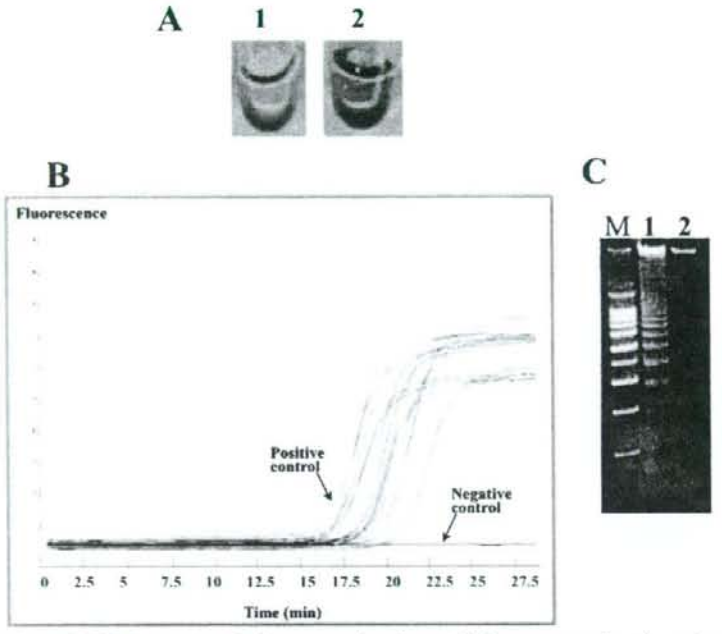

FIG. 3. LAMP assay to detect MDR *P. aeruginosa* isolates possessing the *aac(6')-Iae* gene encoding the aminoglycoside acetyltransferase AAC(6')-Iae. *P. aeruginosa* IMCJ2.S1 and ATCC 27853 were used as positive and negative controls, respectively. (A) Visual inspection analysis of LAMP products. Lane 1, *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853. (B) Real-time amplification monitoring of *aac(6')-Iae*-specific LAMP. The amplification signal was detected at an average of 18 min, as indicated by the continuous increase in fluorescence. Increased fluorescence was not observed in the negative control. (C) Acrylamide gel electrophoresis of LAMP product. Lane 1, LAMP product of the 204-bp target sequence of the *aac(6')-Iae* gene of *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853 negative control; lane M, 1-kbp ladder.

resistance associated with these mutations remains to be determined.

Analysis of the *aac(6')-Iae* gene by the LAMP method. To detect *aac(6')-Iae*, we developed a gene-specific LAMP assay. The index strain IMCJ2.S1 was used to standardize the method. Visual inspection showed that the LAMP assay successfully amplified the target sequence of the *aac(6')-Iae* gene of *P. aeruginosa* IMCJ2.S1 (Fig. 3A). Real-time kinetics of the LAMP reaction showed that the amplification signal could be detected on average by 18 min; fluorescence increased in the positive samples, following a sigmoid curve (Fig. 3B). Agarose gel electrophoresis of the LAMP products (Fig. 3C) showed a ladder-like pattern on the gel due to the formation of a mixture of stem-loop DNAs of various stem lengths, which are characteristic of LAMP products.

A total of 284 isolates, including 214 MDR *P. aeruginosa* isolates, were tested by the LAMP assay (Table 3). A total of 212 isolates were positive by the LAMP assay (Table 3). The results of the LAMP assay were in complete concordance with the PCR data, indicating that the PCR can be replaced by the LAMP method for detection of *aac(6')-Iae*-carrying *P. aeruginosa*. These results, together with ones of the agglutination test (Table 3), indicate that multidrug resistance was strongly associated with the presence of *aac(6')-Iae* and AAC(6')-Iae production in the *P. aeruginosa* isolates ($P < 0.0001$).

Genotyping by PFGE. The 284 isolates, including 214 MDR isolates, were typed by PFGE. One hundred thirty-three different PFGE types, designated from A1 to AL, were distinguished (Table 1). Fourteen types, A1, A2, A12, A14, A18, A21, A25, A27, A37, A41, A42, A43, A44, and A60, were identified in more than 2 isolates (Fig. 1), and type A1, which represented 83 of the isolates (29%), was the most prevalent and widely disseminated (Fig. 1), suggesting prefecture-wide clonal dissemination. Types A1, A12, A14, A21, A27, A37, and A38 were identified at two or more hospitals. Cluster analysis of the PFGE restriction patterns showed three large clusters, A, B, and C, sharing ≥70% similarity (Fig. 4). Of the 214 MDR isolates, 211 belonged to cluster A, comprising types A1 to A67, indicating that multidrug resistance was associated with one genotype, cluster A (Fig. 4 and Table 3). Fifteen isolates belonged to cluster B comprising types B1 to B14, and 10 isolates belonged to cluster C, comprising types C1 to C8. The PFGE patterns of the 35 non-MDR isolates varied greatly.

DISCUSSION

A clonal expansion of *P. aeruginosa* resistant to three antibiotics, carbapenems, amikacin, and fluoroquinolones, has been reported (4, 14, 36, 37, 46). However, previous surveillance studies in Japan have not shown clonal expansion involving multiple hospitals (19, 52). The present study showed clonal expansion of MDR *P. aeruginosa* in hospitals in the Tohoku area of Japan. To our knowledge, this is the first description of a large-scale, community-wide outbreak of nos-

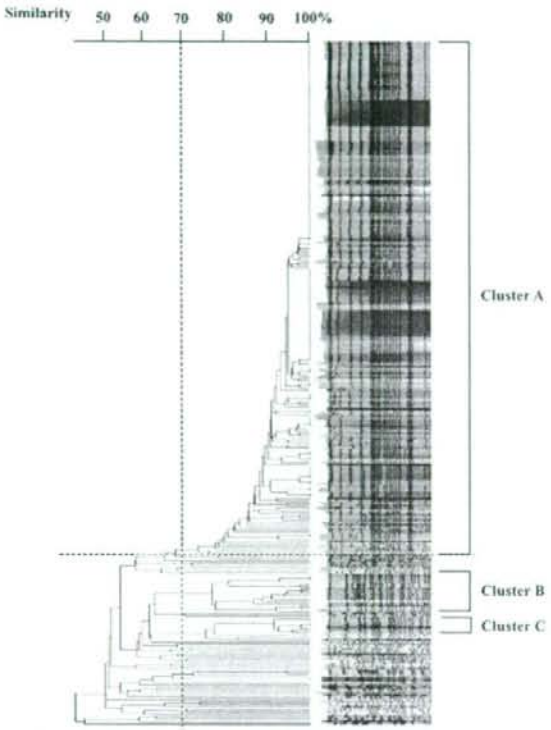

FIG. 4. Cluster analysis based on the PFGE patterns of 284 clinical isolates of *P. aeruginosa* from the 13 hospitals in the present study. Clustering was carried out with Molecular Analyst FingerprintingPlus software, version 1.6, as described in Materials and Methods.

ocomial infection caused by a single *P. aeruginosa* clone with high-level resistance to a large number of antibiotics. The routes of transmission of the MDR *P. aeruginosa* clone remain unclear. *P. aeruginosa* that can be recovered from the hospital environment could be a possible source of nosocomial infection (6, 42, 54). Patient-to-patient transmission has been documented among patients with cystic fibrosis (5, 42, 54). Catheter-associated urinary tract infections appeared widespread among the hospitals in our study; the majority of the isolates (approximately 70%) were obtained from urine specimens, and approximately 80% of these were from patients with urinary catheters.

Most MDR isolates tested (205 of 214; Table 1) showed a serotype of O11. This was not surprising because these isolates belonged to a single cluster, as revealed by PFGE analysis (Fig. 4). *P. aeruginosa* is categorized into 31 chemotypes, including 20 IATS serotypes and subtypes (48). Thus far, however, particular serotypes, such as serotypes O12 and O11, appear to have been preferentially associated with *P. aeruginosa* outbreaks (9, 23, 38, 41). A clone of *P. aeruginosa* belonging to serogroup O12, which was resistant to both carbenicillin and gentamicin, was predominant in outbreaks involving six hospitals in Athens in 1987 (23). Later, O12 isolates resistant to these two drugs were reported in European countries (9, 38, 41). *P. aeruginosa* O12 resistant to ciprofloxacin and ceftazidime and/or fosfomycin was implicated in hospital outbreaks in France during the period 1993 to 1994 (3). *P. aeruginosa* serotype O11 caused hospital outbreaks in the 1980s in the United States (8) and in 1994 and 1995 in Greece (50). *P. aeruginosa* O11 was implicated in folliculitis caused by the use of whirlpools and hot tubs in the 1970s and 1980s in the United States and Canada (40). More recently, hospital outbreaks caused by MDR *P. aeruginosa* serotype O11 occurred in Belgium (5) and in Japan (46). Different strains of serotype O11 were involved in the above-mentioned outbreaks because their PFGE profiles were quite different. In addition, the Japanese strains produced IMP-1 carbapenemase (46), but the Belgian strains did not (5). It is not known why *P. aeruginosa* strains belonging to particular serotypes of O12 and O11 were involved in these outbreaks.

We analyzed several features including serotype, antimicrobial susceptibility, MBL production, prevalence of *aac(6′)-Iae*, structure of class 1 integrons, resistance to fluoroquinolones, and genotype based on PFGE analysis for MDR *P. aeruginosa* isolates. Results indicated that *aac(6′)-Iae* is a good candidate marker for MDR *P. aeruginosa* infection. To detect the *aac(6′)-Iae* gene and its product, we developed a LAMP-based detection assay and an agglutination assay. LAMP is a nucleic acid amplification method which relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (31). The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. LAMP assays are simple and short and do not require expensive equipment. LAMP assays have been applied to the analysis of various infectious agents such as hepatitis B virus (7), *Mycobacterium tuberculosis* (15), severe acute respiratory syndrome coronavirus (13), *E. coli* O157:H7 (27), *Clostridium difficile* (18), *Bordetella pertussis* (17), *Salmonella enterica* (32), *Mycoplasma pneumoniae* (43), and *Streptococcus pneumoniae* (45). The LAMP assay developed in this study was as sensitive and specific as PCR. Though less sensitive and specific than the LAMP assay, the agglutination assay for AAC(6′)-Iae is sufficiently accurate to detect MDR *P. aeruginosa* (98% of MDR *P. aeruginosa* isolates were positive). The agglutination assay is simpler and cheaper than the LAMP assay and is also useful in detecting MDR *P. aeruginosa* in the clinical setting.

MDR *P. aeruginosa* may have spread across Japan as a result of the increasing use of carbapenems such as IPM, aminoglycosides such as AMK, and fluoroquinolones such as CIP. Nationwide surveillance for MDR *P. aeruginosa* is under way. At the hospital level, monitoring for environmental sources of bacteria, cleaning of contaminated surfaces of treatment rooms and bathrooms, review of infection control measures in the treatment of urine, and avoidance of unnecessary measurements of urine are considered effective in preventing *P. aeruginosa* nosocomial infections. Although the mode of transmission between hospitals is unknown, the movement of infected patients from one hospital to another is a possibility. Thirty-one patients infected with MDR *P. aeruginosa* had been transferred from other hospitals to the hospitals participating in the present study.

ACKNOWLEDGMENTS

We thank M. Nakano (Jichi Medical School, Japan) for comments on the manuscript and Y. Arakawa and N. Shibata (National Institute of Infectious Diseases, Japan) for providing *P. aeruginosa* M207 possessing bla_{IMP-1}, *P. aeruginosa* NCB326 possessing bla_{IMP-2}, and *Acinetobacter baumannii* NCB0211-439 possessing bla_{VIM-2}. We also thank H. Oikawa, T. Suzuki, I. Kurokawa, T. Sato, N. Onodera, K. Hiratsuka, M. Chiba, M. Utagawa, H. Mikami, M. Tachiya, K. Sasaki, S. Nakanowatari, M. Sasaki, Y. Abe, M. Koseki, N. Yaguchi, T. Nakamura, Y. Sato, and Y. Suzuki for providing clinical isolates of *P. aeruginosa*. Doripemen was kindly provided by Shionogi & Co., Ltd., Osaka, Japan.

This study was supported by Health Sciences Research grants from the Ministry of Health, Labor, and Welfare of Japan (H16-JRYO-IPPAN-011 and H18-SHINKO-11).

REFERENCES

1. **Akasaka, T., M. Tanaka, A. Yamaguchi, and K. Sato.** 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. **45:** 2263–2268.
2. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38:**40–43.
3. **Bingen, E., S. Bonacorsi, P. Rohrlich, M. Duval, S. Lhopital, N. Brahimi, E. Vilmer, and R. V. Goering.** 1996. Molecular epidemiology provides evidence of genotypic heterogeneity of multidrug-resistant *Pseudomonas aeruginosa* serotype O:12 outbreak isolates from a pediatric hospital. J. Clin. Microbiol. **34:**3226–3229.
4. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M07-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Deplano, A., O. Denis, L. Poirel, D. Hocquet, C. Nonhoff, B. Byl, P. Nordmann, J. L. Vincent, and M. J. Struelens.** 2005. Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J. Clin. Microbiol. **43:**1198–1204.
6. **Doring, G., S. Jansen, H. Noll, H. Grupp, F. Frank, K. Botzenhart, K. Magdorf, and U. Wahn.** 1996. Distribution and transmission of *Pseudomonas acruginosa* and *Burkholderia cepacia* in a hospital ward. Pediatr. Pulmonol. **21:**90–100.
7. **Enomoto, Y., T. Yoshikawa, M. Ihira, S. Akimoto, F. Miyake, C. Usui, S. Suga, K. Suzuki, T. Kawana, Y. Nishiyama, and Y. Asano.** 2005. Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. J. Clin. Microbiol. **43:**951–955.
8. **Farmer, J. J., III, R. A. Weinstein, C. H. Zierdt, and C. D. Brokopp.** 1982. Hospital outbreaks caused by *Pseudomonas aeruginosa*: importance of serogroup O11. J. Clin. Microbiol. **16:**266–270.
9. **Grattard, F., O. G. Gaudin, B. Pozzetto, A. Ros, and A. D. Mbida.** 1993. Genotypic homogeneity of nosocomial *Pseudomonas aeruginosa* O12 strains demonstrated by analysis of protein profiles, DNA fingerprints and rRNA gene restriction patterns. Eur. J. Clin. Microbiol. Infect. Dis. **12:**57–61.
10. **Grundmann, H., C. Schneider, D. Hartung, F. D. Daschner, and T. L. Pitt.** 1995. Discriminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. J. Clin. Microbiol. **33:**528–534.
11. **Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plesiat.** 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. **47:**1887–1894.
12. **Homma, J. Y.** 1982. Designation of the thirteen O-group antigens of *Pseudomonas aeruginosa*; an amendment for the tentative proposal in 1976. Jpn. J. Exp. Med. **52:**317–320.
13. **Hong, T. C., Q. L. Mai, D. V. Cuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, and K. Morita.** 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42:**1956–1961.
14. **Hsueh, P. R., L. J. Teng, P. C. Yang, Y. C. Chen, S. W. Ho, and K. T. Luh.** 1998. Persistence of a multidrug-resistant *Pseudomonas aeruginosa* clone in an intensive care burn unit. J. Clin. Microbiol. **36:**1347–1351.
15. **Iwamoto, T., T. Sonobe, and K. Hayashi.** 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. **41:**2616–2622.
16. **Jones, R. N., T. R. Anderegg, J. M. Swenson, and The Quality Control Working Group.** 2005. Quality control guidelines for testing gram-negative control strains with polymyxin B and colistin (polymyxin E) by standardized methods. J. Clin. Microbiol. **43:**925–927.
17. **Kamachi, K., H. Toyoizumi-Ajisaka, K. Toda, S. C. Socung, S. Sarath, Y. Nareth, Y. Horiuchi, K. Kojima, M. Takahashi, and Y. Arakawa.** 2006. Development and evaluation of a loop-mediated isothermal amplification method for rapid diagnosis of *Bordetella pertussis* infection. J. Clin. Microbiol. **44:**1899–1902.
18. **Kato, H., T. Yokoyama, and Y. Arakawa.** 2005. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. J. Clin. Microbiol. **43:**6108–6112.
19. **Kimura, S., J. Alba, K. Shiroto, R. Sano, Y. Niki, S. Maesaki, K. Akizawa, M. Kaku, Y. Watanuki, Y. Ishii, and K. Yamaguchi.** 2005. Clonal diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in geographically diverse regions of Japan. J. Clin. Microbiol. **43:**458–461.
20. **Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan.** 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **38:**1944–1952.
21. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J. M. Frere, and G. M. Rossolini.** 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
22. **Lee, J. K., Y. S. Lee, Y. K. Park, and B. S. Kim.** 2005. Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents **25:**290–295.
23. **Legakis, N. J., N. Koukoubanis, K. Malliara, D. Michalitsianos, and J. Papavassiliou.** 1987. Importance of carbenicillin and gentamicin cross-resistant serotype 0:12 *Pseudomonas aeruginosa* in six Athens hospitals. Eur. J. Clin. Microbiol. **6:**300–303.
24. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
25. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
26. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33:**256–264.
27. **Maruyama, F., T. Kenzaka, N. Yamaguchi, K. Tani, and M. Nasu.** 2003. Detection of bacteria carrying the *stx2* gene by in situ loop-mediated isothermal amplification. Appl. Environ. Microbiol. **69:**5023–5028.
28. **Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**62–66.
29. **Naas, T., L. Poirel, and P. Nordmann.** 1999. Molecular characterisation of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, aadA6, in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **1489:**445–451.
30. **Nakamura, A., M. Hosoda, T. Kato, Y. Yamada, M. Itoh, K. Kanazawa, and H. Nouda.** 2000. Combined effects of meropenem and aminoglycosides on *Pseudomonas aeruginosa* in vitro. J. Antimicrob. Chemother. **46:**901–904.
31. **Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase.** 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. **28:**E63.
32. **Ohtsuka, K., K. Yanagawa, K. Takatori, and Y. Hara-Kudo.** 2005. Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. Appl. Environ. Microbiol. **71:**6730–6735.
33. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo-β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
34. **Pagani, L., C. Colinon, R. Migliavacca, M. Labonia, J. D. Docquier, E. Nucleo, M. Spalla, M. Li Bergoli, and G. M. Rossolini.** 2005. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-beta-lactamase. J. Clin. Microbiol. **43:**3824–3828.
35. **Partridge, S. R., G. D. Recchia, C. Scaramuzzi, C. M. Collis, H. W. Stokes, and R. M. Hall.** 2000. Definition of the attI1 site of class 1 integrons. Microbiology **146:**2855–2864.
36. **Pellegrino, F. L. P. C., L. M. Teixeira, M. da Glória Siqueira Carvalho, S. Aranha Nouer, M. Pinto De Oliveira, J. L. Mello Sampaio, A. D'Ávila Freitas, A. L. P. Ferreira, E. de Lourdes Teixeira Amorim, L. W. Riley, and B. M. Moreira.** 2002. Occurrence of a multidrug-resistant *Pseudomonas aeruginosa* clone in different hospitals in Rio de Janeiro, Brazil. J. Clin. Microbiol. **40:**2420–2424.
37. **Pirnay, J. P., D. De Vos, C. Cochez, F. Bilocq, J. Pirson, M. Struelens, L. Duinslaeger, P. Cornelis, M. Zizi, and A. Vanderkelen.** 2003. Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. J. Clin. Microbiol. **41:**1192–1202.
38. **Pitt, T. L., D. M. Livermore, D. Pitcher, A. C. Vatopoulos, and N. J. Legakis.** 1989. Multiresistant serotype O 12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe. Epidemiol. Infect. **103:**565–576.
39. **Pournaras, S., M. Maniati, E. Petinaki, L. S. Tzouvelekis, A. Tsakris, N. J. Legakis, and A. N. Maniatis.** 2003. Hospital outbreak of multiple clones of

Pseudomonas aeruginosa carrying the unrelated metallo-beta-lactamase gene variants bla_{VIM-2} and bla_{VIM-4}. J. Antimicrob. Chemother. **51:**1409–1414.
40. **Ratnam, S., K. Hogan, S. B. March, and R. W. Butler.** 1986. Whirlpool-associated folliculitis caused by *Pseudomonas aeruginosa*: report of an outbreak and review. J. Clin. Microbiol. **23:**655–659.
41. **Richard, P., R. Le Floch, C. Chamoux, M. Pannier, E. Espaze, and H. Richet.** 1994. *Pseudomonas aeruginosa* outbreak in a burn unit: role of antimicrobials in the emergence of multiply resistant strains. J. Infect. Dis. **170:**377–383.
42. **Saiman, L., and J. Siegel.** 2004. Infection control in cystic fibrosis. Clin. Microbiol. Rev. **17:**57–71.
43. **Saito, R., Y. Misawa, K. Moriya, K. Koike, K. Ubukata, and N. Okamura.** 2005. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*. J. Med. Microbiol. **54:**1037–1041.
44. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, NY.
45. **Seki, M., Y. Yamashita, H. Torigoe, H. Tsuda, S. Sato, and M. Maeno.** 2005. Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. J. Clin. Microbiol. **43:**1581–1586.
46. **Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, T. Fujino, I. Kobayashi, K. Morita, Y. Kikuchi, T. Kuratsuji, and T. Kirikae.** 2005. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. **49:**3734–3742.
47. **Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa.** 2003. 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. **41:** 5407–5413.
48. **Stanislavsky, E. S., and J. S. Lam.** 1997. *Pseudomonas aeruginosa* antigens as potential vaccines. FEMS Microbiol. Rev. **21:**243–277.
49. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669–1683.
50. **Tassios, P. T., V. Gennimata, A. N. Maniatis, C. Fock, N. J. Legakis, and The Greek *Pseudomonas aeruginosa* Study Group.** 1998. Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O:11. J. Clin. Microbiol. **36:**897–901.
51. **Tsuchizaki, N., K. Ishino, F. Saito, J. Ishikawa, M. Nakajima, and K. Hotta.** 2006. Trends of arbekacin-resistant MRSA strains in Japanese hospitals (1979 to 2000). J. Antibiot. **59:**229–233.
52. **Tsuji, A., I. Kobayashi, T. Oguri, M. Inoue, E. Yabuuchi, and S. Goto.** 2005. An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *Pseudomonas aeruginosa* isolated at medical institutes nationwide in Japan. J. Infect. Chemother. **11:**64–70.
53. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
54. **Zimakoff, J., N. Hoiby, K. Rosendal, and J. P. Guilbert.** 1983. Epidemiology of *Pseudomonas aeruginosa* infection and the role of contamination of the environment in a cystic fibrosis clinic. J. Hosp. Infect. **4:**31–40.

J Infect Chemother (2007) 13:418–422
DOI 10.1007/s10156-007-0560-5

CASE REPORT

**Jun-ichiro Sekiguchi · Katsuji Teruya · Kumi Horii
Emi Kuroda · Hisami Konosaki · Yukie Mizuguchi
Minako Araake · Akihiko Kawana · Hiroshi Yoshikura
Tadatoshi Kuratsuji · Hisayoshi Miyazaki · Teruo Kirikae**

Molecular epidemiology of outbreaks and containment of drug-resistant *Pseudomonas aeruginosa* in a Tokyo hospital

Received: March 23, 2007 / Accepted: August 31, 2007

Abstract We witnessed outbreaks of multidrug-resistant (MDR) and drug-resistant *Pseudomonas aeruginosa* at a hospital in Tokyo, Japan, during the period September 2004 through May 2005. The first outbreak occurred in September and October 2004. Three isolates of MDR *P. aeruginosa* were identified from urine samples obtained from three nonambulatory immunodeficient patients in one ward. After 3 weeks, another outbreak of *P. aeruginosa* occurred in the hematology ward on the same floor of the hospital. During the outbreaks, environmental surveys were conducted twice in each of the two wards, at 2-week intervals, to identify the sources of the pathogens. A total of 23 *P. aeruginosa* isolates, including 11 from environmental sources, were analyzed for chromosomal DNA typing by pulsed-field gel electrophoresis, for O-antigen serotyping, and for other typing. Results revealed two causative clones, as well as environmental contamination by *P. aeruginosa* clones on the surfaces of urine volume-measuring devices in rooms where urine is handled, which may have been sources of the pathogens during the outbreaks. To prevent further outbreaks, we performed the following: (a) environmental surface monitoring for drug-resistant *P. aeruginosa*, (b) active surveillance of specimens, (c) strict isolation of infected patients or carriers of MDR *P. aeruginosa*, (d) rigorous contact precautions, and (e) disinfection with 70% alcohol on the surfaces of apparatuses contaminated by MDR or drug-resistant *P. aeruginosa* and in the rooms where urine is handled. As a result, the outbreaks were contained.

Key words Multidrug resistant · *Pseudomonas aeruginosa* · Environment · Survey · Infection control

J. Sekiguchi · K. Teruya · K. Horii · E. Kuroda · H. Konosaki · Y. Mizuguchi · M. Araake · A. Kawana · T. Kuratsuji · T. Kirikae (✉)
International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan
Tel. +81-3-3202-7181 (ext. 2838); Fax +81-3-3202-7364
e-mail: tkirikae@ri.imcj.go.jp

H. Yoshikura
Ministry of Health, Labor and Welfare, Tokyo, Japan

T. Kuratsuji
National Research Institute for Child Health and Development, Setagaya, Japan

H. Miyazaki
National Hospital Organization, Kumamoto Medical Center, Kumamoto, Japan

Introduction

Nosocomial infection caused by *Pseudomonas aeruginosa* has been recognized as an important problem in hospitals in recent years because of the danger posed to immunocompromised patients.[1] Nosocomial *P. aeruginosa* is usually multidrug-resistant (MDR), which is not well-defined internationally,[2] but is defined in Japan as resistance to imipenem (IPM; minimum inhibitory concentration [MIC], ≥16 μg/ml), amikacin (AMK; MIC, ≥32 μg/ml), and ciprofloxacin (CPFX; MIC, ≥4 μg/ml).[3]

Recently, we experienced outbreaks of *P. aeruginosa* at the 925-bed International Medical Center Hospital in Tokyo. We successfully controlled these outbreaks within several months. Here, we report how we controlled the outbreaks and determined the cause, the method of transmission, the patterns of drug resistance, and genotyping by pulsed-field gel electrophoresis (PFGE) of the causative *P. aeruginosa* isolates from clinical and environmental sources in the hospital.

Methods

The first outbreak occurred in one ward (5S, the south part of the fifth floor) in September and October 2004. Three isolates of MDR *P. aeruginosa* were obtained from urine taken from three nonambulatory immunodeficient patients. The three patients were hospitalized during overlapping periods (Fig. 1). After 3 weeks, another outbreak of *P. aeruginosa* occurred in the hematology ward (5N, the north part of the fifth floor) on the same floor in the hospital. The

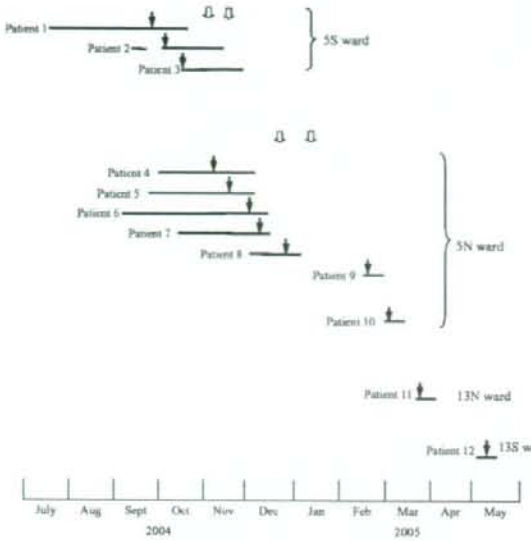

Fig. 1. Time course of *Pseudomonas aeruginosa* O11 detection in the hospital. The *parallel lines* indicate the periods of hospitalization. *Black arrows*, day of *P. aeruginosa* O11 detection; *white arrows*, day of environmental investigation for multidrug-resistant *P. aeruginosa*

causative isolates were not MDR *P. aeruginosa*, but they were AMK-sensitive and IPM- and CPFX-resistant. *P. aeruginosa* was consecutively isolated from three urine samples, one sputum sample, and one pharynx swab from five different patients in the ward. Each of these five patients had a serious underlying disease but was ambulatory. Two more patients were infected with *P. aeruginosa* 1 month later (Fig. 1). Two patients in other wards (13S and 13N, the south and north parts of the thirteenth floor, respectively) were found to have sporadic infections with *P. aeruginosa* during the same period or several weeks later (Fig. 1). The infections were contained by May 2005.

To determine the cause of the outbreaks and to prevent additional cases, an epidemiologic investigation was initiated by the hospital's infection-control team. Environmental surveys were conducted twice in each of the two wards (5S and 5N), at 2-week intervals, to identify the source(s) of the pathogens. Multiple samples from environmental surfaces were tested to detect *P. aeruginosa* on NAC agar medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with or without ceftazidime at a final concentration of 16 μg/ml. A total of 23 *P. aeruginosa* isolates, including 11 from environmental sources, were analyzed for chromosomal DNA typing with a counter-clamped homogeneous electric field system (CHEF Mapper; Bio-Rad Laboratories, Hercules, CA, USA), for O-antigen serotyping with control serum (Denka Seiken, Tokyo, Japan), and for antibiotic resistance by a microdilution method according to the guidelines of the Clinical Laboratory Standards Institute.[4] DNA sequences of the quinolone resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC*, and *parE* genes were determined as described previously.[5] Resistance genes other than quinolone-resistance genes were detected by polymerase chain reaction with three primer pairs designed to amplify the sequences of the metallo β-lactamase bla_{IMP-1} gene,[6] aminoglycoside 6′-acetyltransferase *aac(6′)-Iae* gene,[5] and the aminoglycoside 2″-adenylyltransferase *aadB* gene.[7]

The minimum inhibitory concentrations (MICs) of eight antibiotics tested against *P. aeruginosa* isolates are shown in Table 1. Five of the 12 clinical isolates and 3 of the 11 environmental isolates were MDR *P. aeruginosa* and were resistant to all antibiotics tested, except for gentamicin (GM) and polymixin B (PL-B). Six clinical isolates and 3 environmental isolates were resistant to IPM and CPFX but sensitive to AMK (Table 1). The remaining 6 isolates (IMCJ nos. 333, 262, 264, 268, 326, and 330) were each sensitive to four to eight antibiotics tested.

The five clinical and three environmental isolates of MDR *P. aeruginosa* carried the bla_{IMP-1} and *aac(6′)-Iae* genes, and showed mutations in T83I and S87L in the QRDRs of *gyrA* and *parC*, respectively (Table 1). Among these eight MDR isolates, two (IMCJ nos. 335 and 361) had an additional D87G mutation in the QRDR of *gyrA*. Six other clinical and two other environmental isolates carried the bla_{IMP-1} and *aadB* genes, and showed mutations in T83I, E468D, and S87W in the QRDRs of *gyrA*, *gyrB*, and *parC*, respectively (Table 1). The presence of these genes and their alterations in the QRDRs in *gyrA*, *gyrB*, and *parC* are consistent with their resistance phenotypes. These genetic analyses of drug-resistant genes in MDR *P. aeruginosa* could provide useful information about the evolution of nosocomial pathogens.

Of the total 23 isolates, 18 expressed the O11 antigen, 4 expressed the O1 antigen, and 1 expressed the O6 antigen (Table 1). Notably, all clinical isolates and 6 environmental isolates expressed the O11 antigen (Table 1).

The PFGE patterns of the isolates are shown in Fig. 2. Among the 23 *P. aeruginosa* isolates, 13 different PFGE patterns were detected. Three clinical isolates (IMCJ nos. 254–256) and 3 environmental isolates (IMCJ nos. 260, 261, and 267) from the 5S ward were of the same pattern (PA1; Fig. 2 and Table 1). Six clinical isolates (IMCJ nos., 257, 270, 321–323, and 332) and 2 environmental isolates (IMCJ nos. 324 and 331) in the hematology ward (5N) were of the same pattern (PE). Taken together, the data indicate that the first outbreak was caused by one strain of MDR *P. aeruginosa*, but that the second outbreak was caused by a separate strain of drug-resistant *P. aeruginosa*. Three other MDR *P. aeruginosa* isolates, obtained from patients 10 (ward 5N), 11 (ward 13N), and 12 (ward 13S), had PFGE patterns (shown in lanes 8, 10, and 9, respectively, in Fig. 2) that differed from those of isolates obtained from patients 1, 2, and 3 (ward 5S) and from the isolates obtained from patients 4, 5, 6, 7, 8, and 9 (ward 5N), indicating that the infections in patients 10, 11, and 12 were sporadic.

In the environmental survey, three isolates of MDR *P. aeruginosa* (IMCJ260, IMCJ267, and IMCJ261; see Table 1) were obtained from wet surfaces in ward 5S; from a rack for urinals, from a urine volume-measuring device in a room for the handling of urine, and from a bath drain in a bathing room, respectively. Three *P. aeruginosa* isolates,

Table 1. Origins and characteristics of the *Pseudomonas aeruginosa* isolates from clinical and environmental sources

Strain	Source (specimen)	Date isolated	Ward	O serotype	MICs against[a]:								PCR for resistance genes			Mutations in QRDRs[b]			PFGE type (lane in Fig. 2)
					PIPC	CAZ	IPM	AMK	GM	TOB	CPFX	PL-B	bla_{IMP-1}	*aac(b')-Iae*	*aadB*	GyrA	GyrB	ParC	
IMCJ254	Patient 1 (urine)	30-Sept	5S	O11	256	>256	16	128	8	256	>32	4	+	+	−	T83I		S87L	PA1 (2)
IMCJ255	Patient 2 (urine)	30-Sept	5S	O11	>256	>256	32	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (3)
IMCJ256	Patient 3 (urine)	18-Oct	5S	O11	>256	256	16	64	4	128	>32	4	+	+	−	T83I		S87L	PA1 (4)
IMCJ257	Patient 4 (urine)	8-Nov	5N	O11	>256	>256	>64	2	32	4	>32	4	+	−	+	T83I	E468D	S87W	PE (14)
IMCJ270	Patient 5 (sputum)	12-Nov	5N	O11	>256	>256	>64	1	32	2	>32	4	+	−	+	T83I	E468D	S87W	PE (15)
IMCJ321	Patient 6 (urine)	24-Nov	5N	O11	256	>256	>64	1	32	2	>32	4	+	−	+	T83I	E468D	S87W	PE (16)
IMCJ322	Patient 7 (pharynx)	8-Dec	5N	O11	>256	>256	>64	4	128	16	>32	4	+	−	+	T83I	E468D	S87W	PE (17)
IMCJ323	Patient 8 (urine)	16-Dec	5N	O11	>256	>256	>64	8	256	16	>32	4	+	−	+	T83I	E468D	S87W	PE (18)
IMCJ332	Patient 9 (urine)	2-Feb	5N	O11	>256	>256	>64	2	128	8	>32	4	+	−	+	T83I	E468D	S87W	PE (21)
IMCJ333	Patient 10 (pharynx)	28-Feb	5N	O11	>256	>256	16	2	4	8	>32	4	−	−	−	T83I		S87L	PA2 (8)
IMCJ335	Patient 11 (urine)	23-Mar	13N	O11	>256	>256	>64	64	8	128	>32	4	+	+	−	T83I + D87G		S87L	PA4 (10)
IMCJ361	Patient 12 (urine)	11-May	13S	O11	>256	>256	>64	64	8	64	>32	4	+	+	−	T83I + D87G		S87L	PA3 (9)
	Environmental																		
IMCJ260	Rack for urinal	28-Oct	5S	O11	256	>256	64	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (5)
IMCJ261	Bath drain	28-Oct	5S	O11	>256	>256	>64	64	8	256	>32	4	+	+	−	T83I		S87L	PA1 (6)
IMCJ264	Sink in P1 room	28-Oct	5S	O1	8	2	4	16	8	2	0.5	4	−	−	−				PD (13)
IMCJ262	Sink in staff room	28-Oct	5S	O1	2	0.5	1	1	0.5	0.5	0.064	2	−	−	−				PG (23)
IMCJ267	Urine volume-measuring device	12-Nov	5S	O11	256	>256	32	64	>256	>256	>32	4	+	+	−	T83I		S87L	PA1 (7)
IMCJ268	Sink in staff room	12-Nov	5S	O1	1	1	1	2	0.5	0.25	0.064	2	−	−	−				PH (24)
IMCJ324	Sink in lavatory	21-Dec	5N	O11	>256	>256	>64	4	256	16	>32	4	+	−	+	T83I	E468D	S87W	PE (19)
IMCJ325	Urine volume-measuring device	21-Dec	5N	O11	>256	>256	>64	4	64	16	>32	4	+	−	−	T83I		S87L	PF (22)
IMCJ326	Sink in staff room	21-Dec	5N	O6	>256	32	8	16	128	128	0.19	4	−	−	−				PB (11)
IMCJ330	Sink in lavatory	11-Jan	5N	O1	>256	>256	>64	4	2	2	0.064	4	−	−	−				PC (12)
IMCJ331	Urine volume-measuring device	11-Jan	5N	O11	>256	>256	>64	4	>256	16	>32	4	+	−	+	T83I	E468D	S87W	PE (20)
IMCJ2.S1[c]				O11	>128	>28	128	128	16	64	32	2	+	+	−	T83I		S87L	PA (1)

[a] PIPC, piperacillin; CAZ, ceftazidime; IPM, imipenem; AMK, amikacin; GM, gentamicin; TOB, tobramycin; CPFX, ciprofloxacin; PL-B, polymixin B

[b] T83I, Thr at position 83 of GyrA changed to Ile (ACC→ATC); S87L, Ser at position 87 of ParC changed to Leu (TCG→TTG); E468D, Glu at position at 468 changed to Asp (GAG→GAT); S87W, Ser at position 87 of ParC changed to Trp (TCG→TGG); D87G, Asp at position 87 changed to Gly (GAC→GGC). The numbering of the amino acids is based on that of *P. aeruginosa* PAO1 (Genbank accession no. NC_002516)

[c] Nosocomial strain that caused outbreaks among hospitals in an area of Japan[3]

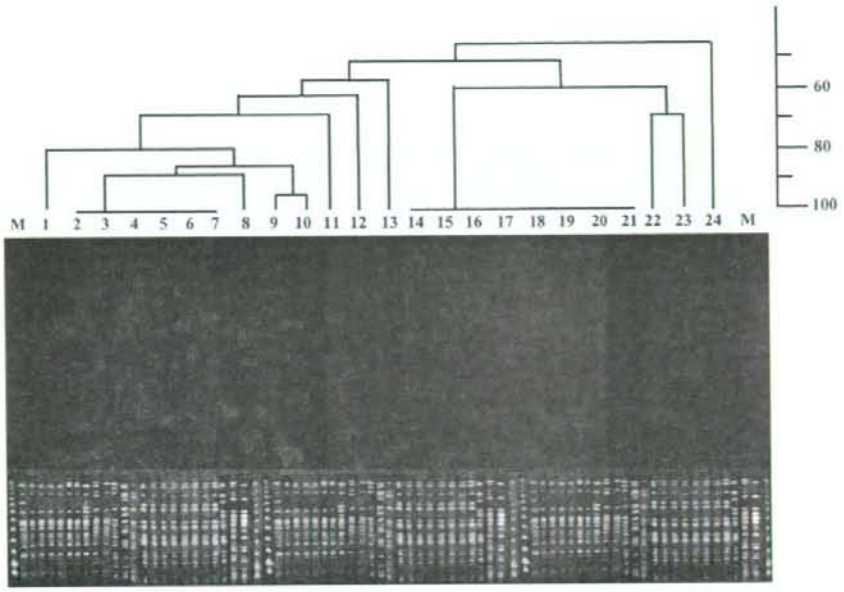

Fig. 2. Pulsed-field gel electrophoresis (PFGE) patterns and dendrogram for *P. aeruginosa* isolates from clinical and environmental sources. The isolates corresponding to *each lane* are listed in Table 1

resistant to IPM and CPFX, but sensitive to AMK, were obtained in ward 5N. One isolate (IMCJ324) was from a sink in a lavatory, and two (IMCJ325 and IMCJ331) were obtained on different days from the surfaces of a urine volume-measuring device in a room for the handling of urine. As mentioned above, PFGE analysis revealed that three of the MDR *P. aeruginosa* isolates and two of three isolates of the IPM- and CPFX-resistant but AMK-sensitive *P. aeruginosa* were causative pathogens of the outbreaks. The data pointed to environmental contamination by drug-resistant *P. aeruginosa* in the bathing room, the lavatory, and the rooms for the handling of urine, which may have been sources of the pathogens during the outbreaks.

During the two outbreaks, we took the following steps: (a) environmental surface monitoring as described, (b) active surveillance for drug-resistant *P. aeruginosa* obtained from the samples of patients, (c) strict isolation of infected patients or carriers of MDR *P. aeruginosa*, (d) rigorous contact precautions, especially during the handling of urine and urinary catheters, and (e) disinfection with 70% alcohol on the surfaces of apparatuses contaminated by MDR or drug-resistant *P. aeruginosa* and in rooms where urine is handled. As a result, the outbreaks were contained. Each patient with MDR *P. aeruginosa* was isolated in a single room during the two outbreaks. However, if a single room is not available, an area in a ward that is separate from other patients could be used for patients with MDR *P. aeruginosa*. Inadequate use of antimicrobial agents against *P. aeruginosa* was not found in the wards where the outbreaks occurred, although prior-approval programs, including pre-approved indications for antibiotics against *P. aeruginosa*, such as carbapenems and aminoglycosides, were not performed, but were in the planning stage in this hospital.

However, sporadic MDR *P. aeruginosa* will continue to be isolated from inpatients who may bring the pathogens into the hospital. In fact, we have reported outbreaks of MDR *P. aeruginosa* in hospitals in a prefecture in Japan.[3] Analysis indicated that the *P. aeruginosa* IMCJ.S1 strain responsible for the outbreaks underwent clonal expansion.[3] The PFGE patterns of MDR *P. aeruginosa* isolates from the first outbreak described herein had 80% similarity to the pattern of *P. aeruginosa* IMCJ.S1. In addition, MDR isolates from the sporadic cases in the present study (patients 10, 11, and 12) had close similarity to the IMCJ.S1 and the strains associated with the first outbreak. These results suggest that some dominant MDR *P. aeruginosa* strains may be prevalent in Japan.

Acknowledgments We thank Dr. M. Nakano (Jichi Medical School, Tochigi, Japan) for comments on the manuscript. This study was supported by Health Sciences Research Grants from the Ministry of Health, Labour, and Welfare of Japan (H16-IRYO-IPPAN-011 and H18-SHINKO-11).

References

1. Kirikae T, Tokunaga O, Inoue Y, Fujino T, Saruta K, Yoshikura H, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens* in a long-term care facility for patients with severe motor and intellectual disabilities. Jpn J Infect Dis 2004;57:226–8.
2. Falagas ME, Koletsi PK, Bliziotis IA. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. J Med Microbiol 2006;55:1619–29.
3. Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Kasai A, Mizuguchi Y, Araake M, et al. Outbreaks of multidrug-resistant *Pseudomonas*

aeruginosa in community hospitals in Japan. J Clin Microbiol 2007;45:979–89.
4. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Approved standard M07-A7. Wayne, PA: Clinical and Laboratory Standards Institute; 2006.
5. Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Fujino T, Kobayashi I, Morita K, et al. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob Agents Chemother 2005;49:3734–42.
6. Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, et al. Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob Agents Chemother 1994;38:71–8.
7. Camaron FH, Groot Obbink DJ, Ackerman VP, Hall RM. Nucleotide sequence of the AAD(2") aminoglycoside adenylyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. Nucleic Acids Res 1986;14:8625–35.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2006, p. 791–795
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.2.791–795.2006

Vol. 50, No. 2

PCR Classification of CTX-M-Type β-Lactamase Genes Identified in Clinically Isolated Gram-Negative Bacilli in Japan

Naohiro Shibata, Hiroshi Kurokawa, Yohei Doi, Tetsuya Yagi, Kunikazu Yamane, Jun-ichi Wachino, Satowa Suzuki, Kouji Kimura, Satoshi Ishikawa, Haru Kato, Yoshiyuki Ozawa, Keigo Shibayama, Kumiko Kai, Toshifumi Konda, and Yoshichika Arakawa*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan

Received 26 March 2005/Returned for modification 15 June 2005/Accepted 1 November 2005

Of 1,456 strains isolated from 2001 to 2003 demonstrating resistance to either oxyimino-cephalosporin, 317 strains, isolated in 57 of 132 clinical facilities, were found to harbor bla_{CTX-M} genes by PCR. Fifty-seven, 161, and 99 strains harbored bla_{CTX-M} genes belonging to the $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ clusters, respectively.

In recent years, CTX-M-type β-lactamases have been recognized as a growing family possessing a high level of hydrolyzing activities, especially against cefotaxime (CTX) and ceftriaxone. Nearly 40 variants of the CTX-M-type enzymes have been identified (2, 4, 13, 25) and registered to date (http://www.lahey.org/studies/other.asp#table_1). Further proliferation of CTX-M-type β-lactamase-producing gram-negative bacteria has become a great concern (6), since a large number of nosocomial outbreaks caused by such bacteria have so far been recognized and reported in various medical facilities in many countries (1, 3, 5, 7–9, 19, 21).

In Japan, FEC-1 and Toho-1 were initially identified (12, 15) and were later included in CTX-M-type enzymes. Since then, various strains that produce a Toho-1-like β-lactamase have been identified in Japanese clinical settings (26, 28). Almost all of them, however, were found to be CTX-M-2 by sequence analyses (N. Shibata, et al. Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2235, 2001). However, the trends for several CTX-M-type β-lactamases other than CTX-M-2 have remained unclear. In the present study, we investigated the molecular types of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated in Japanese clinical facilities using PCR methods.

From January 2001 to December 2003, 1,456 gram-negative bacterial isolates demonstrating resistance to oxyimino-cephalosporins were submitted from 132 hospitals to the reference laboratory at our institute. These strains were then subjected to screening for β-lactamases, including TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs), CTX-M-type β-lactamases, AmpC- and CMY-type class C cephalosporinases and cephamycinases, and class B metallo-β-lactamases (MBLs). The strains were checked for ESBL production by the double-disk diffusion synergy test recommended by the CLSI (formerly the NCCLS) (18). The MICs of ceftazidime (CAZ) and CTX for the clinical isolates were determined by the agar dilution method recommended by the CLSI guidelines. When a clinical isolate demonstrated resistance to either oxyimino-cephalosporin, the strain was then subjected to PCR analyses for detection of bla_{CTX-M} genes. PCR analysis was performed by the method reported previously (27). The four sets of PCR primers used for detection of bla_{CTX-M} genes in the present study were as follows: primers CTX-M-1-F (5′-GCT GTT GTT AGG AAG TGT GC-3′) and CTX-M-1-R (5′-CCA TTG CCC GAG GTG AAG-3′), primers CTX-M-2-F (5′-ACG CTA CCC CTG CTA TTT-3′) and CTX-M-2-R (5′-CCT TTC CGC CTT CTG CTC-3′), primers CTX-M-8-F (5′-CGG ATG ATG CTA ATG ACA AC-3′) and CTX-M-8-R (5′-GTC AGA TTG CGA AGC GTC-3′), and primers CTX-M-9-F (5′-GCA GAT AAT ACG CAG GTG-3′) and CTX-M-9-R (5′-CGG CGT GGT GGT GTC TCT-3′). Only one strain was selected from an individual patient and subjected to the PCR test.

As shown in Table 1, the inhibition patterns by combination of the double-disk diffusion synergy test for ESBL detection and the sodium mercaptoacetic acid (SMA) disk test for MBL detection were classified into four groups. Of 1,456 strains tested, 59 were resistant only to CAZ and susceptible to clavulanic acid. It was speculated that these strains produce mainly SHV- or TEM-derived ESBLs, because SHV-12-producing strains have been prevalent in Japan (27). On the other hand, 276 strains showed resistance to CTX but were susceptible to CAZ. The MIC of CTX was significantly decreased in the presence of clavulanic acid. It was speculated that these strains chiefly produce CTX-M-type β-lactamases. Five hundred forty-eight isolates demonstrated resistance to both CAZ and CTX; but the inhibitory effect of clavulanic acid was not clear in these strains, and the production of MBL was suggested, because the MICs of CAZ and CTX were reduced in the presence of SMA, which is a specific inhibitor of metallo-β-lactamase (23). The remaining 573 strains, which demonstrated resistance to either of the oxyimino-cephalosporins, did not become susceptible to these agents in the presence of SMA, suggesting the production of some AmpC-type enzymes, including plasmid-mediated CMY-type enzymes.

Of 1,397 strains subjected to the PCR analyses, 317 strains were suggested to harbor bla_{CTX-M} genes. Of these strains, 57 appeared to carry genes of the $bla_{CTX-M-1}$ group, including

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

TABLE 1. Results of screening by double-disk diffusion synergy tests

Bacterial species	Pattern of double-disk diffusion synergy test				Total no. of strains tested
	Resistant to CAZ and susceptible to clavulanic acid (no. of strains)	Resistant to CTX and susceptible to clavulanic acid[b]	Resistant to CAZ and CTX and susceptible to SMA[c]	Resistant to either oxyimino-cephalosporin and not susceptible to SMA	
Escherichia coli	33	157/157	7/24	4/4	218
Proteus mirabilis	0	71/71	0/1	0/0	72
Klebsiella pneumoniae	15	42/42	7/31	1/2	90
Klebsiella oxytoca	4	5/5	1/3	0/2	14
Serratia marcescens	7	0/0	0/65	10/77	149
Enterobacter cloacae	0	0/0	2/11	1/20	31
Enterobacter aerogenes	0	0/0	0/2	1/8	10
Citrobacter freundii	0	0/0	0/4	2/15	19
Citrobacter koseri	0	0/0	0/0	1/1	1
Providencia rettgeri	0	1/1	0/2	0/0	3
Acinetobacter baumannii	0	0/0	1/49	3/40	89
Other bacterial species[d]	0	0/0	0/356	0/404	760
Total[e]	59	276/276	18/548[f]	23/573[f]	1,456

[a] The data represent the number of bla_{CTX-M}-positive strains by PCR/total number of strains demonstrating each inhibition pattern and subjected to PCR.
[b] Strains that produce metallo-β-lactamase are included.
[c] Strains that produce plasmid-mediated CMY-type cephalosporinase or chromosomal AmpC hyperproducers are included.
[d] *Pseudomonas* spp., *Alcaligenes* spp., *Achromobacter* spp., and *Burkholderia* spp. demonstrating resistance to ceftazidime or cefotaxime were included; but *Stenotrophomonas* spp. and *Chryseobacterium* spp. that produce intrinsic metallo-β-lactamase were excluded.
[e] Out of the total number of strains being subjected to PCR analysis (1,397; represented in columns 2, 3, and 4), 317 were found to be bla_{CTX-M} positive.

$bla_{CTX-M-1}$, $bla_{CTX-M-3}$, and $bla_{CTX-M-15}$, as shown in Table 2. Moreover, 161 strains were suggested to harbor the genes encoding the CTX-M-2 group of enzymes, such as CTX-M-2, CTX-M-20, and CTX-M-31. Furthermore, 99 strains appeared to carry the genes for the CTX-M-9 group of enzymes, such as CTX-M-9, CTX-M-14, and CTX-M-16. No strain harboring genes for the CTX-M-8 or the CTX-M-25 group of enzymes was found among the strains tested.

As shown in Table 3, strains that harbored genes for the CTX-M-type enzymes were isolated from 57 of 132 hospitals across Japan, except for the Hokkaido region, throughout the 3-year investigation period. Fourteen and 24 strains that harbored genes for the CTX-M-1 group of enzymes were identified in 7 and 10 hospitals located in the Kanto and Chubu regions, respectively (Table 3). However, no strain harboring genes for the CTX-M-1 group of enzymes were found in the Chugoku and Shikoku regions (Table 3). In 22 of 57 hospitals, genes for multiple CTX-M-type β-lactamases belonging to different groups were identified during the investigation period (Fig. 1). Interestingly, genes for all three groups of CTX-M-type enzymes were identified in 7 of 57 hospitals (Fig. 1; Table 3).

TABLE 2. Number of strains that produce CTX-M-type β-lactamases as detected by PCR

Bacterial species	No. of strains by the following PCR type:			Total
	CTX-M-1 group[a]	CTX-M-2 group[b]	CTX-M-9 group[c]	
Escherichia coli	33	46	89	168
Proteus mirabilis	0	71	0	71
Klebsiella pneumoniae	10	31	9	50
Klebsiella oxytoca	2	3	1	6
Serratia marcescens	9	1	0	10
Enterobacter cloacae	0	3	0	3
Enterobacter aerogenes	1	0	0	1
Citrobacter freundii	2	0	0	2
Citrobacter koseri	0	1	0	1
Providencia rettgeri	0	1	0	1
Acinetobacter baumannii	0	4	0	4
Total	57	161	99	317

[a] The PCR primers used can detect genes for CTX-M-1 and several variants, such as CTX-M-3 and CTX-M-15.
[b] The PCR primers used can detect genes for CTX-M-2 and several variants, such as CTX-M-20 and CTX-M-31.
[c] The PCR primers used can detect genes for CTX-M-9 and several variants, such as CTX-M-14 and CTX-M-16.

After the first description of Toho-1 in Japan in 1995, several outbreaks caused by CTX-M-type β-lactamase producers have been reported in there (17, 26, 28). In the present investigation, it became clear that gram-negative nosocomial bacilli producing the CTX-M-1, CTX-M-2, or CTX-M-9 group of enzymes have already been dispersed in various clinical settings in Japan, although strains that produce TEM- or SHV-derived ESBLs are not predominant to date.

Recently, the CTX-M-1 group of enzymes, such as CTX-M-3 and CTX-M-15, have emerged in Europe and Asia (3, 8–10, 14, 22, 28). In the present study, we also identified the genes for the CTX-M-1 group of enzymes in various bacterial species, including *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, in addition to *Providencia rettgeri*, *Citrobacter freundii*, *Citrobacter koseri*, and *Enterobacter cloacae*. This finding may be suggestive of the lateral transfer of very similar plasmids bearing bla_{CTX-M} genes among different bacterial species. Actually, probable nosocomial transmissions of CTX-M-producing bacterial strains were suspected in several medical facilities, as shown in Fig. 1 and Table 3. Especially in hospitals D18, D20, and E5, all three groups of genes for CTX-M enzymes were identified; and genes for CTX-M-type enzymes were detected in various gram-negative bacterial species, suggesting the horizontal transfer of the bla_{CTX-M} genes among different bacterial species. Interestingly,

TABLE 3. Bacterial species that produce each group of CTX-type β-lactamases

Region	PCR type	Bacterial species (no. of isolates)	Hospital (no. of isolates)
Hokkaido (0[a]/7[b])		None	None
Tohoku (4/17)	CTX-M-1	*K. pneumoniae* (2[c])	B4 (2[c])
	CTX-M-2	*E. coli* (1)	B1 (1)
		P. mirabilis (10)	B4 (10)
	CTX-M-9	*E. coli* (6)	B2 (1), B3 (4), B4 (1)
Kanto (9/26)	CTX-M-1	*E. coli* (7)	C1 (1), C3 (2), C9 (4)
		K. pneumoniae (6)	C2 (2), C6 (1), C7 (3)
		K. oxytoca (1)	C8 (1)
	CTX-M-2	*P. mirabilis* (28)	C4 (9), C5 (19)
		A. baumannii (3)	C5 (3)
	CTX-M-9	*K. pneumoniae* (1)	C7 (1)
		E. coli (11)	C2 (1), C3 (1), C4 (1), C7 (4), C8 (4)
Chubu (22/37)	CTX-M-1	*E. coli* (12)	D2 (1), D3 (5), D6 (3), D7 (1), D20 (1), D22 (1)
		K. pneumoniae (2)	D1 (1), D20 (1)
		C. freundii (2)	D18 (2)
		E. aerogenes (1)	D19 (1)
		S. marcescens (5)	D18 (5)
	CTX-M-2	*E. coli* (29)	D5 (1), D6 (2), D8 (1), D13 (4), D14 (1), D15 (5), D18 (1), D20 (14)
		K. pneumoniae (21)	D20 (20), D22 (1)
		K. oxytoca (3)	D6 (1), D15 (1), D20 (1)
		P. mirabilis (17)	D14 (4), D16 (11), D17 (1), D18 (1)
		S. marcescens (1)	D20 (1)
		E. cloacae (3)	D18 (1), D20 (2)
		A. baumannii (1)	D20 (1)
	CTX-M-9	*E. coli* (34)	D4 (1), D5 (1), D6 (4), D7 (4), D8 (4), D9 (3), D10 (1), D11 (1), D12 (1), D14 (1), D16 (4), D18 (1), D20 (3), D21 (5)
		K. pneumoniae (4)	D12 (4)
		K. oxytoca (1)	D12 (1)
Kinki (10/19)	CTX-M-1	*E. coli* (6)	E5 (4), E7 (1), E10 (1)
		K. oxytoca (1)	E4 (1)
		S. marcescens (4)	E1 (4)
	CTX-M-2	*E. coli* (8)	E3 (1), E5 (6), E8 (1)
		K. pneumoniae (6)	E5 (6)
		P. mirabilis (15)	E2 (1), E5 (14)
		P. rettgeri (1)	E8 (1)
	CTX-M-9	*E. coli* (11)	E2 (2), E3 (1), E5 (6), E6 (1), E9 (1)
		K. pneumoniae (2)	E2 (1), E5 (1)
Chugoku (5/13)	CTX-M-2	*E. coli* (3)	F2 (2), F5 (1)
		K. pneumoniae (2)	F3 (2)
	CTX-M-9	*E. coli* (8)	F1 (4), F4 (1), F5 (3)
Shikoku (3/5)	CTX-M-2	*E. coli* (1)	G2 (1)
		C. koseri (1)	G3 (1)
	CTX-M-9	*E. coli* (15)	G2 (15)
		K. pneumoniae (2)	G1 (1), G2 (1)
Kyushu and Okinawa (4/8)	CTX-M-1	*E. coli* (8)	H1 (1), H2 (6), H3 (1)
	CTX-M-2	*E. coli* (4)	H1 (1), H4 (3)
		K. pneumoniae (2)	H4 (2)
		P. mirabilis (1)	H2 (1)
	CTX-M-9	*E. coli* (4)	H1 (3), H2 (1)
Total (57/132)			

[a] Number of medical facilities where bla_{CTX-M}-harboring strains were detected.
[b] Number of medical facilities that submitted strains to our laboratory.
[c] Number of clinical isolates harboring bla_{CTX-M} gene.

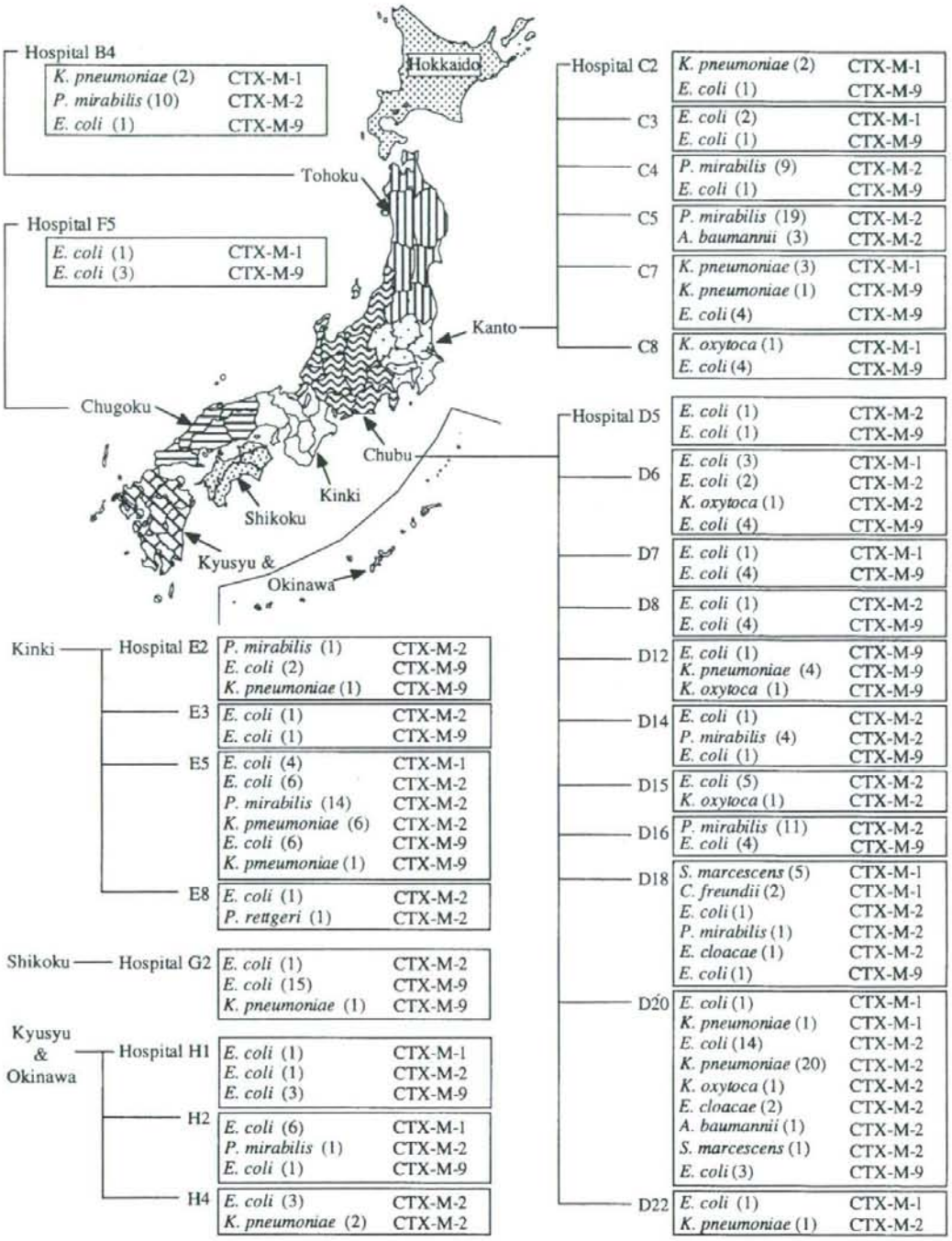

FIG. 1. Clinical facilities where multiple bla_{CTX-M} genes belonging to different genetic clusters were identified. Facilities where multiple bacterial species that bear bla_{CTX-M} genes were isolated are also added. The numbers in parentheses demonstrate the number of clinical isolates of each bacterial species.

all 71 *Proteus mirabilis* strains were identified as CTX-M-2 producers, and they were isolated in widely separate medical facilities located far apart in Japan, implying a close relatedness between CTX-M-2 and *P. mirabilis* in Japanese clinical environments. The plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ may be very adaptive for *P. mirabilis*, which may either serve as a reservoir for plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ gene (16, 17) or have preferentially accepted $bla_{CTX\text{-}M\text{-}2}$ genes from some environmental *Kluyvera* spp. (11, 20). Comparative analyses of plasmids that bear the $bla_{CTX\text{-}M\text{-}2}$ gene would provide a clue to elucidate the relatedness and origins of the plasmids.

The CTX-M-9 group of enzymes, including CTX-M-14, have so far been found worldwide in the species belonging to the family *Enterobacteriaceae* (7–9). However, almost all of the CTX-M-9 group of enzymes were found in *E. coli* in the present study, and some of them were suggested to be CTX-M-14. Precise analysis of the genetic environments mediating the $bla_{CTX\text{-}M\text{-}9}$ group of genes among these strains as well as their genome profiles would explain the presence of CTX-M-producing pandemic strains in Japan.

In conclusion, the aim of the present study was to make a rough estimate of the current status of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated from Japanese medical facilities. The findings obtained imply that various plasmid-mediated genetic determinants for CTX-M-type β-lactamases have already been disseminated in Japanese clinical environments. Since CTX-M-2 was also identified in livestock (24), we must take special precautions against the further proliferation of gram-negative bacterial strains that harbor plasmids carrying genes for CTX-M-type β-lactamases, together with the other classes of plasmid-mediated β-lactamases, such as CMY-type cephamycinases and MBLs.

We thank all medical institutions for submitting bacterial strains to the national reference laboratory for performance of this study.

This work was mainly supported by two grants (grants H15-Shinko-9 and H15-Shinko-10) from the Ministry of Health, Labor and Welfare of Japan. PCR typing of *bla* genes was supported by grants (grants 1017221 and 13770141) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. **Barthelemy, M., J. Peduzzi, H. Bernard, C. Tancrede, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122**:15–22.
2. **Bonnet, R.** 2004. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. **48**:1–14.
3. **Boyd, D. A., S. Tyler, S. Christianson, A. McGeer, M. P. Muller, B. M. Willey, E. Bryce, M. Gardam, P. Nordmann, and M. R. Mulvey.** 2004. Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum β-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrob. Agents Chemother. **48**:3758–3764.
4. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14**:933–951.
5. **Brenwald, N. P., G. Jevons, J. M. Andrews, J. H. Xiong, P. M. Hawkey, and R. Wise.** 2003. An outbreak of a CTX-M-type β-lactamase-producing *Klebsiella pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum β-lactamases. J. Antimicrob. Chemother. **51**:195–196.
6. **Bush, K.** 2002. The impact of β-lactamases on the development of novel antimicrobial agents. Curr. Opin. Investig. Drugs **3**:1284–1290.
7. **Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey.** 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. Antimicrob. Agents Chemother. **46**:630–637.
8. **Dutour, C., R. Bonnet, H. Marchandin, M. Boyer, C. Chanal, D. Sirot, and J. Sirot.** 2002. CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from *Enterobacteriaceae* isolated in France. Antimicrob. Agents Chemother. **46**:534–537.
9. **Edelstein, M., M. Pimkin, I. Palagin, I. Edelstein, and L. Stratchounski.** 2003. Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob. Agents Chemother. **47**:3724–3732.
10. **Gniadkowski, M., I. Schneider, A. Palucha, R. Jungwirth, B. Mikiewicz, and A. Bauernfeind.** 1998. Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob. Agents Chemother. **42**:827–832.
11. **Humeniuk, C., G. Arlet, V. Gautier, P. Grimont, R. Labia, and A. Philippon.** 2002. β-Lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother. **46**:3045–3049.
12. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. **39**:2269–2275.
13. **Jacoby, G. A.** 1997. Extended-spectrum β-lactamases and other enzymes providing resistance to oxyimino-β-lactams. Infect. Dis. Clin. N. Am. **11**:875–887.
14. **Markovska, R., I. Shneider, E. Keuleyan, and A. Bauerfeind.** 2004. Extended-spetrum β-lactamase (ESBL) CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae* in Sofia, Bulgaria. Clin. Microbiol. Infect. **10**:752.
15. **Matsumoto, Y., F. Ikeda, T. Kamimura, Y. Yokota, and Y. Mine.** 1988. Novel plasmid-mediated β-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrob. Agents Chemother. **32**:1243–1246.
16. **Nagano, N., Y. Nagano, C. Cordevant, N. Shibata, and Y. Arakawa.** 2004. Nosocomial transmission of CTX-M-2 β-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. J. Clin. Microbiol. **42**:3978–3984.
17. **Nagano, N., N. Shibata, Y. Saitou, Y. Nagano, and Y. Arakawa.** 2003. Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type β-lactmase. J. Clin. Microbiol. **41**:5530–5536.
18. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial susceptibility testing. Twelfth informational supplement. Approved standard M100-S12. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Palucha, A., B. Mikiewicz, W. Hryniewicz, and M. Gniadkowski.** 1999. Concurrent outbreaks of extended-spectrum β-lactamase-producing organisms of the family Enterobacteriaceae in a Warsaw hospital. J. Antimicrob. Chemother. **44**:489–499.
20. **Poirel, L., P. Kampfer, and P. Nordmann.** 2002. Chromosome-encoded Ambler class A β-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **46**:4038–4040.
21. **Radice, M., C. Gonzalez, P. Power, M. C. Vidal, and G. Gutkind.** 2001. Third-generation cephalosporin resistance in *Shigella sonnei*, Argentina. Emerg. Infect. Dis. **7**:442–443.
22. **Rodriguez, M. M., P. Power, M. Radice, C. Vay, A. Famiglietti, M. Galleni, J. A. Ayala, and G. Gutkind.** 2004. Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. Antimicrob. Agents Chemother. **48**:4895–4897.
23. **Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa.** 2003. 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. **41**:5407–5413.
24. **Shiraki, Y., N. Shibata, Y. Doi, and Y. Arakawa.** 2004. *Escherichia coli* producing CTX-M-2 β-lactamase in cattle, Japan. Emerg. Infect. Dis. **10**:69–75.
25. **Walther-Rasmussen, J., and N. Hoiby.** 2004. Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum β-lactamases. Can. J. Microbiol. **50**:137–165.
26. **Yagi, T., H. Kurokawa, K. Senda, S. Ichiyama, H. Ito, S. Ohsuka, K. Shibayama, K. Shimokata, N. Kato, M. Ohta, and Y. Arakawa.** 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like β-lactamase genes. Antimicrob. Agents Chemother. **41**:2606–2611.
27. **Yagi, T., H. Kurokawa, N. Shibata, K. Shibayama, and Y. Arakawa.** 2000. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol. Lett. **184**:53–56.
28. **Yamasaki, K., M. Komatsu, T. Yamashita, K. Shimakawa, T. Ura, H. Nishio, K. Satoh, R. Washidu, S. Kinoshita, and M. Aihara.** 2003. Production of CTX-M-3 extended-spectrum β-lactamase and IMP-1 metallo-β-lactamase by five gram-negative bacilli: survey of clinical isolates from seven laboratories collected in 1998 and 2000, in the Kinki region of Japan. J. Antimicrob. Chemother. **51**:631–638.

Jpn. J. Infect. Dis., 59, 2006

Laboratory and Epidemiology Communications

A Nosocomial Outbreak Due to Novel CTX-M-2-Producing Strains of *Citrobacter koseri* in a Hematological Ward

Tsuyoshi Muta*, Nobuko Tsuruta[1], Yumiko Seki[2], Rika Ota[2], Satowa Suzuki[3], Naohiro Shibata[3], Koji Kato, Tetsuya Eto, Hisashi Gondo and Yoshichika Arakawa[3]

Department of Hematology, [1]Department of Respiratory Disease and [2]Department of Clinical Microbiology, Hamanomachi Hospital, Fukuoka 810-8539, and [3]Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011, Japan

Communicated by Kiyosu Taniguchi

(Accepted January 10, 2006)

Citrobacter koseri is a member of the family *Enterobacteriaceae*. Urinary tract infections caused by *C. koseri* have been observed in as many as 12% of all isolates in adults (1). In compromised hosts, *Citrobacter* spp. could cause pneumonitis, empyema (2), biliary infection (3), and bacteremia (4). *Citrobacter* spp. were formerly susceptible to oximinocephalosporins including cefotaxime (3), but recently, *C. koseri* has been reported to have developed resistance to some cephalosporins and cephamycins through the production of an inducible chromosomally-encoded cephalosporinase that can inactivate these agents (5). Most clinically isolated *C. koseri* are susceptible to oximinocephalosporins and carbapenems. Recently, oximinocephalosporin resistance among Gram-negative bacteria has been developed due to the hydrolysis of beta-lactams by beta-lactamases including extended-spectrum beta-lactamases (ESBLs). ESBLs show variable levels of resistance to cefotaxime, ceftazidime, and other broad-spectrum cephalosporins and monobactams. Nosocomial outbreaks due to SHV-4-type ESBL-producing strains and TEM-type ESBL-producing strains of *C. koseri* have already been reported (6,7). We have identified a novel CTX-M-2-type of ESBL among nosocomially isolated *C. koseri* strains, causing a probable outbreak in the hematological ward.

Sixty-eight strains of *C. koseri* were isolated from the blood, urine, feces, sputum, ascites, and pharynx of 31 patients with a hematological malignancy that had lasted over 18 months (Figure 1). *C. koseri* not only colonized but also caused bacteremia, urinary tract infection, enteritis, and peritonitis. These strains showed similar antibiotic susceptibility profiles (Table 1). We collected 5 strains of *C. koseri* from 4 patients (Table 2) and used the double-disk synergy test and plasmid profiling to screen for ESBL-producing strains as reported previously (8,9). All of the 5 strains harbored a plasmid mediating the CTX-M-2 type beta-lactamase gene. Epidemiological study using pulsed-field gel electrophoresis (PFGE) of total DNA prepared from the 5 strains revealed patterns that were indistinguishable from each other (Figure 2). The results suggested that the 5 strains characterized belong to a single epidemic strain.

In general, multiple factors may help to decrease the immunity of patients with hematological malignancies, including impairment of phagocytosis, impaired cellular immunity, and defective production of antibodies. Moreover, intensive chemotherapies usually induce severe granulocytopenia. Thus, bacterial infections are a major cause of complications and death in patients with hematological malignancies. Recently, two studies (10,11) revealed the efficacy of the prophylactic use of quinolon by neutropenic patients. As for febrile neutropenia, empirical antibiotic therapy using cefepime or cefotaxime has been emphasized (12,13). All 31 patients in this study had hematological malignancy and underwent intensive chemoradiotherapy. After that, most of the patients in our ward were administered prophylactic and therapeutic systemic antibiotics such as quinolon, cefepime, and cefotaxime, which might well be associated with the selection of antibiotic-resistant microorganisms. Unlike other members of the family *Enterobateriaceae*, CTX-M-2-producing *C. koseri* might survive in a patient's bowel flora, because of its resist-

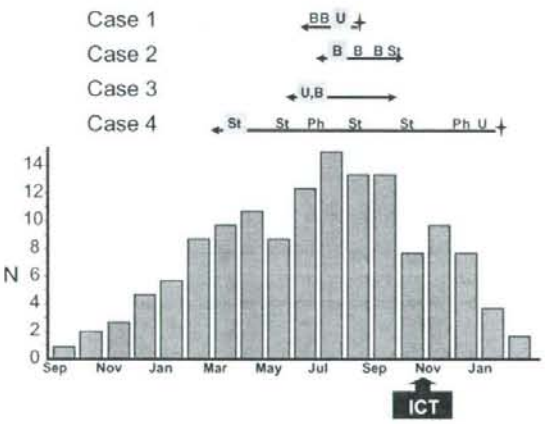

Fig. 1. The number of patients infected and/or colonized with *C. koseri*. Bars indicate the number of patients infected and/or colonized per month with *C. koseri*, the antibiotic susceptibility of which showed the same pattern. Case numbers are identical to those in Table 1. Arrows indicate the duration of each patient's hospitalization in the ward. The bald signs on each arrow indicate the samples, from which *C. koseri* was isolated. The network-breaking characters indicate the samples, from which genetically identical strains were isolated in our study. An infection control team (ICT) intervened in the ward to resolve the outbreak (see article). B, blood; U, urine; St, stool; Ph, pharynx.

*Corresponding author: Mailing address: Department of Hematology, Hamanomachi Hospital, 3-5-27 Maizuru, Chuo-ku, Fukuoka 810-8539, Japan. Tel: +81-92-721-0831, Fax: +81-92-714-3262, E-mail: muta-t@hamanomachi.jp

Table 1. Antibiotics susceptibility profile of *C. koseri* isolated in this outbreak

ampicillin	>16
ampicillin/ clavulanate	16
piperacillin	>64
cefazolin	>16
cefotaxime	>16
cefotiam	>32
ceftazidime	>16
cefpirome	>16
cefpodoxime proxetil	>4
cefcapene pivoxil	>1
cefmetazole	>32
flomoxef	32
sulbactam/cefoperazone	>32
aztoreonam	>16
imipenem	<1
gentamicin	<1
amikacin	<1
minomicin	2
levofloxacin	>4

MICs were determined by microdilution method recommended by NCCLS (currently CLSI) guideline with Muller-Hinton broth (Difco, Detroit, Mich., USA) using MicroScan-kit (Dade Behring, West Sacramento, Calif., USA).

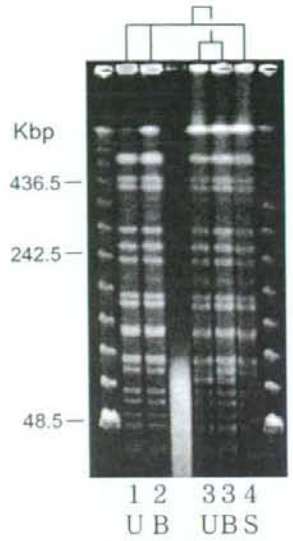

Fig. 2. PFGE analysis of *C. koseri* strains. Bacterial DNA was extracted, digested and subjected to PFGE, as previously described (8,9). Lanes 1, 2, 3, and 4, were sampled from patients Nos. 1, 2, 3, and 4, respectively. U, B, and S indicate urine, blood, and stool, respectively.

Table 2. Profiles of cases involved in the outbreak

No.	Age/Sex	Underlying disease	Therapy/Outcome	Infection	Sources of *C. koseri*
1	71/M	malignant lymphoma	chemotherapy/refractory	sepsis	Urine
2	61/M	adult T-cell leukemia	chemotherapy/partial response	sepsis	Blood
3	25/F	acute lymphoblastic leukemia	bone marrow transplantation	sepsis	Urine, Blood
4	63/F	acute lymphoblastic leukemia	chemotherapy/complete remission	enteritis	Stool

ance to quinolon, cefepime, and cefotaxime. In addition, urinary tract infections tended to be easily associated with urinary catheterization in our cases. We speculated that the situation was as follows. Once *C. koseri* colonizes in the bladder or intestine, it will then disseminate into the blood stream causing severe bacteremia during intensive chemotherapy. The symptoms of sepsis caused by *C. koseri* were often very serious, and could only be cured by appropriate and immediate administration of carbapenem. However, the use of carbapenem in high amounts and at high frequency in our ward could create a grave epidemiological problem.

The number of *C. koseri* infections increased significantly, and standard infection control measures were not effective to stop this outbreak. Therefore, we began to enforce the following precautions. We introduced barrier precautions against not only infected patients but also colonized patients, using disposable gloves and drapes. Mandatory hand washing was done immediately before and after any manipulation involved in the nursing care. Hand hygiene using commercial alcoholic disinfectant (Welpas; Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) was promoted not only for medical workers but also for patients. As for the environment, the water taps were converted to the hands-free types, and all doorknobs and bars for drip injection were sterilized using 70% alcohol twice daily. We also tried to restrict the prophylactic use of quinolon for high-risk patients with neutropenia decreasing under 100/μL which was keeping for more than 1 week. After these procedures, the incidence of *C. koseri* isolation decreased, but this type of infection has not yet been eradicated, as shown in Figure 1. We continue to make an effort to prevent nosocomial transmission of *C. koseri*.

In this report, we emphasize the appearance of *C. koseri* and its new type of drug resistance. We also warn that it is quite difficult to control the outbreak of such antimicrobial-resistant microorganisms in a hematological ward. In the future, we must pay close attention to the nosocomial spread of this type of *C. koseri*, which has demonstrated resistance to a broad spectrum of cephalosporins, cephamycins, and carbapenems.

Genetic characterization of beta-lactamase genes were supported by the grants (H15-Shinko-9 and -10) from the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

1. Whitby, J. L. and Muir, G. G. (1961): Bacteriological studies of urinary tract infection. Br. J. Urol., 33, 130-134.
2. Madrazo, A., Geiger, J. and Lauter, C. B. (1975): Citrobacter diversus at grace hospital, Detroit, Michigan. Am. J. Med. Sci., 270, 497-501.
3. Lew, P. D., Baker, A. S., Kunz, L. J. and Moellering, R. C., Jr. (1984): Intra-abdominal Citrobacter infections: association with biliary or upper gastrointestinal source. Surgery, 95, 398-403.
4. Kim, B. N., Woo, J. H., Ryu, J. and Kim, Y. S. (2003): Resistance to extended-spectrum cephalosporins and

mortality in patients with *Citrobacter freundii* bacteremia. Infection, 31, 202-207.
5. Underwood, S. and Avison, M. B. (2004): *Citrobacter koseri* and *Citrobacter amalonaticus* isolates carry highly divergent beta-lactamase genes despite having high levels of biochemical similarity and 16S rRNA sequence homology. J. Antimicrob. Chemother., 53, 1076-1080.
6. El Harrif-Heraud, Z., Arpin, C., Benliman, S. and Quentin, C. (1997): Molecular epidemiology of a nosocomial outbreak due to SHV-4-producing strains of *Citrobacter diversus*. J. Clin. Microbiol., 35, 2561-2567.
7. Perilli, M., Mugnaioli, C., Luzzaro, F., Fiore, M., Stefani, S., Rossolini, G. M. and Amicosante, G. (2005): Novel TEM-type extended-spectrum beta-lactamase, TEM-134, in a *Citrobacter koseri* clinical isolate. Antimicrob Agents Chemother., 49, 1564-1566.
8. Nagano, N., Nagano, Y., Cordevant, C., Shibata, N. and Arakawa, Y. (2004): Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. J. Clin. Microbiol., 42, 3978-3984.
9. Nagano, N., Shibata, N., Saitou, Y., Nagano, Y. and Arakawa, Y. (2003): Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type beta-lactamase. J. Clin. Microbiol., 41, 5530-5536.
10. Bucaneve, G., Micozzi, A., Menichetti, F., Martino, P., Dionisi, M. S., Martinelli, G., Allione, B., D'Antonio, D., Buelli, M., Nosari, A. M., Cilloni, D., Zuffa, E., Cantaffa, R., Specchia, G., Amadori, S., Fabbiano, F., Deliliers, G. L., Lauria, F., Foa, R. and Del Favero, A. (2005): Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) Infection Program. Levofloxacin to prevent bacterial infection in patients with cancer and neutropenia. N. Engl. J. Med., 353, 977-987.
11. Cullen, M., Steven, N., Billingham, L., Gaunt, C., Hastings, M., Simmonds, P., Stuart, N., Rea, D., Bower, M., Fernando, I., Huddart, R., Gollins, S. and Stanley, A. (2005): Simple Investigation in Neutropenic Individuals of the Frequency of Infection after Chemotherapy +/- Antibiotic in a Number of Tumours (SIGNIFICANT) Trial Group. Antibacterial prophylaxis after chemotherapy for solid tumors and lymphomas. N. Engl. J. Med., 353, 988-998.
12. Cherif, H., Bjorkholm, M., Engervall, P., Johansson, P., Ljungman, P., Hast, R. and Kalin, M. (2004): A prospective, randomized study comparing cefepime and imipenem-cilastatin in the empirical treatment of febrile neutropenia in patients treated for haematological malignancies. Scand. J. Infect. Dis., 36, 593-600.
13. Raad, I. I., Escalante, C., Hachem, R. Y., Hanna, H. A., Husni, R., Afif, C., Boktour, M. R., Whimbey, E. E., Kontoyiannis, D., Jacobson, K., Kantarjian, H., Levett, L. M. and Rolston, K. V. (2003): Treatment of febrile neutropenic patients with cancer who require hospitalization: a prospective randomized study comparing imipenem and cefepime. Cancer, 98, 1039-1047.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2006, p. 6955–6964 Vol. 72, No. 11
0099-2240/06/$08.00+0 doi:10.1128/AEM.00934-06

Genetic Analysis of Bacteriocin 43 of Vancomycin-Resistant *Enterococcus faecium*▽

Daisuke Todokoro,[1,2] Haruyoshi Tomita,[1]* Takako Inoue,[1] and Yasuyoshi Ike[1,3]

Department of Bacteriology and Bacterial Infection Control,[1] Department of Ophthalmology,[2] and Laboratory of Bacterial Drug Resistance,[3] Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Received 20 April 2006/Accepted 9 August 2006

A total of 636 vancomycin-resistant *Enterococcus faecium* (VRE) isolates obtained between 1994 and 1999 from the Medical School Hospital of the University of Michigan were tested for bacteriocin production. Of the 277 (44%) bacteriocinogenic strains, 21 were active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *Listeria monocytogenes*. Of those 21 strains, a representative bacteriocin of strain VRE82, designated bacteriocin 43, was found to be encoded on mobilizable plasmid pDT1 (6.2 kbp). Nine open reading frames (ORFs), ORF1 to ORF9, were presented on pDT1 and were oriented in the same direction. The bacteriocin 43 locus (bac43) consists of the bacteriocin gene *bacA* (ORF1) and the immunity gene *bacB* (ORF2). The deduced *bacA* product is 74 amino acids in length with a putative signal peptide of 30 amino acids at the N terminus. The *bacB* gene encodes a deduced 95-amino-acid protein without a signal sequence. The predicted mature BacA protein (44 amino acids) showed sequence homology with the membrane-active class IIa bacteriocins of lactic acid bacteria and showed 86% homology with bacteriocin 31 from *E. faecalis* YI717 and 98% homology with bacteriocin RC714. Southern analysis with a bac43 probe of each plasmid DNA from the 21 strains showed hybridization to a specific fragment corresponding to the 6.2-kbp EcoRI fragment, suggesting that the strains harbored the pDT1-like plasmid (6.2 kb) which encoded the bacteriocin 43-type bacteriocin. The bac43 determinant was not identified among non-VRE clinical isolates.

Bacteriocins are produced by a wide variety of gram-positive and gram-negative bacteria. They are bacterial proteins which inhibit the growth of other bacteria that are closely related to the producer strains, and they usually exhibit a relatively narrow spectrum of activity. Bacteriocins are thought to provide the producer strain with an ecological or selective advantage over other strains. Bacteriocin production has been described for several genera of lactic acid bacteria (LAB) (12, 33). LAB bacteriocins can be classified into two main classes (34), i.e., class I, modified bacteriocins (the lantibiotics) and class II, the small, heat-stable nonlantibiotics, which are divided into subgroups IIa, pediocin-like bacteriocins with a strong antilisterial effect, and IIb, non-pediocin-like bacteriocins and those with two peptides that require the complementary action of both peptides for full antimicrobial activity. In the genus *Enterococcus*, *Enterococcus faecalis* and *E. faecium* bacteriocins have been genetically and biochemically well characterized. *E. faecalis* bacteriocins include the β-hemolysin/bacteriocin (cytolysin) (9, 17, 18, 21, 23, 24), the peptide antibiotic AS-48 (28), bacteriocin 21 (Bac 21) (43), and Bac 31 (42). These bacteriocins have been identified from *E. faecalis* clinical isolates (29, 42, 43, 47). The well-characterized *E. faecium* bacteriocins have been isolated from food grade organisms (6, 19) and include enterocins A (1), B (3), P (4), I (15), L50A, and L50B (5). These bacteriocins belong to the LAB class II bacteriocins and are active against *Listeria monocytogenes* (34). Enterocins A and P are pediocin-like bacteriocins (12).

* Corresponding author. Mailing address: Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan. Phone: 81-27-220-7992. Fax: 81-27-220-7996. E-mail: tomitaha@med.gunma-u.ac.jp.

▽ Published ahead of print on 21 August 2006.

In contrast to the bacteriocins obtained from *E. faecalis* clinical isolates, there have been few reports describing either the bacteriocins present in *E. faecium* clinical isolates, including vancomycin-resistant *E. faecium* (VRE), or the relationship between the bacteriocin determinant and a plasmid (10, 11, 25).

In our previous study (25), a total of 636 VRE strains were tested for bacteriocin production against various indicator strains. Two hundred seventy-seven (44%) of the 636 strains tested were bacteriocinogenic. The bacteriocinogenic strains were classified into four groups on the basis of their bacteriocin activity. Of the 277 bacteriocin producers tested for activity against enterococci, 21 strains (3.3%) showed bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *L. monocytogenes*, and *L. denitrificans*; 193 strains (69.7%) showed activity against *E. faecium*, *E. hirae*, and *E. durans*; and 4 strains (0.6%) showed activity against *E. faecalis*. The remaining 59 bacteriocinogenic strains produced a small zone of bacteriolysis against *E. hirae*. In this study, we present an analysis of Bac 43, which was active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *L. monocytogenes*, and *L. denitrificans*.

MATERIALS AND METHODS

Bacteria, media, and reagents. The strains and plasmids used in this study are listed in Table 1. A total of 640 VRE clinical isolates were obtained from different patients who had been admitted to the University of Michigan Medical School Hospital, Ann Arbor, between 1994 and 1999. The bacteriocinogenic strains among these isolates were previously classified into three groups on the basis of their bacteriocin activity (25). Of the 636 VRE clinical isolates tested, 21 strains showing bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *L. monocytogenes*, and *L. denitrificans* were used in this study. The indicator strains used for the bacteriocin assay were *Staphylococcus aureus* FDA209P (32), *E. faecalis* FA2-2 (8) and OG1S (7), *E. faecium* BM4105RF (44), *E. hirae* ATCC 9790 (38), *E. durans* ATCC 49135, *E. raffinosus* JCM8733, *E.*

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmid	Genotype or phenotype	Description	Reference or source
Strains			
Enterococcus faecalis			
FA2-2	Rif^r Fus^r	Derivative of JH2	8
JH2SS	Str^r Spc^r	Derivative of JH2	41
OG1-10 (OG1S)	Str^r	Derivative of OG1	7
Enterococcus faecium			
BM4105RF	Rif^r Fus^r	Derivative of plasmid-free *E. faecium* BM4105	44
BM4105SS	Str^r Spc^r	Derivative of plasmid-free *E. faecium* BM4105	44
VRE82	pDT1(Bac 43) Kam^r Gen^r Tei^r Van^r Amp^r	Bacteriocinogenic clinical isolate	44, this study
Enterococcus hirae ATCC 9790	Penicillin susceptible	Wild type	38
Enterococcus durans ATCC 49135	Penicillin susceptible	Wild type	
Enterococcus raffinosus JCM8733	Penicillin susceptible	Wild type	
Enterococcus gallinarum BM4174	Penicillin susceptible	Wild type	27
Staphylococcus aureus FDA209P	Penicillin susceptible	Wild type	32
Escherichia coli			
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44* ϕ80 *lacZ*Δ M15		Bethesda Research Laboratories
TH688	CSH57b *thr*::Tn*5*		40
Plasmids			
pDT1	Bac 43	Mobilizable plasmid (6.2 kb)	This study
pAM401	Cm^r Tc^r	*E. coli-E. faecalis* shuttle vector	46
pHTβ	Van^r	pMG1-like highly conjugative plasmid (53.7 kb)	45
pUC18	Amp^r *lacZ*	*E. coli* vector	Nippon Gene Co.

gallinarum BM4174 (27), *S. agalactiae*, *S. pyogenes*, and *L. monocytogenes*. *Enterococcus* strains were grown in Todd-Hewitt broth (THB; Difco, Detroit, Mich.) or antibiotic medium 3 (Difco). *Escherichia coli* strains were grown in Luria-Bertani medium. Solid and soft media were prepared by the addition of 1.5% or 0.75% (wt/vol) agar, respectively. All cultures were grown at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml for *Enterococcus* and 50 μg/ml for *E. coli*; vancomycin, 12.5 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 250 μg/ml; gentamicin, 250 μl/ml; spectinomycin, 250 μg/ml; kanamycin, 500 μg/ml for *Enterococcus* and 40 μg/ml for *E. coli*; tetracycline, 12.5 μg/ml.

Soft-agar assay for bacteriocin production and immunity. The bacteriocin production assay was performed as described previously (24). Fifty microliters of an overnight culture of the indicator strains grown in antibiotic medium 3 was added to 5 ml of molten soft agar (0.75%), which was then poured onto a THB plate. After solidification, each strain to be tested was inoculated into the soft agar with a toothpick. The halos of inhibition around the inoculated test strains were monitored after overnight culture.

To test immunity to the bacteriocin, a modification of the bacteriocin production test was performed. The indicator strain was used to test immunity. Inhibition of halo formation means that the indicator strain has immunity against the bacteriocin produced by the inoculated strain.

Mating procedures. Solid-surface matings were performed as previously described (44). Overnight cultures of the donor and the recipient were mixed at a donor/recipient ratio of 1:10, and 10 μl of the mixed culture was dripped onto THB agar without drug. The plates were then incubated overnight (18 h) at 37°C. After incubation, the bacteria grown on the agar plates were scraped off and transferred into 1 ml of fresh broth and then 0.1-ml samples of the suspension were inoculated onto the appropriate selective agar plates. Colonies were counted after 48 h of incubation at 37°C.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method (36). Plasmid DNA was treated with restriction enzymes and subjected to agarose gel electrophoresis for analysis of DNA fragments. Restriction enzymes were obtained from Nippon Gene (Toyama, Japan); New England Biolabs, Inc. (Massachusetts); and Takara (Tokyo, Japan) and used in accordance with the suppliers' specifications. Agarose was obtained from Wako Chemicals (Osaka, Japan) and used at a 0.8% agarose concentration in agarose gel electrophoresis. DNA fragments were eluted from agarose gels with the Wizard SV Gel and the PCR Clean-Up System (Promega Corporation, Madison, WI). The eluted fragments were ligated to dephosphorylated, restriction enzyme-digested vector DNA with the DNA Ligation Kit Ver.2 (Takara, Tokyo, Japan) and then introduced into *E. coli* by electrotransformation (16). Transformants were selected on Luria-Bertani medium agar containing suitable antibiotics.

PCR methodology. The PCR program, with an Ex *Taq* DNA polymerase (Takara), comprised 2 min at 95°C followed by 30 cycles of 2 min at 95°C, 2 min at 56°C, and 2 min at 72°C and then a final incubation at 4°C with a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer).

Determination of pDT1 restriction map. The restriction map of pDT1 was constructed by double digestion and analysis by agarose gel electrophoresis. The restriction enzymes EcoRI, HindIII, EcoRV, XbaI, and BamHI were used for digestion.

Generation of transposon insertional mutants. Tn*5* (Km^r) insertion into pDT1 was performed as described elsewhere (39, 42). pDT1 was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) (40) by electrotransformation. Ten of the transformants were selected and spread onto selective medium containing 40 μg of kanamycin and 12.5 μg of tetracycline per ml, and the plates were left at room temperature for 10 days. The bacteria which grew on the selective plates were scraped off, and the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin (40 μg/ml) and tetracycline (12.5 μg/ml) for selection of Tn*5* kanamycin resistance and pTD1-borne tetracycline resistance, respectively. The transformants were purified and examined to determine the specific location of Tn*5* within the plasmid. The precise location of the Tn*5* insertion was determined by direct nucleotide sequencing with a synthetic primer shown in Table 2, which hybridized to the end of Tn*5* (43).

DNA sequence analysis. Nucleotide sequence analysis was carried out as previously described (37). To determine the entire sequence of pDT1, shotgun sequencing was performed. Fragmented DNA libraries were constructed by sonication of EcoRI-digested pDT1, followed by ligation into the SmaI-digested pUC18 vector plasmid. pUC18 plasmids containing 0.5- to 1.0-kb inserts were used to transform *E. coli* DH5α. The resulting constructs were sequenced in both orientations with an ABI Prism 377 sequencer (Applied Biosystems). The BigDye Terminator Ver.1.1 cycle sequencing kit (Applied Biosystems) and primers 21M13 and M13Rev (Perkin-Elmer) were used for the sequencing reaction.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence and restriction sites[a]	Description
Bac43-1	GAATTCAAAACTACTTTTTATGACG	Analysis of *bac* determinant
Bac43-2	GAATTCTAGGAACTTGTCTAGCTGG	
Bac43-5′	GAATTCTATGATAATTTTTCGGCTC	
Bac43-4′	GAATTCGATAGTCATCTATAGTTGC	
Bac43-6′	GAATTCAAGCCCATCCTCTATATAC	
Tn5	CAGATTTAGCCCAGTCGG	Analysis of Tn*5* insetion mutant
J1	GAGTATTGCAACTTGCTCGC	Analysis of EcoRI junction of pTD1
J2	GCTACAAGAAGTGGTTCGGC	
C	TTGGTACAGGCGTTACTTGG	Analysis of *bacA* gene
E2	ATCCGAATTCATAACCTCCCTACCACTACC	
H1	CGAAAAGGAAAAACAATCATG	Analysis of bac43 determinants
H2	TCCCATTTTCATTTTATTCC	
M1	AAGGGTGGGACTTATGAGCG	Analysis of *mob* genes
M2	TTGTTGGTAGTCTGCTCCTC	

[a] Underlined letters indicate restriction sites (GAATTC; EcoRI).

Open reading frame (ORF) analysis was performed with Genetyx, version 6.1 (Genetyx Corp., Tokyo, Japan). The DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan) was used for homology analysis of nucleotide and amino acid sequences.

Direct sequencing was performed to confirm the sequence near the EcoRI junction of pDT1 and the structures of the insertion and deletion mutants. The PCR products were eluted from agarose gels as described above and sequenced in both orientations with an ABI Prism 310 sequencer (Applied Biosystems). The BigDye Terminator Ver.1.1 cycle sequencing kit (Applied Biosystems) was used for the sequencing reaction with PCR primers (Table 2).

Deletion mutant analysis. The deletion mutants shown in Fig. 3 were constructed by PCR with pDT1 as the template. The primers used to construct each subclone are listed in Table 2. Subclones of pMG502, pMG503, pMG504, and pMG505 were constructed with primer pairs Bac43-1 and -5′, Bac43-1 and -4′, Bac43-2 and -5′, and Bac43-2 and -6′, respectively. PCR products were digested with EcoRI and cloned into shuttle vector pAM401. Each subclone was introduced into *E. faecalis* FA2-2 and tested in the soft-agar assay. The sequences of all subclones were confirmed by direct DNA sequencing.

Southern hybridization. Southern hybridization was performed with the digoxigenin (DIG)-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols (36). Hybridization was performed overnight at 42°C in the presence of 50% formamide. The PCR product generated between primers C and E2 with the PCR DIG synthesis kit (Roche Diagnostics, Mannheim, Germany) was used as the probe for *bacA*. The nucleotide sequences of the primer pair are shown in Table 2. Signals were detected with the DIG chemiluminescence detection kit (Boehringer GmbH). CSPD (Boehringer GmbH) was used as a substrate for alkali phosphatase conjugated to the anti-DIG antibody.

Pulsed-field gel electrophoresis. Genomic DNA was prepared as previously described (31). A gel block containing genomic DNA was incubated overnight with 10 U of SmaI. Electrophoresis was then carried out with a 1% agarose gel with 0.5% Tris-borate-EDTA, and the settings applied were 1 to 21 s, 6 V/cm^2, and 20 h. The gel was stained with ethidium bromide for UV observation. The results were classified as closely related, possibly related, or different types (31).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article are available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB178871.

RESULTS

Identification of a plasmid-coded bacteriocin active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes*. Of the 277 (44%) bacteriocinogenic strains identified among a total of 636 VRE strains, 21 bacteriocinogenic strains that are active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes* (3.3%) were used in this study. The drug resistance patterns of the 21 strains are shown in Table 3. The enterococcal bacteriocins are usually carried on plasmids, some of which are self-transferable and some of which can be mobilized by coresident conjugative plasmids. To examine whether bacteriocin production was cotransferred with drug resistance, mating experiments were performed between each of the 21 bacteriocinogenic strains and recipient strain *E. faecium* BM4105RF on a solid surface (filter mating). Vancomycin or gentamicin was used as a selective marker for transconjugants (Table 3), and rifampin and fusidic acid were used for counterselection against the donor strain. Vancomycin- or gentamicin-resistant transconjugants were obtained at frequencies of 10^{-5} to 10^{-8} per donor cell with 12 of the 21 strains (Table 3). Bacteriocin activities were examined in the transconjugants from each of these strains. The drug resistance transconjugants exhibited bacteriocin activities at a relatively high frequency (Table 3). The bacteriocin activities were identical to that of the donor strain. Of these bacteriocinogenic strains, VRE82 was chosen as a representative for further analysis. With the VRE and bacteriocinogenic *E. faecium* BM4105RF transconjugant of VRE82 as the initial donor, repeated experiments to transfer bacteriocin production were performed between *E. faecium* BM4105RF and *E. faecium* BM4105SS. Vancomycin resistance was used as a selective marker for the transconjugants. Vancomycin-resistant transconjugants were tested for bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes*. About 80 to 95% of the transconjugants were bacteriocinogenic and showed bacteriocin activity identical to that of donor strain VRE82, and the remaining transconjugants showed no bacteriocin activity. Plasmid DNA was isolated from each of the transconjugants, treated with the EcoRI restriction enzyme, and analyzed by agarose gel electrophoresis. All of the nonbacteriocinogenic vancomycin-resistant transconjugants exhibited two major bands, and all of the bacteriocinogenic and vancomycin-resistant transconjugants exhibited an additional DNA band with a molecular size of 6.2 kbp in their agarose gel electrophoresis profiles (Fig. 1).

The 6.2-kb EcoRI fragment obtained by agarose gel electrophoresis was eluted from the agarose gel and ligated with shuttle vector pAM401. The cloned 6.2-kbp EcoRI fragment was introduced into *E. coli* DH5α, and the clone pAM401::6.2-