

Fig. 2. Southern blot profile of IS1642 of *Mycobacterium avium* strains. M, molecular size marker, lambda HindIII. lanes 1–9, human *M. avium* isolates; lane 10, *M. avium* ssp. *paratuberculosis* K-10; lane 11, *M. avium* ssp. *avium* ATCC25291; lane 12, *M. avium* ssp. *avium* ATCC15769. Numbers on the left indicate the sizes of the DNA marker.

respectively. The IS with the 5 bp direct repeat was found to be within the coding region of MAP2026 gene. The sequence flanking the 59 bp direct repeat sequence did not match with any gene in the database. We sought to find a conserved sequence at the insertion sites, but sequence specificity was not obvious among these three target sites.

Distribution of IS1642

The distribution of IS1642 among *M. avium* strains was examined by Southern blot analysis with the following strains: *M. avium* ssp. *paratuberculosis* K-10, *M. avium* ssp. *avium* ATCC25291, *M. avium* ssp. *avium* ATCC15769, and nine *M. avium* strains, isolated from different patients at NHO Higashi Nagoya National Hospital. The DNA bands were found in only some of the *M. avium* strains isolated from human patients, but not in *M. avium* ssp. *paratuberculosis* K-10, *M. avium* ssp. *avium* ATCC25291, and *M. avium* ssp. *avium* ATCC15769 (Fig. 2). The Southern blot profile indicated that the band patterns of IS1642 were very diverse and the copies were multiplied. *Mycobacterium intracellulare* ATCC13950, *M. intracellulare* ATCC25225, five *M. intracellulare* clinical isolates, and seven other mycobacterial species were negative for IS1642 on the Southern analysis profile (data not shown). The prevalence of IS1642 among *M. avium* strains was further examined with eight strains isolated from human patients at the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases by PCR with the three sets of specific primers (primers IS1642-1F and IS1642-1R, IS1642-2F and IS1642-2R, IS1642-3F, and IS1642-3R) (Table 1). All strains were positive for IS1642 (data not shown), suggesting that IS1642 is widely distributed among *M. avium* clinical strains.

Mobility of IS1642

Because the Southern blot profile of IS1642 was very different among strains, we explored whether or not the IS1642 exhibits frequent mobility by examining changes in the Southern blot pattern during repeated passages *in vitro*. One *M. avium* clinical strain was streaked on a 7H10 agar

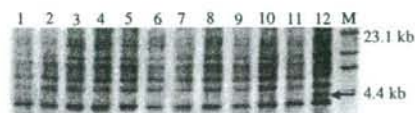


Fig. 3. Change in the Southern blot profile of IS1642 of the human *Mycobacterium avium* isolate by *in vitro* repeated passages. lane 1, original *M. avium* cells; lanes 2–11, *M. avium* cells subcultured once from 10 individual colonies from the original culture plate; lane 12, *M. avium* cells passed 10 times from the original culture plate. M, molecular size marker, lambda HindIII. The additional band that appeared after the repeated passage is indicated by the arrow. Numbers on the right indicate the sizes of the DNA marker.

plate, and passed 10 times. A single colony was used for inoculation at each passage. After the passages, genome DNA was extracted. For reference, 10 colonies were individually subcultured once from the initial culture plate and genome DNA samples were extracted. The genome DNA samples were subjected to Southern blot analysis. While the original cells and the cells of 10 colonies with a single subculture showed identical patterns, the passed cells contained an additional band (Fig. 3), indicating that transposition of the IS occurred during the *in vitro* repeated culture.

Discussion

In this study, we discovered a new IS, IS1642, in *M. avium* clinical strains isolated from human patients. Our results showed that multiple copies of IS1642 are present in several *M. avium* strains. The deduced amino acid sequence of the ORF of IS1642 was highly homologous to the transposase of *M. smegmatis* IS1549 (Plikaytis *et al.*, 1998). IS1549 is an insertion element whose transposases exhibited homology to that of IS1623 and IS1634, and it was suggested that these transposases represent an emerging group in the IS4 family because they exhibit a characteristic lack of the typical conserved N3 region of IS4 family and yet possess unique N2 and N3 motifs (Vilei *et al.*, 1999; Alexander *et al.*, 2003). A notable feature of IS1549 and IS1634 is that they are flanked by unusual long direct repeats that may vary in length (Plikaytis *et al.*, 1998; Vilei *et al.*, 1999). The lengths of the direct repeats of these ISs range up to 500 bp, in contrast to most usual ISs flanked by short direct repeats of 2–14 bp. IS1642 found in this study also exhibited these characteristics. IS1642 contained the C1 region and unique N2 and N3 motifs, but not the N3 region of the IS4 family, suggesting that IS1642 belongs to this new group. IS1642 was flanked by direct repeats of variable lengths ranging up to 161 bp. Considering that IS1642 was homologous to the ISs, which form variable length direct repeats, it would be likely that the direct repeat sequences found in this study were actually created by IS1642. The actual range of lengths

of direct repeats may be larger as reported in other ISs. Because the homology level of the entire region of the amino acid sequence was relatively low among ISs of this group (e.g. 38% identity between IS1549 and IS1634), the conserved structures of the amino acid sequence might play an important role in the formation of the characteristic long direct repeats of variable lengths. There was no conserved sequence at the insertion sites among the three target sites, suggesting that the insertion events by IS1642 take place randomly on the genome.

To date, the advantage of formation of long, variable-length direct repeats is not well elucidated. While insertion with a long target duplication may decrease the likelihood of destroying essential genes at the target sites on the host genome, it would be expected that an insert flanked by long direct repeats could be easily removed by homologous recombination between the repeats. Nobusato *et al.* (2000) reported insertions with long target duplications in the restriction and modification enzyme genes of *Helicobacter pylori* strains. They considered that the long duplication may control the copy number of the genes, thus keeping expression of the genes at an appropriate level. Further studies are required to elucidate the significance of the long, variable-length direct repeats.

Our results suggested that IS1642 is widely distributed among *M. avium* clinical strains. In addition, the experiment of repeated passage suggested that IS1642 is indeed capable of frequent transposition within the genome. This is consistent with the observation that the Southern blot profile was very divergent among the strains tested. Considering this polymorphism, it would be rather inappropriate to use the Southern blot pattern of IS1642 as a genetic typing tool for classification of different clinical strains. Alternatively, genotyping by IS1642 could be useful to confirm the clonality of strains because it would enable high-precision discrimination.

IS elements reportedly often carry an outward-directed promoter sequence, bringing about a constitutive expression of downstream genes at insertion sites (Safi *et al.*, 2004; Soto *et al.*, 2004). IS1549 was found to show promoter activity (Plikaytis *et al.*, 1998). Because IS1642 was homologous with IS1549, we examined IS1642 for promoter activity. However, the expression of GFP was at an undetectable level. IS1642 would have no or very low outward-directed promoter activity, or the promoter sequence could be formed as a hybrid of the IS sequence and genome sequence at insertion sites, thus turning on the expression of otherwise silent genes on the genome, as reported by Szevevényi *et al.* (1996). It might also be possible that promoters of IS1642 could not be recognized by *M. smegmatis*.

IS1642 would be involved in the gene rearrangements on the genome, thus contributing to evolution of the organism, like many other ISs. Although *M. avium* is an opportunistic

pathogen, treatment is often very difficult once the infection is established, despite long-term administration of antibiotics. It may be possible that IS1642, which has a relatively high mobility, serves to facilitate establishment of chronic infection by adapting the phenotype including the pathogenicity and drug resistance of the organism for conditions at the focus of the infection *in vivo*.

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CASE REPORTS

Human Fulminant Gas Gangrene Caused by *Clostridium chauvoei*[†]

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The first human case of fulminant gas gangrene caused by *Clostridium chauvoei*, a pathogen causing ruminant blackleg, was confirmed for a 58-year-old man suffering from diabetes mellitus. The patient developed conspicuous emphysematous gangrene in the right chest wall as well as intravascular gas entrapments and died 2 h after hospital arrival.

CASE REPORT

A 58-year-old man sustained a minor trauma to his anterior to right lateral chest region from accidentally running into an iron pipe at a construction site in late February 2006. He also had a 2-day history of fever over 39°C, cough, and nasal discharge prior to the chest injury. On the morning of the day after his chest injury, the man sought medical attention at a local hospital for a persistent high fever and an increase in pain and swelling of the bruised regions. He was diagnosed with two cracked ribs. In the evening, he suddenly collapsed while going to the bathroom. Upon the arrival at his home of an ambulance with a doctor, he went into cardiopulmonary arrest. Spontaneous circulation was restored, and he was transported to the emergency department of our medical center. He had earlier been treated for mild diabetes mellitus and hypertension with oral medication.

On arrival, he had a Glasgow Coma Scale score of 3, bilateral pupils dilated 6 mm in diameter with no light reflex. His heart rate was 108 beats/min, and blood pressure was undetectable except for palpable slight pulsation of the common carotid artery. Examination of the patient's chest revealed dark red cutaneous ecchymoses measuring approximately 5 by 5 cm in the right anterolateral chest with marked swelling. A computed tomographic (CT) scan was done immediately while the patient was supported with fluid resuscitation. The chest CT scan showed striking subcutaneous emphysema and destruction of muscle tissue centering around the anterior to lateral region of the right chest and rich gaseous contamination in the right subclavian vein and pulmonary artery (Fig. 1A to C).

Laboratory tests showed a mildly elevated leukocyte count and elevation of C-reactive protein (CRP), creatinine kinase,

and blood glucose levels. Arterial blood gas analysis revealed severe mixed acidosis (Table 1). The patient died 2 h after arrival. No autopsy was performed. Culture of the antemortem specimen from the subcutaneous emphysematous lesions by needle aspiration later grew clostridia.

Small to medium rough, grayish white colonies with beta-hemolysis were grown anaerobically in pure culture (Fig. 2A) from a needle aspirate specimen on *Brucella* HK (hemin, vitamin K₁) RS (rabbit, sheep) agar (Kyokuto Pharmaceutical, Tokyo, Japan) by 48-h incubation at 37°C. Abundant gas production and motility were noted with thioglycolate medium (Becton Dickinson Microbiology Systems, Cockeysville, MD). Staining with Gram and spore stains (Fig. 2B and C) demonstrated pleomorphic gram-positive rods with ovoid, subterminal endospores. The organism gave varied results with biochemical identification systems; some yielded *Clostridium septicum* and others *Clostridium clostridioforme* or *Clostridium histolyticum*. The 1,441-base nucleotide sequence of the 16S rRNA gene of the microbe tested was completely identical to that of the 16S rRNA gene of *Clostridium chauvoei* ATCC 10092 (GenBank accession no. U51843) by a BLAST homology search (www.ncbi.nlm.nih.gov/BLAST/). But 13 base mismatches (99% identity) were found between the 1,441-base sequence and that of *C. septicum* ATCC 12464 (GenBank accession no. U59278) (6). Then the 16S–23S rRNA gene intergenic spacer region was amplified by PCR as reported previously (9, 10), generating a 522-bp *Clostridium chauvoei*-specific product coinciding with that of *C. chauvoei* ATCC 10092, as shown in Fig. 2D. Bacterial swarming was indeed observed on soft agar, but no amplicon was observed by PCR using a set of primers for detecting the flagellin gene of *C. chauvoei* (4, 8). This observation may suggest the production of genetically different flagella by this strain.

Clostridial gas gangrene or myonecrosis, characterized by rapid progress of tissue necrosis with gas formation, is a life-threatening infection caused by toxin- and gas-producing clostridia.

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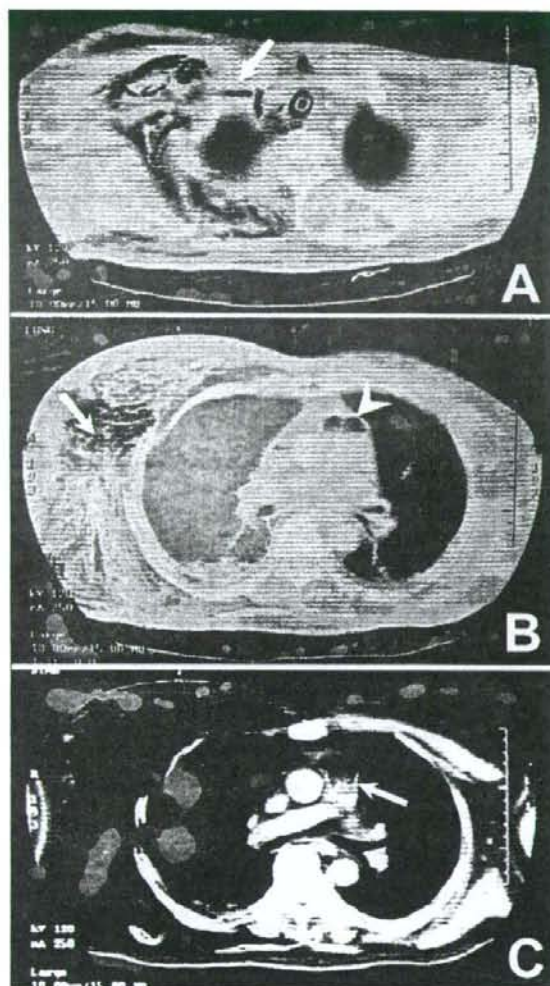


FIG. 1. Chest CT scan imaging 20 min after arrival. (A) Right subclavian vein filled with entrapped gas (arrow). (B) Marked subcutaneous emphysema with destruction of muscle tissue around right chest (arrow) and massive gas entrapped in the pulmonary artery (arrowhead). (C) CT scan revealing many bubbles below the massive gas entrapment in the pulmonary artery.

tridia. The most common histotoxic clostridia in humans include *Clostridium perfringens*, *Clostridium novyi*, and *Clostridium septicum*, followed by *Clostridium histolyticum* and *Clostridium bifermentans* (1). These clostridia mainly inhabit the soil and the intestinal tracts of humans and animals (1). Human clostridial gas gangrene often results from spore contamination in wound infections or in surgical operations under nonsterile conditions, while spontaneous, nontraumatic, or intrinsic infections from a bowel source have been increasingly reported recently (5, 11). Spontaneous infection is mostly associated with predisposing factors of hematologic or colorectal malignancies and with diabetes mellitus (1, 5).

C. chauvoei is of veterinary importance as a causative pathogen of blackleg, a highly fatal gas gangrenous infection of cattle and sheep (7). Other susceptible hosts reported so far are limited to animals, including goats and swine (2). No human gas gangrene case caused by this bacterial species has been recognized to date. So it is the rare clinician who notices the presence of *C. chauvoei* as a possible causative pathogen of human gas gangrene and invasive clostridial infections. *C. septicum* and *C. chauvoei* are genetically very similar, making them difficult to distinguish in biochemical testing. In the present study, however, the human fulminant gas gangrene case was confirmed to be caused by *C. chauvoei* after precise genetic characterizations including analyses of the 16S rRNA gene (6) and the 16S-23S rRNA gene intergenic spacer region (9, 10). Therefore, strains from human patients with devastating clostridial infections that have been identified as *C. septicum* are worth checking out by further genetic identification, as was done in the present study.

In the present case, we initially suspected that cardiac arrest might have been caused by cardiac tamponade, massive bleeding of the chest wall, and/or hemopneumothorax triggered by trauma, which was not evident by CT examination. Although subcutaneous emphysema was noted and the immediate cause of sudden cardiac arrest was thought to be pulmonary air embolism, it was still difficult to consider that the minor trauma observed in the contusion area led to cardiac arrest. Moreover, clinical findings of high fever before and after trauma accom-

TABLE 1. Patient clinical laboratory data

Variable (unit)	Value
Hematology	
Leukocyte count (cells/ μ l)	8,800
Erythrocyte count (cells/ μ l)	365×10^4
Hemoglobin (g/dl)	12.4
Hematocrit (%)	39.2
Platelet count (cells/ μ l)	89,000
Blood chemistry	
Aspartate aminotransferase (U/liter)	213
Alanine aminotransferase (U/liter)	72
Lactate dehydrogenase (U/liter)	870
Creatine kinase (U/liter)	9,985
Amylase (U/liter)	86
Creatinine (mg/dl)	1.90
Urea nitrogen (mg/dl)	20
Albumin (g/dl)	3.0
Total bilirubin (mg/dl)	1.1
Sodium (mEq/liter)	137
Potassium (mEq/liter)	6.0
Chloride (mEq/liter)	90
Glucose (mg/dl)	384
CRP (mg/dl)	35.80
Arterial-blood gases	
pH	6.471
Partial pressure of carbon dioxide (mm Hg)	96.1
Partial pressure of oxygen (mm Hg)	57.8
Bicarbonate (mmol/liter)	6.6
Base excess (mmol/liter)	-28.1
Detection of influenza virus antigens from nasal swab specimen^a	
	Negative

^a Conducted at a local hospital.

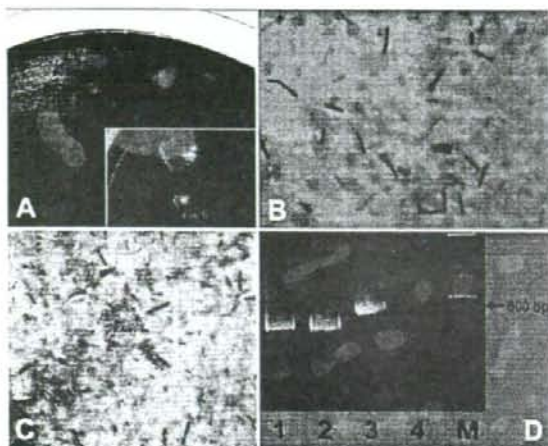


FIG. 2. Microbiological and PCR amplification findings for *Clostridium chauvoei*. (A) Grayish white, rough colonies with beta-hemolysis around bacterial colonies after 48 h of culture on *Brucella* HK RS agar under anaerobic conditions. (B and C) Gram stain morphology and Wirtz-Conklin spore stain, respectively, from growth on *Brucella* HK RS agar (magnification, $\times 1,000$). Gram-positive rods with subterminal endospores were noted. (D) Five percent polyacrylamide gel electrophoresis of PCR products of the 16S–23S rRNA gene intergenic spacer region. Lane 1, patient's isolate; lane 2, *Clostridium chauvoei* ATCC 10092; lane 3, *Clostridium septicum* ATCC 12464; lane 4, negative control; lane M, 100-bp ladder as a molecular marker. A 522-bp amplicon is observed in both lanes 1 and 2.

panied by suspected respiratory infection, an abnormal CRP level, and severe mixed acidosis prompted us to perform a microbiological examination of the needle aspirate specimen from the subcutaneous emphysematous lesions. Finally, the diagnosis of gas gangrene was exactly confirmed by isolation and identification of *C. chauvoei* from the specimen.

C. chauvoei is a common microbe both in soil and in the guts of ruminants. Blackleg usually appears to be a nontraumatic endogenous infection in cattle harboring the organism in their gastrointestinal tracts (2), probably due to ingestion of spores. The spores or trophozoites may translocate from the intestine into humoral circulation and lie dormant in muscle and other tissues until some ectogenic causes induce bacterial proliferation. In sheep, the disease is frequently associated with wounding caused by shearing, castrating, or tail docking (3). Since gas gangrene occurred at the site of blunt trauma in our case, it was likely the result of wound contamination. However, there was no apparent open wound at the contusion area, so the

possibility of a nontraumatic or intrinsic infection could not be excluded. It is also not known whether *C. chauvoei* resides in the intestinal tracts of humans as transitory microbial flora. The patient had suffered from mild diabetes mellitus prior to the onset of gas gangrene, but no obvious colon cancer, a possible means of entrance of microbes, was found on the basis of the abdominal CT scan. It was also unclear whether the high fever and symptoms of upper respiratory tract infections preceding the blunt trauma were associated with the gas gangrene.

In conclusion, we encountered a case of human fulminant gas gangrene caused by *C. chauvoei* that was exactly identified by genetic characterization. Thus, the possibility of *C. chauvoei* should be considered hereafter in human gas gangrene cases diagnosed as caused by *C. septicum*, a species that is difficult to distinguish from *C. chauvoei* by the routine clinical microbiology laboratory tests. *C. chauvoei* may well be recognized as a new zoonotic pathogen.

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Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material

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Objectives: In clinical *Acinetobacter* species, the reduction effects of organic material on bactericidal activities of four major disinfectants were investigated: chlorhexidine gluconate (CHX), benzethonium chloride (BZT), benzalkonium chloride (BZK) and alkyl diaminoethyl glycine hydrochloride (ADH).

Methods: The bactericidal activities of the four disinfectants against 283 strains of *Acinetobacter* species recovered from 97 Japanese hospitals in March 2002 were investigated by four different tests: MIC measurements, MBC measurements, time-killing assays and adaptation assays. Moreover, disinfectant efficacy was examined in the presence of BSA in two tests: MBC measurements and time-killing assays.

Results: No clinical isolates were able to withstand the in-use concentrations of the four disinfectants, although the MIC₉₀ of ADH reached 100 mg/L. Strains for which MICs of at least two disinfectants were higher than MIC₉₀ measured by the broth microdilution method were defined as isolates with 'disinfectant reduced susceptibility (DRS)'. In the presence of 3.0% BSA, the MBCs of BZK, BZT and ADH for DRS isolates rose to 512 and 1024 mg/L, which were about half their in-use concentrations. Moreover, the times for bacterial complete killing were remarkably prolonged in DRS isolates even after a 10 min of exposure to 1000 mg/L of ADH, a half of its in-use concentration. The MICs of CHX for DRS isolates rose to 640 mg/L after repetitive passages in subinhibitory concentrations of CHX.

Conclusions: Given that the bactericidal effects of the four major disinfectants were considerably reduced in the presence of organic material (BSA) and DRS isolates tended to adapt to CHX, continuous surveys of the profiles of susceptibility to disinfectants among clinically isolated *Acinetobacter* species are very necessary from the standpoint of nosocomial infection control.

Keywords: susceptibility profiles, bovine serum albumin, adaptation

Introduction

Acinetobacter species have recently been recognized as one of the major hospital-acquired pathogens that cause opportunistic infections such as pneumonia, urinary tract infections, septicaemia and surgical site infections, particularly in immunocompromised patients^{1,2} accommodated in intensive care units where they commonly undergo invasive medical procedures and tend to receive various broad-spectrum antimicrobial agents.^{3,4} Moreover, *Acinetobacter* species have rapidly developed multi-drug resistance capabilities over the past 10 years, and the

increasing difficulties encountered in the treatment of infections caused by this opportunistic pathogen have become a serious clinical concern.^{5–7} The ability of this microbe to survive long-term in hospital environments even on dry surfaces has also been considered to play a crucial role in hospital-acquired infection.^{8,9}

Biocides, including quaternary ammonium compounds (QACs) and bisbiguanides, have been assiduously used in hospitals and healthcare facilities, and have significantly contributed to maintaining sanitary conditions and preventing hospital-acquired infections.¹⁰ However, concerns have also been raised that widespread use of disinfectants could serve to select disinfectant-insusceptible

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microbes among hospital-acquired pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.^{11,12} Evidence for the development of reduced susceptibility due to exposure to disinfectants has been reported. For example, a steady increase in MICs of chlorhexidine for *P. aeruginosa* has resulted from the exposure to residual disinfectants at subinhibitory concentrations.¹³ It is possible that *Acinetobacter* species may also have developed insusceptibility to disinfectants because of their innate ability to survive long-term on body surfaces and in hospital environments in which various disinfectants have been consumed.

Another cause for concern is that organic materials are known to reduce the effectiveness of disinfectants and antiseptics. Because these agents have been extensively used in medical and healthcare facilities for the disinfection of mucous membranes and wounds, and for the sterilization of medical instruments and equipment surfaces, which often tend to be contaminated with organic materials, the influence of such materials should not be ignored from the viewpoint of practical use of disinfectants. Therefore, it is very important to understand the susceptibility status of *Acinetobacter* species against disinfectants, as well as the influences of organic materials upon reduction in their efficacies. Since little is known about the present susceptibility status of *Acinetobacter* species to disinfectants, the aim of this study was to assess the susceptibility profiles of clinically isolated *Acinetobacter* species, isolated in 2002. For the isolates with 'disinfectant reduced susceptibility (DRS)' selected from clinical isolates, the bactericidal activities of QACs, bisbiguanides and ampholytic detergents used widely in medical facilities were evaluated both with and without the organic materials. In addition, the adaptive resistance to four disinfectants was also investigated to predict the potential development of resistance to disinfectants in *Acinetobacter* species.

Materials and methods

Bacterial strains and culture media

In March 2002, 283 non-repetitive clinical isolates identified as *Acinetobacter* species were collected from 97 hospitals located in different geographical areas of Japan. Since these isolates were speculated to be major causative microbes of infection in each patient, they were subjected to identification and antibiotic-susceptibility tests. They were identified as 273 *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex, 7 *Acinetobacter lwoffii* and 3 *Acinetobacter junii* by their biochemical identification using the API20NE system (bioMérieux Japan, Ltd, Tokyo, Japan) and a complementary test for the ability to grow at 37, 41 and 44°C. After re-identification, each isolate was grown on Luria-Bertani (LB) agar plates or in LB broth (Becton-Dickinson Diagnostic System, Sparks, MD, USA) for further studies.

Disinfectants and susceptibility testing

The disinfectants were obtained from the following sources: chlorhexidine gluconate (CHX), benzethonium chloride (BZT), Wako Pure Chemical Industries, Ltd, Osaka, Japan; benzalkonium chloride (BZK), Kanto Chemical Co., Inc., Tokyo, Japan; alkyl diaminoethyl glycine hydrochloride [ADH; TEGO 51TM, 10% (w/v) solution]. The MICs of the disinfectants were determined by the broth micro-dilution method using Mueller-Hinton broth (Becton-Dickinson

Diagnostic System) according to the protocol recommended by the CLSI (formerly NCCLS) in document M100-S14.¹⁴

Assay of bactericidal activity: quantitative suspension test

The bactericidal effects of disinfectants on *Acinetobacter* species were measured using a slightly modified quantitative suspension test referring to the European Standard EN 1040.¹⁵ The neutralizer solution used in the suspension test contained the following: 10% Tween 80 (v/v), 3% lecithin (w/v), 0.1% histidine (w/v), 0.5% sodium thiosulphate (w/v) and phosphate-buffered saline (PBS, pH 7.4), all obtained from Wako Pure Chemical Industries.

Each isolate was cultivated in LB broth until its optical density (OD) of 0.90 at 660 nm was reached and washed once with PBS (pH 7.4). The bacterial test suspensions were then adjusted to an optical density of 0.08 at 660 nm ($\sim 10^8$ cfu/mL), and bacterial test suspensions were prepared for each strain. A 100 μ L of test suspension was added to tubes containing 900 μ L of disinfectant solutions at different concentrations and left for 3 min at $20 \pm 2^\circ\text{C}$. Since the test results obtained at room temperature demonstrated poor reproducibility, all reactions were performed as much as possible at $20 \pm 2^\circ\text{C}$ in an incubator using bacterial suspensions, disinfectant solutions and neutralizer solutions preincubated at $20 \pm 2^\circ\text{C}$. Aliquots of the reaction mixture (100 μ L) of containing bacterial cells and disinfectant were then added to 900 μ L of neutralizer solution at $20 \pm 2^\circ\text{C}$ for 3 min and serially diluted in PBS (pH 7.4). After dilution, 50 μ L of the mixture was spread immediately onto LB agar plates and incubated for 18 h at 35°C . The numbers of colonies surviving on each plate were counted, and cell survival rates were calculated with those obtained by a test using a bacterial suspension treated with PBS (pH 7.4) instead of disinfectant as the control. BSA (Sigma) was used to imitate organic soiling, thus ensuring that the 'dirty' test simulated practical 'in-use' conditions. The quantitative suspension tests with BSA were performed according to recommendation EN 1276.¹⁶ Each test was repeated to simulate 'dirty' conditions by mixing the test suspension with 0.3% or 3.0% (w/v) BSA solution before adding the 100 μ L of test suspension to the 900 μ L of disinfectant solution. The experiments were repeated three times on different days. In addition, the neutralizer was checked for its possible toxicity for the test organisms. Aliquots of a diluted bacterial suspension (100 μ L) containing $1-3 \times 10^4$ cfu/mL was added to 900 μ L of neutralizer and left at $20 \pm 2^\circ\text{C}$ for 3 min. Two samples of 100 μ L of mixture described above were then spread onto LB agar plates and incubated as above. The values obtained on each plate were 100-300 cfu/100 μ L. Finally, the inactivation of the bactericidal activity of each disinfectant by neutralizer was also validated. Nine hundred microlitres of the solutions containing different concentrations of each disinfectant was added to 100 μ L of the PBS (pH 7.4) and left at $20 \pm 2^\circ\text{C}$ for 3 min. Then, 100 μ L of the mixture was transferred into 800 μ L of neutralizer and left at $20 \pm 2^\circ\text{C}$ for 3 min. Aliquot of a diluted bacterial suspension (100 μ L) containing $1-3 \times 10^4$ cfu/mL was then added and left at $20 \pm 2^\circ\text{C}$ for 3 min. Two samples of 100 μ L mixture, containing disinfectant, neutralizer and bacteria, were then spread onto LB agar plates and incubated as described above. The numbers of colonies grown on each plate ranged from 100 to 300 cfu/100 μ L.

MBC

MBC was determined using the quantitative suspension test described above. The disinfectant solutions at different concentrations were made by serial 2-fold dilutions of each disinfectant.

MBC was defined as the lowest concentration of disinfectants that completely suppressed bacterial growth in each disinfectant.

Measurement of bactericidal activity: time-killing assay

Time-killing assays were performed to evaluate the bactericidal effects of disinfectants using a modified quantitative suspension test referring to the European Standards EN1040 and EN1276. Bacterial cell suspension (100 µL) was added to 900 µL of disinfectant solution and 50 µL aliquots of the mixtures were sampled at 0.5, 1, 2.5, 5, 10, 20, 30, 60, 90 and 120 min, respectively. Each sample was immediately added to 450 µL of neutralizer solution at $20 \pm 2^\circ\text{C}$ and left for 3 min, then serially diluted in PBS (pH 7.4). Fifty microlitres of each diluent was spread onto LB agar plate and incubated for 18 h at 35°C . The numbers of colonies that grew on each plate were counted, and cell survival rates were calculated by similar methods using a bacterial suspension as the control treated with PBS (pH 7.4) instead of the mixture containing disinfectant and neutralizer solution. The test was repeated to simulate 'dirty' conditions using the test suspension containing a 0.3% or 3.0% (w/v) BSA by the same method employed in the MBC measurement. The experiments were repeated three times on different days.

Adaptation to disinfectants

Twelve strains, including five disinfectant-susceptible clinical isolates and seven DRS isolates, strains 1, 2, 4, 5, 6, 7 and 10 shown in Table 2, were subjected to the adaptation test. Five susceptible clinical isolates were randomly selected from the group of strains for which the MICs of all four disinfectants were less than MIC_{50} as shown in Table 1. Aliquots of the overnight culture (100 µL) were added to nutrient broth containing each agent at 1/2 of the MIC for each strain. The 1/2 MIC culture was incubated at 37°C with shaking for 72 h, and bacterial growth was assessed visually. When a culture density of higher than $\sim 1 \times 10^8$ cfu/mL was observed, 100 µL of the 1/2 MIC culture was spread onto nutrient agar plates containing the same concentration of disinfectant, and the agar plates were incubated overnight at 37°C . The colonies grown on each agar plate were selected and cultured for further testing using nutrient broth containing 1/2 MIC disinfectant. An aliquot of cell culture was stored at -80°C as passage 1 (P1), and MICs and MBCs for P1 were re-determined. Any isolates that had shown an increase in MICs and MBCs were then inoculated into the broth

media containing twice the original concentration of disinfectant, and the others not shown augmented MICs and MBCs were inoculated into media containing the disinfectant with the previous concentration. This procedure was repeated four times every 5 days (including 3 days for culture and 2 days for assay) after the selection of P1. If culture density was lower than 1×10^8 cfu/mL, the passage was not advanced to the next step, because no $>5 \log_{10}$ reduction could be detected in this condition. The stabilities of the MICs and MBCs for P5 isolates demonstrating highest disinfectant-reduced susceptible profile were checked four times every 5 days by repetitive culture with disinfectant-free broth. Moreover, the bactericidal activities of each disinfectant for these adapted isolates were evaluated by the quantitative suspension tests. The experiments were repeated three times on different days.

Statistical analysis

Data were analysed using the statistical program SPSS for Windows version 11.0J (SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U*-test was performed to compare bactericidal activities measured by both MBC and time-killing assays. Dunnett's and Bonferroni's multiple comparison tests were performed to determine statistically significant differences between passage groups of the adaptation test. A *P* value of ≤ 0.05 was considered statistically significant.

Results

Susceptibility to disinfectants and selection of DRS isolates

Distributions of MICs of CHX, BZK, BZT and ADH for 283 clinical isolates are shown in Table 1. *Acinetobacter* species tended to be susceptible to CHX, BZK and BZT, and MIC_{90} s obtained by the broth microdilution method in the absence of BSA were ≤ 25 mg/L. However, MICs of ADH were relatively higher than those of the other three disinfectants, and the MIC_{90} of ADH obtained was 100 mg/L (Table 1).

The isolates with DRS possessing relatively high MICs of CHX, BZK, BZT and/or ADH were selected. In the present study, the DRS isolates were defined as those for which MICs of at least two among the four disinfectants were higher than MIC_{90} when measured by the broth microdilution method. As a result, 14 isolates (8 *A. baumannii*, 4 *A. calcoaceticus* and 2

Table 1. Distributions of MICs of various disinfectants by the broth microdilution method

Disinfectant	Number of strains at each MIC (mg/L) of disinfectant								MIC_{50}	MIC_{90}	
	2.5	5	10	25	50	100	200	400			800
CHX	0	126 ^a	80	62	14 ^b	<u>1</u>	0	0	0	10	25
BZK	0	168 ^a	104	<u>9</u>	<u>2</u>	0	0	0	0	5	10
BZT	0	2	196 ^a	68	<u>15</u>	<u>2</u>	0	0	0	10	25
ADH	0	0	1	21	176 ^a	85	0	0	0	50	100

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride.

^aNumbers indicate a group of clinical isolates demonstrating the phenotype susceptible to each disinfectant, and five strains used for adaptation tests were randomly selected in each group.

^bStandard MIC_{50} and MIC_{90} measurements (quantal measurement of 50% and 90% of the population) are given. Underlined numbers indicate number of isolates for which MICs of each disinfectant were higher than MIC_{90} . MIC_{90} of ADH for all isolates was 100 mg/L. Fourteen isolates for which MICs of at least CHX, BZK and BZT were above MIC_{90} were defined as 'isolates with reduced disinfectant susceptibility' and selected as candidates for MBC and time-killing assays.

Reduction in bactericidal activity of disinfectants by BSA

Table 2. Susceptibility profiles of 14 isolates with 'disinfectant-reduced susceptible' properties

Strain number	MIC (mg/L) of disinfectant ^a				Species
	CHX	BZK	BZT	ADH	
1 ^b	50	10	50	100	<i>A. baumannii</i>
2 ^b	25	25	50	50	<i>A. junii</i>
3	25	25	50	100	<i>A. baumannii</i>
4 ^b	50	25	50	50	<i>A. baumannii</i>
5 ^b	25	25	100	100	<i>A. baumannii</i>
6 ^b	25	25	100	100	<i>A. baumannii</i>
7 ^b	50	50	50	100	<i>A. calcoaceticus</i>
8	50	10	50	100	<i>A. baumannii</i>
9	50	10	50	100	<i>A. baumannii</i>
10 ^b	100	25	50	100	<i>A. calcoaceticus</i>
11	10	50	50	50	<i>A. junii</i>
12	25	25	50	100	<i>A. baumannii</i>
13 ^c	50	10	50	100	<i>A. calcoaceticus</i>
14 ^c	50	10	50	100	<i>A. calcoaceticus</i>

^aMICs of disinfectants were determined using the broth microdilution method.

^bStrains 1, 2, 4, 5, 6, 7 and 10 were selected for adaptation tests.

^cStrains 13 and 14 were isolated from different patients in the same hospital.

A. junii) out of 283 were provisionally defined as DRS isolates in the present study (Table 2).

MBCs of four disinfectants

To evaluate the phenotypes of DRS isolates selected, the MBC and bactericidal activity of the four disinfectants were measured under both the so-called 'clean' and 'dirty' conditions. Figure 1 shows cell survival rates of DRS isolates after exposure to CHX, BZK, BZT and ADH, with concentrations ranging from 1 to 1024 mg/L. The MBC values of the four disinfectants in the presence of BSA were higher than those in the absence of BSA, and MBC values of BZK, BZT and ADH obtained by the addition of 3.0% BSA showed high values (512 and 1024 mg/L), which were about half of the in-use concentration of these agents (Figure 1).

Time-killing assay

Time-killing assays were also performed to evaluate the bactericidal effects of the four disinfectants on DRS isolates from the viewpoint of exposure duration. As can be seen in Figure 2, at low concentrations (MIC₅₀ of each disinfectant obtained by the broth microdilution method), the bacterial cell count in the absence of BSA reached a 5 log₁₀ reduction after 10 min. On the other hand, the presence of 0.3% BSA simulating the 'dirty' condition elevated the cell survival rate, and, even after 120 min of exposure, reduction of bacterial cells was less than 1 log₁₀ under the test conditions employed for BZK, BZT and ADH.

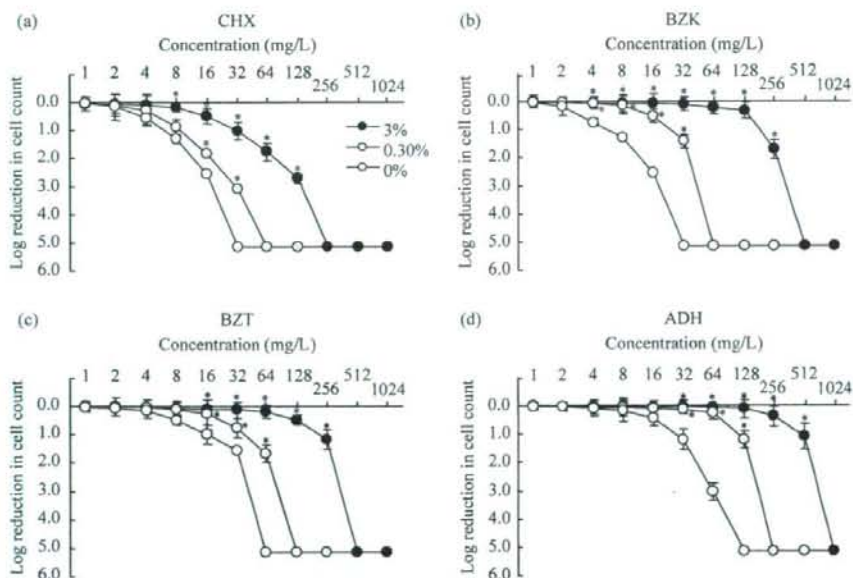


Figure 1. Comparison of MBC. Fourteen isolates for which MICs of at least two disinfectants were more than MIC₉₀ measured by the broth microdilution method were defined as isolates with DRS. MBCs of the four disinfectants tested on the DRS isolates are shown. Results are expressed as log₁₀ reduction in cell counts compared with those of the control sample treated with PBS. The viable cell count before the exposure to disinfectant was $2.21 \pm 0.323 \times 10^7$ cfu/mL. White circles, 'clean' condition (0% BSA); grey circles, 'dirty' condition (0.3% BSA); black circles, 'dirty' condition (3% BSA). Error bars represent standard deviations of results from three experiments, and the asterisks indicate a significant difference ($*P < 0.05$, as determined by Mann-Whitney *U*-test). (a) Chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH).

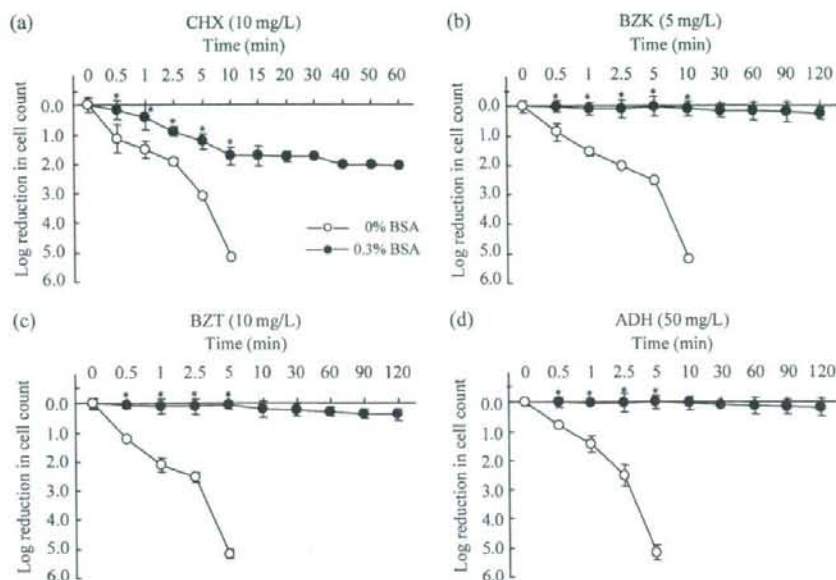


Figure 2. Results of time-killing assays. Time-kill assays were performed for the 14 DRS isolates selected in the same manner as in the MBC assay. The concentration of each disinfectant is as follows: (a) chlorhexidine gluconate (CHX), 10 mg/L; (b) benzalkonium chloride (BZK), 5 mg/L; (c) benzethonium chloride (BZT), 10 mg/L; (d) alkyl diaminoethyl glycine hydrochloride (ADH), 50 mg/L. Bacterial cells were treated with each disinfectant at $20 \pm 2^\circ\text{C}$. Each bacterial test sample was removed at 0.5, 1, 2.5, 5, 10, 20, 30, 60, 90 and 120 min, respectively. Cell viabilities were determined by plating serially diluted cell suspensions on LB plates. Results are expressed as log₁₀ reduction in cell counts compared with those of control sample treated with PBS. The viable cell count before the exposure to disinfectant was $2.60 \pm 0.530 \times 10^7$ cfu/mL. White circles, 'clean' condition (0% BSA); black circles, 'dirty' condition (0.3% BSA). Error bars represent standard deviations of results from three experiments, and the asterisks indicate a significant difference (* $P < 0.05$, as determined by Mann-Whitney U -test).

Indeed, higher concentrations of disinfectants were reasonably more effective, especially under 'clean' conditions (Table 3). However, the bacterial cell numbers failed to show a 5 log₁₀ reduction after a 1 min of exposure to all four disinfectants at 1/5 of the in-use concentrations in the presence of 3.0% BSA. As for ADH, bacterial cells survived even after a 10 min of exposure to 1000 mg/L, a half of the in-use concentration, with 3.0% BSA (Table 3).

Adaptation to disinfectants

Five susceptible isolates and 7 DRS isolates selected from 283 isolates were subjected to an adaptation test. The adaptation profiles of these isolates are shown in Figure 3. For five susceptible strains, the MICs of BZK, BZT and ADH were not significantly elevated after repetitive passages through the broth media containing each disinfectant. These five susceptible isolates were most susceptible to CHX among the four disinfectants, but these isolates could adapt to only 2-fold higher concentrations of CHX after five passages. The MICs of BZK, BZT and ADH for DRS isolates were only slightly elevated (<2-fold) during five passages. However, MICs of CHX were significantly elevated ~10-fold (up to 640 mg/L) after the repeated passages. Similar findings were observed in the elevation of MBC values throughout the experimental process of passage as were seen in MIC (data not shown). The stability of disinfectant-reduced susceptible profile among strains demonstrating the highest adaptation ability was checked by culturing for 20 days in disinfectant-free

broth. In some cases, the MIC or MBC of CHX for the adapted strains decreased approximately to 50%; however, no case declined to the level of parent strains (data not shown). Time-killing assays by suspension test were also performed to evaluate the bactericidal effect of CHX on both DRS and adapted isolates obtained through each passage process (Table 4). No considerable changes in the bactericidal activity of BZK, BZT and ADH for adapted isolates obtained after passage in CHX were observed (data not shown). On the other hand, an apparent elevation of the resistance level to CHX in the adapted isolates was observed by suspension test. As can be seen in Table 4, the bacterial cell counts of both DRS and adapted isolates showed a 5 log₁₀ reduction after a 1 min of exposure to 5000 mg/L CHX, that is, its in-use concentration. However, in P4 and P5 strains adapted for disinfectants, a 5 log₁₀ reduction of bacterial cells failed after a 1 min of exposure to 1000 mg/L CHX, 1/5 of its in-use concentration (Table 4).

Discussion

In healthcare settings, it has become more difficult to treat infections caused by *Acinetobacter* species because of their acquisition of consistent resistance to major groups of antimicrobial agents.⁵⁻⁷ Difficulties in the infection control practices as well as in chemotherapy of infectious diseases are due to the intrinsic capacities of *Acinetobacter* species for long-term survival in

Reduction in bactericidal activity of disinfectants by BSA

Table 3. Bactericidal effects of four disinfectants on 'disinfectant reduced-susceptible' isolates

Condition and exposure time	Reduction in live bacterial cell numbers ^a							
	CHX (mg/L)				BZK (mg/L)			
	5000 ^b	2500	1000	500	1000 ^b	500	200	100
0% BSA, 1 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23
0.3% BSA, 1 min	>5.23	>5.23	>5.23	4.86 ± 0.08	>5.23	>5.23	>5.23	4.55 ± 0.09
3.0% BSA, 1 min	>5.23	>5.23	4.86 ± 0.09	3.89 ± 0.10	>5.23	>5.23	2.86 ± 0.16 ^c	2.50 ± 0.10
3.0% BSA, 10 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	4.16 ± 0.09	2.53 ± 0.10
	BZT (mg/L)				ADH (mg/L)			
	2000 ^b	1000	400	200	2000 ^b	1000	400	200
0% BSA, 1 min	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23	>5.23
0.3% BSA, 1 min	>5.23	>5.23	>5.23	4.35 ± 0.09	>5.23	>5.23	3.82 ± 0.10	3.22 ± 0.14
3.0% BSA, 1 min	>5.23	>5.23	2.77 ± 0.16 ^c	2.53 ± 0.12	>5.23	2.82 ± 0.14 ^c	2.15 ± 0.14	2.06 ± 0.18
3.0% BSA, 10 min	>5.23	>5.23	3.85 ± 0.12	2.72 ± 0.10	>5.23	4.89 ± 0.14	2.27 ± 0.21	2.32 ± 0.13

CHX, chlorhexidine gluconate; BZK, benzalkonium chloride; BZT, benzethonium chloride; ADH, alkyl diaminoethyl glycine hydrochloride.

The bacterial cell concentration exposed to each disinfectant was $3.40 \pm 0.81 \times 10^7$ cfu/mL.

^aShown as log₁₀ reduction in bacterial cell number in both 'clean' (0% BSA) and 'dirty' (0.3% and 3% BSA) conditions; mean ± SD.

^bNumbers indicate 'in-use' concentration (mg/L) of each disinfectant.

^cDisinfectants failed to provide a 5 log₁₀ reduction in live bacterial cell counts in the presence of BSA.

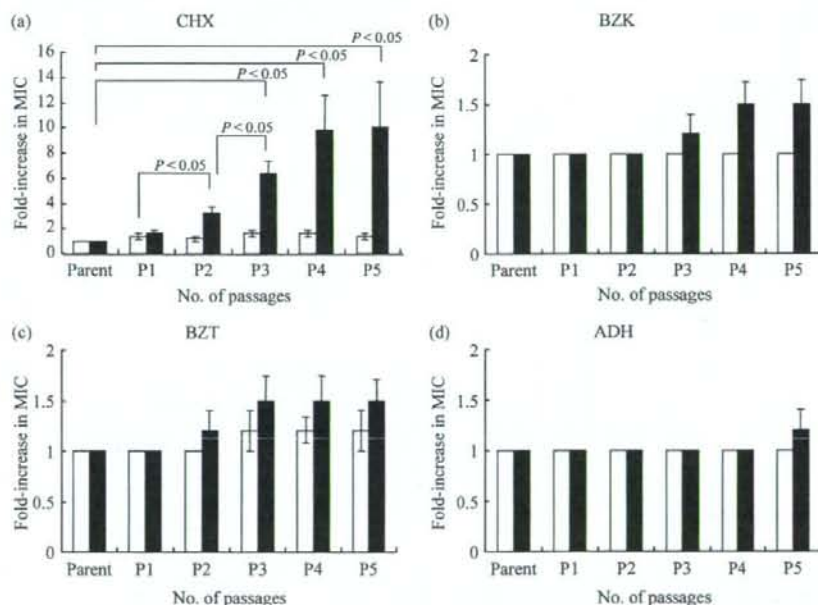


Figure 3. Adaptation to four disinfectants after persistent passages. Adaptations to disinfectants were generated by repeated subculture in nutrient broths containing each disinfectant (a) chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl glycine hydrochloride (ADH). Five disinfectant-susceptible clinical isolates selected randomly and seven DRS isolates (strains 1, 2, 4, 5, 6, 7 and 10 shown in Table 2) were subjected to this test. The 1/2 MIC cultures were incubated at 37°C with shaking for 72 h, and growth was assessed visually for cultures grown in media containing disinfectants. MICs were checked, and any isolates that showed an increase in MICs were then inoculated into media containing twice the original concentration. This procedure was repeated a total of five times every 5 days (including 3 days for culture and 2 days for assay). Stabilities of adaptive resistances of most resistant bacterial cells (P5) were determined by further passages through disinfectant-free broths and checked four times every 5 days. The experiments were performed three times on different days. White bars, five disinfectant-susceptible isolates selected; black bars, seven DRS isolates. Error bars represent standard deviations, and a significant difference is indicated by $P < 0.05$ (as determined by Dunnett's and Bonferroni's multiple-comparison tests).

Table 4. Bactericidal activity of CHX against adapted isolates with reduced susceptibility to CHX

Passage ^a	Bacterial cells exposed to disinfectant ^b	CHX (5000 mg/L) ^c					
		exposure time (30 s)		exposure time (60 s)		exposure time (180 s)	
		viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction
Parent	5.36 ± 1.20 × 10 ⁷	NC	>5.43	NC	>5.43	NC	>5.43
P1	4.43 ± 1.73 × 10 ⁷	NC	>5.34	NC	>5.34	NC	>5.34
P2	8.00 ± 1.00 × 10 ⁷	NC	>5.60	NC	>5.60	NC	>5.60
P3	8.85 ± 2.06 × 10 ⁷	NC	>5.65	NC	>5.65	NC	>5.65
P4	6.34 ± 1.73 × 10 ⁷	NC	>5.50	NC	>5.50	NC	>5.50
P5	3.37 ± 0.70 × 10 ⁷	NC	>5.23	NC	>5.23	NC	>5.23
		CHX (1000 mg/L) ^c					
		exposure time (30 s)		exposure time (60 s)		exposure time (180 s)	
		viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction
Parent	5.36 ± 1.20 × 10 ⁷	NC	>5.43	NC	>5.43	NC	>5.43
P1	4.43 ± 1.73 × 10 ⁷	2.54 ± 1.05 × 10 ²	5.24	NC	>5.34	NC	>5.34
P2	8.00 ± 1.00 × 10 ⁷	5.54 ± 1.25 × 10 ²	5.16	NC	>5.60	NC	>5.60
P3	8.85 ± 2.06 × 10 ⁷	3.10 ± 1.43 × 10 ³	4.46	NC	>5.65	NC	>5.65
P4	6.34 ± 1.73 × 10 ⁷	1.88 ± 0.88 × 10 ³	4.53	9.80 ± 0.78 × 10 ^{1e}	5.50	NC	>5.50
P5	3.37 ± 0.70 × 10 ⁷	3.64 ± 0.89 × 10 ³	3.97	3.60 ± 0.68 × 10 ²	4.97	NC	>5.23
		CHX (500 mg/L) ^c					
		exposure time (30 s)		exposure time (60 s)		exposure time (180 s)	
		viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction	viable cell concentration ^d	log ₁₀ reduction
Parent	5.36 ± 1.20 × 10 ⁷	2.26 ± 0.99 × 10 ³	4.38	NC	>5.43	NC	>5.43
P1	4.43 ± 1.73 × 10 ⁷	2.50 ± 1.21 × 10 ³	4.25	NC	>5.34	NC	>5.34
P2	8.00 ± 1.00 × 10 ⁷	4.68 ± 1.31 × 10 ³	4.23	NC	>5.60	NC	>5.60
P3	8.85 ± 2.06 × 10 ⁷	5.46 ± 1.56 × 10 ³	4.21	2.80 ± 0.92 × 10 ²	5.28	NC	>5.65
P4	6.34 ± 1.73 × 10 ⁷	4.75 ± 0.65 × 10 ³	4.12	4.37 ± 1.31 × 10 ²	5.31	NC	>5.50
P5	3.37 ± 0.70 × 10 ⁷	2.56 ± 0.41 × 10 ⁴	3.12	3.60 ± 0.68 × 10 ²	3.99	2.71 ± 1.65 × 10 ²	5.09

CHX, chlorhexidine gluconate.

For seven DRS isolates, the experiments were repeated three times on different days. First, the viable cell counts of each isolate were calculated by the average of three test results performed on different days, and then average and distribution of the viable cell counts of the seven DRS isolates were again calculated.

NC indicates negative culture. Viable cell counts were estimated to be <2 × 10² cfu/mL in all three tests for seven DRS isolates selected.

^bStrains were obtained throughout adaptation process passaged in increasing concentrations of CHX as shown in Figure 3.

^cAverage viable cell concentration (cfu/mL ± SE) measured in PBS instead of disinfectant solution.

^dConcentration of CHX to which bacterial cells were exposed before inoculation onto agar plate for counting surviving bacterial colonies.

^eAverage viable cell concentration (cfu/mL ± SE) after the exposure to disinfectant.

^fSince no colonies grew on several plates, viable cell count became below the detection limit after the statistical analysis.

Reduction in bactericidal activity of disinfectants by BSA

hospital environments and transmission among patients. Therefore, performance of contact-precautions including hand-hygiene and disinfection is crucial to interrupt patient-to-patient transmission of this microbe. Indeed numerous studies have focused on the issues of *Acinetobacter* species resistant to antimicrobial agents,^{17,18} but only a few have so far explored the susceptibility profiles to antiseptics and disinfectants in this microbe. Thus, investigations on the susceptibility status of clinically isolated *Acinetobacter* species' susceptibility to antiseptics and disinfectants assessed by four different test methods, MIC measurements, MBC measurements by quantitative suspension tests, time-killing assays and adaptation tests, would provide instructive new insights into coping with *Acinetobacter* species in various healthcare settings.

In the present study, the MIC₉₀s of the four disinfectants tested for 283 *Acinetobacter* species isolates were ≤ 100 mg/L, which is lower than the actual in-use concentration of each disinfectant. Moreover, the 14 DRS isolates selected were also confirmed to be susceptible to disinfectants by multiple tests, including MBC measurement, and time-killing assay, in the absence of organic materials. The results are consistent with those of Martró *et al.*¹⁹ and Wisplinghoff *et al.*²⁰ who found no apparent development of resistance to disinfectants among clinically isolated *Acinetobacter* species. They assessed the susceptibility of *A. baumannii* to respective disinfectants and antiseptics by suspension test without adding organic materials. However, since *Acinetobacter* species inhabit hospital environments often contaminated with a variety of organic materials and colonize various body sites of patients, one must never fail to take into account the reduction effects of organic materials on antiseptics and disinfectants in practical use. Therefore, we further extended their studies to evaluate the properties of DRS isolates against antiseptics and disinfectants by MBC measurements and time-killing assays in the presence of BSA. The 10 min of exposures of DRS isolates to CHX at much lower than its in-use concentration in the presence of 3.0% BSA provided a 5 log₁₀ reduction in bacterial cell numbers, whereas 10 min of exposure of these isolates to 200 mg/L BZK or 400 mg/L BZT failed to produce a 5 log₁₀ reduction of bacterial cells in the presence of 3.0% of BSA. In addition, the 10 min of exposure to 1000 mg/L ADH, half of its in-use concentration, failed to eliminate the live bacterial cells in the presence of 3.0% BSA. This finding is crucial, because ADH is one of the most frequently used disinfectants for medical instruments and hospital environments.

Acinetobacter species usually cause hospital-acquired infections, including urinary- and respiratory-tract infections, and particularly ventilator-associated pneumonia, especially in debilitated individuals.^{1,2,21} Indeed, no apparent resistance properties of these DRS isolates against disinfectants were observed from the viewpoints of MIC and MBC measurements in the absence of organic materials, but the results obtained by the suspension test in the presence of BSA suggested that these DRS isolates may well survive in conditions of contamination by organic materials such as blood and exudation. Thus, care should be taken in monitoring the susceptibility profile of *Acinetobacter* species against disinfectants, especially when this microbe is frequently or continuously isolated from clinical samples.

To our knowledge, no adaptive resistance to disinfectants in strains belonging to *Acinetobacter* species has been reported to date. Our results demonstrate that repeated exposure to

subinhibitory concentrations of CHX gradually elevated its MIC (at most 10-fold, up to 640 mg/L) for the DRS isolates. Furthermore, P4 and P5 strains obtained after several passages in 1/2 MICs of CHX survived after a 1 min of exposure to 1000 mg/L CHX, 20% of its in-use concentration (0.5% = 5000 mg/L), in suspension test. CHX has been demonstrated to have a persistent or residual effect after applications to skin²² and mucous membranes.²³

Izarry *et al.*²⁴ suggested that environmental residues of CHX and cetylpyridinium chloride might confer some selective advantage on MRSA, an organism that has also been known as relatively resistant to CHX on dry surfaces. A recent study by Thomas *et al.*¹³ gives some experimental support to the idea that repetitive exposure to subinhibitory concentrations of CHX results in a stable increase in MICs of CHX for *P. aeruginosa*. *Acinetobacter* species have also become very common in hospital environments as well as MRSA and *P. aeruginosa* known as the major hospital-acquired pathogens. Our results suggest that the DRS isolates, which have been exposed to some extent to subinhibitory amounts of disinfectants remaining on environmental surfaces or even on the skin, would also develop the properties of adaptive resistance to CHX. It would therefore be necessary to carefully screen and select appropriate disinfectants based on a sufficient understanding of each for use in medical facilities.

In conclusion, no resistance to CHX, BZK, BZT and ADH was detected among clinically isolated *Acinetobacter* species by MIC measurements. However, the bactericidal effects of BZK, BZT and ADH, especially on the DRS isolates, were remarkably reduced in the presence of an organic material (3% BSA). Furthermore, the DRS isolates tended to adapt a higher concentration of CHX after repetitive passages in 1/2 MIC concentrations of CHX. To prevent hospital-acquired infections caused by this kind of microbe, the profile of susceptibility to disinfectants, as well as to antimicrobial agents, must be carefully monitored and checked among *Acinetobacter* species isolated from both clinical specimens and environments. Disinfectants are indispensable to perform appropriate infection control. Hence, this study highlights the need to ensure that these agents are being used appropriately in practice at the correct concentrations and for adequate contact times.

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Transparency declarations

None to declare.



SHORT COMMUNICATION

Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan

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KEYWORDS

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Summary Recently, the incidence of reported pertussis cases of adults has dramatically increased in Japan. In the present study, we analyzed seven *Bordetella pertussis* isolates recovered from adults in Japan using pulsed-field gel electrophoresis (PFGE) and sequencing of their antigenic and virulence-associated proteins, compared with those from children. PFGE analysis demonstrated that the adult strains were closely related to the child strains (78–100% genetic similarity). On the other hand, the genotyping revealed that 71% (5/7) of the adult strains and 47% (25/53) of the child strains had the same combination of antigenic/virulence-associated allelic variants (*ptxS1B/prn1/fim2-1/fim3A/fhaB1/tcfA2*) as the Japanese vaccine strain Tohama, respectively. In comparison to the child strains, there was no apparent antigenic and genetic shift in the adult strains. Our result suggests that (i) there is no *B. pertussis* circulating strain specific to adults and (ii) the antigenic/virulence-associated proteins are unrelated to the rise in adult pertussis incidence in Japan.

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Introduction

Bordetella pertussis, a highly communicable gram-negative coccobacillus, causes whooping cough which has been a major acute respiratory infection resulting in severe child-

hood illness and infant death [1]. Although, childhood pertussis immunization programs have been contributing to the reduction in morbidity and mortality in infants and children, the incidence of the disease in the U.S. has increased over the past 20 years, most notably in previously immunized adults and adolescents [2–4]. Recently, pertussis adults/adolescents are thought to be the primary reservoir of *B. pertussis* and play a crucial role in the transmission of the microbe to children, especially unvaccinated young infants [5–7]. In Japan, the incidence of reported pertussis cases of adults has dramatically increased from

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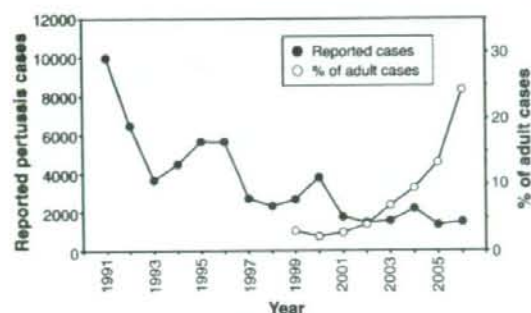


Figure 1 Reported pertussis cases and % of adult cases (≥ 20 years old) in Japan. The data were obtained from Infectious Disease Surveillance data of the Ministry Health, Labor and Welfare of Japan. The adult cases before 1998 were not monitored.

2002 to the present, although there has been no significant change in the diagnostic method. Based on the Infectious Disease Surveillance data of the Ministry of Health, Labor and Welfare of Japan, the pertussis incidence rates of adults (≥ 20 years old) were 4.0, 6.9, 9.5, 13.4 and 24.3% of the total reported cases in 2002–2006, respectively (Fig. 1). Although, high attack rates of pertussis have been observed in adults/adolescents even in the high vaccinating countries, it is not clear whether the adult/adolescent pertussis and *B. pertussis* recently circulating strain are associated with each other.

During the last decade, antigenic and genetic divergences in *B. pertussis* circulating strains have been observed in many countries [8–16]. The antigenic variants have been found in the S1 subunit of pertussis toxin (*ptxS1*) and pertactin (*prn*), which are important virulence factors of *B. pertussis*. In the United States, Canada, Russia and European countries, vaccine-types *ptxS1B* and *prn1* alleles of circulating strains have been replaced mainly with nonvaccine-types *ptxS1A* and *prn2*, respectively [8, 9, 14]. In Japan, the antigenic shifts have been observed from the mid-1990s, and two *ptxS1* (*ptxS1A* and *ptxS1B*) and 4 *prn* (*prn1*, *prn2*, *prn3* and *prn12*) allelic variants have been identified so far [17, 18]. The novel variant, *prn12*, was found in one isolate collected in 1991 (GenBank accession no AB278117). Besides the antigenic shifts, the genetic divergence of *B. pertussis* virulence factors, tracheal colonization factor (*tcfA*), fila-

mentous hemagglutinin (*fhaB*) and serotype 2 and 3 fimbriae (*fim2* and *fim3*), has also been found in several countries [13, 19–21]. It has been suggested that the shifts in *B. pertussis* recently circulating strains have been induced as a vaccine-driven evolution [9, 11]. However, it is unclear whether or not the divergence in the antigenic/virulence-associated proteins is indeed implicated in the increase of the adult/adolescent pertussis patients.

The aims of the present study were (i) to determine the genetic similarity in *B. pertussis* isolates recovered from adults and children by pulsed-field gel electrophoresis (PFGE) analysis, and (ii) to investigate the divergence in the antigenic/virulence-associated allelic variants in the adult strains and to compare this with the child strains. We also wanted to investigate the relationship between the allelic variation and the adult pertussis.

Materials and methods

Isolates

Seven and 53 *B. pertussis* clinical isolates recovered from adults (≥ 20 years old) and children [0–11 year(s) old], respectively, were investigated in the present study. All of the isolates were collected from nonepidemiologically related cases of pertussis during the period of 1991–2007 in Japan. The number of available adult strains from the laboratory collection of the National Institute of Infectious Diseases (NIID, Japan) was 7 due to the difficulty in isolating the organism from previously immunized persons, whereas the 53 child strains were randomly selected from the laboratory collections so as to reflect the same temporal distribution. Among the adult strains, three (BP173, BP183 and BP313) were recovered from individual familial infection cases, but the clinical associations of the others (BP241, BP239, BP245 and BP316) were unknown (Table 1). The isolates were cultured on Bordet-Gengou agar (Difco) supplemented with 1% glycerol and 15% defibrinated horse blood and incubated at 37 °C for 2–3 days.

PFGE analysis

PFGE was performed on a CHEF Mapper apparatus (Bio-Rad, Hercules, CA, USA) according to standardized recommenda-

Table 1 *Bordetella pertussis* isolates recovered from adults in Japan

Isolate	Isolation year	PFGE type	MLST	Patient		
				Age	Sex	Infection route
BP173	1991	A	MLST-1	32	Female	Household contact
BP183	1991	A	MLST-1	>20 ^a	Male	Household contact
BP241	2003	A	MLST-1	27	Male	NA ^b
BP239	2004	B	MLST-2	38	Female	NA
BP245	2004	A	MLST-1	73	Female	NA
BP313	2006	A	MLST-1	58	Female	Household contact
BP316	2007	A	MLST-3	53	Female	NA

^a Precise age unknown.

^b Data not available.

tions for typing of *B. pertussis* [22]. The run condition was selected by the autoalgorithm mode of the system with a size range of 20–300 kb. The PFGE patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using Diversity Database version 1.1 software (PDI, Inc).

Genotyping

PtxS1 and *prn* of *B. pertussis* isolates were genotyped as described previously [18]. For *fim2*, *fim3* and *tcfA*, the complete open reading frames were sequenced [21]. On the other hand, *fhaB* was sequenced between positions 2250 to 2750, which contains the single polymorphic site identified previously [21]. Sequence reactions were carried out with BigDye terminator v.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and the products were sequenced on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Multilocus sequence typing (MLST)

Among 60 *B. pertussis* isolates tested, allelic variation was observed only in the three genes, *ptxS1*, *prn* and *fim3*. These combined allelic profiles were used to define MLSTs.

Statistic analysis

The chi-square test was employed. A value of $P < 0.05$ was considered to be significant.

Results

Genetic relationship between *B. pertussis* adult and child strains

To investigate the genetic relationship between adult and child strains, their PFGE profiles were analyzed in 7 and 53 clinical isolates recovered from adults and children, respectively. As shown in Fig. 2, 36 PFGE profiles were identified among the adult and child strains, and the profiles were classified into two major groups (type A and B) using UPGMA. The adult strains showed 6 different PFGE profiles, but the strains could be classified into the two groups; 6 isolates (BP173, BP183, BP241, BP245, BP313 and BP316) were type A and 1 isolate (BP239) was type B (Table 1 and Fig. 2). Of the 7 adult strains, 2 isolates (BP183 and BP316) had the same PFGE profile and 3 isolates (BP183, BP313 and BP316) had PFGE profiles to specific adults. However, the phylogenetic analysis of PFGE profiles demonstrated that all of the adult strains were closely related to the child strains (78–100% genetic similarity).

Distribution of antigenic and virulence-associated allelic variants in *B. pertussis* adult and child strains

To identify the antigenic/virulence-associated allelic variants in the adult strains, we carried out sequencing analyses of *ptxS1*, *prn*, *fim2*, *fim3*, *fhaB* and *tcfA* genes. Table 2 shows

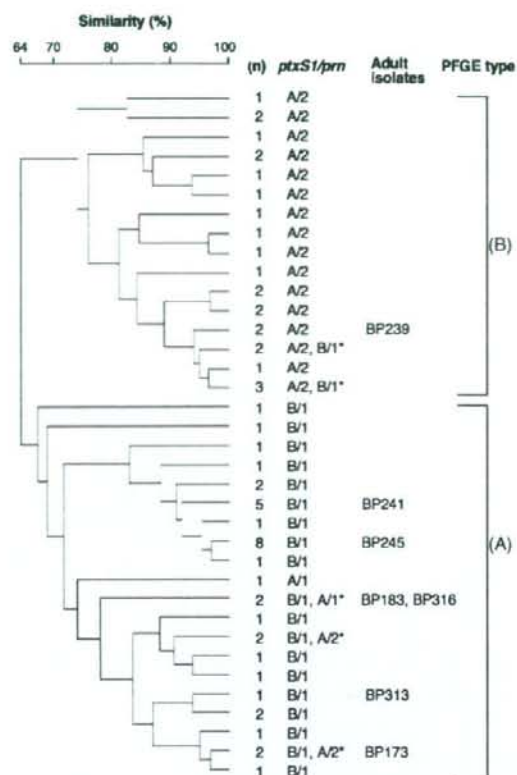


Figure 2 Phylogenetic tree of PFGE profiles of *Bordetella pertussis* isolates recovered from adults and children. The dendrogram was calculated by UPGMA. The adult isolates are indicated by their strain number (BP173, BP183, BP239, BP241, BP245, BP313 and BP316). The regions including the *ptxS1* and *prn* repeat were sequenced, and the combination of *ptxS1* and *prn* alleles is shown as *ptxS1/prn*, e.g., B/1 indicates *ptxS1B/prn1*. * One isolate had different *ptxS1/prn* alleles in the PFGE profile: BP173, B/1; BP183, B/1; BP316, A/1.

the distribution of the antigenic/virulence-associated allelic variants between the adult and child strains. Among the adult strains, *ptxS1B* and *ptxS1A* were detected in 71.4% ($n=5$) and 28.6% ($n=2$) of the isolates, respectively, and *prn1* and *prn2* in 85.7% ($n=6$) and 14.3% ($n=1$), respectively. For *fim3* gene, only *fim3A* was detected in the adult strains. On the other hand, among the child strains, *ptxS1B* and *ptxS1A* were detected in 54.7% ($n=29$) and 45.3% ($n=24$) of the isolates, *prn1* and *prn2* in 56.6% ($n=30$) and 43.4% ($n=23$), and *fim3A* and *fim3B* in 84.9% ($n=45$) and 15.1% ($n=8$), respectively. The distributions of *ptxS1*, *prn* and *fim3* alleles between the adult and child strains were not statistically significant ($P=0.42$, 0.14, and 0.27, respectively), although the number of adult strains was somewhat low for meaningful analysis. Interestingly, *fim3B* was first observed in 1994 in Canada and then in Taiwan and Russia [8, 19, 20], whereas the variant was first identified from 2004 in Japan.

Table 2 Comparison of *Bordetella pertussis* antigenic and virulence-associated allelic variants between adult strains and child strains

Gene	Allele	Type ^a	% Frequency (n)	
			Adult strain (n=7)	Child strain (n=53)
<i>ptxS1</i>	<i>ptxS1B</i>	Vaccine	71.4 (5)	54.7 (29)
	<i>ptxS1A</i>	Nonvaccine	28.6 (2)	45.3 (24)
<i>prn</i>	<i>prn1</i>	Vaccine	85.7 (6)	56.6 (30)
	<i>prn2</i>	Nonvaccine	14.3 (1)	43.4 (23)
<i>fim2</i>	<i>fim2-1</i>	Vaccine	100 (7)	100 (53)
<i>fim3</i>	<i>fim3A</i>	Vaccine	100 (7)	84.9 (45)
	<i>fim3B</i>	Nonvaccine		15.1 (8)
<i>fhaB</i>	<i>fhaB1</i>	Vaccine	100 (7)	100 (53)
<i>tcfA</i>	<i>tcfA2</i>	Vaccine	100 (7)	100 (53)

^a The Japanese vaccine strain Tohama harbors *ptxS1B*, *prn1*, *fim2-1*, *fim3A*, *fhaB1* and *tcfA2* alleles.

For *fim2*, *fhaB* and *tcfA* genes, each variant (*fim2-1*, *fhaB1* and *tcfA2*) was identified among the adult and child strains. There was no allelic variation in *fim3*, *fhaB* and *tcfA*. The Japanese vaccine strain Tohama harbored *ptxS1B*, *prn1*, *fim2-1*, *fim3A*, *fhaB1* and *tcfA2* alleles.

MLSTs of *B. pertussis* adult and child strains

Three genes (*ptxS1*, *prn* and *fim3*) were used to define MLSTs. Among 60 *B. pertussis* isolates tested, five MLSTs (MLST-1 to -5) were identified by the combined allelic profiles (Table 3). Three (MLST-1 to -3) and 5 MLSTs (MLST-1 to -5) were found in the adult and child strains, respectively. MLST-1 was predominant in both strains. Five (71.4%) of 7 adult strains and 25 (47.2%) of 53 child strains were MLST-1, respectively. Among the adult strains, MLST-1 strains were found from 1991 to 2006, and MLST-2 and MLST-3 in 2004 and 2007, respectively (Table 1). The differences in frequencies of the MLSTs between adult and child strains were not statistically significant ($P=0.28$). The Japanese vaccine strain Tohama was MLST-1.

Table 3 MLST frequencies between *Bordetella pertussis* adult strains and child strains

MLST	Alleles	Type ^a	% Frequency (n)	
			Adult strain (n=7)	Child strain (n=53)
MLST-1	<i>ptxS1B/prn1/fim3A</i>	Vaccine	71.4 (5)	47.2 (25)
MLST-2	<i>ptxS1A/prn2/fim3A</i>		14.3 (1)	35.8 (19)
MLST-3	<i>ptxS1A/prn1/fim3A</i>		14.3 (1)	1.9 (1)
MLST-4	<i>ptxS1A/prn2/fim3B</i>			7.5 (4)
MLST-5	<i>ptxS1B/prn1/fim3B</i>			7.5 (4)

^a The Japanese vaccine strain Tohama is MLST-1.

Discussion

In the present study, we demonstrated that (i) PFGE profiles of adult strains were closely related to those of child strains (78–100% genetic similarity), and (ii) there were no significant differences in the distribution of the antigenic/virulence-associated allelic variants between the adult and child strains. These results indicate that *B. pertussis* adult strains are indistinguishable from the child strains by molecular typing methods. To estimate the possibility of acquisition of novel virulence factor(s) in adult strains, we also performed a comparative proteomic analysis of the adult and child strains. However, no apparent difference was observed in their protein profiles (data not shown). Our observations strongly suggest that there is no *B. pertussis* circulating strain specific to adults, and that the circulating strains have an ability to infect not only children but also adults.

Previously, De Schutter et al. [23] reported that Belgian *B. pertussis* adult strains, recovered from familial infections in the period of 1999–2002, harbored nonvaccine-types *ptxS1A* and *prn2* (or *prn3*) alleles. However, in European countries and the United States, the genetic shifts observed in *ptxS1* and *prn* alleles had already occurred since the 1990s [8, 9, 14]. The most circulating strains therefore harbored *ptxS1A* and *prn2* (or *prn3*) alleles in those countries. In contrast, approximately half of the Japanese circulating strains, collected in the period of 1991–2007, harbored vaccine-types *ptxS1B* and *prn1* alleles, indicating that the shifts have now progressed in Japan (this study). Therefore, the present study showed the actual relationship between the antigenic shift and *B. pertussis* adult strains, i.e., there was no apparent antigenic shift in the adult strains. In a previous report, Mastrantonio et al. [24] demonstrated that the frequency of vaccine-type *prn1* allele in strains recovered from unvaccinated children was higher than that of vaccinated children. They suggested that *B. pertussis* strains harboring *prn1* allele might be more affected by vaccine-induced immunity than the strains harboring nonvaccine-type *prn2* or *prn3* allele. However, in the present study, no such difference in the distribution of *prn* allelic variants was observed between adult and child strains.

In Japan, the acellular pertussis vaccines introduced in 1981 have successfully controlled pertussis in children. However, the incidence of reported pertussis cases of adults has considerably increased from 2002 to the present. The waning of vaccine-acquired immunity and decreased oppor-

tunities for boosting of immunity because of reduced levels of circulation of *B. pertussis* have been proposed as possible reasons for adult/adolescent pertussis [25]. However, the Japanese adult pertussis cannot be thus explained, since U.S. adult/adolescent pertussis has steadily increased during the last two decades [2–4]. The reason for the rising pertussis incidence in Japanese adult population has remained unclear. Further analyses are warranted to determine the cause of the adult pertussis.

In conclusion, *B. pertussis* isolates recovered from Japanese adults and children were indistinguishable by molecular typing methods. In Japan, the antigenic and genetic shifts, especially *ptxS1* and *prn* alleles, would be unrelated to the rise of the adult pertussis incidence.

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Genetic Analysis of the *Enterococcus* Vancomycin Resistance Conjugative Plasmid pHT β : Identification of the Region Involved in Cell Aggregation and *traB*, a Key Regulator Gene for Plasmid Transfer and Cell Aggregation[†]

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The *Enterococcus* plasmid pHT β (63.7 kbp) is a pheromone-independent, highly conjugative pMG1-like plasmid that carries a Tn1546-like transposon encoding vancomycin resistance. The transfer-related regions (Tra I, Tra II, and Tra III) containing *oriT* and a putative nickase gene (*traI*) have previously been identified in pHT β , and in this study, we found that the plasmid conferred the ability to self-aggregate on the host strain *Enterococcus faecalis* FA2-2. A region where mutation resulted in the impairment of aggregation was identified and mapped to a point upstream of the transfer-related Tra I region. This region consisted of an approximately 6-kbp segment that contained the five open reading frames (ORFs) ORF9 to ORF13. These ORFs are considered to encode the aggregation function, although the precise mode of action of each ORF has not yet been elucidated. An in-frame deletion mutant of ORF10 resulted in reduced aggregation and decreased transfer frequency in broth mating. Transcription analysis of the aggregation region showed that the five ORFs from ORF9 to ORF13 form an operon structure, and a long transcript that started from a promoter region located upstream of ORF9 was identified. Tra II spans a 1.7-kbp region containing ORF56 and ORF57. Tn917-*lac* insertions into or an in-frame deletion mutant of ORF56 (187 amino acids) resulted in impaired transfer and aggregation. The cloned ORF56 complemented these functions in *trans*. The transcription levels of ORF10 and ORF13 were reduced in the in-frame mutants of ORF56, but this reduction was complemented by a cloned ORF56 in *trans*. The results indicated that ORF56 positively regulated the aggregation and plasmid transfer in the host strain, and ORF56 was designated *traB*.

The genes and systems required for bacterial conjugation have been classified into two functional groups: the DNA transfer and replication system and the mating pair formation (Mpf) system (24, 40). The DNA transfer and replication system is also referred to as the relaxosome system and is involved in the multiprotein-plasmid DNA complex that is generated at the plasmid origin of transfer (*oriT*) and the generation of the single strand to be transferred to the recipient cell (40).

The Mpf system is a plasmid-encoded multiprotein structure that is involved in the protein complex that establishes intimate contact between donor and recipient, such as the sex pili in gram-negative bacteria, and the formation of the transmembrane channel for the donor plasmid DNA strand to move from donor to recipient cell. The Mpf system is linked with a coupling protein, such as those encoded by *traD* of the F plasmid, *traG* of RP4, and *virD4* of pTi, to deliver the protein-DNA complex generated by the relaxosome system to the entrance of the Mpf channel. A macromolecular transfer system present in gram-negative bacteria that is similar to the

conjugal Mpf system has been classified as a type IV secretion system (24).

The nucleotide sequence data for the *tra* region of the gram-positive bacterial conjugative plasmid show homologies with certain protein components of type IV secretion systems (14), and an Mpf system corresponding to the type IV secretion system has recently been identified in the conjugative plasmid pIP501 isolated from gram-positive bacteria (1). The conjugation systems in gram-negative and gram-positive bacteria differ mainly in the mechanisms by which the intimate contact between donor and recipient cell that is a prerequisite for the initiation of conjugative transfer is established. In gram-negative bacteria, this initial contact between donor and recipient cells is mediated by the sex pili. In the majority of gram-positive bacteria, the mechanisms by which the initial cell-to-cell contact is established have not been identified.

Two types of highly efficient conjugative plasmids that are effectively transferred in broth mating have been identified in the gram-positive enterococci (4). One is the pheromone-responsive plasmid found in *Enterococcus faecalis*, and the other is the non-pheromone-responsive plasmid found in *Enterococcus faecium* strains. The pheromone-responsive conjugative plasmids of *E. faecalis* have a unique conjugative system that is the best example of the mechanism of initial cell-to-cell contact in gram-positive bacteria to be elucidated to date. The regulatory process is highly unusual among gram-positive bacteria and is instrumental in the conjugative transfer of the phero-

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