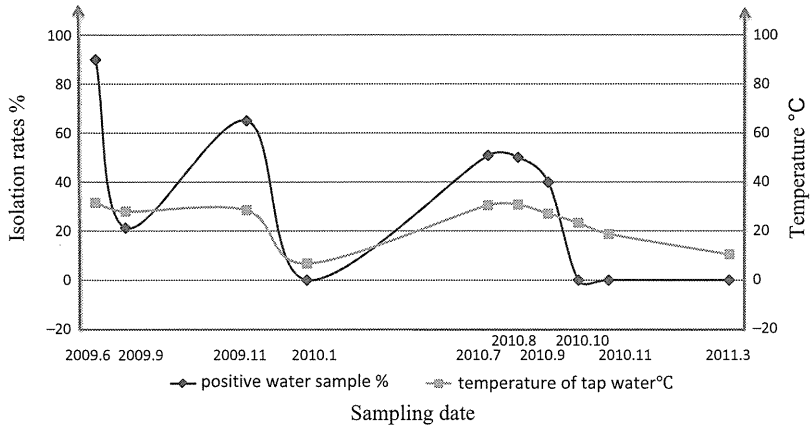

FIG. 2. Relationship between contamination rate of tap water and temperature (*Legionella* spp.).

TABLE 2. Growth of different pathogens and HPC bacteria in unfiltered, pre-filtered and POU-filtered water samples

			Mean concentration of organisms in positive samples (CFU/L)			
	Total No. of samples	**No. of positive samples (%)**	**HPC bacteria**	**Legionella**	**Mycobacterium**	**Filamentous fungi**
Unfiltered water	57	57 (100)	1.2×10^8	1.0×10^3	4.0×10^2	16.5
Pre-filtered water	43	43 (100)	3.7×10^8	1.8×10^3	7.0×10^2	12.7
Filtered water	90	34 (37.7)	3.4×10^4	0	0	0
Filtered water after 3-day interval	34	1 (2.9)	0	0	0	0
Filtered water after 7-day interval	19	8 (42.1)	1.3×10^4	0	0	0
Filtered water after 10-day interval	21	7 (33.3)	5.4×10^4	0	0	0
Filtered water after 14-day interval	26	18 (69.2)	2.6×10^4	0	0	0

LTU. During the interventional control strategy using POU water filter, we found that POU filters completely eliminated these waterborne pathogens from the water supply over 14 days of use. Furthermore, the rate of Gram-negative bacterium infection/colonization patients per 1000 patient-days of hospitalization in the post-filtration period (1.70 ± 0.95) decreased significantly compared with the pre-filtration period (3.20 ± 1.25; $\chi^2 = 2.119$, $p = 0.067$), a 47% reduction! Our study demonstrated that POU water filters provided a barrier against various waterborne pathogens that can further reduce the rate of nosocomial infections. However, the retrograde contaminations may occur by either splash water from the water basin during use or by direct contact with contaminated hands and dirty clothes of staff or patients [14,15]. In our surveillance, HPC bacteria were recovered from the filtered water after 1 week of use. The source of the HPC bacteria from filtered water remains unclear, and molecular typing may be useful to track the dissemination.

Due to the poor quality of the supplied tap water in the LTU, we installed pre-filtration fixtures on three taps upstream of POU filters in order to remove particulate debris. Before the POU filter study, laboratory and field tests were conducted for evaluating the performance of pre-filtration of various materials and styles and removal ratings, and 1.2-μm pore size was chosen for the pre-filtration fixture (data not shown). The pre-filtration fixture would not prohibit the waterborne pathogens from tap water. However, the concentration of various pathogens after pre-filtration was surprisingly higher than that in unfiltered water in some samples. The reason may be the growth of pathogens within the pre-filtration media because of higher nutrient content from the trapped debris in the water.

Although only 27% of distal sites (3/11) were equipped with POU filters, the incidence of Gram-negative bacterium colonization/infection decreased significantly, possibly because of the use of filtered tap water for perineal washing of patients, the bed environment and the hands of nursing personnel. In this study, it remains unclear whether such filters contribute to the reduction of non-tuberculosis mycobacterium and filamentous fungi infections in high-risk patients. Therefore, more research is needed to evaluate the efficacy and cost-effectiveness of POU filters in preventing specific colonization/infection of hospitalized patients. Many studies have focused primarily upon recognized outbreaks of *Legionella* spp. and *P. aeruginosa* [16,17]. However, other opportunistic waterborne pathogens may also cause nosocomial infections, outbreaks or sporadic infections [18,19]. There is controversy over whether it is economical to invest medical resources in preventing opportunistic waterborne pathogen-associated nosocomial infections, especially because the disposable POU water filters have a limited effective life and could be very expensive. We propose a modest approach whereby removal of waterborne

pathogens is targeted towards areas of highest risk of nosocomial infections, such as our transplant unit. In such settings the costs are likely to be justifiable. We should remove waterborne pathogens from transplantation units because these patients are at the highest risk of nosocomial. If an organ transplant patient dies from nosocomial infection, a valuable organ is also being destroyed. Given such a high potential cost associated with nosocomial infections in transplant patients, POU water filters may be a viable economical option [20,21]. Instead of treating the entire hospital water supply with systematic chemical disinfection (e.g. chlorination), POU filters can be easily installed at a few sites for prevention of infection [22–24]. Furthermore, based on our data, we suggest installing POU water filters only in the hot season (June to October) in countries with limited medical resources.

In conclusion, hospital water supplies were highly contaminated by various waterborne pathogens. Using POU filters appeared to be one of the most simple and cost-effective methods to reduce the risk of waterborne pathogen-associated infections in hospitals.

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Transparency Declaration

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ORIGINAL ARTICLE

Comparison study of single and concurrent administrations of carbapenem, new quinolone, and macrolide against in vitro nontypeable *Haemophilus influenzae* mature biofilms

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Abstract Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen and a common cause of otitis media in children, chronic bronchitis, and pneumonia in patients with chronic obstructive pulmonary disease. Many studies have reported that NTHi is capable of producing biofilms, which may be one of the important factors involved in chronic diseases and accelerating antimicrobial resistance. Unfortunately, there is still no consensus about the elimination of biofilms. In this study, concurrent administrations of levofloxacin (LVFX)-imipenem (IPM) and clarithromycin (CAM)-IPM, as well as the single administration of IPM, LVFX, and CAM, were performed to treat the mature biofilms produced by NTHi, respectively. Biofilm inhibition was quantified using microtiter biofilm assay (MBA), and relative biomass was calculated as the ratio compared to that of untreated control biofilms. The relative biomasses of biofilms treated with IPM, LVFX-IPM, and CAM-IPM against a β-lactamase-negative ampicillin-resistant strain was 1.10, 0.08, and 0.13 at 1× minimum inhibitory concentration (MIC), 0.90, 0.05, and 0.07 at 10× MIC, and 0.80, 0.06, and 0.07 at 100× MIC, respectively. Biofilms were also visually observed by scanning electron microscopy, and a focused ion-beam system showed that high concentrations of combined administration strongly inhibited the biofilms, which was consistent with the results of MBA. Our data demonstrated the antibiofilm effect of concurrent administration against mature NTHi biofilms, which indicated a rationale for the potential use of concurrent administrations in diseases involving chronic NTHi biofilms.

Keywords Biofilm · BLNAR · Concurrent administration · *Haemophilus influenzae*

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Introduction

Haemophilus influenzae is a fastidious gram-negative and pleomorphic bacterium that initially colonizes the human nasopharynx. Nontypeable *H. influenzae* (NTHi) is reported to cause otitis media, sinusitis in children, and is associated with community-acquired pneumonia (CAP) and acute exacerbations of chronic bronchitis [1]. Ampicillin has long been used as the drug of first choice for the treatment of infections caused by *H. influenzae*. Although TEM-1 and ROB-1-type β-lactamase-positive ampicillin-resistant (BLPAR) strains are responsible for almost all isolates with decreased susceptibility to ampicillin, β-lactamase-nonproducing ampicillin-resistant (BLNAR) strains have also increased in some countries recently [2–5]. Therefore, otitis media, paranasal sinusitis, and lower respiratory tract infection caused by *H. influenzae* have become more and more difficult to be cured with oral antibiotics therapy [6].

Also, it has been reported that NTHi produces biofilms that are associated with a variety of diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and middle ear infections [7, 8]. As is well

known, biofilms are defined as communities of microorganisms that attach to a surface and are enveloped in a hydrated polymeric matrix of their own synthesis [9, 10], and bacteria within biofilms are more resistant to antibiotics than in the planktonic state [11]. Although many studies have discussed biofilm treatment with single or concurrent antibiotics, such as quinolones, macrolides, aminoglycoside, penicillin, and cephems [12–16], there is still no consensus about the treatment of biofilm diseases. Here we evaluated the combinatory efficacy of a carbapenem with a new quinolone and a macrolide against NTHi biofilms compared to each single administration.

Methods

Bacteria strains and culture conditions

Two *H. influenzae* strains were isolated from different Japanese patients with respiratory tract infections. These strains were reconstituted from frozen stocks and propagated on chocolate agar or brain heart infusion (Becton-Dickinson, USA) supplemented with 10 μg/ml hemin (Sigma, USA) and β-nicotinamide adenine nucleotide (β-NAD) (Sigma) (sBHI) 5 % CO_2 at 37 °C [17], and β-lactamase production was detected by means of a disc impregnated with nitrocefin (Becton–Dickinson, USA). Serotyping was performed by slide agglutination with antisera purchased from Difco Laboratories (Detroit, MI, USA).

Antimicrobial susceptibility test

Minimum inhibitory concentrations (MICs) were determined using the broth dilution method according to guidelines from the Clinical and Laboratory Standards Institute [18]. NTHi strains were tested against four antibiotics: ampicillin (ABPC) (Wako, Tokyo, Japan); imipenem (IPM) [C_{max}, 40.1 (μg/ml; healthy adult, 0.5 g, intravenous drip, 30 min) (Merck, USA); clarithromycin (CAM) [C_{max}, 1.16 (μg/ml; healthy adult, 200 mg, oral administration (p.o.) (Abbot Japan, Japan)]; levofloxacin (LVFX) [C_{max}, 2.04 ± 0.21 (μg/ml; healthy adult, 200 mg, p.o.) (Daiichi Sankyo, Japan)].

Genetic identification of antimicrobial resistance-related genes

Polymerase chain reaction (PCR) was performed to identify antimicrobial resistance-related genes by using mixed primers following the manufacturer's manual (Wakunaga Pharmaceutical, Hiroshima, Japan), as described previously [19, 20]. Briefly, we used P6 primers to amplify the P6 gene that encodes the P6 membrane protein specific for *H. influenzae* (198 bp); TEM-I primers to amplify a part of the bla_{TEM-1} gene (458 bp); PBP3-S primers to identify an Asn526 → Lys amino acid substitution in the *ftsI* gene (551 bp); and PBP3-BLN primers to identify an Asn526 → Lys and Ser385 → Thr amino acid substitution in the *ftsI* gene (465 bp).

Quantification of biofilms

NTHi biofilms were investigated by a modified microtiter biofilm assay (MBA) [21]. Bacteria cells were suspended in sBHI approximately at 0.1 OD_{490}, and a 200-μl aliquot was inoculated into a well of a 96-well microplate. Biofilm formation was confirmed after 48 h culture, medium was changed, and then 200 μl fresh sBHI with one of the following antibiotics (IPM, CAM, LVFX, or combined administration of LVFX-IPM and CAM-IPM) was inoculated into each tested well with a dose-dependent MIC concentration (0.01× MIC, 0.1× MIC, 1× MIC, 10× MIC, 100× MIC). After 2 h exposure to the antibiotics, all tested wells were washed and refilled with 200 μl sBHI for continuous culture. Antibiotics administration was repeated every 12 h for a total of four times. After the last antimicrobial exposure, biofilms were stained with 1 % freshly adjusted crystal violet (Merck, USA) at room temperature for 15 min and vigorously washed with water three times. After extraction with 230 μl 95 % ethanol, biofilms were measured at OD_{595} with a microplate reader. Additionally, intact biofilms (without any antibiotics exposure) were measured as the baseline data. All studies were tested in triplicate, and the average ± SD of each experiment was calculated.

Preparations and conditions of scanning electron microscopy

The KL-1 strain was suspended in sBHI at approximately 0.1 OD_{490}. A 1-ml aliquot of bacteria solution was inoculated into a flat bottle with glass coverslips inside and statically cultured for 48 h at 37 °C with 5 % CO_2. After biofilm formation was confirmed, 1 ml fresh sBHI containing one of the following antibiotics (IPM, CAM, LVFX, combined administration of LVFX-IPM and CAM-IPM) was inoculated into each tested bottle with a dose-dependent MIC concentration (0.1× MIC, 1× MIC, 10× MIC). After 2 h exposure to the antibiotics, all tested bottles were washed and refilled with 1 ml sBHI for continuous culture. Antibiotics administration was repeated every 12 h for a total four times.

The glass coverslips were taken from the bottles and incubated in 2.5 % glutaraldehyde for 1 h at room temperature. After washing with 7.5 % sucrose (1 h), fixation

was performed by incubation in 1 % OsO_4 (2 % OsO_4 1:1 7.5 % sucrose) for 1 h (4 °C). Specimens were continuously dehydrated by critical-point drying, which the water in the cells is replaced with graded ethanol from 50 % to 100 %, with use of 100 % *t*-butyl alcohol for freeze drying. Samples were coated with gold in an ion-sputter coater [21, 22]. The specimens were observed with a scanning electron microscopy (SEM) (S-800; Hitachi, Tokyo, Japan), as well as a focused ion-beam system (FIB) (Quanta 3D; FEI, USA) [23].

Statistical evaluation

Data were analyzed using the Student's paired *t* test. A *P* value < 0.05 was considered statistically significant.

Results

Characteristics of the strains

According to the characteristics of resistant genes, KL-1 was confirmed as BLNAR, and KL-2 was a beta-lactamase-nonproducing ampicillin-susceptible (BLNAS) strain: these were verified as NTHi strains and without β-lactamase production. The MICs values (μg/ml) for ABPC, IPM, LVFX, and CAM against KL-1 were 16, 0.5, 0.032, and 8, and those against KL-2 were 0.25, 1, 0.032, and 8, respectively.

Effect of single and combination treatments against biofilms

Biofilms were treated with IPM, LVFX, and CAM, as well as the concurrent administration of LVFX-IPM and CAM-IPM, respectively. Biofilm formation was measured by MBA as previously mentioned in the Methods, and the biofilm inhibition effect was defined as the ratio of antibiotics-treated biofilm to untreated biofilm. When effects of single-agent administrations against the KL-1 biofilms were investigated, LVFX and CAM showed a significant biofilm inhibition effect compared to IPM from 10× MIC administration. For KL-2, single-agent administration of IPM, LVFX, and CAM showed high biofilm inhibition effect from 10× MIC, respectively. IPM administration at 100× MIC seems to be more significant to inhibit the biofilm formation compared to LVFX and CAM ($P = 0.001$ and $P = 0.002$). On the other hand, administration of IPM, LVFX-IPM, and CAM-IPM against KL-1 biofilms showed a high biofilm inhibition effect with results of 1.10, 0.08, and 0.13 at 1× MIC, 0.90, 0.05, and 0.07 at 10× MIC, and 0.80, 0.06, and 0.07 at 100× MIC, respectively. IPM-LVFX showed significant biofilm inhibition effect against biofilms produced by both stains from 1× MIC (Fig. 1a, b). CAM-LVFX also obtained a similar effect against KL-1 biofilms (Fig. 1c); however, there was no difference among single-agent administration of CAM and IPM and combined administration of CAM-IPM against KL-2 biofilms at 1× MIC (Fig. 1d).

Architectures of biofilms

Biofilms of KL-1 were treated with different antibiotics and visually observed by SEM. A large of amount of biofilm materials was evident after exposure to the single-agent administration of IPM, LVFX, and CAM at 0.1× and 1× MIC, as well as the combined administrations of LVFX-IPM and CAM-IPM at 0.1 MIC (Fig. 2). Strands of fibrin with many fewer bacteria cells were obviously present in the biofilms exposed to the combined-agent administration from 1× MIC (Fig. 2i, j, n, o). Fewer bacteria cells were seen in a fibrous matrix of biofilms treated with IPM or LVFX compared to CAM at 10× MIC (Fig. 2k–m).

Biofilms treated with IPM and LVFX-IPM at 1× MIC were also observed by the FIB/SEM system (Fig. 3). The dense matrix contributing to the outer shapes of biofilms were observed before milling the biofilms (Fig. 3a), and after biofilms were sliced at the same site, the strands of materials from interconnected cells were detected inside the biofilms (Fig. 3b). Higher magnification showed filamentous material links bacteria cells to each other (Fig. 3c). Clusters of bacteria cells associated with the flocculent materials on the surface were observed at low magnification (Fig. 3d). At higher magnification, biofilms were seen to be composed of large numbers of cells intimately associated with a fibrous material resembling fibrin, and cells of different sizes can be seen (Fig. 3e).

Discussion

Biofilms have become the major concern for clinicians in the treatment of infectious diseases. It was reported that bacteria in a biofilm can survive antibiotic concentrations up to 1,000 fold higher than the same bacteria in a planktonic state [24]. Some studies reported that azithromycin (AZM) administered alone had no effect when *Pseudomonas aeruginosa* biofilms became established, and either CAM or LVFX alone had no statistical effect in a biofilm-associated murine model [13, 25], but the use of subinhibitory concentrations of AZM seemed to significantly decrease biomass and maximal thickness in both forming and established NTHi biofilms [26]. These results indicated the efficacy of single antibiotic treatment for biofilms is controversial. IPM became the first carbapenem

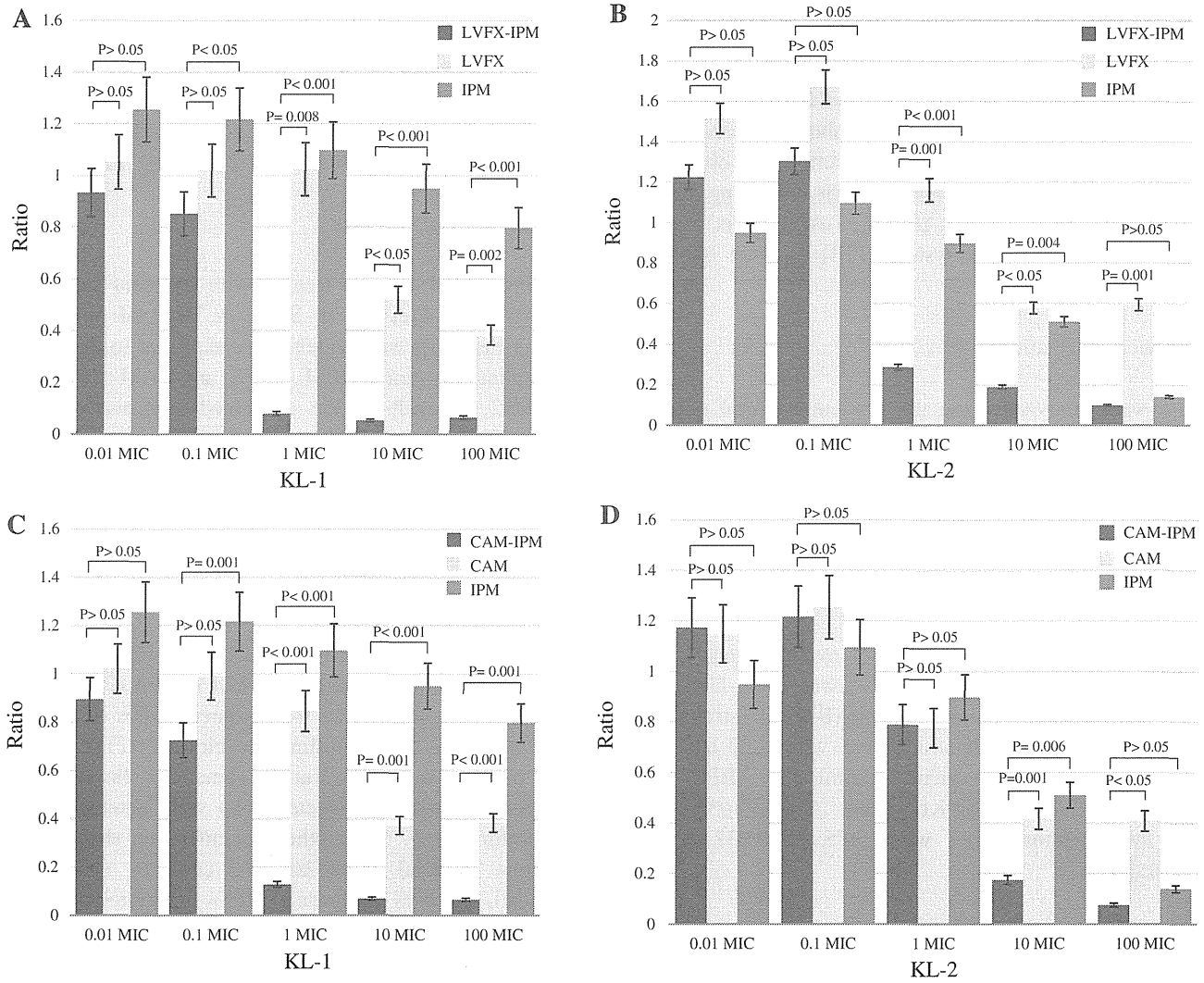

Fig. 1 Effects of single-agent and combined-agents administration against biofilms. Biofilm formation was measured by microtiter biofilm assay (MBA), as previously mentioned in Methods, and the biofilm inhibition effect was defined as the ratio of antibiotics-treated biofilm to untreated biofilm. All studies were tested in triplicate and the average ± SD of each experiment was calculated

with an extremely broad spectrum of activity that is available for the treatment of complex microbial infections [27], which was thought to be a candidate for eliminating biofilms. In this study, only BLNAS biofilms were effectively inhibited at concentrations higher than 10× MIC after the single administration of CAM, LVFX, or IPM, which indicated that clinically attainable high tissue concentrations or blood concentrations are necessary to clear bacteria inside biofilms. Also, these findings indicated that carbapenem may penetrate an established biofilm well, but the decreased antibiofilm effect may be induced by the alternate PBPs in the BLNAR strain or the increased expression of efflux pumps.

Many studies have reported the combined administrations were more effective against biofilm formation than a single antibiotic by decreasing the volume and height of biofilms. The antimicrobial effects of the combined administration of macrolide-quinolone, macrolide-cephem, and macrolides-aminoglycoside were well demonstrated [12, 13, 15]. Our results from drug intervention showed that LVFX-IPM at concentrations higher than 1× MIC were capable of inhibiting both established BLNAR and BLNAS NTHi biofilms. The combined use of CAM-IPM seemed to effectively inhibit the BLNAR (from 1× MIC) and BLNAS biofilms (from 10× MIC), respectively. These findings indicated the synergic therapeutic effect of quinolone-carbapenem and macrolide-carbapenem at clinically attainable concentrations. There is still limited information about the mechanisms of combined administration in eliminating biofilms. Previous studies presented several

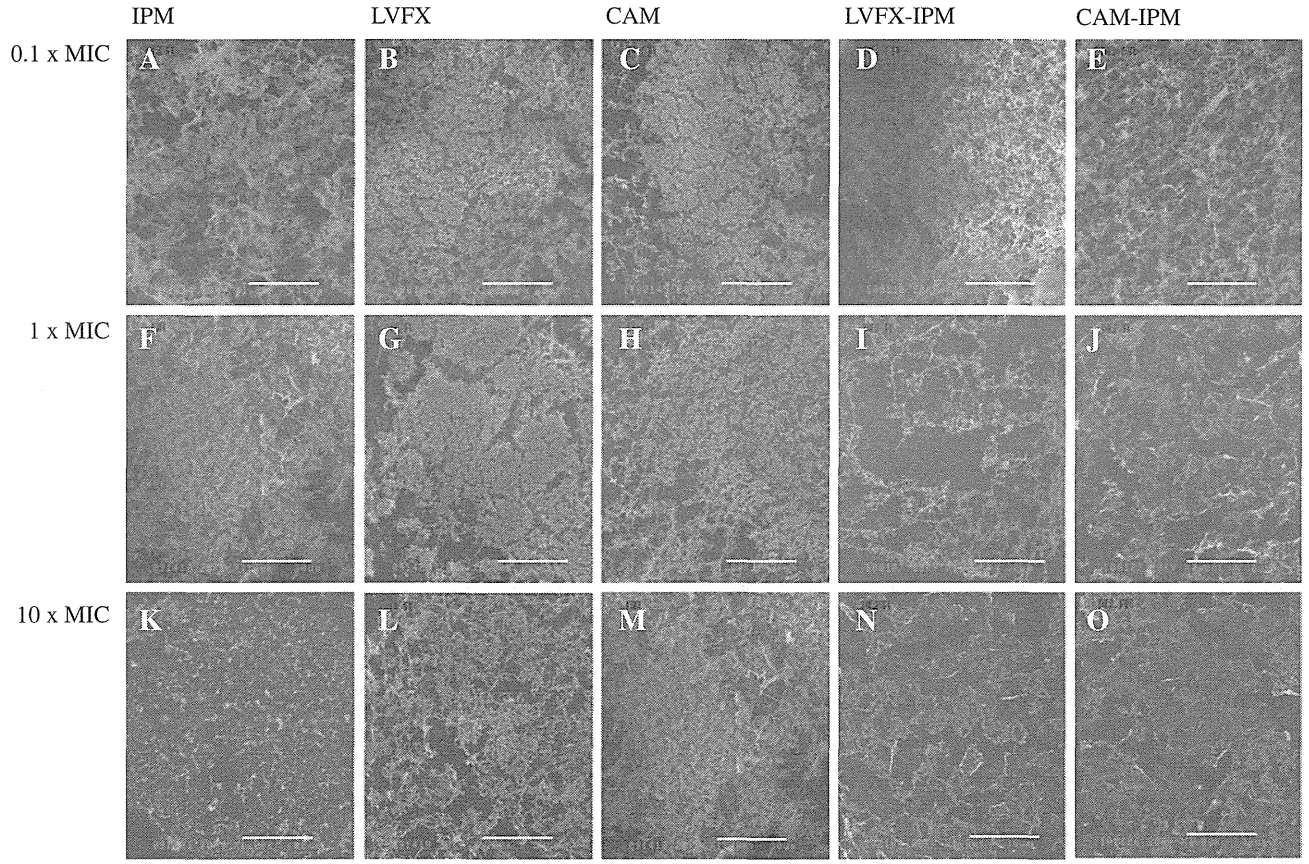

Fig. 2 Antibiotics-treated biofilms observed by scanning electron microscopy (SEM). Biofilms produced by KL-1 were exposed to the different antibiotics at 0.1× minimal inhibitory concentration (MIC) (**a–e**), 1× MIC (**f–j**), and 10× MIC (**k–o**), respectively. Magnification ×1,000, *Bars* 30 μm

hypotheses that the synergy of CAM and LVFX may originate from the activity of CAM in removing the polysaccharide glycocalyx in or on bacterial biofilms in a murine model [13], fluoroquinolones may penetrate exopolysaccharides [16, 28], and macrolides may have a subinhibitory effect on biofilm formation and survival of cells in biofilms [29, 30]. All these hypotheses may not be sufficient to demonstrate the mechanisms, and future study should focus on this aspect.

Biofilms were also observed by a newly developed FIB/SEM system, a focused-ion beam combined with a scanning electron microscope (Quanta 3D), which brings transmission electron microscope sample preparation or structural modification of sample surfaces at the nanometer scale. The FIB/SEM system is thought to be with a versatile high resolution and low vacuum for 2D and 3D material characterization and analysis, which has already been utilized in many studies [23, 31, 32]. Understanding of the inside architecture of biofilms, including bacteria cells and fibrous structure construction, seems to be important in eliminating biofilms. SEM is usually used to observe the external form of biofilms but is not suitable for exploring the inside of biofilms. The FIB/SEM system provided a novel method, and a field emission electron source delivered clear and sharp 3D electron images of the inner and outer morphology of biofilms in this study. To our knowledge, this is the first use of an FIB/SEM in the observation of biofilms. Our results suggested that FIB/SEM, as a powerful tool, may extend the applications range for 3D characterization and nanoanalysis in future biofilm studies.

There are still several limitations in our study, such as a relatively small number of strains, and our in vitro model is a relative simple and static one in comparison with some other reported methods [16]. Several studies that have demonstrated the effect of combined administration against biofilms in vivo [13, 14, 33] provide good examples for us to extend our study in the next step. In conclusion, our study demonstrated that the combined use of antibiotics, compared with the single-agent administration, seemed to be more effective in eliminating established NTHi biofilms. Our data provide a rationale for the potential use of concurrent administration in diseases involving chronic NTHi biofilms, such as COPD, otitis media, and early infections in CF.

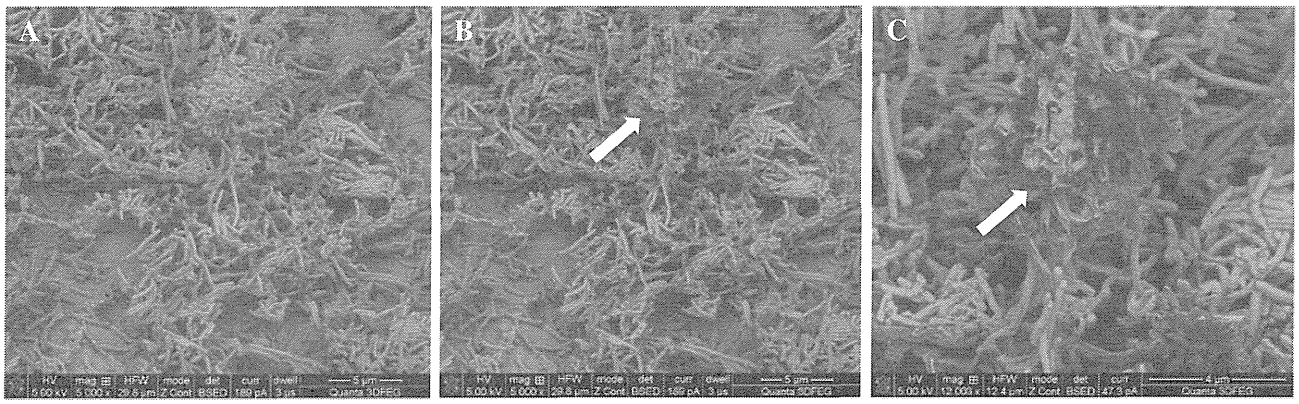

Fig. 3 Biofilms observed by focused ion-beam (FIB/SEM) system after exposure to the different antibiotics. *Upper panel* biofilms exposed to IPM at 1× MIC (**a**–**c**); *lower panel* biofilms exposed to the combined administration of IPM-LVFX at 1× MIC (**d**, **e**). *Arrows* indicate milled sites of biofilms with filamentous material linkage between cells

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Infection control for a methicillin-resistant *Staphylococcus aureus* outbreak in an advanced emergency medical service center, as monitored by molecular analysis

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Abstract A methicillin-resistant *Staphylococcus aureus* (MRSA) outbreak occurred in an advanced emergency medical service center between 2010 and 2011. Our objective was to evaluate the status of the MRSA outbreak, as monitored by molecular analysis. Twenty-eight MRSA strains were isolated from blood samples from 11 patients, from other specimens (pharynx, nasal cavity, etc.) from 12 patients, from two environmental samples, and from the skin, middle nasal meatus, and urine of one patient each from other wards. Pulsed-field gel electrophoresis (PFGE) was performed to evaluate horizontal transmission. Molecular typing by PFGE showed that the 28 MRSA strains presented 7 patterns in total, and that 11 of the MRSA strains had the same PGFE pattern. Unselective use of intranasal mupirocin ointment, MRSA monitoring for new inpatients, and prevention of direct or indirect contact infection were performed. However, the number of inpatients with MRSA did not quickly decrease, and additional molecular typing by PFGE showed that 10 of 19 MRSA strains found (5 of 6 from blood, 5 of 13 from other specimens) were the same as those found previously. Lectures and ward rounds were performed repeatedly, and staff participation in ward rounds was suggested. Finally, the number of inpatients with MRSA significantly decreased more than 6 months after the intervention. Although the MRSA outbreak was thought to have ended, follow-up molecular typing by PFGE showed that horizontal transmission persisted. Our data suggest that various combinations of infection control measures are essential when dealing with an MRSA outbreak, and monitoring by molecular analysis using PFGE is useful to identify the status of the outbreak.

Keywords MRSA · Outbreak · PFGE · Infection control

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major causative organism of hospital-acquired infection. MRSA strains easily colonize a host, particularly immunodeficient patients, and can cause a variety of serious infections [1–3]. The principal mode of transmission is via the transiently colonized hands of hospital personnel [4]. An outbreak of MRSA in intensive care units or neonatal intensive care units is often prolonged and can result in substantial morbidity and mortality [5, 6]. Although there are reports concerning the efficacy of the unselective use of intranasal mupirocin ointment to control MRSA outbreaks [7, 8], effective infection control measures still have not been established.

Molecular analysis is essential for evaluating horizontal transmission during an MRSA outbreak, and pulsed-field

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gel electrophoresis (PFGE) remains the gold standard technique in this context [9–11].

In this report, we describe how an MRSA outbreak in our advanced emergency medical service center (hereafter referred to as the ICU) was brought under control through early recognition of the outbreak, monitoring by molecular analysis, and the stepwise addition of infection control measures.

Materials and methods

Ethical approval

All studies described herein were approved by the Human Ethics Review Boards of Kurume University (10268).

Setting and outbreak description

In Kurume University Hospital, there are 29 diagnosis and treatment departments, and 25 wards with 1,098 beds, including an ICU with 44 beds. The ICU in our hospital receives many severe patients via the ambulance and helicopter emergency medical services. The number of patients per month with newly identified MRSA colonization or infections including a positive blood culture in the ICU increased at the beginning in June 2010 (Fig. 1). Since an outbreak is defined as at least twice the usual number of cases in the same ward, the Infection Control Team (ICT) initiated an intervention for an MRSA outbreak in the ICU in August 2010.

Bacterial strains and patients

The first PFGE was performed against 28 MRSA isolates from blood samples of 11 patients (11 strains), and from other specimens (pharynx, nasal cavity, stool, sputum, ascites, and pus) of 12 patients (12 strains), the environment (2 strains), and the skin, middle nasal meatus, and urine of one patient each (3 strains) from other wards between April and September 2010. Since the number of inpatients with MRSA did not decrease quickly despite the initiation of various infection control measures, such as the unselective application of intranasal mupirocin ointment to inpatients, MRSA monitoring for new inpatients and measures for the prevention of direct or indirect contact were implemented. A second PFGE was performed against 19 MRSA isolates from blood, sputum, nasal cavity, skin, and pus in the ICU, and 3 MRSA strains each in neurosurgery, reconstructive and maxillofacial surgery, and cardiovascular medicine, where inpatients were occasionally transferred from the ICU between October 2010 and January 2011. Finally, even after the end of the MRSA outbreak in the ICU, a follow-up PFGE was performed

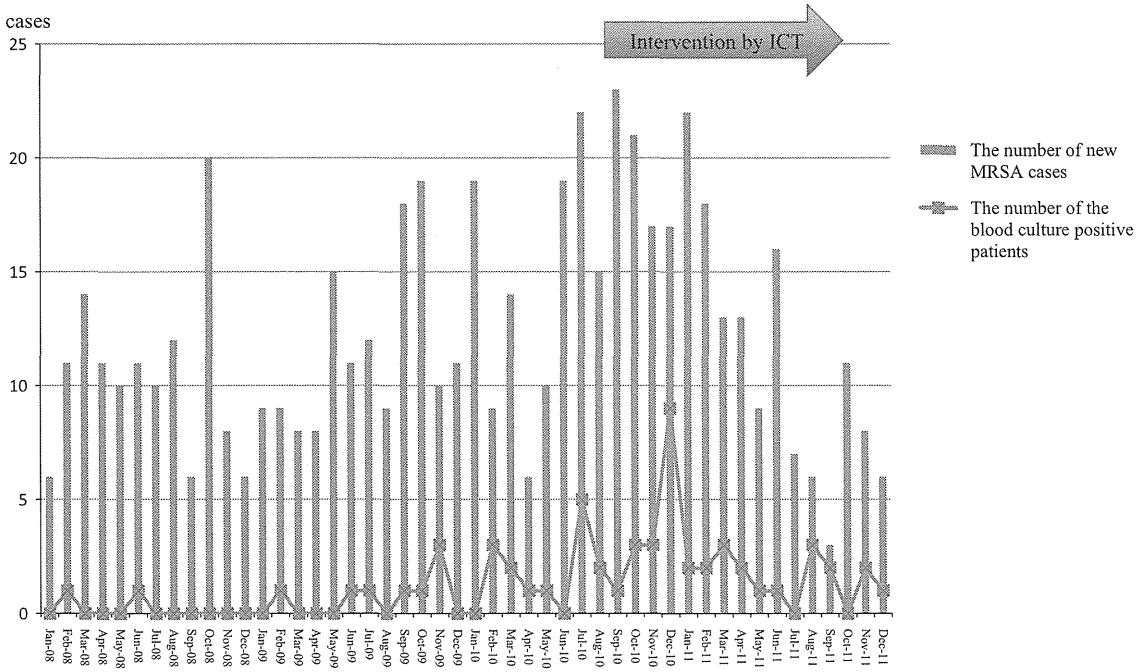


Fig. 1 Changes in the number of patients per month with newly identified MRSA colonization or infection (including a positive blood culture) in the ICU from January 2008 to December 2011. MRSA cases in the ICU with a positive blood culture increased dramatically from June 2010. After performing various kinds of interventions, the rate of MRSA infections decreased significantly more than 6 months after intervention was initiated

Fig. 2 Post-intervention improvements by the ICT. *Left*: organizing a desk for injection manufacture. *Center*: clarification of waste containers. *Right*: placement of a rack for gloves and gowns

against 28 MRSA isolates from blood, nasal cavities, sputum, pus, pharynx, catheter tip, and ascites in the ICU between August and November 2011.

Pulsed-field gel electrophoresis

PFGE was performed as described previously [1]. The DNA was digested with *Sma*I (Takara Shuzo Co., Shiga, Japan). CHEF Mapper pulsed field electrophoresis systems (Bio-Rad Life Science Group, Hercules, CA, USA) were used for the electrophoresis, with a potential of 6 V/cm, switch times of 0.47 and 63 s, and run-times of 20 h and 18 min. After staining with ethidium bromide, the PFGE patterns were interpreted based on the criteria described by Tenover et al. [12].

Intervention by the ICT

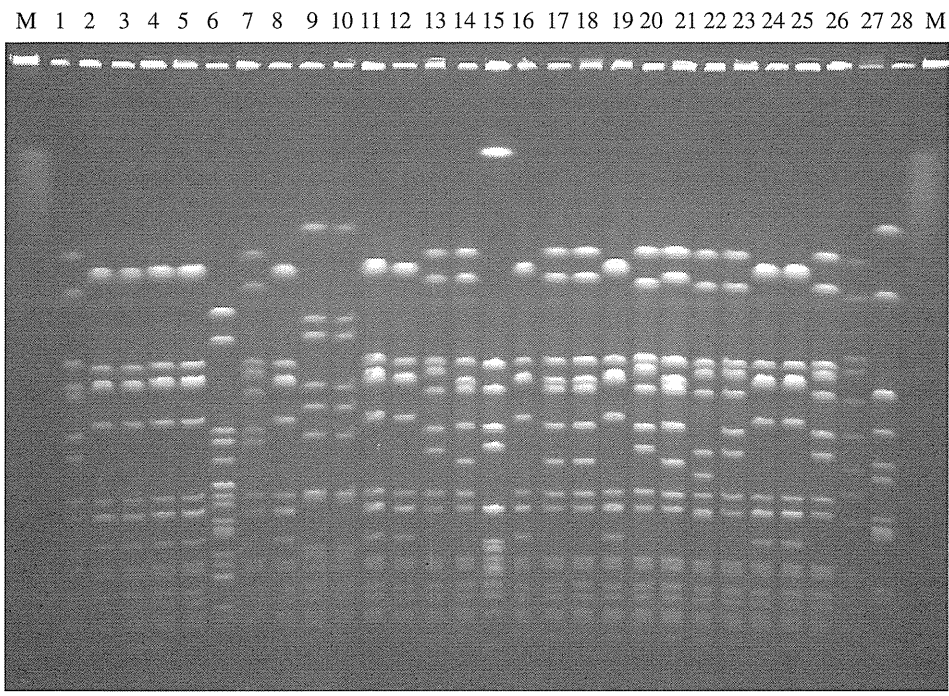
Weekly MRSA monitoring cultures for all inpatients in the ICU were implemented from August 2010, and environmental bacterial cultures from 38 locations in the ICU were performed in September 2010. MRSA was detected in 2 of the 38 locations (5.3 %) around the MRSA-colonized patients. As molecular analysis by the first PFGE showed horizontal transmission of MRSA strains in the ICU, the ICT carried out the following measures: (1) active surveillance and unselective application of intranasal mupirocin ointment to all inpatients for 3 days beginning in November 2010, (2) reinforcement of direct or indirect contact infection measures, (3) reinforcement of bloodstream infection measures, and (4) action to monitor reinforcement by the ICT and education for the ICU staff (Fig. 2).

Results

Interpretation of PFGE

Molecular typing by the first PFGE showed that the 28 MRSA strains presented 7 patterns in total, and that 11 of these MRSA strains (six from blood, two from the environment, and one each from the pharynx, ascites, and pus) exhibited PFGE pattern b. Since those patients got PFGE pattern b of the MRSA strains after admission to the ICU, horizontal transmission of the predominant PFGE pattern b of the MRSA strains must have occurred in the ICU (Fig. 3).

Molecular typing by a second PFGE performed during the MRSA outbreak showed 19 MRSA strains in the ICU with 7 patterns in total, and 10 of these strains (5 of the 6 from blood; 5 of the 13 from other specimens) still presented the predominant PFGE pattern b (Fig. 4). Moreover, PFGE pattern b of the MRSA strains was confirmed in the



No.	Date (mo/day/yr)	Source	Location
1	04.12.10	Blood	ICU
2	05.10.10	Blood	ICU
3	06.16.10	Blood	ICU
4	06.18.10	Blood	ICU
5	06.22.10	Blood	ICU
6	06.22.10	Blood	ICU
7	06.24.10	Blood	ICU
8	07.20.10	Blood	ICU
9	07.26.10	Blood	ICU
10	08.10.10	Blood	ICU
11	09.21.10	Blood	ICU
12	08.25.10	Pharynx	ICU
13	08.28.10	Stool	ICU
14	08.30.10	Pharynx	ICU
15	08.31.10	Pharynx	ICU
16	08.31.10	Pus	ICU
17	08.31.10	Pharynx	ICU
18	08.31.10	Nasal cavity	ICU
19	09.01.10	Ascites	ICU
20	09.04.10	Nasal cavity	ICU
21	09.07.10	Nasal cavity	ICU
22	09.08.10	Stool	ICU
23	09.14.10	Sputum	ICU
24	09.10.10	Suction tube	ICU
25	09.10.10	Ventilator panel	ICU
26	08.30.10	Skin	Dermatology
27	09.02.10	Middle nasal meatus	Otolaryngology
28	09.02.10	Urine	Pediatrics

ICU: advanced emergency medical service center

Fig. 3 PFGE patterns of *Sma*I-digested DNAs of MRSA isolates obtained between April and September 2010. Molecular typing of the first PFGE showed that the 28 MRSA strains had 7 patterns in total, and that 11 of the MRSA strains (six in blood, two from the environment, and one each from the pharynx, ascites, and pus) exhibited PFGE pattern b. This PFGE pattern was not observed in other wards (nos. 26–28)

patients who were transferred from the ICU to the neurosurgery and reconstructive and maxillofacial surgery wards, which meant that the MRSA outbreak had potentially spread from the ICU to other wards.

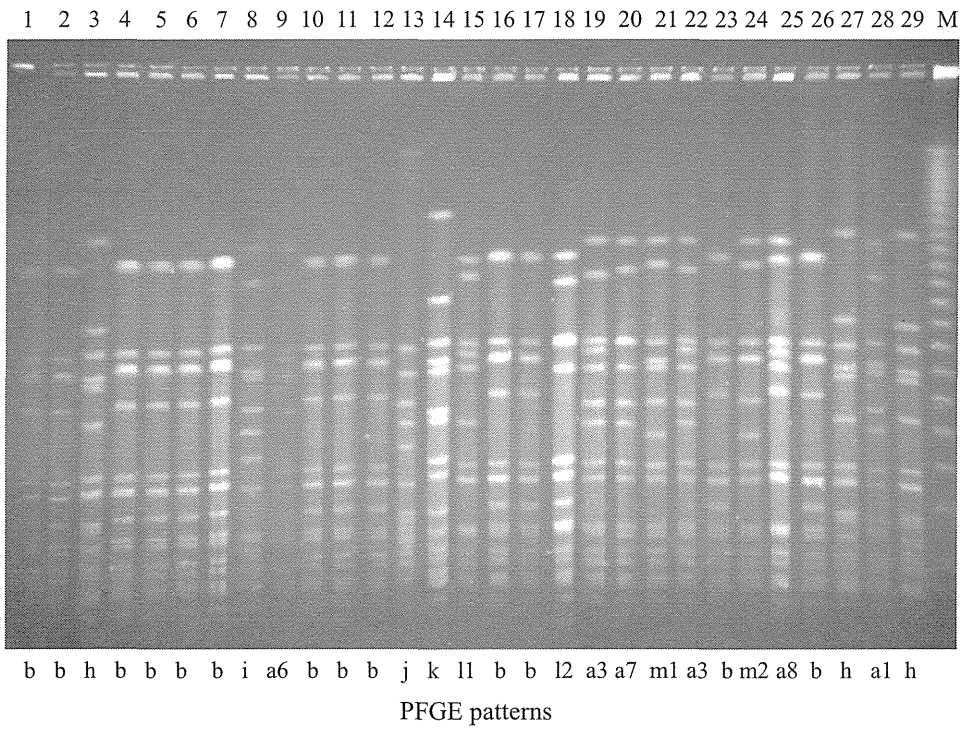
Molecular typing by a follow-up PFGE performed after the end of the MRSA outbreak in the ICU showed 28 MRSA strains with 7 patterns in total, and 21 of those 28 MRSA strains (2 of the 4 from blood; 19 of the 24 from other specimens) were still PFGE pattern b (Fig. 5), which meant that horizontal transmission of the predominant PFGE pattern b of MRSA strains had continued even after the end of the MRSA outbreak in the ICU.

Additional intervention by the ICT

The ICT performed various kinds of interventions aimed at preventing an MRSA outbreak in the ICU, as described above. However, the number of inpatients with MRSA did not quickly decrease. Lectures and ward rounds were performed repeatedly, and participation in the ward rounds by ICU staff members was suggested as a means to enhance levels of precaution against the MRSA outbreak. As a result of these various kinds of interventions, the number of inpatients with MRSA significantly decreased more than 6 months later (Fig. 1).

Discussion

MRSA outbreaks occasionally occur as a result of hospital-acquired infection, particularly in intensive care units [12], neonatal intensive care units [6], and burns units [13], and often prolong and increase the incidences of hospital-acquired pneumonia and bloodstream infection, as well as the risk of death [5]. Since the MRSA strain is known to spread in a ward via transiently colonized hands of hospital personnel [4], hand hygiene is a basic preventive measure [14, 15]. Although a previous report demonstrated that simple control measures (including contact isolation of colonized patients and reinforcement of hand-washing practices among personnel) effectively controlled MRSA outbreaks [12], some specific preventive measures, such as the unselective use of intranasal mupirocin ointment [7, 8] and enteral vancomycin [16, 17] have been used in the clinical setting in the past. Indeed, various kinds of interventions, including the unselective use of intranasal



No.	Date (mo/day/yr)	Source	Location
1	05.10.10	Blood	ICU
2	12.09.10	Blood	ICU
3	12.11.10	Blood	ICU
4	12.27.10	Blood	ICU
5	01.07.11	Blood	ICU
6	01.17.11	Blood	ICU
7	01.17.11	Blood	ICU
8	01.02.11	Sputum	ICU
9	01.04.11	Sputum	ICU
10	01.04.11	Nasal cavity	ICU
11	01.04.11	Skin	ICU
12	01.05.11	Pus	ICU
13	01.06.11	Sputum	ICU
14	01.11.11	Nasal cavity	ICU
15	01.11.11	Nasal cavity	ICU
16	01.12.11	Sputum	ICU
17	01.12.11	Sputum	ICU
18	01.12.11	Nasal cavity	ICU
19	01.17.11	Nasal cavity	ICU
20	01.17.11	Pus	ICU
21	11.01.10	Sputum	Neurosurgery
22	11.05.10	Pus	Neurosurgery
23	11.07.10	Spinal fluid	Neurosurgery
24	10.22.10	Pharynx	Reconstructive and Maxillofacial Surgery
25	11.01.10	Sputum	Reconstructive and Maxillofacial Surgery
26	11.10.10	Pus	Reconstructive and Maxillofacial Surgery
27	10.21.10	Pus	Cardiovascular Medicne
28	11.01.10	Sputum	Cardiovascular Medicne
29	11.04.10	Pharynx	Cardiovascular Medicne

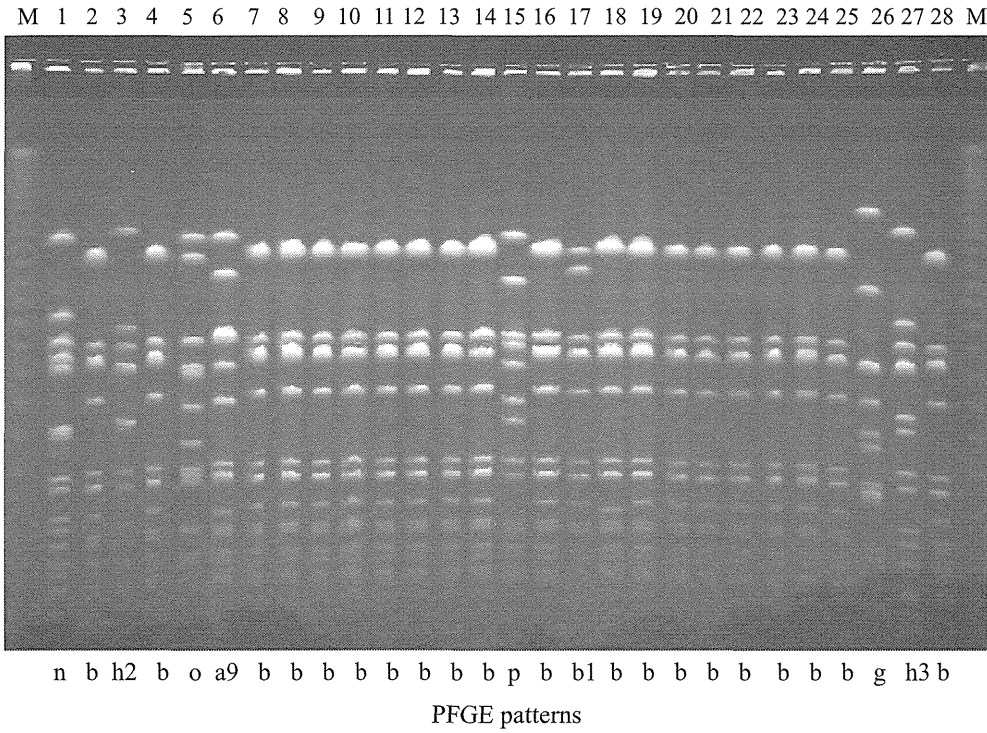
ICU: advanced emergency medical service center

Fig. 4 PFGE patterns of *Sma*I-digested DNAs of MRSA isolates obtained between October 2010 and January 2011. Molecular typing by a second PFGE showed 19 MRSA strains in the ICU with 7 patterns in total, and 10 of the 19 MRSA strains (5 of the 6 from blood; 5 of the 13 from other specimens) presented the predominant PFGE pattern b. PFGE pattern b of MRSA strains was also identified in the patients who were transferred from the ICU to neurosurgery and to the reconstructive and maxillofacial surgery wards (nos. 23 and 26)

mupirocin ointment to control MRSA outbreaks in the ICU, have been performed. However, the number of inpatients with MRSA did not decrease quickly, and the MRSA outbreak ended after additional interventions were performed, such as repeated lectures and ward rounds and the participation of the ICU staffs in ward rounds in order to enhance the levels of precaution against further MRSA outbreaks.

During this particular MRSA outbreak, molecular analysis by PFGE was performed repeatedly to evaluate horizontal transmission, and the ICU staff were immediately informed of the results. PFGE seems to be useful for evaluating the presence of cross-transmission in hospital-acquired infection [9–11]. In addition, a previous study indicated that PFGE studies may be helpful when assessing the effectiveness of interventions during a MRSA outbreak [18]. In our study, a predominant MRSA isolate was thought to be spread in the ICU via the transiently colonized hands of hospital personnel, and repeatedly informing the ICU staff of the results of PFGE studies appeared to be a useful way of reminding them of the status of the MRSA infection in the ICU. However, a follow-up PFGE performed after the MRSA outbreak in the ICU had ended showed that the predominant MRSA isolate continued to spread as a horizontal transmission, although the number of inpatients with newly identified MRSA colonizations or infections had decreased. That means that the predominant MRSA strain could potentially cause another MRSA outbreak in the ICU, so the precautions should be continued.

In conclusion, our data suggest that it is essential to apply various combinations of infection control measures in order to protect against an MRSA outbreak, and that monitoring by molecular analysis using PFGE is useful for identifying the status of a MRSA outbreak. Therefore, a



No.	Date (mo/day/yr)	Source	Location
1	08.18.11	Blood	ICU
2	10.10.11	Blood	ICU
3	10.13.11	Blood	ICU
4	11.04.11	Blood	ICU
5	08.16.11	Nasal cavity	ICU
6	08.17.11	Sputum	ICU
7	08.19.11	Sputum	ICU
8	08.19.11	Sputum	ICU
9	08.20.11	Pus	ICU
10	08.29.11	Sputum	ICU
11	09.09.11	Sputum	ICU
12	09.12.11	Pharynx	ICU
13	09.27.11	Catheter tip	ICU
14	09.28.11	Pus	ICU
15	10.05.11	Ascites	ICU
16	10.03.11	Sputum	ICU
17	10.11.11	Sputum	ICU
18	10.11.11	Sputum	ICU
19	10.12.11	Sputum	ICU
20	10.13.11	Sputum	ICU
21	10.15.11	Sputum	ICU
22	10.17.11	Sputum	ICU
23	10.21.11	Ascites	ICU
24	10.31.11	Pus	ICU
25	11.07.11	Sputum	ICU
26	11.21.11	Sputum	ICU
27	11.21.11	Sputum	ICU
28	11.21.11	Sputum	ICU

ICU: advanced emergency medical service center

Fig. 5 PFGE patterns of *Sma*I-digested DNAs of MRSA isolates obtained between August and November 2011. Molecular typing by a follow-up PFGE performed after the end of the MRSA outbreak in the ICU showed 28 MRSA strains with 7 patterns, and 21 of those 28 MRSA strains (2 of the 4 from blood; 19 of the 24 from other specimens) were still PFGE pattern b

follow-up PFGE—even one performed after the end of an MRSA outbreak—should be considered in order to prevent a recurrence.

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Conflict of interest None declared.

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Original Article

Determination of Epidemiology of Clinically Isolated *Cryptococcus neoformans* Strains in Japan by Multilocus Sequence Typing

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SUMMARY: *Cryptococcus neoformans* and *Cryptococcus gattii* are the causative agents of cryptococcosis. Despite its importance, our knowledge of the epidemiology of cryptococcosis in Japan remains limited. To establish an epidemiological database on cryptococcosis in Japan, we determined the genetic variability of 44 Japanese clinical isolates of *C. neoformans* (*var. grubii*: serotype A) by multilocus sequence typing (MLST). The strains were clinically isolated from 1992 to 2011 in 5 different areas of Japan (the Hokkaido region [n = 1], Kanto region [n = 32], Chubu region [n = 1], Kansai region [n = 1], and Kyushu region [n = 9]). According to the method recommended by the International Society for Human and Animal Mycology cryptococcal genotyping working group, 36 isolates (82%) were identified as sequence type (ST)46. The remaining strains belonged to ST45 (n = 1) and ST47 (n = 1), and 6 isolates belonged to novel independent STs. There was little geographic difference in the ST population. Our present data are still limited; however, because most clinical isolates showed the same MLST profile in Japan, applying the current MLST scheme for *Cryptococcus* may at times be insufficient for investigating the infection route among outbreak cases. To solve this problem, it may be necessary to investigate other gene loci or develop a novel method with greater discriminatory power. However, in cases in which a strain belongs to a minor ST, our data may serve as useful epidemiological information in Japan.

INTRODUCTION

Cryptococcosis is a systemic mycosis caused by the haploid, encapsulated, basidiomycetous yeast *Cryptococcus neoformans* (serotypes A, D, and AD) and *Cryptococcus gattii* (serotypes B and C), which are commonly associated with pigeon excreta and plant materials. Cryptococcosis typically involves lung diseases and central nervous system infection. *C. neoformans* causes approximately 1 million cases of meningitis globally per year in human immunodeficiency virus (HIV)-infected patients, resulting in approximately 624,700 deaths within 3 months after infection (1).

C. neoformans is presently divided into 2 varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (2). In addition, diploid and aneuploid AD hybrids have been isolated from the environment and patients (3,4). *C. neoformans* var. *grubii* is responsible for more than 90% cases of cryptococcosis worldwide (5). The sexual reproduction of *Cryptococcus* has a two-allele mating system comprising MATa and MATα (6). The α mating type of *C. neoformans* is predominant among clinical and environmental isolates (>98%–99.9%) (5). Mating basically occurs between opposite mating types. Recently, unisexual mating between 2 α cells has been suggested to occur naturally in *C. gattii* (7) and *C. neoformans* (8).

Genotypic analysis such as M13 DNA fingerprinting, amplified fragment length polymorphism, and multilocus sequence typing (MLST) of *C. neoformans* var. *grubii* has identified 3 molecular types: VNI, VNII, and

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VNB (9–13). VNI strains are globally dominant, and VNII isolates are less common. VNB is a novel molecular type discovered as a unique cryptococcal population in Botswana (13).

Recently, a standardized MLST scheme for *C. neoformans* and *C. gattii* has been established by the Cryptococcal Working Group I (Genotyping of *C. neoformans* and *C. gattii*) of the International Society for Human and Animal Mycology (ISHAM) to enable global standardization and overcome problems arising from interlaboratory reproducibility (10). The scheme uses 7 unlinked genetic loci, including the housekeeping genes *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, and *URA5* and the IGS1 region. Based on the ISHAM scheme, 183 clinical and environmental isolates from Thailand and 77 isolates from the global collection of *C. neoformans* var. *grubii* were analyzed, and the MLST database was established (14). The majority of Thailand isolates exhibit 3 sequence types (STs): ST44 (33%, n = 70), ST45 (43%, n = 78), and ST46 (14%, n = 26). ST44 and ST45 are unique to Thailand, and ST46 has been identified in Thailand and Japan (14). A recent epidemiological report has shown that 31 of the 35 *C. neoformans* var. *grubii* isolates from non-HIV patients in Nagasaki, Japan exhibited the same ST based on MLST using the same 7 loci as those used in the ISHAM scheme (15).

In Japan, cryptococcosis caused by *C. neoformans* occurs in all regions. Despite its importance, our knowledge of the epidemiology of cryptococcosis in Japan remains limited. The establishment of an epidemiological database on cryptococcosis in Japan is urgently required to manage epidemics and potential outbreaks of cryptococcosis. In this study, we used MLST, a rapid, reproducible, and discriminatory methodology for genotyping isolates of *C. neoformans* to investigate the genetic relatedness among isolates from several medical facilities in Japan. The MLST database constructed in this study could be a useful resource for global epidemiologic studies and for the recognition and tracking of the inter or intrahospital spread of *C. neoformans*.

MATERIALS AND METHODS

Clinical isolates: Forty-four clinical isolates of *C. neoformans* var. *grubii* were collected from 11 facilities from 5 regions comprising 8 prefectures in Japan. The regional distribution of the isolates was as follows: 1 clinical isolate was collected from 1 facility in the Hokkaido region, 32 clinical isolates were collected from 5 facilities in the Kanto region, 1 clinical isolate was collected from 1 facility in the Chubu region, 1 clinical isolate was collected from 1 facility in the Kansai region, and 9 clinical isolates were collected from 3 facilities in the Kyushu region (Table 1, Fig. 1). Species identification was initially performed by sequencing internal transcribed spacer (ITS) 1–2 and D1/D2 26S ribosomal DNA (rDNA) sequences.

DNA extraction: The isolates were cultured in liquid yeast extract-peptone-dextrose (YPD) medium at 30°C. DNA was extracted from the collected cells using Dr. GenTLE (TakaraBio, Otsu, Japan). Alternatively, a suspension of freshly grown cells was prepared in lysis buffer (DNeasy kit; QIAGEN, Valencia, Calif., USA) and subjected to mechanical lysis using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). DNA was then purified from the lysed cells using the DNeasy kit (QIAGEN) according to the manufacturer's protocol.

Serotype and mating type analysis: The mating type of each isolate was determined by 4 different polymerase chain reaction (PCR) amplification reactions. Primers specific to the MATα or MATa allele of the *STE20* locus for either serotype A or D isolates were used, namely primers JOHE7270 and JOHE7272 (aA), JOHE7273/JOHE7275 (aD), JOHE7264/JOHE7265 (αA), and JOHE7267/JOHE7268 (αD), as described previously (16).

MLST analysis: MLST analysis was essentially performed according to a previously described protocol (10). Each isolate was PCR amplified in 30 μl of reaction volumes for each of the 7 MLST loci using MightyAmp (TakaraBio). The primers used in this study are the same as a previously described protocol (10), except that a primer LAC1R2 (5′-TCGGACTA TTAATCTCCAAACTC) was used instead of the primer LAC1R (10). The PCR reaction procedure included denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 10 s, 60°C for 15 s, and 68°C for 1 min for all the primers. PCR products were run on 1% agarose gel and purified using the DNA purification kit (TakaraBio). Each locus was subsequently sequenced using the Applied Biosystems 3730 sequencer with the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, Calif., USA) or by ordering from FASMAC Co., Atsugi, Japan. Sequences were manually edited using EnzymeX (mekentosj.com). Alleles at each locus were assigned numbers (allele types: ATs) in comparison with those identified in the global collection (13). All nucleotide sequences obtained in this study, including novel ATs, were deposited in the DNA Data Bank of Japan (DDBJ accession nos. AB744719–AB745026). Each unique allelic profile was concatenated and assigned an ST using the archived online *C. neoformans* database on MLST.net (http://cneoformans.mlst.net/) (14).

Phylogenetic analysis: Evolutionary analyses were performed on concatenated sequences from 7 loci (*CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and IGS1) in MEGA5 (17). The evolutionary history was inferred by the maximum-likelihood method based on the Tamura-Nei model (18).

RESULTS

All the 44 isolates collected in this study belonged to serotype A and mating type α according to PCR classification. Sequence data were obtained for all the 44 Japanese *C. neoformans var. grubii* isolates typed at the 7 loci (Table 1). The 7 loci yielded 26 ATs (*CAP59*, 4; *GPD1*, 3; IGS1, 3; *LAC1*, 5; *PLB1*, 4; *SOD1*, 3; and *URA5*, 4), 3 of which were novel loci (*LAC1*, *SOD1*, and *URA5*). The novel AT of *LAC1* (accession no. AB744879) is the closest to AT 1 (addition of C to AT1), the novel AT of *SOD1* (accession no. AB744945) is the closest to AT12 (addition of GGA to AT12), and the novel AT of *URA5* (accession no. AB744994) is the closest to AT1 (replacement of A with C).

We identified 9 multilocus STs within the Japanese

Table 1. Clinical isolates in this study

Strain ID no.	Region	Facility	Isolated year	Serotype	Mating type	AT[1)]							ST[1)]
						CAP59	*GPD1*	IGS1	*LAC1*	*PLB1*	*SOD1*	*URA5*	
NIIDCr0001	Hokkaido	A	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0002	Kanto	B	1992	A	α	1	3	19	5	2	13	1	46
NIIDCr0003	Kanto	B	1993	A	α	1	3	19	5	2	13	1	46
NIIDCr0004	Kanto	B	1994	A	α	1	3	19	5	2	13	1	46
NIIDCr0005	Kanto	B	1994	A	α	1	3	19	5	2	13	1	46
NIIDCr0006	Kanto	B	1995	A	α	1	3	19	5	2	13	1	46
NIIDCr0007	Kanto	B	1997	A	α	10	9	21	8	11	Nov	4	Nov
NIIDCr0008	Kanto	B	2000	A	α	1	3	19	5	2	13	1	46
NIIDCr0009	Kanto	B	2001	A	α	1	3	19	5	2	13	1	46
NIIDCr0010	Kanto	B	2006	A	α	1	1	20	3	4	13	1	47
NIIDCr0011	Kanto	B	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0012	Kanto	C	2011	A	α	1	3	19	5	2	13	Nov	Nov
NIIDCr0013	Kanto	D	2005	A	α	1	1	19	4	2	13	5	45
NIIDCr0014	Kanto	D	2006	A	α	1	1	20	3	2	13	1	Nov
NIIDCr0015	Kanto	D	2007	A	α	1	3	19	5	2	13	1	46
NIIDCr0016	Kanto	D	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0017	Kanto	D	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0018	Kanto	D	2009	A	α	2	9	21	8	11	14	4	Nov
NIIDCr0019	Kanto	D	2009	A	α	1	3	19	5	2	13	1	46
NIIDCr0020	Kanto	D	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0021	Kanto	E	2005	A	α	1	3	19	5	2	13	1	46
NIIDCr0022	Kanto	E	2005	A	α	1	3	19	5	2	13	1	46
NIIDCr0023	Kanto	E	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0024	Kanto	E	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0025	Kanto	E	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0026	Kanto	E	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0027	Kanto	E	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0028	Kanto	E	2002	A	α	1	3	19	5	2	13	1	46
NIIDCr0029	Kanto	E	2004	A	α	7	1	19	Nov	1	13	1	Nov
NIIDCr0030	Kanto	E	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0031	Kanto	E	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0032	Kanto	E	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0033	Kanto	F	2010	A	α	1	3	19	5	2	13	1	46
NIIDCr0034	Kyushu	G	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0035	Kyushu	G	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0036	Kyushu	G	2008	A	α	7	1	19	5	1	13	1	Nov
NIIDCr0037	Kyushu	G	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0038	Kyushu	G	2008	A	α	1	3	19	5	2	13	1	46
NIIDCr0039	Kyushu	G	2009	A	α	1	3	19	5	2	13	1	46
NIIDCr0040	Kyushu	G	2009	A	α	1	3	19	5	2	13	1	46
NIIDCr0041	Kyushu	H	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0042	Chubu	I	2011	A	α	1	3	19	5	2	13	1	46
NIIDCr0043	Kansai	J	2011	A	α	1	3	19	5	2	13	1	46
YC-13	Kyushu	Nagasaki Univ.	Ref. (21)	A	α	1	3	19	5	2	13	1	46

[1)]: Nov, novel AT and ST.

isolates, consisting of ST45, ST46, ST47, and 6 novel STs. Thirty-six of the 44 isolates belonged to ST46 (81%) (Table 1). The remaining strains belonged to ST45 (n = 1) and ST47 (n = 1), and 6 isolates belonged to novel independent STs. A phylogenetic tree was constructed by the maximum-likelihood method based on a concatenated data set from 7 loci for MLST (Fig. 2). Among the novel STs, 2 strains (NIIDCr0007 and NIIDCr0018) were located in a different cradle, which indicated the VNII genetic type. The other 42 strains belonged to the VNI type, a major type of *C. neoformans*. No clinical isolate belonged to the VNB type.

From a geographic perspective, 27 of 34 isolates (79%) from Eastern Japan, including the Hokkaido, Kanto, and Chubu regions, and 9 of 10 isolates (90%) from Western Japan, including the Kansai and Kyushu regions, belonged to ST46, indicating that there is little geographical bias between Eastern and Western Japan in the contributions of ST46 isolates (Fisher's exact test; P = 0.659). Two isolates (NIIDCr0007 and NIIDCr0018), which belong to the molecular type VNII, were isolated from the Kanto region of Eastern Japan.

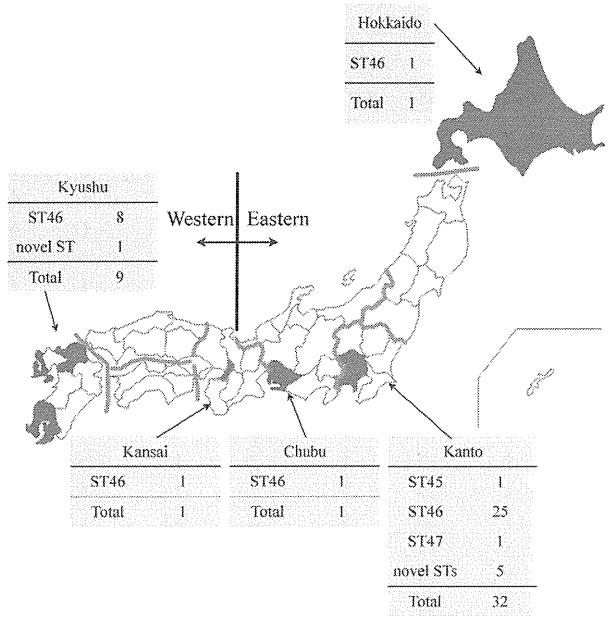

Fig. 1. Map of Japan showing the distribution of multilocus sequence typing (MLST) profile and the number of isolates according to the region involved in the study. Details are shown in Table 1.

DISCUSSION

There are no previous studies of MLST epidemiology by the ISHAM standard method for clinical isolates of *C. neoformans* var. *grubii* obtained from multiple facilities in Japan. In this study, we analyzed 44 clinical strains of *C. neoformans* from 11 facilities. Most isolates were classified as the same ST, ST46 (81%). This result indicates that it may be difficult to predict the origin of potential cryptococcosis outbreaks using the MLST scheme if the isolates belong to ST46. However, STs of other isolates are phylogenetically independent of each other (Fig. 2), and these minor STs will be useful for global epidemiologic studies, recognition, and tracking the inter or intrahospital spread of *C. neoformans* in Japan.

A recent epidemiological study has shown that clinical and environmental isolates in Thailand predominantly consist of 3 STs, ST44, ST45, and ST46, accounting for up to 90% of the total isolates investigated (14), indicating low genetic diversity. In Japan, Mihara et al. (15) has reported that 31 of the 35 isolates in Nagasaki, Japan exhibited ST5. ST46 in this study and ST5 in the study of Mihara et al. are derived from the same nucleotide sequences in the 7 loci. This discrepancy of ST might be due to the MLST database;

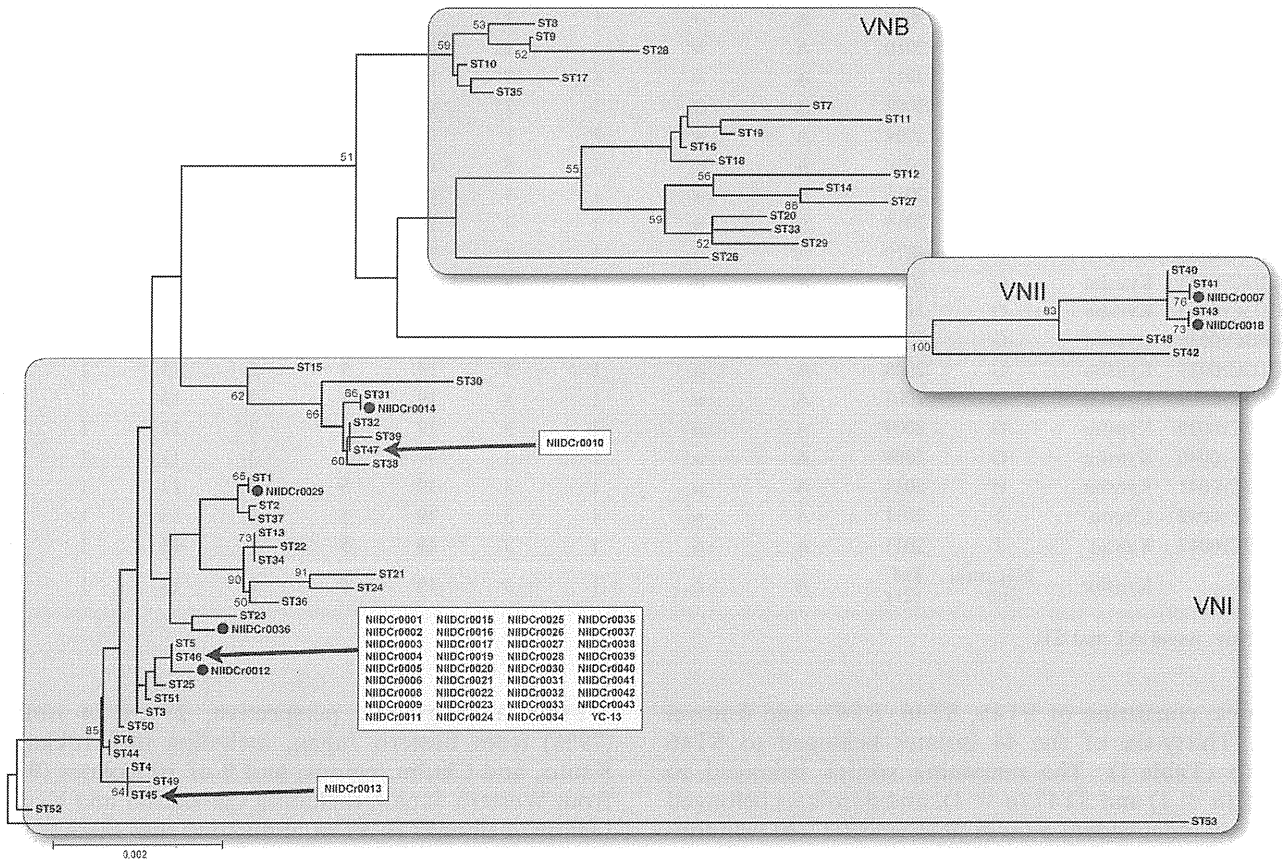

Fig. 2. Molecular phylogenetic analysis by the maximum-likelihood method of the 44 Japanese clinical isolates and known sequence types (STs) of *Cryptococcus neoformans* using a concatenated data set derived from seven loci (*CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and IGS1). The tree with the highest log likelihood (−7730.9017) is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage (>50%) of trees in which the associated taxa clustered together is shown next to the branches. Analysis involved 61 nucleotide sequences. There were 3901 positions in the final dataset. VN molecular types (VNI, VNII, and VNB) are represented by gray rectangles.