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RESEARCH LETTER

# A novel insertion sequence, IS*1642*, of *Mycobacterium avium*, which forms long direct repeats of variable length

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## Abstract

A new insertion sequence (IS), IS*1642*, was identified in a *Mycobacterium avium* strain isolated from a human patient. IS*1642* had a size of 1642 bp and contained a single ORF encoding a probable transposase of 503 amino acid residues homologous (79% identity) to that of IS*1549* found in *Mycobacterium smegmatis*. The IS*1642* included imperfect inverted repeats (5′-cctgacttttatca-3′, 5′-tgataaaagtcggg-3′) on its ends, and was flanked by direct repeats of variable length ranging from 5 to 161 bp. It was suggested that the IS*1642* was widely distributed in many *M. avium* strains of human patients, and the Southern blot profile of IS*1642* was very diverse among the strains examined. The transposition event of IS*1642* was observed by *in vitro* repeated passages, showing that the IS*1642* is actually a transposable element. In light of these characteristics, IS*1642* could be a new useful marker when genotyping with high discrimination is required.

## Introduction

The insertion sequence (IS) on a bacterial genome facilitates gene rearrangements, which could contribute to the evolution of the organism (Mahillon & Chandler, 1998). ISs are widely distributed in most microorganisms, including the *Mycobacterium* species. For example, genome sequence analysis revealed that the *Mycobacterium avium* ssp. *paratuberculosis* strain K-10 contained 19 kinds of ISs, with 58 total copies in the genome (Li *et al.*, 2005). The genome of *Mycobacterium tuberculosis* H37Rv contained 56 loci with homology to ISs (Gordon *et al.*, 1999).

Bacterial ISs usually contain one or several ORFs, which encode enzymes such as transposases that catalyze the movement within the genome. ISs typically contain short terminal inverted repeat sequences (IRs), ranging from 10 to 40 bp. Upon insertion into the host genome, ISs are flanked on either side by short directly repeated sequences (DRs). The length of a direct repeat, which is usually a fixed characteristic of each IS, generally ranges between 2 and 14 bp (Mahillon & Chandler, 1998).

ISs are used as markers in restriction fragment length polymorphism studies for species typing and for molecular epidemiological purposes [e.g. IS*6110* in *M. tuberculosis* (Otal *et al.*, 1991; Small & van Embden, 1994) and IS*1245* in *M. avium* (Guerrero *et al.*, 1995; Pestel-caron & Arbeit, 1998; Ritacco *et al.*, 1998; van Soolingen *et al.*, 1998; Motiwala *et al.*, 2006)]. When isolates from different sources have few distinguishing phenotypic characteristics, the use of ISs as probes could enable powerful discrimination. On the other hand, because of their mobility, IS could be potential useful markers for identifying substrains or tracking the genetic drift (Hernandez Perez *et al.*, 1994; Laurent *et al.*, 2002).

In this study, we identified a novel IS, designated IS*1642*, which was flanked by unusually long, variable-length direct repeats, in *M. avium* clinical isolates. Here, we present molecular and genetic characterizations of this IS.

## Materials and methods

### Bacterial strains and growth conditions

*Mycobacterium avium* clinical strains isolated from human patients were kindly provided by Dr K. Ogawa of NHO Higashi Nagoya National Hospital and Dr Matsumoto of the

Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Japan. These strains were identified as the *Mycobacterium avium/intracellulare* complex by the *Mycobacterium* identification kit (Kyokuto Pharmaceutical Industrial Co. Ltd), and further identified *as M. avium* by PCR (Nishimori *et al.*, 1995). *Mycobacterium avium* ssp. *paratuberculosis* K-10 (BAA968), two *M. avium* strains (25291 and 15769), and two *M. intracellulare* strains (13950 and 25225) were obtained from the American Type Culture Collection (ATCC). Five *M. intracellulare* strains, *Mycobacterium marinum*, *Mycobacterium szulgai*, *Mycobacterium simiae*, *Mycobacterium fortuitum*, and *Mycobacterium abscessus*, and *Mycobacterium bovis* BCG Japanese strain were from our laboratory. The laboratory strain *M. tuberculosis* H37Rv was also included for analysis. These strains were cultured on Middlebrook 7H10 supplemented with 10% (v/v) OADC enrichment (BD) at 37 °C.

### DNA manipulations

Mycobacterial genomic DNA was isolated as described (Pelicic *et al.*, 1997), with a minor modification. Bacterial cells were harvested and suspended in 1 mL of acetone. The cells were pelleted by centrifugation (10 min at 5000 *g*). The pellet was resuspended in 500 μL solution I (25% sucrose/50 mM Tris · HCl, pH 8.0/50 mM EDTA/500 mg $mL^{-1}$ lysozyme) and incubated overnight at 37 °C. Then 500 μL of solution II (100 mM, Tris · HCl, pH 8.0/1% SDS/400 μg $mL^{-1}$ proteinase K) was added, and the samples were incubated for 5 h at 55 °C. Genomic DNA was extracted from the lysate using the bacteria genomicPrep Kit (GE Healthcare).

### PCR and nucleotide sequencing

The primers used in this study are listed in Table 1. PCRs were performed with Phusion high-fidelity DNA polymerase (New England Biolabs). Thermal cycling conditions comprised preincubation at 98 °C for 30 s, followed by 30 cycles at 98 °C for 10 s, at 68 or 55 °C for 30 s, and at 72 °C for 2 min, and a final extension at 72 °C for 10 min. For examination of direct repeats, genome DNA was digested by PvuII or NotI, and self-ligated. The direct repeat region was amplified by PCR with the primer set IS1642-DR1 and IS1642-DR2 (Table 1), which match the ends of IS*1642* and face the outwards. Two amplified products were obtained, and the nucleotide sequences were determined by sequencing. For sequencing, PCR products were purified using the GFX PCR purification kit (GE Healthcare). Sequencing was carried out using the Big Dye DNA sequencing kit (Applied Biosystems) and the ABI Prism 3130XL Genetic Analyzer. Nucleotide sequences and deduced amino acid sequences were analyzed by GENETYX-MAC software, version 14.0.1 (Genetyx Co., Tokyo, Japan).

**Table 1.** List of primers used in this study

| Primer | Sequence (5′–3′) |
|---|---|
| IS1642-1F | TTGTGTAGGGCTGTGACCTG |
| IS1642-1R | ACGTAGGCTGTGGATGTTTG |
| IS1642-2F | TGACCTGTGTCTTCGGTTG |
| IS1642-2R | TGTGCTTGCCGGCTTGGATAG |
| IS1642-3F | TCATCGAACCGACCAGCAAG |
| IS1642-3R | CACCACATCAGGTAAAAACG |
| IS1642-DR1 | CTACCGTACCGTCACTATCC |
| IS1642-DR2 | TCTTCACCGTGCGCACATAG |
| PIS-2 | CGGGATCCCCGCCATTTCACCTGAAACC |
| PIS-3 | CCCAAGCTTGCGAACAAAAAATCGACGCC |

### Southern blot analysis

Genome DNA was digested with restriction endonuclease PvuII (New England Biolabs), which had no recognized sites in the IS*1642* sequence. The digested DNA samples were electrophoresed on a 0.8% agarose gel. DNA fragments were transferred onto a nylon membrane and hybridized with a digoxigenin-labelled probe prepared using the PCR DIG Probe Synthesis Kit (Roche) with primer sets IS1642-3F and IS1642-3R designed to amplify a 515-bp portion of IS*1642*. Hybridized bands were visualized by chemiluminescence detection (Labeling and Detection Starter Kit II, Roche).

### Promoter activity analysis

The entire IS*1642* region was amplified by PCR with the primer set PIS-2 and PIS-3 (Table 1), and cloned upstream of a promoterless green fluorescent protein (GFP) gene on a cloning vector pVV16 (a kind gift from Dr Vissa). The plasmids were transformed into *Mycobacterium smegmatis* $mc^2$ 155, and the expression of these genes was assessed by measuring the fluorescence of GFP with a fluorescence plate reader (Perkin Elmer, Wallac 1420 ARVO MX).

### Nucleotide sequence accession numbers

AB453386 is the GenBank accession number for the nucleotide sequence of the entire region of insertion element IS*1642*. The GenBank accession number for the nucleotide sequence of the region containing insertion element IS*1642* and the 161-bp direct repeats is AB453387.

## Results

### Identification of new IS, IS*1642*

A new IS was identified in an *M. avium* strain isolated from a human patient. The IS was found 673 bp upstream of the start codon of the gene corresponding to the MAP0076 gene of the *M. avium* ssp. *paratuberculosis* K-10 genome. This

sequence contained a nucleotide sequence of 1642 bp, with a GC content of 64%, which approximates those of the mycobacterial genomes (62–70%) (Wayne & Kubica, 1986). The sequence contained a single ORF coding for a protein of 503 amino acids and 14-bp imperfect inverted repeats (5′-cctgacttttatca-3′, 5′-tgataaaagtcggg-3′) at its ends. The complete nucleotide sequence of the IS and the deduced amino acid sequence is shown in Fig. 1a. Database searches revealed that this IS was 79% identical to that of IS*1549* of *M. smegmatis* (accession number AF006614) (Plikaytis *et al.*, 1998) at the amino acid sequence level. Hence, we assigned IS*1642* to the newly identified IS.

IS*1549* is an insertion element that was reported to be distantly related to the IS*4* family (Plikaytis *et al.*, 1998). Transposases of the IS*4* family typically contain conserved regions, N3 and C1, but IS*1549* lacks the N3 region (Plikaytis *et al.*, 1998). Analysis of alignment indicated that IS*1642* also contained the C1 region, but not the N3 region. The C1 signature sequence of the IS*4* family is Y-($X_2$)-R-($X_3$)-E-($X_6$)-K (Rezsöhazy *et al.*, 1993), and the corresponding IS*1642* sequence was Y-($X_2$)-L-($X_3$)-E-($X_6$)-K. It was reported that IS*1549* possesses unique N2 and N3 regions. These sequences were found in IS*1642* as well (Fig. 1a). While the N2 signature sequence was D-($X_2$)-T-(X)-YFE-($X_{10}$)-G-(X)-SK (Alexander *et al.*, 2003), the corresponding IS*1642* sequence was D-($X_2$)-T-(X)-HFE-($X_{10}$)-G-(X)-SK. The N3 signature sequence was AD-(X)-G-($X_5$)-N (Alexander *et al.*, 2003), and the corresponding IS*1642* sequence was identical.

## Identification of direct repeats

IS*1642* identified upstream of the MAP0076 gene was flanked by direct repeats of 161 bp, which was a duplication of the nucleotide sequence of the region upstream of the MAP0076 gene. Compared with the usual direct repeats of general ISs, this direct repeat is extremely long. Because Southern blot analysis indicated that the *M. avium* strain contained multiple copies of IS*1642* (Fig. 2, lane 1), we sought to determine the length of the direct repeats of IS*1642* of other copies. Two direct repeats were successfully identified by the method described in Materials and methods. The length of the direct repeats was 5 and 59 bp,

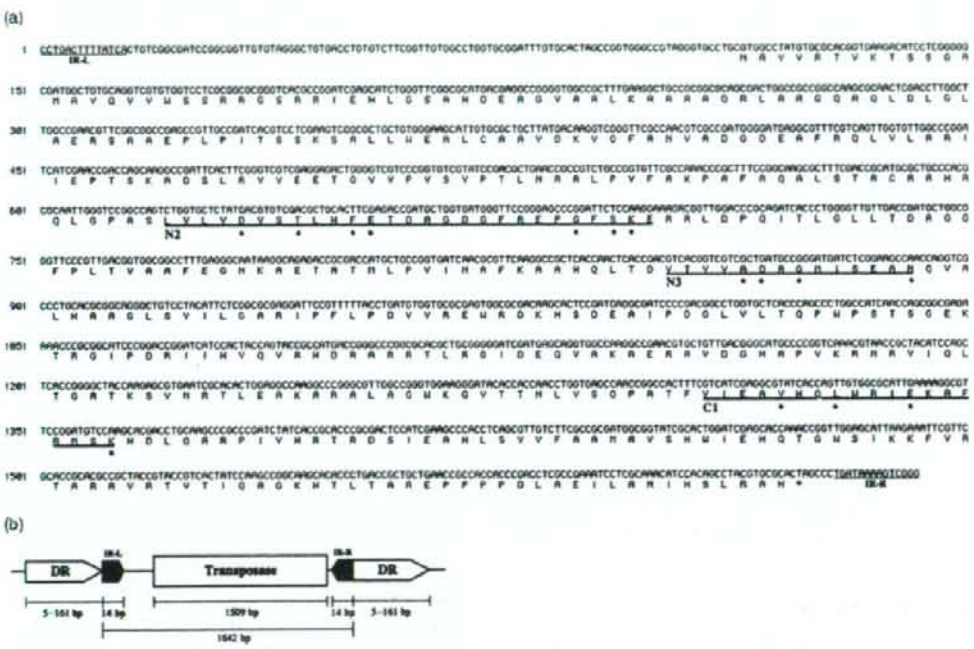


**Fig. 1.** Nucleotide sequence and structure of IS*1642*. (a) Nucleotide sequence of IS*1642* and deduced amino acid sequence of ORF. Inverted repeats, designated IR-R and IR-L, are underlined. Conserved regions, N2, N3, and C1, are underlined in bold. Dots below amino acids indicate the conserved signature sequences of these regions. (b) Schematic representation of the IS*1642* and the DR. The IR and the DR, and the beginning and end of the ORF encoding a putative transposase are shown.


**Fig. 2.** Southern blot profile of IS*1642* 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.


**Fig. 3.** Change in the Southern blot profile of IS*1642* 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 passaged 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.

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 IS*1642*

The distribution of IS*1642* 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 IS*1642* 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 IS*1642* on the Southern analysis profile (data not shown). The prevalence of IS*1642* 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 IS*1642* (data not shown), suggesting that IS*1642* is widely distributed among *M. avium* clinical strains.

### Mobility of IS*1642*

Because the Southern blot profile of IS*1642* was very different among strains, we explored whether or not the IS*1642* 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 plate, and passaged 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 passaged 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, IS*1642*, in *M. avium* clinical strains isolated from human patients. Our results showed that multiple copies of IS*1642* are present in several *M. avium* strains. The deduced amino acid sequence of the ORF of IS*1642* was highly homologous to the transposase of *M. smegmatis* IS*1549* (Plikaytis *et al.*, 1998). IS*1549* is an insertion element whose transposases exhibited homology to that of IS*1623* and IS*1634*, and it was suggested that these transposases represent an emerging group in the IS*4* family because they exhibit a characteristic lack of the typical conserved N3 region of IS*4* family and yet possess unique N2 and N3 motifs (Vilei *et al.*, 1999; Alexander *et al.*, 2003). A notable feature of IS*1549* and IS*1634* 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. IS*1642* found in this study also exhibited these characteristics. IS*1642* contained the C1 region and unique N2 and N3 motifs, but not the N3 region of the IS*4* family, suggesting that IS*1642* belongs to this new group. IS*1642* was flanked by direct repeats of variable lengths ranging up to 161 bp. Considering that IS*1642* 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 IS*1642*. 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 IS*1549* and IS*1634*), 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 IS*1642* 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 IS*1642* is widely distributed among *M. avium* clinical strains. In addition, the experiment of repeated passage suggested that IS*1642* 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 IS*1642* as a genetic typing tool for classification of different clinical strains. Alternatively, genotyping by IS*1642* 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). IS*1549* was found to show promoter activity (Plikaytis *et al.*, 1998). Because IS*1642* was homologous with IS*1549*, we examined IS*1642* for promoter activity. However, the expression of GFP was at an undetectable level. IS*1642* 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 Szeverényi *et al.* (1996). It might also be possible that promoters of IS1642 could not be recognized by *M. smegmatis*.

IS*1642* 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 IS*1642*, 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 $K_1$) 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 clos-

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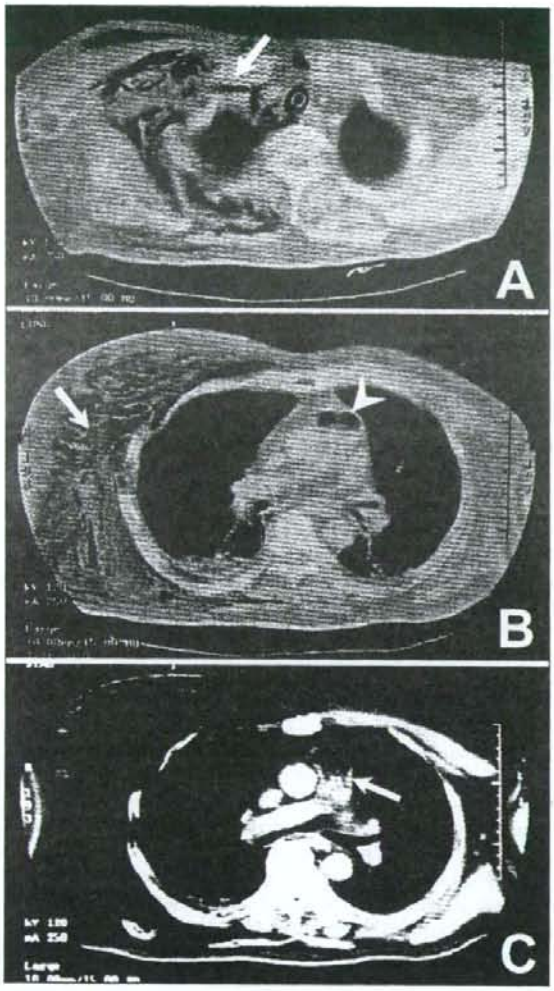


FIG. 1. Chest CT scan imaging 20 min after arrival. (A) Right subclavian vein filled with entrapped gas (arrow). (B) Marked subcutaneous emphysema with destruction of muscle tissue around right chest (arrow) and massive gas entrapped in the pulmonary artery (arrowhead). (C) CT scan revealing many bubbles (arrow) 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/μl) | 8,800 |
| Erythrocyte count (cells/μl) | $365 \times 10^4$ |
| Hemoglobin (g/dl) | 12.4 |
| Hematocrit (%) | 39.2 |
| Platelet count (cells/μl) | 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.

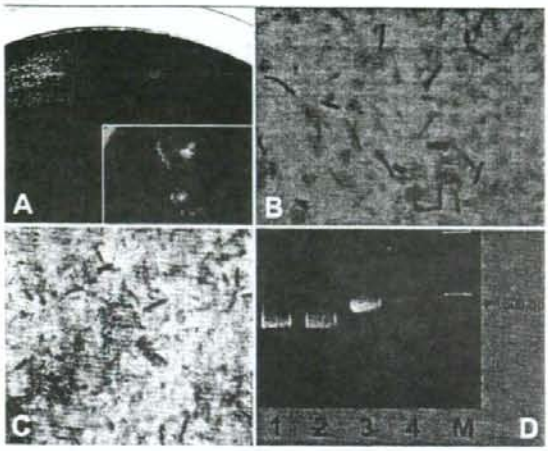


FIG. 2. Microbiological and PCR amplification findings for *Clostridium chauvoei*. (A) Grayish white, rough colonies with beta-hemolysis around bacterial colonies after 48 h of culture on *Brucella* HK RS agar under anaerobic conditions. (B and C) Gram stain morphology and Wirtz-Conklin spore stain, respectively, from growth on *Brucella* HK RS agar (magnification, ×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 $MIC_{90}$ of ADH reached 100 mg/L. Strains for which MICs of at least two disinfectants were higher than $MIC_{90}$ 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 multidrug 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 microdilution 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 μL of test suspension was added to tubes containing 900 μL of disinfectant solutions at different concentrations and left for 3 min at 20 ± 2°C. Since the test results obtained at room temperature demonstrated poor reproducibility, all reactions were performed as much as possible at 20 ± 2°C in an incubator using bacterial suspensions, disinfectant solutions and neutralizer solutions preincubated at 20 ± 2°C. Aliquots of the reaction mixture (100 μL) of containing bacterial cells and disinfectant were then added to 900 μL of neutralizer solution at 20 ± 2°C for 3 min and serially diluted in PBS (pH 7.4). After dilution, 50 μL 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 μL of test suspension to the 900 μL 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 μL) containing $1-3 \times 10^4$ cfu/mL was added to 900 μL of neutralizer and left at 20 ± 2°C for 3 min. Two samples of 100 μL 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 μL. 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 μL of the PBS (pH 7.4) and left at 20 ± 2°C for 3 min. Then, 100 μL of the mixture was transferred into 800 μL of neutralizer and left at 20 ± 2°C for 3 min. Aliquot of a diluted bacterial suspension (100 μL) containing $1-3 \times 10^4$ cfu/mL was then added and left at 20 ± 2°C for 3 min. Two samples of 100 μL 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 μL.

### *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 ± 2°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 $1 \times 10^8$ 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 $\leq 0.05$ 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] | 1 | 0 | 0 | 0 | 10 | 25 |
| BZK | 0 | 168[a] | 104 | 9 | 2 | 0 | 0 | 0 | 0 | 5 | 10 |
| BZT | 0 | 2 | 196[a] | 68 | 15 | 2 | 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.
[a]Numbers 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.
[b]Standard $MIC_{50}$ 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.

**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 | *A. baumannii* |
| 2[b] | 25 | 25 | 50 | 50 | *A. junii* |
| 3 | 25 | 25 | 50 | 100 | *A. baumannii* |
| 4[b] | 50 | 25 | 50 | 50 | *A. baumannii* |
| 5[b] | 25 | 25 | 100 | 100 | *A. baumannii* |
| 6[b] | 25 | 25 | 100 | 100 | *A. baumannii* |
| 7[b] | 50 | 50 | 50 | 100 | *A. calcoaceticus* |
| 8 | 50 | 10 | 50 | 100 | *A. baumannii* |
| 9 | 50 | 10 | 50 | 100 | *A. baumannii* |
| 10[b] | 100 | 25 | 50 | 100 | *A. calcoaceticus* |
| 11 | 10 | 50 | 50 | 50 | *A. junii* |
| 12 | 25 | 25 | 50 | 100 | *A. baumannii* |
| 13[c] | 50 | 10 | 50 | 100 | *A. calcoaceticus* |
| 14[c] | 50 | 10 | 50 | 100 | *A. calcoaceticus* |

[a]MICs of disinfectants were determined using the broth microdilution method.
[b]Strains 1, 2, 4, 5, 6, 7 and 10 were selected for adaptation tests.
[c]Strains 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 ($MIC_{50}$ of each disinfectant obtained by the broth microdilution method), the bacterial cell count in the absence of BSA reached a 5 $log_{10}$ 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 $log_{10}$ under the test conditions employed for BZK, BZT and ADH.

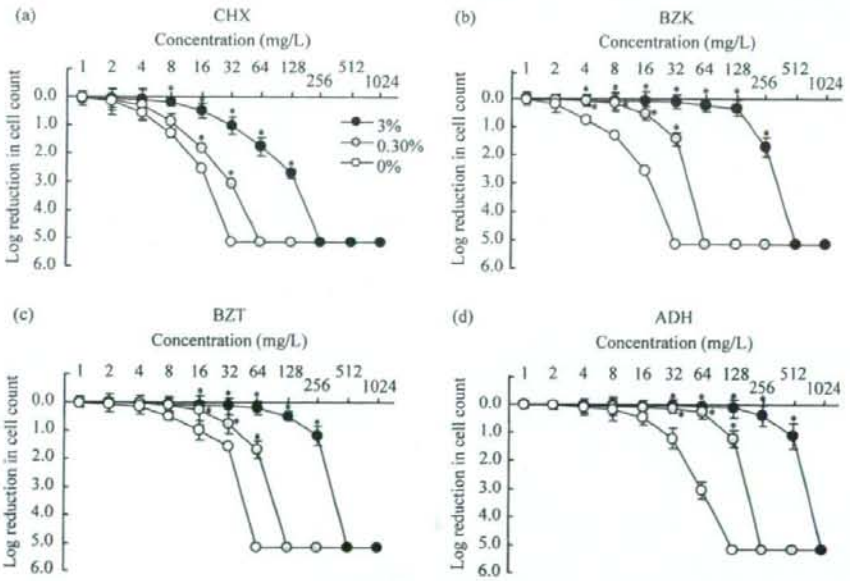


**Figure 1.** Comparison of MBC. Fourteen isolates for which MICs of at least two disinfectants were more than $MIC_{90}$ measured by the broth microdilution method were defined as isolates with DRS. MBCs of the four disinfectants tested on the DRS isolates are shown. Results are expressed as $log_{10}$ 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).

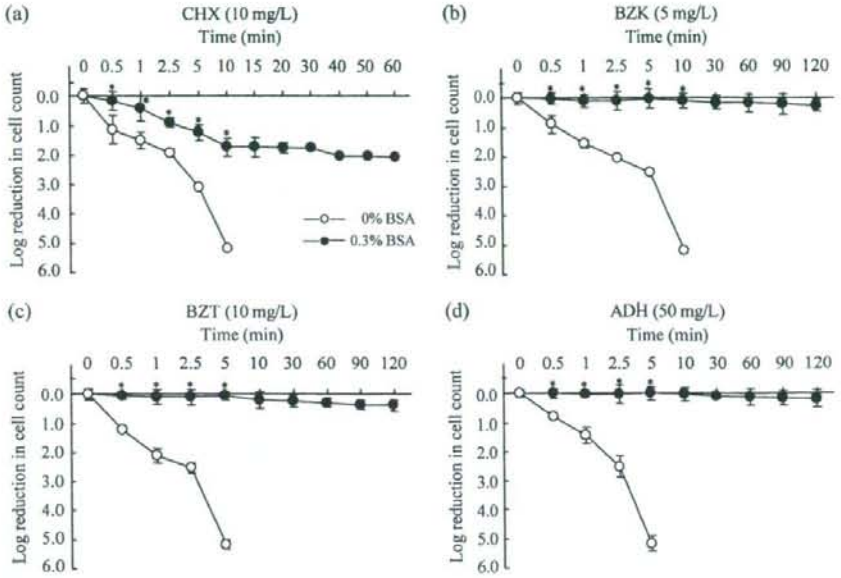


**Figure 2.** Results of time-killing assays. Time–kill assays were performed for the 14 DRS isolates selected in the same manner as in the MBC assay. The concentration of each disinfectant is as follows: (a) chlorhexidine gluconate (CHX), 10 mg/L; (b) benzalkonium chloride (BZK), 5 mg/L; (c) benzethonium chloride (BZT), 10 mg/L; (d) alkyl diaminoethyl glycine hydrochloride (ADH), 50 mg/L. Bacterial cells were treated with each disinfectant at $20 \pm 2$°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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ 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

**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.
[a]Shown as $\log_{10}$ reduction in bacterial cell number in both 'clean' (0% BSA) and 'dirty' (0.3% and 3% BSA) conditions; mean ± SD.
[b]Numbers indicate 'in-use' concentration (mg/L) of each disinfectant.
[c]Disinfectants failed to provide a 5 $\log_{10}$ reduction in live bacterial cell counts in the presence of BSA.

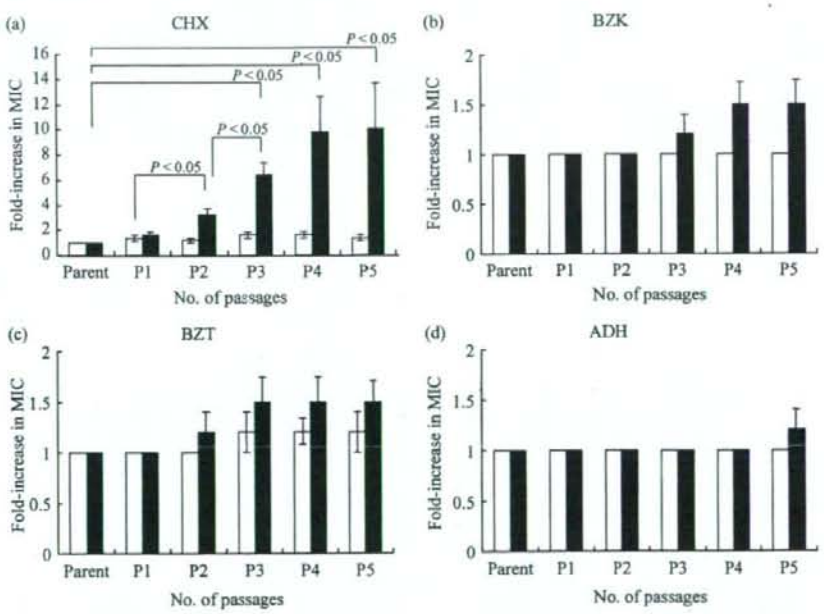


**Figure 3.** Adaptation to four disinfectants after persistent passages. Adaptations to disinfectants were generated by repeated subculture in nutrient broths containing each disinfectant (a) chlorhexidine gluconate (CHX); (b) benzalkonium chloride (BZK); (c) benzethonium chloride (BZT); (d) alkyl diaminoethyl 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 | $5.36 \pm 1.20 \times 10^7$ | NC | >5.43 | NC | >5.43 | NC | >5.43 |
| P1 | $4.43 \pm 1.73 \times 10^7$ | NC | >5.34 | NC | >5.34 | NC | >5.34 |
| P2 | $8.00 \pm 1.00 \times 10^7$ | NC | >5.60 | NC | >5.60 | NC | >5.60 |
| P3 | $8.85 \pm 2.06 \times 10^7$ | NC | >5.65 | NC | >5.65 | NC | >5.65 |
| P4 | $6.34 \pm 1.73 \times 10^7$ | NC | >5.50 | NC | >5.50 | NC | >5.50 |
| P5 | $3.37 \pm 0.70 \times 10^7$ | 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 | $5.36 \pm 1.20 \times 10^7$ | NC | >5.43 | NC | >5.43 | NC | >5.43 |
| P1 | $4.43 \pm 1.73 \times 10^7$ | $2.54 \pm 1.05 \times 10^2$ | 5.24 | NC | >5.34 | NC | >5.34 |
| P2 | $8.00 \pm 1.00 \times 10^7$ | $5.54 \pm 1.25 \times 10^2$ | 5.16 | NC | >5.60 | NC | >5.60 |
| P3 | $8.85 \pm 2.06 \times 10^7$ | $3.10 \pm 1.43 \times 10^3$ | 4.46 | NC | >5.65 | NC | >5.65 |
| P4 | $6.34 \pm 1.73 \times 10^7$ | $1.88 \pm 0.88 \times 10^3$ | 4.53 | $9.80 \pm 0.78 \times 10^1$[e] | 5.50 | NC | >5.50 |
| P5 | $3.37 \pm 0.70 \times 10^7$ | $3.64 \pm 0.89 \times 10^3$ | 3.97 | $3.60 \pm 0.68 \times 10^2$ | 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 | $5.36 \pm 1.20 \times 10^7$ | $2.26 \pm 0.99 \times 10^3$ | 4.38 | NC | >5.43 | NC | >5.43 |
| P1 | $4.43 \pm 1.73 \times 10^7$ | $2.50 \pm 1.21 \times 10^3$ | 4.25 | NC | >5.34 | NC | >5.34 |
| P2 | $8.00 \pm 1.00 \times 10^7$ | $4.68 \pm 1.31 \times 10^3$ | 4.23 | NC | >5.60 | NC | >5.60 |
| P3 | $8.85 \pm 2.06 \times 10^7$ | $5.46 \pm 1.56 \times 10^3$ | 4.21 | $2.80 \pm 0.92 \times 10^2$ | 5.28 | NC | >5.65 |
| P4 | $6.34 \pm 1.73 \times 10^7$ | $4.75 \pm 0.65 \times 10^3$ | 4.12 | $4.37 \pm 1.31 \times 10^2$ | 5.31 | NC | >5.50 |
| P5 | $3.37 \pm 0.70 \times 10^7$ | $2.56 \pm 0.41 \times 10^4$ | 3.12 | $3.60 \pm 0.68 \times 10^2$ | 3.99 | $2.71 \pm 1.65 \times 10^2$ | 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 $<2 \times 10^2$ cfu/mL in all three tests for seven DRS isolates selected.
[a]Strains were obtained throughout adaptation process passaged in increasing concentrations of CHX as shown in Figure 3.
[b]Average viable cell concentration (cfu/mL $\pm$ SE) measured in PBS instead of disinfectant solution.
[c]Concentration of CHX to which bacterial cells were exposed before inoculation onto agar plate for counting surviving bacterial colonies.
[d]Average viable cell concentration (cfu/mL $\pm$ SE) after the exposure to disinfectant.
[e]Since no colonies grew on several plates, viable cell count became below the detection limit after the statistical analysis.

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 $MIC_{90}$s of the four disinfectants tested for 283 *Acinetobacter* species isolates were $\leq$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 $\log_{10}$ 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 $\log_{10}$ 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 skin[22] and mucous membranes.[23]

Irizarry *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, BZX, 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.

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SHORT COMMUNICATION

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

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*Bordetella pertussis*;
Adult;
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PFGE profile

**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.


## Introduction

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