Keigo Shibayama[1]*

[1]*Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo 208-0011;*
[2]*Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan; and*
[3]*National Institute of Hygiene and Epidemiology, Hanoi, Vietnam*

Communicated by Makoto Ohnishi

(Accepted September 24, 2013)

New Delhi metallo-β-lactamase (NDM)-1 carbapenemase-producing bacteria are resistant to antibiotics of the carbapenem family, which are used as a last resort for the treatment of infectious diseases caused by drug-resistant bacteria. Therefore, the emergence of these bacteria presents a serious public health issue. This is particularly true given that NDM-1 carbapenemase has been detected in many clinical isolates worldwide since it was first identified in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a patient hospitalized in New Delhi (1). The NDM-1 carbapenemase gene has been predominantly identified in *Enterobacteriaceae*, but it can also occur in non-fermenters (2). Hence, it is necessary to monitor the emergence and spread of NDM-1 producers with a convenient and effective screening method.

NDM-1 carbapenemase is a class B zinc metallo-β-lactamase (MBL) (3). A variety of techniques have already been developed to detect MBL producers (4), including a disk-based synergy test, the sodium mercaptoacetate (SMA) test, which is conventionally used in Japanese clinical microbiology laboratories (5). This test uses a Kirby-Bauer (KB) disk containing a β-lactam antibiotic (ceftazidime [CAZ] recommended) and a disk containing SMA, an MBL inhibitor that can bind to the MBL active site through interactions with zinc ions (6). Although this test works well for the detection of IMP- and VIM-type MBL producers, which are the predominant MBL types in Japan (7), preliminary results have indicated that it may fail to detect NDM-1 producers when using the SMA test with the CAZ disk according to general recommendations (8). Hence, the present study aimed at improving the SMA test by replacing the CAZ disk for detecting NDM-1 producers among *Enterobacteriaceae* and *Acinetobacter baumannii*.

A collection of 15 NDM-1-positive bacterial isolates (5 *E. coli*, 4 *K. pneumoniae*, 1 *Enterobacter cloacae*, 1 *Citrobacter freundii*, and 4 *A. baumannii*), obtained from hospitals in Vietnam in 2010, and 1 NDM-1-positive *K. pneumoniae* strain (MRY10-722) isolated in a hospital in Japan in 2010, were used in this study. These isolates were identified using the API 20E and Vitek2 systems (bioMerieux, Marcy l'Etoile, France). Identification of *A. baumannii* isolates was further confirmed by *rpoB* gene sequencing (9). The NDM-1 gene was detected using PCR analysis with specific primers as described previously (10).

A total of 16 isolates were subjected to the SMA test. The inhibitory effect of CAZ was compared with that of the carbapenems, imipenem (IPM) and meropenem (MPM). Suspensions of the bacterial isolates were adjusted and spread on Mueller-Hinton agar plates according to the protocol recommended by the Clinical and Laboratory Standards Institute guidelines (11). The KB disks containing β-lactam antibiotics (CAZ [30 μg] or IPM [10 μg] or MPM [10 μg]) (Eiken Chemical Co., Ltd., Tokyo, Japan) were placed on the plates, and disks containing SMA (3 mg) (Eiken Chemical) were placed close to 1 β-lactam disk as shown in Fig. 1. The center-to-center diameter between the KB disk and the SMA disk was 16 mm. The plate was incubated at 35°C for 18 h and the growth-inhibitory zone around the KB disk close to the SMA disk was compared with that around the KB disk alone. An isolate was considered MBL-positive when an apparent expansion was observed, i.e., an enlargement of 5 mm or greater of the growth-inhibitory zone around the KB disk close to the SMA disk compared with that around the KB disk alone (Fig. 1, middle and lower panels). An isolate was considered MBL-negative if there was no expansion of the growth-inhibitory zone or if the expansion was less than 5 mm (Fig. 1, upper panel).

The results are summarized in Table 1. The highest sensitivity was obtained with a combination of the MPM and SMA disks. Of the 16 strains, 15 (93.8%) were confirmed as positive. The combination of the IPM and SMA disks resulted in the positive identification of 14 strains (87.5%). On the other hand, a combination of the CAZ and SMA disks was considerably less sensitive, as only 7 strains (43.8%) had positive results.

These results indicated that the carbapenems, IPM and MPM, are generally more suitable than CAZ for

*Corresponding author: Mailing address: Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Tel: +81-42-561-0771, ext. 3500, Fax: +81-42-561-7173, E-mail: keigo@niid.go.jp

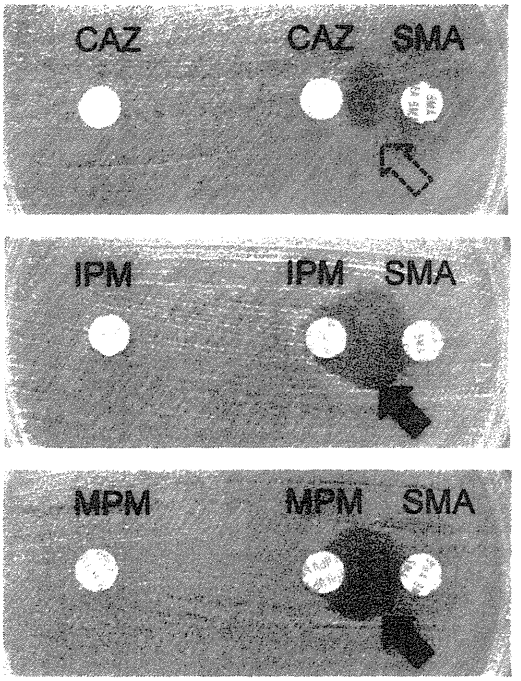

Fig. 1. Results of the disk-based synergy test, the sodium mercaptoacetate (SMA) test, for the New Delhi metallo-β-lactamase (NDM)-1-producing *Acinetobacter baumannii* isolate V-275. Apparent expansion of the growth-inhibitory zone (black arrows) between SMA and imipenem (IPM)/meropenem (MPM) disks were observed. On the other hand, a very slight inhibitory effect (arrow with dashed black line) was observed between the ceftazidime (CAZ) and SMA disks.

detecting NDM-1-producing bacteria. The low sensitivity of the CAZ disk could be ascribed to the coproduction of other β-lactamases such as extended-spectrum β-lactamases and plasmid-mediated or chromosomally encoded AmpC β-lactamases, which inactivate CAZ without being inhibited by SMA. In fact, it has been reported that NDM-1-producers often simaultaneously carry other β-lactamase genes, such as $bla_{\text{CTX-M}}$, bla_{CMY}, or bla_{DHA} (12,13). Nevertheless, 1 *E. cloacae* isolate (V-87) produced a clearly enlarged inhibitory zone between the SMA and CAZ disks but not between SMA and MPM or IPM, suggesting the importance of CAZ. We hypothesized that these findings may have been owing to the coproduction of OXA-48 carbapenemase, which is usually resistant to MPM but not to CAZ. The OXA-48 carbapenemase is a member of the serine-β-lactamases, whose activities are not inhibited by SMA. We used PCR to screen all isolates for the presence of $bla_{\text{OXA-48}}$. As expected, only the *E. cloacae* isolate V-87 carried the $bla_{\text{OXA-48}}$ gene; however, further examinations with additional isolates are required to confirm our hypothesis.

On the basis of these results, we conclude that the SMA test using both the MPM and CAZ disks is the most suitable method for screening carbapenem-resistant isolates for NDM-1-type MBL producers. As reported previously (8), this combination also allows for highly sensitive and specific detection of the IMP- and VIM-type MBL producers. Effective screening of MBL producers, including the NDM-1 type and other MBL types, can therefore be performed by the SMA test using MPM and CAZ disks in clinical laboratories followed by confirmation of the MBL genes by PCR analysis in specialized laboratories.

Table 1. Inhibitory activity of SMA disks for NDM-1-producing bacterial isolates

Bacterial isolate	Antibiotic disk		
	CAZ	IPM	MPM
E. coli V-22	+	+	+
E. coli V-48	+	+	+
E. coli V-91	−	+	+
E. coli V-102	−	+	+
E. coli V-134	−	+	+
K. pneumoniae MRY10-722	+	−	+
K. pneumoniae V-17	+	+	+
K. pneumoniae V-21	+	+	+
K. pneumoniae V-90	−	+	+
K. pneumoniae V-182	−	+	+
E. cloacae V-87	+	−	−
C. freundii V-868	−	+	+
A. baumannii V-275	−	+	+
A. baumannii V-303	+	+	+
A. baumannii V-320	−	+	+
A. baumannii V-357	−	+	+

SMA, sodium mercaptoacetate; NDM, New Delhi metallo-β-lactamase; CAZ, ceftazidime; IPM, imipenem; MPM, meropenem; +, positive; −, negative.

Acknowledgments We thank the Saitama City Institute of Health Science and Research for providing the NDM-1-positive *K. pneumoniae* strain MRY10-722. We are grateful to Yoshichika Arakawa, Professor at the Nagoya University Graduate School of Medicine, for the valuable comments on the manuscript.

This study was supported by the Ministry of Health, Labour and Welfare of Japan (grant no. H23-Shinkou-Shitei-020 and H24-Shinkou-Ippan-010) and by a research grant of National Institute of Hygiene and Epidemiology, Hanoi, Vietnam.

Conflict of interest None to declare.

REFERENCES

1. Yong, D., Toleman, M.A., Giske, C.G., et al. (2009): Characterization of a new metallo-β-lactamase gene, $bla_{\text{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother., 53, 5046-5054.
2. Johnson, A.P. and Woodford, N. (2013): Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. J. Med. Microbiol., 62, 499-513.
3. Nordmann, P., Naas, T. and Poirel, L. (2011): Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerg. Infect. Dis., 17, 1791-1798.
4. Willems, E., Verhaegen, J., Magerman, K., et al. (2013): Towards a phenotypic screening strategy for emerging β-lactamases in gram-negative bacilli. Int. J. Antimicrob. Agents, 41, 99-109.
5. Arakawa, Y., Shibata, N., Shibayama, K., et al. (2000): Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol., 38, 40-43.
6. Wachino, J., Yamaguchi, Y., Mori, S., et al. (2013): Structural insights into the subclass B3 metallo-β-lactamase SMB-1 and the mode of inhibition by the common metallo-β-lactamase inhibitor mercaptoacetate. Antimicrob. Agents Chemother., 57, 101-109.
7. Shibata, N., Doi, Y., Yamane, K., et al. (2003): PCR typing of genetic determinants for metallo-β-lactamases and integrases car-

ried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol., 41, 5407–5413.
8. Hattori, T., Kawamura, K. and Arakawa, Y. (2013): Comparison of test methods for detecting metallo-β-lactamase-producing gram-negative bacteria. Jpn. J. Infect. Dis., 66, 512–518.
9. La Scola, B., Gundi, V.A., Khamis, A., et al. (2006): Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. J. Clin. Microbiol., 44, 827–832.
10. Hoang, T.H., Wertheim, H., Minh, N.B., et al. (2013): Carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains containing New Delhi metallo-beta-lactamase isolated from two patients in Vietnam. J. Clin. Microbiol., 51, 373–374.
11. Clinical and Laboratory Standards Institute (2010): Performance standards for antimicrobial disk susceptibility tests; 9th informational supplement. Approved standard M2-A10. Wayne, Pa.
12. Sekizuka, T., Matsui, M., Yamane, K., et al. (2011): Complete sequencing of the bla_{NDM-1}-positive IncA/C plasmid from *Escherichia coli* ST38 isolate suggests a possible origin from plant pathogens. PLoS One, 6, e25334.
13. Nordmann, P., Poirel, L., Walsh, T.R., et al. (2011): The emerging NDM carbapenemases. Trends Microbiol., 19, 588–595.

genome
Journals.ASM.org

Genome Sequence of a Strain of the Human Pathogenic Bacterium *Pseudomonas alcaligenes* That Caused Bloodstream Infection

Masato Suzuki,[a] Satowa Suzuki,[a] Mari Matsui,[a] Yoichi Hiraki,[b] Fumio Kawano,[c] Keigo Shibayama[a]

Department of Bacteriology II, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan[a]; Department of Pharmacy, National Hospital Organization Kumamoto Medical Center, Chuo-ku, Kumamoto, Japan[b]; Department of Hematology, National Hospital Organization Kumamoto Medical Center, Chuo-ku, Kumamoto, Japan[c]

***Pseudomonas alcaligenes*, a Gram-negative aerobic bacterium, is a rare opportunistic human pathogen. Here, we report the whole-genome sequence of *P. alcaligenes* strain MRY13-0052, which was isolated from a bloodstream infection in a medical institution in Japan and is resistant to antimicrobial agents, including broad-spectrum cephalosporins and monobactams.**

Received 2 October 2013 **Accepted** 4 October 2013 **Published** 31 October 2013

Citation Suzuki M, Suzuki S, Matsui M, Hiraki Y, Kawano F, Shibayama K. 2013. Genome sequence of a strain of the human pathogenic bacterium *Pseudomonas alcaligenes* that caused bloodstream infection. Genome Announc. 1(5):e00919-13. doi:10.1128/genomeA.00919-13.

Address correspondence to Masato Suzuki, suzuki-m@nih.go.jp.

Pseudomonas alcaligenes is a Gram-negative aerobic rod belonging to the bacterial family *Pseudomonadaceae* and is a common inhabitant of soil and water. A recent study showed that *P. alcaligenes* is useful as a microbial inoculant for the biodegradation of toxic polycyclic aromatic hydrocarbons (1). *P. alcaligenes* has also been known as a rare opportunistic human pathogen (2). Based on 16S rRNA gene sequence analysis, *P. alcaligenes* was classified in the *Pseudomonas aeruginosa* group (3). However, little is known about the clinical importance of *P. alcaligenes* and its virulence factors, mainly because of the difficulties in identifying and distinguishing this bacterium from closely related *Pseudomonas* species in medical settings.

In this report, we announce the availability of the first draft genome sequence of *P. alcaligenes*. *P. alcaligenes* strain MRY13-0052 was recovered from a bloodstream infection in a medical institution in Japan in 2013 and was resistant to broad-spectrum cephalosporins and monobactams. Whole-genome shotgun (WGS) sequencing of strain MRY13-0052 was performed using the Roche 454 pyrosequencing platform (500-bp insert size). The reads were assembled *de novo* using Newbler Assembler version 2.3 (Roche). The draft genome sequence of MRY13-0052 consists of 237 contigs, yielding a total of 6,876,944 bp with an N_{50} contig size of 64,175 bp. The mean G+C content was 65.8%. A total of 6,190 coding DNA sequences were identified by the RAST server (http://rast.nmpdr.org) (4). The MRY13-0052 strain carried three class C β-lactamase genes that might confer resistance to β-lactam antibiotics. Any other acquired antimicrobial resistance genes in the WGS data were not detected using a Web-based tool, ResFinder version 1.3 (http://cge.cbs.dtu.dk/services/ResFinder/) (5).

Bacterial pathogens frequently use protein secretions to interact with their hosts. MRY13-0052 contains the type VI secretion system (T6SS) gene cluster and three genes that encode VgrG (valine glycine repeat G) translocator proteins (6). The T6SS, which is conserved among *Pseudomonas* species (7), delivers effectors into neighboring organisms, including bacteria and mammalian cells, leading to cytotoxicity and cell death in the targets (6). The MRY13-0052 strain furthermore contains a set of genes that encode proteins homologous to *P. aeruginosa* Tse1 (type VI effector 1) and Tsi1 (type VI immunity 1) (8) (66.9% and 48.8% identities, respectively). On the other hand, MRY13-0052 is devoid of the virulence-associated type III secretion system (T3SS) gene cluster, whereas a T3SS is the major virulence factor in animal and plant pathogenic *Pseudomonas* species, *P. aeruginosa*, and *Pseudomonas syringae* (9, 10). These data suggest that the T6SS might be an important virulence determinant in *P. alcaligenes* and that *P. alcaligenes* might partially share the same T6SS-dependent effector and immunity system with *P. aeruginosa*.

A more detailed report of the virulence phenotype of *P. alcaligenes* MRY13-0052 will be included in a future publication. A genome-wide comparison of *P. alcaligenes* with related *Pseudomonas* species, such as *P. aeruginosa*, *Pseudomonas mendocina*, and *Pseudomonas pseudoalcaligenes*, will facilitate additional comprehensive bioinformatic and phylogenetic analyses, thus expanding our understanding of fatal nosocomial infections caused by these opportunistic human pathogens.

Nucleotide sequence accession numbers. The WGS projects for *P. alcaligenes* MRY13-0052 have been deposited at DDBJ/EMBL/GenBank under the accession no. BATO00000000. The version described in this paper is the first version, BATO01000000.

ACKNOWLEDGMENTS

We thank Kumiko Kai and Yoshie Taki for technical assistance. We are grateful to the participating medical institution for providing the strain and clinical information.

This work is supported by a Grant-in-Aid for Young Scientists (A) (no. 25713017) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and H24-Shinkou-Ippan-010 from the Ministry of Health, Labour, and Welfare, Japan.

REFERENCES

1. **O'Mahony MM, Dobson AD, Barnes JD, Singleton I.** 2006. The use of ozone in the remediation of polycyclic aromatic hydrocarbon contaminated soil. Chemosphere 63:307–314.

2. **Martino P, Micozzi A, Venditti M, Gentile G, Girmenia C, Raccah R, Santilli S, Alessandri N, Mandelli F.** 1990. Catheter-related right-sided endocarditis in bone marrow transplant recipients. Rev. Infect. Dis. **12**:250–257.
3. **Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H.** 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. **50**(Pt 4):1563–1589.
4. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics **9**:75. doi:10.1186/1471-2164-9-75.
5. **Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV.** 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. **67**:2640–2644.
6. **Silverman JM, Brunet YR, Cascales E, Mougous JD.** 2012. Structure and regulation of the type VI secretion system. Annu. Rev. Microbiol. **66**: 453–472.
7. **Barret M, Egan F, Fargier E, Morrissey JP, O'Gara F.** 2011. Genomic analysis of the type VI secretion systems in *Pseudomonas* spp.: novel clusters and putative effectors uncovered. Microbiology **157**: 1726–1739.
8. **Russell AB, Hood RD, Bui NK, LeRoux M, Vollmer W, Mougous JD.** 2011. Type VI secretion delivers bacteriolytic effectors to target cells. Nature **475**:343–347.
9. **Hauser AR.** 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat. Rev. Microbiol. **7**:654–665.
10. **Lindeberg M, Cunnac S, Collmer A.** 2012. *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. Trends Microbiol. **20**:199–208.

Antimicrobial Agents and Chemotherapy

Isolation of Genetically Indistinguishable Carbapenem-Resistant and -Susceptible Acinetobacter baumannii Isolates from a Single Patient

Mari Matsui, Keigo Shibayama, Yasuhiro Tsuji, Hidetoshi Kamimura, Yoshiharu Karube, Mayumi Yoshida, Yoko Masuda, Yoichi Hiraki, Kazutaka Takaki and Fumio Kawano
Antimicrob. Agents Chemother. **2013, 57(11):5781. DOI: 10.1128/AAC.01631-13.**
Published Ahead of Print 26 August 2013.

AAC
Journals.ASM.org

Isolation of Genetically Indistinguishable Carbapenem-Resistant and -Susceptible *Acinetobacter baumannii* Isolates from a Single Patient

Mari Matsui,[a] Keigo Shibayama,[a] Yasuhiro Tsuji,[b] Hidetoshi Kamimura,[c] Yoshiharu Karube,[c] Mayumi Yoshida,[d] Yoko Masuda,[d] Yoichi Hiraki,[d] Kazutaka Takaki,[d] Fumio Kawano[d]

Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan[a]; Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Japan[b]; Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan[c]; Department of Infection Control Unit, National Hospital Organization Kumamoto Medical Center, Kumamoto, Japan[d]

The efficacy of carbapenems has been threatened by the increasing frequency of carbapenem-resistant bacteria. *Acinetobacter baumannii* carrying the bla_{OXA-23} carbapenemase gene has spread worldwide (1). We isolated carbapenem-resistant and then -susceptible *A. baumannii* from the sputum of a 74-year-old male patient. Here we describe the characterization of these isolates and discuss the implications of these findings and their clinical importance.

The first isolate (MRY12-278) was obtained 3 days after treatment with meropenem (MEPM; 1,500 mg/day) for suspected pneumonia. The isolate was resistant to MEPM (MIC, >8 μg/ml), imipenem (IPM; MIC, >8 μg/ml), amikacin (MIC, >32 μg/ml), gentamicin (MIC, >8 μg/ml), ciprofloxacin (MIC, >2 μg/ml), and levofloxacin (MIC, >4 μg/ml) by the broth microdilution method of the Clinical and Laboratory Standards Institute (2). MEPM was discontinued immediately, as the responsible physicians felt that the clinical scenario was more consistent with colonization rather than infection. Seven days after MEPM discontinuation, the other isolate (MRY12-281) was obtained; its susceptibility profile was identical except that it was susceptible to MEPM and IPM (MICs, <4 μg/ml).

Both isolates yielded identical banding profiles by pulsed-field gel electrophoresis (PFGE) using ApaI (3) (Fig. 1), suggesting their derivation from a single clone. When tested, the isolates were negative for the production of metallo-β-lactamases by a double-disc synergy test (4). OXA-type carbapenemase-encoding genes and the IS*Aba* element, which contains the promoter sequence upstream of the bla_{OXA} genes, were screened by PCR with primers specific for $bla_{OXA-51-like}$, $bla_{OXA-23-like}$, $bla_{OXA-58-like}$, $bla_{OXA-24-like}$, and IS*Aba1* (5, 6). $bla_{OXA-51-like}$ was detected in both isolates, but IS*Aba1* was not found upstream of the gene. $bla_{OXA-58-like}$ and $bla_{OXA-24-like}$ were not detected in either isolate. $bla_{OXA-23-like}$ and the upstream IS*Aba1* element were detected in MRY12-278 but not in MRY12-281. The nucleotide sequence of $bla_{OXA-23-like}$ was identical to that of the published bla_{OXA-23} gene (GenBank accession no. AJ132105). These findings indicate that MRY12-278 carbapenem resistance was conferred by bla_{OXA-23}. To determine

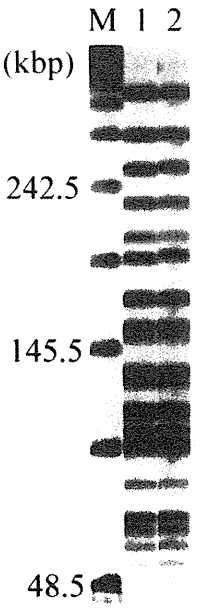

FIG 1 Pulsed-field gel electrophoresis (PFGE) of ApaI-digested DNA from *A. baumannii* isolates. Lane 1, MRY12-278; lane 2, MRY12-281; lane M, contour-clamped homogeneous electric field (CHEF) DNA size standard lambda ladder (Bio-Rad).

Published ahead of print 26 August 2013

Address correspondence to Yoichi Hiraki, hiraki@kumamoto2.hosp.go.jp.

M.M. and Y.H. contributed equally to this article.

doi:10.1128/AAC.01631-13

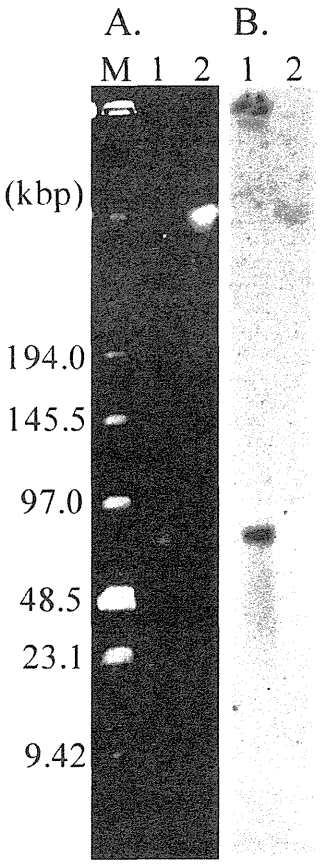

FIG 2 Localization of the bla_{OXA-23} gene in *A. baumannii* isolates MRY12-278 and -281. S1 nuclease-digested DNA was separated by PFGE. (A) Ethidium bromide-stained image; (B) hybridization with a probe specific for the bla_{OXA-23} gene. Lane 1, MRY12-278; lane 2, MRY12-281; lane M, low-range PFG marker (New England BioLabs).

whether bla_{OXA-23} was located on a plasmid, S1 nuclease-digested DNA was separated by PFGE and hybridized with a digoxigenin (DIG)-labeled probe (Roche Applied Science, Mannheim, Germany) specific for bla_{OXA-23} (Fig. 2) (7). The resistant isolate harbored an ~60-kbp plasmid containing bla_{OXA-23}. Thus, MRY12-278 and MRY12-281 are clonal except for the presence of the plasmid. We believe that the carbapenem-susceptible isolate MRY12-281 was derived from resistant isolate MRY12-278 by loss of the plasmid carrying bla_{OXA-23}. Both isolates were grouped into sequence type 92 (ST92) according to the Bartual et al. multilocus sequence typing scheme (8). ST92 is of the most epidemic clonal lineage (international clone II lineage, which frequently causes outbreaks worldwide).

Infections caused by resistant *A. baumannii* strains are a major concern for clinicians, as available treatment options are limited. Our results indicated that transformation of a carbapenem-resistant strain into a susceptible strain indeed occurs in clinical settings. When susceptible strains predominate, resistant strains might be undetectable, and vice versa. It is important to consider such situations carefully during antibiotic therapy.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Health, Labor, and Welfare of Japan (grant no. H24-Shinkou-Ippan-010).

REFERENCES

1. **Mugnier PD, Poirel L, Naas T, Nordmann P.** 2010. Worldwide dissemination of the bla_{OXA-23} carbapenemase gene of *Acinetobacter baumannii*. Emerg. Infect. Dis. **16**:35–40.
2. **Clinical and Laboratory Standards Institute.** 2011. Performance standards for antimicrobial susceptibility testing. Twenty-first international supplement. M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Seifert H, Dolzani L, Bressan R, van der Reijden T, van Strijen B, Stefanik D.** 2005. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. J. Clin. Microbiol. **43**:4328–4335.
4. **Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H.** 2000. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38**: 40–43.
5. **Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S.** 2006. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int. J. Antimicrob. Agents **27**:351–353.
6. **Turton JF, Ward ME, Woodford N, Kaufmann ME, Pike R, Livermore DM.** 2006. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol. Lett. **258**:72–77.
7. **Zhou Z, Du X, Wang L, Yang Q, Fu Y, Yu Y.** 2011. Clinical carbapenem-resistant *Acinetobacter baylyi* strain coharboring bla_{SIM-1} and bla_{OXA-23} from China. Antimicrob. Agents Chemother. **55**:5347–5349.
8. **Bartual SG, Seifert H, Hippler C, Luzon MA, Wisplinghoff H, Rodriguez-Valera F.** 2005. Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. J. Clin. Microbiol. **43**:4382–4390.

Microbiology and Immunology

Microbiol Immunol 2013; 57: 665–673
doi: 10.1111/1348-0421.12089

ORIGINAL ARTICLE

Role of γ-glutamyltranspeptidase in the pathogenesis of *Helicobacter pylori* infection

Emiko Rimbara, Shigetarou Mori, Hyun Kim and Keigo Shibayama

Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-Shi, Tokyo 208-0011, Japan

ABSTRACT

γ-Glutamyltranspeptidase and asparaginase have been shown to play important roles in *Helicobacter pylori* colonization and cell death induced by *H. pylori* infection. In this study, the association of γ-glutamyltranspeptidase and asparaginase was elucidated by comparing activities of both deamidases in *H. pylori* strains from patients with chronic gastritis, gastric and duodenal ulcers, and gastric cancer. γ-Glutamyltranspeptidase activities in *H. pylori* strains from patients with gastric cancer were significantly higher than in those from patients with chronic gastritis or gastric ulcers. There was a wide range of asparaginase activities in *H. pylori* strains from patients with gastric cancer and these were not significantly than those from patients with other diseases. To identify the contributions of γ-glutamyltranspeptidase and asparaginase to gastric cell inflammation, human gastric epithelial cells (AGS line) were infected with *H. pylori* wild-type and knockout strains and inflammatory responses evaluated by induction of interleukin-8 (IL-8). IL-8 response was significantly decreased by knockout of the γ-glutamyltranspeptidase-encoding gene but not by knockout of the asparaginase-encoding gene. Additionally, IL-8 induction by infection with the *H. pylori* wild-type strain was significantly decreased by adding glutamine during infection. These findings indicate that IL-8 induction caused by γ-glutamyltranspeptidase activity in *H. pylori* is mainly attributable to depletion of glutamine. These data suggest that γ-glutamyltranspeptidase plays a significant role in the chronic inflammation caused by *H. pylori* infection.

Key words asparaginase, gastric cancer, γ-glutamyltranspeptidase, *Helicobacter pylori*.

Helicobacter pylori is a pathogenic bacterium that inhabits the stomach (1). Chronic *H. pylori* infection has been proven to cause gastrointestinal diseases such as gastric and duodenal ulcers and gastric cancer (2, 3). Gastric cancer is a common cause of cancer deaths worldwide, including in Japan, and it is well known that the prevalence of *H. pylori* infection does not always correspond to that of patients with gastric cancer (4). Therefore, other factors such as genetic factors, dietary habits (such as salt and alcohol intake) and differences in pathogenicity between *H. pylori* strains contribute to the development of gastric cancer in *H. pylori* -infected individuals. Many virulence factors have thus far been discovered in *H. pylori*, including CagA protein, which is injected by the Type IV secretion system to cause an inflammatory response (5) and VacA protein, which causes vacuolation of gastric cells (6). Polymorphisms of these proteins in *H. pylori* have been reported and shown to be associated not only with differences in inflammatory responses caused by *H. pylori* infection *in vitro* but also with differences in the clinical manifestations of *H. pylori* infection (6, 7). Indeed, the prevalence of polymorphisms has been shown to differ geographically and to correlate with a high rate of gastric cancer in specific regions, such

Correspondence
Emiko Rimbara, Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-Shi, Tokyo 208-0011, Japan.
Tel: +81 42 561 0771; fax: +81 42 561 7173; email: rimbara@nih.go.jp

Received 2 June 2013; revised 17 July 2013; accepted 29 July 2013.

List of Abbreviations: *H. pylori*, *Helicobacter pylori*; IL-8, interleukin-8; OD_{600}, optimal density at 600 nm; PBST, PBS with Tween 20.