FIG. 2. Dendrogram of S3DHFR and DHFR from a variety of organisms. The dendrogram was created by the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the DHFR proteins (accession number and species given in parentheses) of types I (X00926, from *E. coli*), Ib (I40985, from *E. coli*), IV (A60935, from *E. coli*), V (X12868, from enterobacterial plasmid pLMO150), VI (Z86002, from *Proteus mirabilis*), VII (X58425, from *E. coli*), VIII (U10186, from *E. coli*), IX (A49788, from *E. coli*), X (AY123253, from *Klebsiella pneumoniae*), XII (I41043, from *E. coli*), E1 (AF028812, from *E. faecalis*), S1 (X13290, from *S. aureus*), and S2 (Z50141, from *S. haemolyticus* MUR313) and the chromosomal DHFRs of *B. anthracis* Ames (AE017031), *B. cereus* ATCC 14579 (AE017005), *B. cereus* ATCC 10987 (AE017271), *B. subtilis* Marburg (L77246), *Enterobacter aerogenes* (M26022), *E. coli* K-12 (P00379), *E. faecalis* V583 (AE016951), *E. faecium* mutant strain A (741860A), *Haemophilus influenzae* R1047 (X84205), *Salmonella enterica* serovar Paratyphi ATCC 9150 (CP000026), *S. aureus* ATCC 25923 (SaDHFR; Z16422), *S. epidermidis* ATCC 14900 (SeDHFR; Z48233), *Streptococcus pneumoniae* ATCC 49619 (Z74778), and *Vibrio vulnificus* YJ016 (BA000037).

indicating that the region is in an insertion sequence (IS). The DNA sequence, ranging from 275 bp upstream of *dfrG* to the 363 bp of the 5′-terminal region of *dfrG*, was identical to that previously reported for plasmid pMG1 in *Enterococcus faecium* (18). The *dfrG* gene may have been acquired from *E. faecium* via IS-mediated recombination. The ancestral origin of S3DHFR, however, remains unknown; S3DHFR showed little similarity to and considerable phylogenetic distance from intrinsic DHFR of *E. faecium* (Fig. 2).

The MICs of TMP in *E. coli* transformants harboring pSA1 or pT7dfrG carrying *dfrG* were significantly increased than those in control strains (Table 1), indicating that *dfrG* is responsible for TMP resistance. An *E. coli* transformant harboring pT7dfrB carrying *dfrB* also showed increased MIC, but it was not as high as those of *E. coli* strains expressing *dfrG*. *dfrB* is believed to encode a TMP-sensitive DHFR of *S. aureus* because it was found in all *S. aureus* strains, regardless of TMP susceptibility. Similar results were reported for *dfrE* encoding

Enterococcus faecalis DHFR (4). The increased MIC for TMP in *E. coli* carrying *dfrB* may be explained by the multicopy effects of high expression of the housekeeping protein DHFR.

For functional analysis of S3DHFR and DHFR from *S. aureus* CM.S2 (SaDHFR$_{CM.S2}$), overexpression and purification of these recombinant DHFRs were achieved. Overexpression of S3DHFR or SaDHFR$_{CM.S2}$ was accomplished by integration of the respective coding regions downstream of the His-tagged coding region of the pCR/T7NT expression vector and transformation into the *E. coli* strain BL21-AI. Recombinant protein in soluble extracts was purified by affinity chromatography to determine enzymatic activities. The K_m values of recombinant S3DHFR for DHF and NADPH were 2.68 ± 1.09 µM and 2.38 ± 1.97 µM, respectively (Table 2). The K_m values of DHF and NADPH for S3DHFR did not differ from those of SaDHFR$_{CM.S2}$, but the IC$_{50}$ values of TMP for these DHFRs differed significantly. The IC$_{50}$ of TMP for S3DHFR was more than 8,000-fold greater than IC$_{50}$ values for TMP-

sensitive SaDHFR and SaDHFR$_{CM.S2}$, indicating that S3DHFR and SaDHFR$_{CM.S2}$ are indeed DHFRs but that only S3DHFR plays a critical role in TMP resistance. The K_m values of crude extracts for NADPH were sixfold greater than those of recombinant S3DHFR (Table 2). Crude extracts may contain other factor(s) that bind to NADPH.

Detection of *dfrG* was performed by PCR on isolates from Chiang Mai, Thailand, and Tokyo, Japan. All Chiang Mai isolates were resistant to TMP and contained *dfrG*, whereas all Tokyo isolates but one were sensitive to TMP and did not contain *dfrG* (data not shown). The single Tokyo isolate IMCJ934 was resistant to TMP and contained *dfrG* (Table 1).

Pulsed-field gel electrophoresis (PFGE) analysis revealed 13 patterns of SmaI digestion in the 43 MRSA isolates from Chiang Mai, Thailand (data not shown). Cluster analysis showed that 12 of the 13 PFGE patterns formed a cluster (>75% similarity). The PFGE pattern of *S. aureus* CM.S2 genomic DNA was identical to that of 18 MRSA isolates. These results suggest that clonal expansion of MRSA carrying *dfrG* occurred at the hospital in Chiang Mai. The TMP-resistant isolate from Tokyo, Japan, IMCJ934, showed the same PFGE pattern as that of one of the Chiang Mai isolates, *S. aureus* CM.S2 (data not shown).

dfrG was detected by Southern blotting on fragments of SmaI-digested genomic DNA, but it was not detected on plasmids (data not shown). Conjugal transfer of TMP resistance from *S. aureus* CM.S2 to recipient strains *S. aureus* IMCJ565RFP^r or IMCJ644RFP^r was unsuccessful, suggesting that *dfrG* is located on the chromosome and not on a plasmid of these clinical isolates. It remains to be determined whether *dfrG* can be transferred by phages or mobile elements.

A single amino acid substitution (Phe to Tyr) at codon 98 of SaDHFR was reported to be associated with TMP resistance in *S. aureus* (5). Therefore, approximately 390 bp of internal DNA sequence of *dfrB* encoding SaDHFR was determined. When *S. aureus* ATCC 29213 was used as a control (5), all isolates from Chiang Mai, Thailand, exhibited three silent mutations: CAT to CAC in codon 77 and TTT to TTC in codons 91 and 118. All isolates from Tokyo, Japan, contained four silent mutations: AAA to AAG in codon 30, CAT to CAC in codon 77, and TTT to TTC in codons 91 and 118. These results indicate that these mutational changes are not associated with TMP resistance in the isolates from Chiang Mai or Tokyo. Other possible mechanisms of TMP resistance, such as overexpression of intrinsic DHFR, efflux, or impermeability, may be involved.

The CM.S2 strain was the dominant clone from Chiang Mai, Thailand. MRSA surveillance is being carried out in the hospital from which these isolates were obtained. *S. aureus* CM.S2 is resistant to clindamycin, erythromycin, gentamicin, and tetracycline and is less sensitive to arbekacin. Fosfomycin, linezolid, and vancomycin are effective in vitro; quinupristin-dalfopristin and daptomycin were not available for testing. Results of this surveillance will be reported in the future.

Our data strongly suggest that the TMP resistance-associated gene *dfrG* is prevalent in Thailand, and an isolate harboring this gene was found in Japan. This gene may spread worldwide, and measures against this, such as gene monitoring and adequate use of TMP, should be established.

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Survey of human immunodeficiency virus (HIV)-seropositive patients with mycobacterial infection in Japan

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Abstract *Objective.* To assess DNA polymorphisms in mycobacterial isolates obtained from human immunodeficiency virus (HIV)-seropositive patients with tuberculosis in Japan from 1996 to 2003.

Methods. Restriction fragment length polymorphisms (RFLP) from *Mycobacterium tuberculosis* and *Mycobacterium avium* isolates obtained from individual seropositive patients with tuberculosis ($n=78$) were analysed with the use of IS6110 and (CGG)$_5$ or IS1245 and IS1311, respectively, as markers. As a control, the same procedures were applied to isolates from HIV-seronegative tuberculosis patients ($n=87$).

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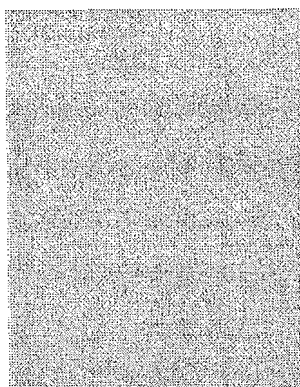
Results. Of 86 mycobacterial strains, *M. tuberculosis, M. avium* and *Mycobacterium chelonae* were identified in 48 (55.8%), 36 (41.9%) and 2 (2.3%) isolates, respectively. The obtained RFLP patterns of *M. tuberculosis* isolates from both the HIV-seropositive and -seronegative groups were variable, suggesting no obvious clustering among the isolates. Similar results were obtained in isolates of *M. avium*.

Conclusions. This is the first report on the molecular epidemiology of *Mycobacterium* spp. isolated from HIV-seropositive patients in Japan. The results indicate that no particular clones of *M. tuberculosis* or *M. avium* prevail in HIV-seropositive patients in Japan. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

Introduction

In recent years, a decline in the number of new patients with acquired immunodeficiency syndrome (AIDS) has been observed in several industrialized countries, including the United States, Western European countries, Australia and New Zealand.[1-6] However, no decline in patients with human immunodeficiency virus (HIV) has been observed in Japan.[7] Mycobacterial infections, such as those of *Mycobacterium tuberculosis* and *Mycobacterium avium*, are important opportunistic infections in HIV-seropositive patients. With respect to tuberculosis (TB), several studies based on clinical observations[8-12] and on epidemiologic surveys[13-17] have provided evidence that HIV infection is a risk factor for the development of active and often lethal TB. Outbreaks of TB among communities of HIV patients have been reported in the United States, but multidrug resistant (MDR) *M. tuberculosis* strains were rarely isolated from these patients.[12,18] In sub-Saharan Africa, TB associated with HIV has played an important role in increasing TB transmission throughout the population.[17,19]

Non-tuberculous mycobacterial infection can be difficult to treat because of primary resistance against most of the commonly used anti-tubercular drugs, such as isoniazid, rifampin, streptomycin, ethambutol, pyrazinamide and kanamycin.[20] A relatively high prevalence of non-tuberculous mycobacterial infections has been observed in HIV/AIDS patients, and 25-50% of patients with AIDS in the United States and Europe are infected with this group of bacteria, primarily with *M. avium*, which mainly causes disseminated mycobacteraemia in AIDS patients.[21]

Japan is considered to have a low prevalence of HIV/AIDS, with a cumulative number of 2556 AIDS cases and 5140 HIV cases reported by the end of 2002.[7] However, the recent trend of HIV cases shows a substantial increase, particularly among men who have sex with men and youth/young adults. A considerable number of HIV patients in Japan have experienced discrimination or breach of confidentiality and they feel insufficiency of social and economical supports.[22] Patients with mycobacterial infection used to be discriminated, but the prejudice toward the patients declines. The medical, social and economic backgrounds of HIV patients in Japan differ considerably from those in regions such as North America, Europe and Africa. The correlation between HIV and mycobacterial infections in Japan may also differ from that in countries where research on AIDS-related diseases is well developed. Survey of the occurrence and clinical profiles of these infections is important for the development of countermeasures against mycobacteria and HIV coinfection. In this study, we analysed the current prevalence, clinical features and epidemiologic findings of mycobacterial infection associated with HIV infection in Japan.

Materials and methods

Bacterial isolates and clinical data

From 1996 to 2003, 86 clinical mycobacterial isolates were obtained from eight hospitals in Japan: the International Medical Centre of Japan (IMCJ) (Tokyo); Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (JATA) (Tokyo); National Tokyo Hospital (Tokyo); Tokyo Metropolitan Komagome Hospital (Tokyo); Social Insurance Central General Hospital (Tokyo); National Nishi-Kofu Hospital (Yamanashi); National Osaka National Hospital (Osaka) and National Kyushu Medical Centre (Fukuoka). Clinical information on individual patients was obtained by the physicians in charge with questionnaire on mycobacterial isolation date, history of previous mycobacterial infection, microscopic observation of

Table 1 Nationality and sex of HIV-positive patients with mycobacterial infection in Japan

Nationality	No. of patients	Male:female	Mycobacteria species
Japanese	33	31:2	M. tuberculosis: 21 M. avium: 11 M. chelonae: 1
Non-Japanese	16	9:7	M. tuberculosis: 9 M. avium: 6 M. chelonae: 1
Unknown[a]	37	33:2, unknown[a]:2	M. tuberculosis: 18 M. avium: 19 M. chelonae: 0
Total	86	74:10, unknown[a]:2	M. tuberculosis: 48 M. avium: 36 M. chelonae: 2

[a] Nationality or sex of these patients was not disclosed due to the ethics code of the corresponding hospital.

sputa, sites of infection (pulmonary or extra-pulmonary), peripheral blood CD4$^+$ lymphocyte number, chemotherapeutic regimens and standard demographic data. The Ethics Committees in each hospital approved this study (IMCJ-H13-54) and all patients gave a written informed consent.

As a control for *M. tuberculosis* genotyping, 87 clinical isolates from adult HIV-seronegative tuberculosis patients without any serious complication at IMCJ were used. Since other hospitals, except JATA, have no ward for TB patients and the RFLP patterns of *M. tuberculosis* isolates from JATA and IMCJ were variable, and showed no obvious clustering among the isolates.

Mycobacterial culture and identification of strains

Bacteria were grown on egg-based Ogawa medium (Kyokuto Pharmaceutical Co., Ltd, Tokyo, Japan) for 3-5 weeks. Cultured organisms were applied to a polymerase chain reaction (PCR) kit for *M. tuberculosis* diagnosis (Amplicor *Mycobacterium tuberculosis* Test, Roche Diagnostic Systems, Inc., Branchburg, NJ), and PCR-negative organisms were further applied to an identification kit for mycobacterial species that uses DNA-DNA hybridization (DDH Mycobacteria, Kyokuto Pharmaceutical Co., Ltd).

Drug sensitivity testing

Drug sensitivity of *M. tuberculosis* strains was tested by two agar proportion methods, one with Middlebrook 7H10 agar medium, as recommended by the U.S. Public Health Service,[23] and the other with egg-based Ogawa medium, as recommended

by the Japanese Society for Tuberculosis (Vit Spectrum-SR™, Kyokuto Pharmaceutical Co., Ltd).

DNA fingerprinting

Chromosomal DNA from mycobacterial isolates was prepared as described previously[24,25] but with slight modification. The DNA was precipitated in isopropanol, and the precipitates were redissolved in 20 μl 0.1X TE buffer.

For IS6110- and (CGG)$_5$-restriction fragment length polymorphisms (RFLP)[26] of *M. tuberculosis*, DNA was digested overnight with restriction enzymes *PvuII* and *AluI* (Takara Bio, Inc., Shiga, Japan), respectively. The digested fragments were separated by electrophoresis on 1% agarose gels. A 1-kb DNA ladder (Promega Corp., Madison, WI) was used as a marker. The agarose gels were stained with ethidium bromide, and the results were recorded photographically. DNA fragments were transferred onto N$^+$ Hybond membrane (Amersham Biosciences, Little Chalfont, UK), and the DNA was fixed to the membrane by UV illumination. The IS6110 probe was a 245-bp DNA fragment amplified by PCR as described previously.[25] The 15-mer oligonucleotide (CGG)$_5$ was synthesized by Nippn Techno Cluster, Inc., Tokyo, Japan. The probes were labelled with horseradish peroxidase by the ECL Direct™ System (Amersham Biosciences). Hybridization was conducted with the ECL Direct™ System, according to the recommendations of the manufacturer. Autoradiographs were obtained by exposing the membranes to X-ray film.

For IS1245-[27] and IS1311-RFLP[28] of *M. avium*, DNA was digested overnight with *PvuII*. The IS1245 and IS1311 probes were 427 and 200-bp DNA fragments, respectively, and were amplified by

PCR as described previously.[27,28] Briefly, the oligo-nucleotides for IS1245, 5'-GCCGCCGAAACGATC-TAC-3' and 5'-AGGTGGCGTCGAGGAAGAC-3',[27] and for IS1311, 5'-GTCGGGTTGGGCGAAGAT-3' and 5'-GTGCAGCTGGTGATCTCTGA-3',[28] were used to amplify the fragments prepared from purified chromosomal DNA from M. avium ATCC 25291 by PCR.

Analysis

Fingerprinting patterns of M. tuberculosis or M. avium were analysed with Molecular Analyst Fingerprinting Plus Software, version 1.6 (Bio-Rad Laboratories, Inc., Hercules, CA). To facilitate comparison of the fingerprinting patterns, normalization was performed relative to the molecular-weight markers. Each dendrogram was calculated according to the unweighted-pair group method with average linkage according to the supplier's instructions.
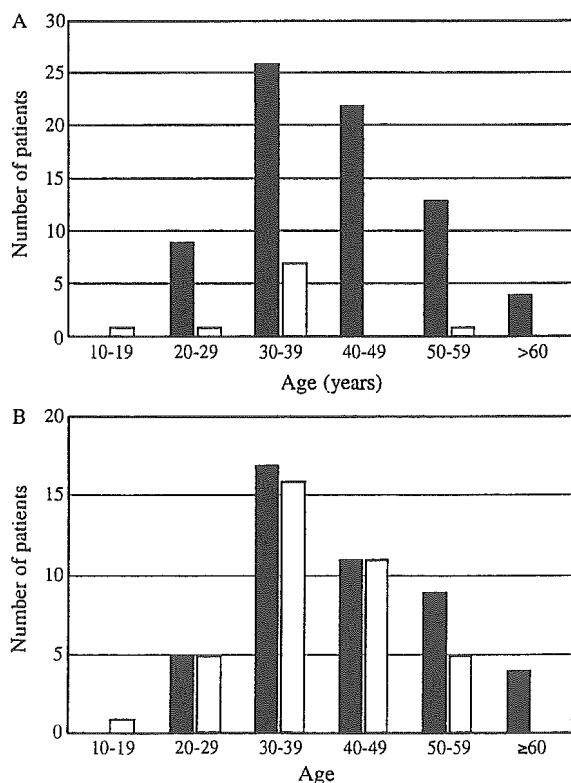
Figure 1   Distribution of 84 mycobacterial infections in HIV-seropositive patients. Panel A: age (years) and sex distribution. Filled bars, male; open bars, female. Panel B: age (years) and pathogenic agent distribution. Filled bars, tuberculosis patients; open bars, non-tuberculous mycobacterial-infected patients.

Results

Mycobacterial infection in HIV-seropositive patients

From 86 HIV-seropositive patients, 48 (55.8%) M. tuberculosis, 36 (41.9%) M. avium, and 2 (2.3%) Mycobacterium chelonae isolates were identified (Table 1).

Nationality and sex are also listed in Table 1. Mean age was 40.5±12.2 years, ranging from 11 to 68 years. Most mycobacteria and HIV coinfected patients were aged 30-39 years (Fig. 1). The most frequent route of HIV infection was sexual transmission (90%); other routes were infection by blood products (5%), drug abuse (5%), mother-to-child infection (1%) and unknown (1%). With respect to mycobacterial infection, 48 and three individuals had primary and recurrent infection, respectively. There was no corresponding record for the remaining patients.

Profile of HIV-seropositive patients with M. tuberculosis

In 46 of the 48 tubeculosis patients, the ratio of males/females was 43/3 (Table 1). Mean age was 42.7±11.9 years, ranging from 22 to 68 years. Twenty-five patients had combined pulmonary and extra-pulmonary infection, mainly due to miliary tuberculosis. A total of 56.3% of the 48 patients had pulmonary tuberculosis, as evidenced by positive microscopy smears. Peripheral blood CD4$^+$ cell counts at the time of TB diagnosis ranged from 6 to 331/mm^3, and the median was 62/mm^3.

According to drug sensitivity testing, 43 isolates (89.6%) were sensitive to anti-tubercular drugs, 3 (6.3%) were resistant to a single drug, and 2 (4.2%) were resistant to 2 and 5 drugs, respectively.

In 87 HIV-seronegative TB patients with tuberculosis, 82 were Japanese and five were non-Japanese. The ratio of males/females was 56/31. Mean age was 53.3±20.5 years (56.1±19.0 years for males and 48.6±22.0 years for females), ranging from 18 to 95 years (18-90 for males and 18-95 for females) and patients over 40 years of age accounted for 66.7% of the total. According to drug sensitivity testing, 75 isolates (86.2%) were sensitive to anti-tubercular drugs, 6 (6.9%) were resistant to a single drug, and 6 (6.9%) were resistant to 2 and 6 drugs, respectively.

RFLP analysis of M. tuberculosis

To determine whether specific strain(s) of tubercular bacilli prevail among HIV-seropositive patients in Japan, we analysed DNA fingerprints of the isolates by RFLP analysis. Thirty-three of the 48 M. tuberculosis clinical isolates were analysed by RFLP, and the patterns are shown in Fig. 2.
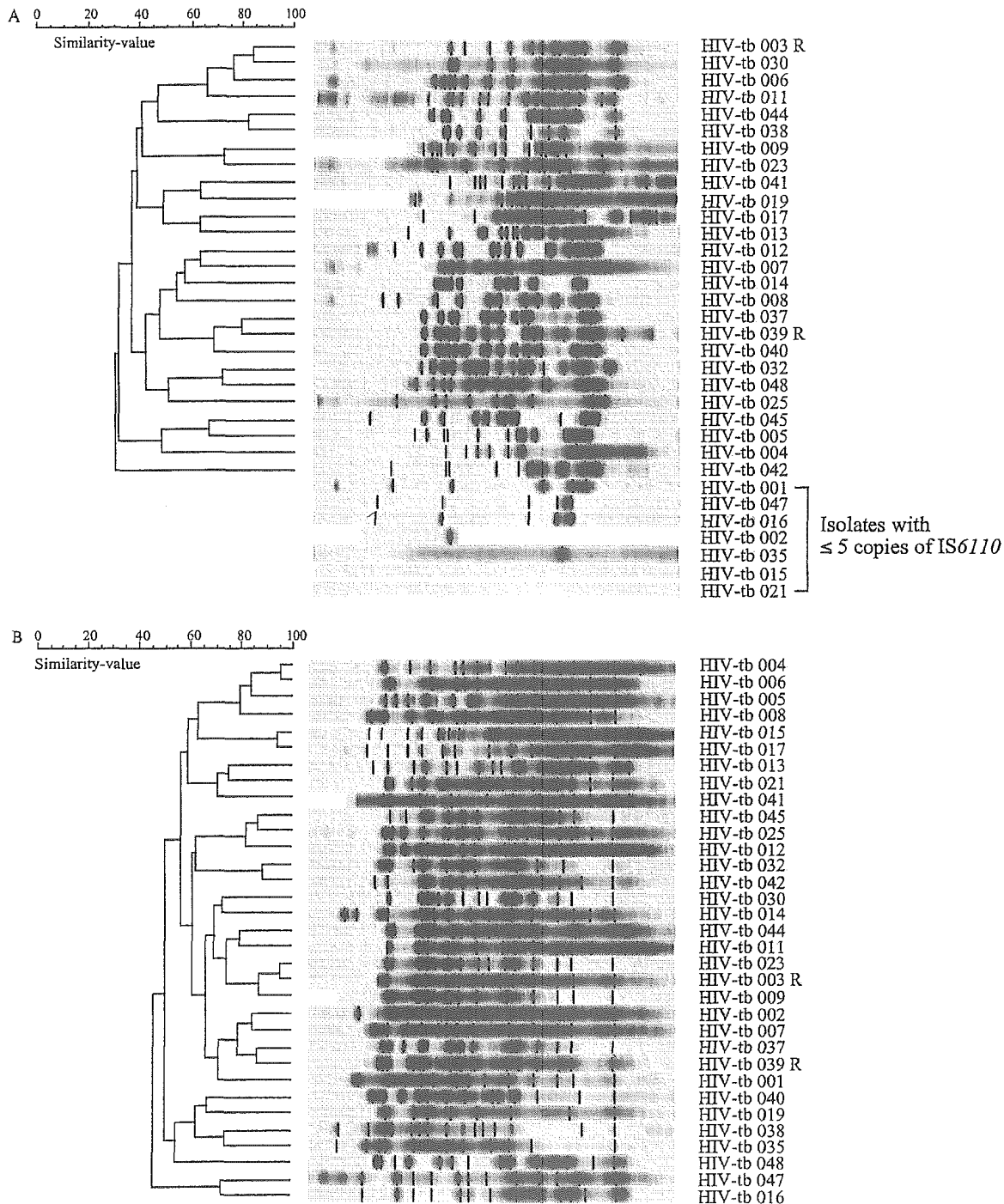
Figure 2 IS6110- and (CGG)$_5$-probed DNA fingerprinting patterns of M. tuberculosis clinical isolates from HIV-seropositive patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS6110 (A) or (CGG)$_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS6110-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, HIV-tb 003 R is an HIV-seropositive patient-derived isolate.
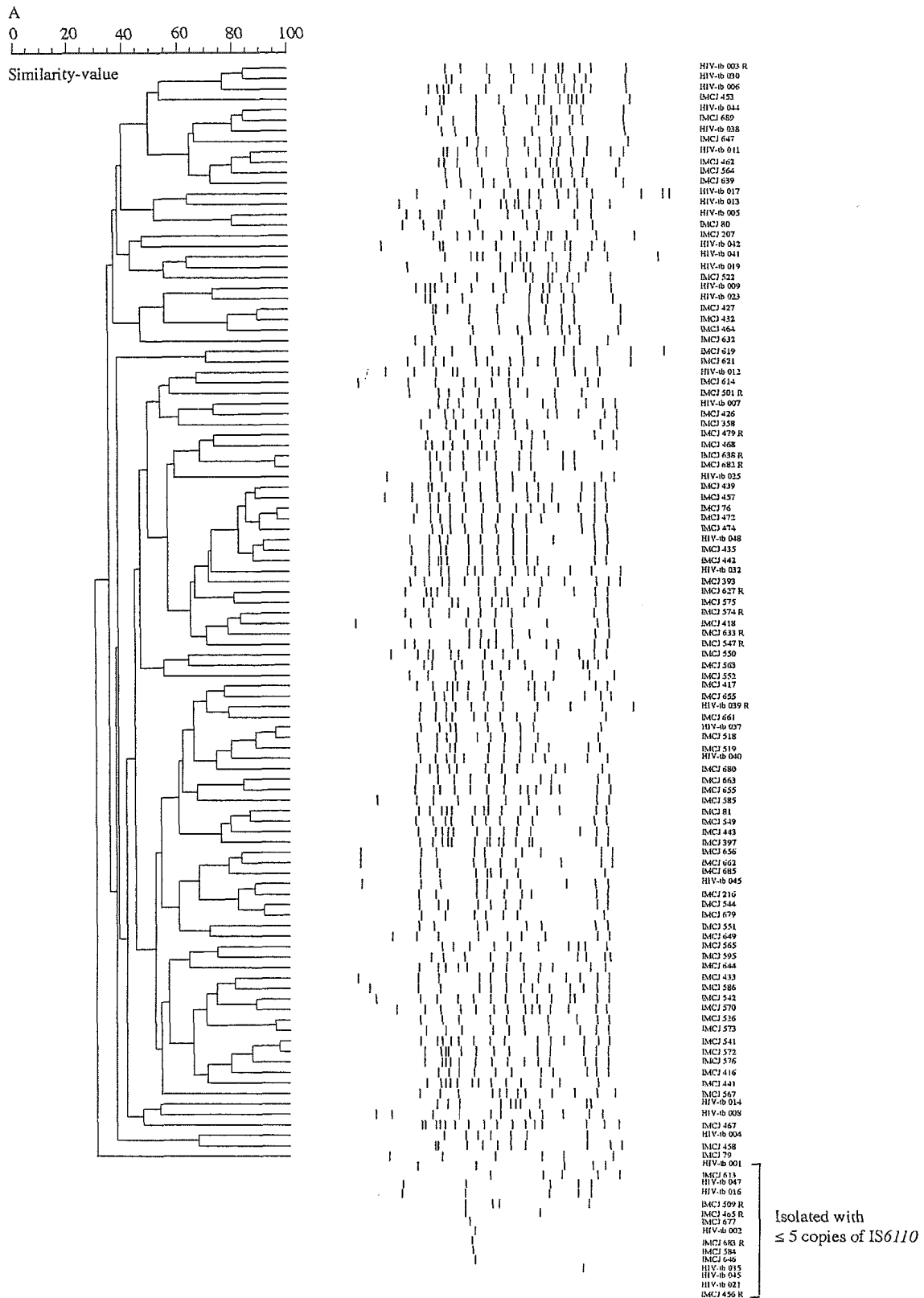
Figure 3. IS6110- and (CGG)₅-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive and HIV-seronegative patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS6110 (A) or (CGG)₅ (B) band is normalized so that the patterns for all strains are comparable. In the IS6110-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, a prefix of 'IMCJ' indicates an HIV-seronegative patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, IMCJ 627 R is an HIV-seronegative patient-derived isolate.

B
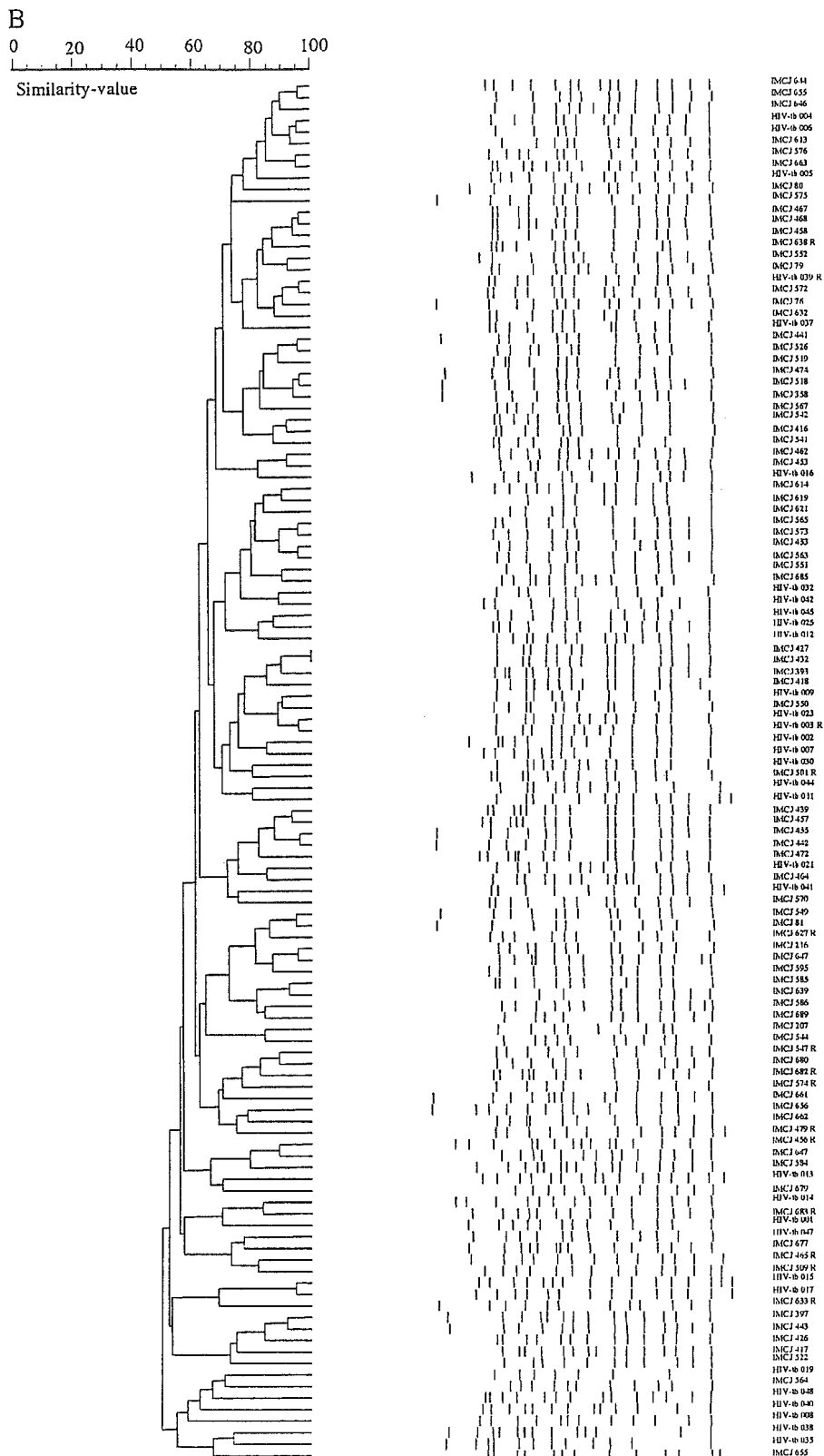
Figure 3 (*continued*)
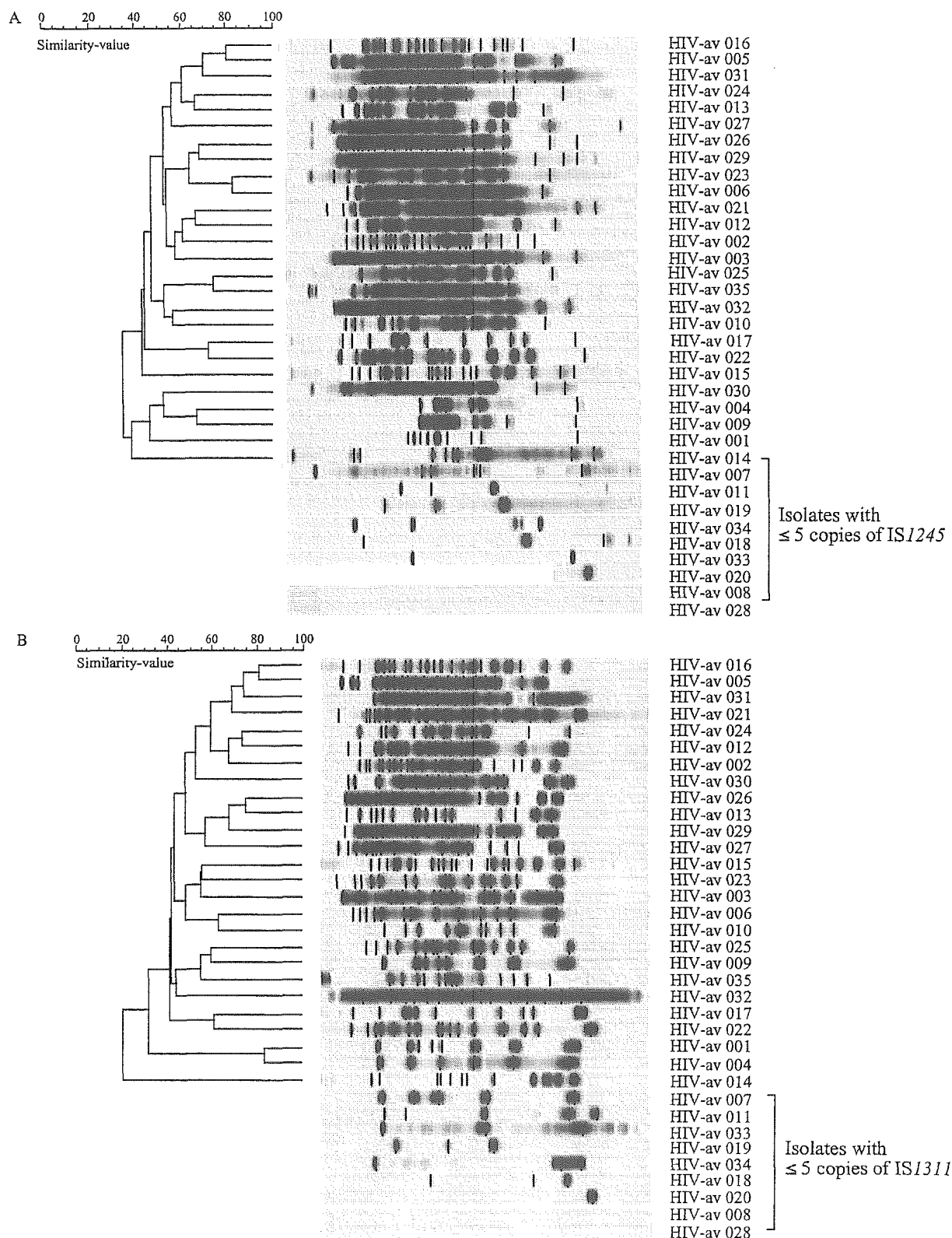
Figure 4   IS1245- and IS1311-probed DNA fingerprinting patterns of *M. avium* clinical isolates from HIV-seropositive patients in Japan and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS1245 (A) or IS1311 (B) band is normalized so that the patterns for all strains are comparable. In both the IS1245- and IS1311-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated. The isolates are named as follows: a prefix of 'HIV-av' indicates an HIV-seropositive patient-derived isolate.

The number of IS6110 bands in the isolates ranged from 0 to 15 (Fig. 2A). Thirty-one different IS6110 fingerprinting patterns were observed in the isolates. Seven isolates (21.2%) showed 0-5 IS6110 bands, these isolates were insufficient in band number for cluster analysis. Identical patterns were not found among the isolates (Fig. 2A).

The number of (CGG)₅ bands of the copy isolates ranged from 8 to 16 (Fig. 2B). Thirty-three different (CGG)₅ fingerprinting patterns were observed in the isolates. Identical patterns were not found among the isolates (Fig. 2B). Three pairs of isolates (HIV-tb 004 and HIV-tb 006, HIV-tb 015 and HIV-tb 017 and HIV-tb 03 and HIV-tb 023) were closely related, with 90% or more similarity. However, the corresponding patients did not show any linkage such as hospital or date of sample isolation.

Comparison of RFLP patterns between HIV-seropositive and -seronegative TB patients

To assess whether the same kinds of mycobacteria prevail in HIV-seropositive and -seronegative patients, RFLP patterns of $M.$ $tuberculosis$ clinical isolates were investigated in both groups. In IS6110- or (CGG)₅-patterns from both groups, the patterns from both HIV-seropositive and -seronegative patients did not consist of apparent clusters and appeared to segregate randomly in the dendrograms (Fig. 3).

Profile of HIV-seropositive patients with $M.$ $avium$ infection

The number of HIV-seropositive patients with $M.$ $avium$ infection was 36 (Table 1). Mean age was 37.4 ± 9.9 years, ranging from 11 to 56 years. Most of the $M.$ $avium$-infected patients (58.3%) suffered from disseminated infection, and the sputa of 88.9% patients were culture-positive but smear-negative upon preliminary mycobacterial examination. Almost all the $M.$ $avium$ isolates were resistant to all anti-TB drugs. Peripheral blood CD4$^+$ cell counts of 34 patients (unknown: 2) at the time of $M.$ $avium$ diagnosis ranged from 0 to 202/mm^3, and the mean CD4$^+$ cell count was 38.6 ± 60.4/mm^3. In 26 of 34 patients (76.4%), the CD4$^+$ cell counts were less than 50/mm^3.

RFLP analysis of $M.$ $avium$

The RFLP patterns of 35 of 36 $M.$ $avium$ isolates were investigated (Fig. 4). The number of IS1245- and IS1311-bands ranged from 0 to 25 and from 0 to

23, respectively, and analysis showed 33 different patterns of each. Nine isolates (25.7%) showed 0-5 bands; these isolates were insufficient for cluster analysis because of few numbers of IS1245 or IS1311 bands. Among the isolates, identical patterns were not found. Cluster analysis revealed no clusters. These results indicate that no particular strain of $M.$ $avium$ prevailed among HIV-seropositive patients.

Discussion

We analysed mycobacterial isolates obtained from HIV-seropositive patients and found that $M.$ $tuberculosis$ and $M.$ $avium$ accounted for a large proportion of HIV-associated mycobacterial infection in Japan. Although $Mycobacterium$ $kansasii$ is also known to be associated with AIDS,[29,30] it was not isolated in this study. Two isolates of $M.$ $chelonae$ were obtained from stool specimens of patients.

It has been suggested that recurrent TB is responsible for most cases of HIV-associated TB, particularly in countries with high-level of transmission.[31] Kanazawa et al.[32] reported that the majority of HIV-positive Japanese patients with TB (83%) were more than 40 years of age and had recurrent TB. In the present study, the age of HIV-seropositive patients shifted to the 30s, suggesting that TB incidence among HIV-positive patients in Japan is transforming from recurrence in older persons to primary infection in younger persons.

With respect to drug resistance, 10.4% of the strains obtained from HIV-seropositive patients showed resistance to one or more anti-TB drugs. Abe et al.[33] reported that 10.3% of $M.$ $tuberculosis$ isolates from patients in Japan were resistant to one or more of the four first-line anti-TB drugs: isoniazid, rifampin, streptomycin and ethambutol. A 1996 report noted that the drug resistance rate in New York City was 33%.[34]

We found that both the IS6110 and (CGG)₅ fingerprinting patterns of $M.$ $tuberculosis$ isolates from HIV-seropositive patients in Japan differed from those of a TB outbreak in New York City[12,18] and of isolates from the patients in Lima, Peru.[16] Comparing RFLP patterns of $M.$ $tuberculosis$ isolates from HIV-seropositive patients with those from HIV-seronegative patients, we found that the DNA fingerprints did not distinguish between these two TB patient groups. These data indicate that TB transmission in Japan occurs via HIV-seronegative TB patients rather than via HIV-seropositive TB patients. The epidemiological studies in Botswana[17] and Tanzania[19] showed no clustering any particular

pattern of DNA fingerprints. These findings are consistent with our present results.

Patients infected with *M. avium* suffer from chronic lung disease. In patients with HIV-associated *M. avium* infection, it is thought that pulmonary symptoms will develop when $CD4^+$ lymphocyte counts fall below $100/mm^3$. The median $CD4^+$ lymphocyte count at *M. avium* diagnosis was $10/mm^3$, and at that time the majority of patients showed disseminated *M. avium* infection. Almost all *M. avium*-infected patients in the present study were in advanced stages of AIDS. *M. avium* organisms can be isolated from environmental sources such as water or soil.[35-37] Because they are capable of causing infection in animals, e.g. birds and pigs, it has been postulated that the source of human infection is either the environment or from animals. Ichiyama et al.[38] searched sources of soil, water and dust in Japan and found *M. avium* isolates in 68.0% of dust samples tested. It is believed that the most frequent mode of *M. avium* infection in humans occurs by inhalation or by deglutition of the agent from environmental sources.[37,39,40] To prevent infection with this agent in HIV-seropositive patients, further studies are needed to identify original sources and to further elucidate infectious routes.

In conclusion, the number of HIV patients in Japan is increasing; according to the latest report,[7] the number is over 10 000. The number of TB patients in Japan remains higher than in other developed countries.[41] However, the number of HIV-infected patients with mycobacterial infection in Japan is limited. With respect to TB, no outbreak among HIV-seropositive patients was found. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

Acknowledgements

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RAPID AWARENESS AND TRANSMISSION OF SEVERE ACUTE RESPIRATORY SYNDROME IN HANOI FRENCH HOSPITAL, VIETNAM

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Abstract. A case-control study was conducted to examine the relationship between severe acute respiratory syndrome (SARS) and the time-dependent precautionary behaviors taken during an outbreak of SARS in Hanoi French Hospital (HFH), Vietnam. Masks (odds ratio [OR] = 0.3; 95% confidence interval [CI]: 0.1, 0.7) and gowns (OR = 0.2; 95% CI: 0.0, 0.8) appeared to prevent SARS transmission. The proportion of doctors and nurses who undertook each measure significantly improved (χ^2 = 9.8551, P = 0.043) after the onset of secondary cases. The impact of individual behaviors on an outbreak was investigated through mathematical approaches. The reproduction number decreased from 4.1 to 0.7 after notification. The basic reproduction number was estimated, and the use of masks alone was shown to be insufficient in containing an epidemic. Intuitive results obtained by means of stochastic individual-based simulations showed that rapid improvements in behavior and isolation would increase the probability of extinction.

INTRODUCTION

Notwithstanding the announcement of containment by the World Health Organization (WHO) in 2003,[1] severe acute respiratory syndrome (SARS) has remained a matter of concern worldwide, and it is not surprising that several cases of SARS have reemerged, for example, in China in April 2004.[2] Although the mode of transmission remains partially unclear, especially with regard to airborne transmission[3] and super-spreading events,[4,5] it appears to occur predominantly by large droplets, direct contact with infectious material, or contact with fomites contaminated with infectious material.[6,7] The most effective containment measures identified to date include the tracing of contacts,[8] quarantine,[9] triage and early case detection,[10,11] and isolation.[12] Further, because the close contact required for transmission easily occurs in hospital settings,[13–15] nosocomial spread was determined as one of the major epidemiologic features of SARS.[7,16,17] The elimination of hospital transmission through enhanced infection control practices is therefore a crucial control measure.

An early study in Hong Kong showed that the practice of droplet and contact precautions was adequate in most clinical settings in significantly reducing the risk of infection after exposure to patients with SARS,[18] and if practiced by a high proportion of susceptible individuals, precautionary measures are expected to significantly reduce transmission.[19] The adoption of routine preventive behaviors based on appropriate training and control among health care workers (HCWs), undertaken prior to the isolation of SARS patients, was shown to be one of the most crucial control measures.[20–22]

In this context, Vietnam is considered to have achieved the first highly successful containment of SARS during the early phase of the outbreak.[23] One reason for this rapid containment is thought to be the prevention of infection leakage from hospitals back into the general community.[24] A second is the successful discontinuation of the chain of nosocomial transmission several days after onset based on the radical control measures of the Ministry of Health, Vietnam.[25] Although several nosocomial transmissions were observed in Hanoi French Hospital (HFH) in the early days of the outbreak,[26,27] none were identified in HFH or another local hospital in the latter phase.[28] In both hospitals, staff instituted stringent precautions, strict isolations, and quarantines under the encouragement of Dr. Carlo Urbani (Dr. Urbani died of SARS before seeing the success of the containment).[29] We therefore consider that a comprehensive understanding of the successful containment measures adopted by HFH and their theoretical underpinnings are crucial to the success of control strategies for any future recurrence. Here, we use a case-control study design to time-dependently examine the relationship between SARS and the precautionary behaviors undertaken by those exposed in HFH. We then use mathematical approaches to develop intuitive analyses of the impact of individual behaviors on the control of a SARS epidemic.

MATERIALS AND METHODS

Case-control study. HFH is a 56-bed secondary care hospital. After the admission of an index case on February 26, 2003, 38 cases in total were confirmed to have symptomatic SARS infection. The occurrence of newly diagnosed SARS cases due to local transmission continued until April 7, 2003, 3 weeks before the date when the Vietnamese government and WHO declared the outbreak successfully contained (April 28, 2003) (Table 1). The duration of the HFH outbreak was analyzed by separating it into three phases: Stage 1, February 26–March 4, from admission of the index case to the onset of secondary cases; Stage 2, March 5–March 10, from the suspicion of nosocomial spread to closure of the hospital; and Stage 3, from March 11 on, from strict isolation to local eradication.

A case-control study of 29 of the 38 laboratory-confirmed SARS cases and 98 controls was performed in HFH. The case group included 22 of 28 (78.6%) individuals admitted and retained in HFH and 7 of 10 (70.0%) individuals transferred to another hospital after first being admitted to HFH (total N = 29). The reasons for nonparticipation were death due to

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Chronology of the outbreak of SARS in Hanoi French Hospital (HFH), Vietnam

Stage 1		
26-Feb-03	Day 0*	An index case complaining of fever, dry cough, and headaches was admitted to HFH.
2-Mar-03	Day 3	After intubation, the index case was isolated in ICU the following day.
4-Mar-03	Day 6	Nine secondary cases were suspected.
Stage 2		
5-Mar-03	Day 7	Seven additional cases were suspected. HFH informed the Ministry of Health, Vietnam, of the strange influenza. The health minister and experts from the World Health Organization (WHO) held a meeting. Dr. Carlo Urbani informed all staff to perform stringent precautions.
8-Mar-03	Day 10	HFH decided to close all outpatient/inpatient services. Visitors were not allowed to enter HFH. The hospital board of directors held an emergency meeting. Dr. Carlo Urbani explained the necessity of precautions and possibility of contamination as a mode of transmission. Health care workers were advised not to return home.
Stage 3		
11-Mar-03	Day 13	All inpatients were transferred to other hospitals. The 2nd floor of HFH was allocated to SARS patients only and strict isolation was enforced. ⊛ Three zones were allocated according to symptoms. ⊛ Nonmission individuals including health care workers were not allowed to enter.
13-Mar-03	Day 15	A special committee for SARS control and prevention was established. WHO issued a "global alert" to worldwide health authorities.
28-Apr-03	Day 60	The Vietnamese government and WHO declared successful containment of SARS in Vietnam.

* Day, days after onset of the outbreak. SARS, severe acute respiratory syndrome; ICU, intensive care unit.

SARS and/or respiratory failure ($N = 5$, 13.2%), refusal to take part ($N = 1$, 2.6%), or relocation ($N = 3$, 7.9%). The case group included 28 HFH employees (3 doctors, 13 nurses and nursing assistants, 10 radiologists and other co-medical workers, and 2 receptionist and administrative staff) and 1 relative of a patient. A further 23 Vietnamese patients who were directly admitted to another hospital were excluded because the detailed source of infection was unknown, although several cases were thought to have been infected in HFH. Detailed descriptions of the laboratory diagnoses were given previously.[28] They were confirmed through serological studies using an indirect enzyme-linked immunosorbent assay (ELISA) (Kirikae T, et al., unpublished data).

Controls were nominated based on employment in HFH and exposure among patients' relatives through HFH. The selection criteria included i) Vietnamese individuals more than 20 years old, ii) those who provided written informed consent based on explanation of our methods and purposes, and iii) those thought to have had contact with confirmed cases inside the hospital based on contact investigations. In total, 98 individuals were included as controls; most were HFH employees (13 doctors, 20 nurses and nursing assistants, 13 radiologists and other co-medical workers, and 11 receptionists and administrative staffs) or relatives of patients ($N = 41$). Although we investigated certain known contacts for inclusion as controls, namely individuals who took care of cases or entered cases' room, those who might have had trivial contact, such as possible exposure outside the hospital during, for example, transportation of SARS cases or in the casualty reception room, were not followed and included. The number of hospital employees investigated represented approximately 55.9% of the total employees used during the outbreak.

All participants were surveyed with regards to their use of personal protective equipment (PPE) and hygiene habits when in contact with patients with SARS; that is, the use of masks, gloves, and gowns, and the practice of hand washing, which were specifically recommended as droplet and contact precautions. In this paper, masks denote surgical masks; N95 masks were not available in the early stage of the outbreak in Vietnam. Individual behaviors were investigated mainly in two separate phases according to time-dependency (in Stage 1 and after entering Stage 2; i.e., Stages 2 and 3) (Table 1) to clarify any behavioral changes that occurred. Standardized questionnaires requiring one of two possible answers for each precaution ("performed" or "not performed") were given to each subject, and all responses were collected. Answers of "sometimes" or "seldom" were defined as "not performed" due to imperfect efficacy. In addition, the frequency of contact with infected individuals was investigated to represent the number of exposures per day. An exposure result of "many times" was recorded for those who had close contact with SARS patients, that is, those who cared for or lived with SARS patients, and those likely to have come into direct contact with the respiratory secretions or body fluids of SARS patients, for example, during close conversation (within 3 feet).[30] After completing the initial primary survey, an identical confirmation survey was performed to confirm the validity of the answers. These surveys were conducted along with other epidemiologic studies (Nishiyama A, et al., unpublished data) until mid-March 2004, almost 1 year after onset of the epidemic. No blood test results showing possible asymptomatic infections were available during the survey period. The participants were informed of how the information would be used and assured of the confidentiality of their responses. The purpose of the study was explained in Vietnamese, and written informed consent was obtained.

Statistical analyses were performed as follows. First, univariate associations between precautionary behaviors and infection were investigated in two separate stages (Stage 1 or Stages 2 and 3). Comparisons between groups were made using the χ^2 or Fisher's exact test for univariate analysis. Multivariate logistic regression was done in Stage 1 using forward stepwise selection (Waldesian) to determine the most significant variable associated with protection among those studied. Significant steps were taken to minimize recall bias with Stages 2 and 3 data. Analysis was restricted to those who had probable contact in these stages. It was further restricted to those cases developing symptoms whose incubation period

was within the greater than 95% confidence interval (95% CI) of having occurred after the beginning of Stage 2; and finally to medical doctors and nurses only, for both cases and controls. Second, univariate associations between sociodemographic variables (sex, age, and occupation) and SARS were investigated, with age and occupation categorized into four different groups each. Third, interactions between the identified most significant protective behavior and other variables significantly associated in univariate analysis were investigated through the use of crosstabs statistics, in which the odds of being infected were stratified according to a comparison of variables, and interactions were sought through the different odds ratio in each strata. Finally, multiple logistic regression analysis was used to determine the protective effect and eliminate confounding variables. As described in the next section, all variables significantly associated in univariate analyses, as well as sociodemographic variables, were selected and entered together in the final model. All data were entered into Microsoft Excel 2000 (Microsoft Co., Redmond, WA), and the statistical data were analyzed using the statistical software "R" (R Development Core Team, Vienna).[31]

Mathematical methods. The predictive effects of the behavioral changes were simulated using an individual-based stochastic model. For ease of understanding, a compartmental model, a type of SEIR (susceptible [S], exposed [E], infected [I], and recovered/removed [R]) model, which considered the process of transmission according to the protective behaviors taken against infectious contact among susceptible individuals, was applied. Instead of assuming "exposed (latent)" and "infectious" periods, E and I were defined as "incubation" and "symptomatic" periods, respectively, as the infectious period of SARS has not been fully clarified. Although SEIR models are usually deterministic and use mean estimations as model parameters, even with regard to SARS,[12,32] stochastic simulations were performed in this study because of the need to consider the stochasticity of each protective behavior, and also because of the small sample population size. The infectious lifetime of each individual was presented as an absorbing Markov chain. The simulations start with an individual index case (Day 0) in a population of 300 in which all individuals are susceptible.

Of the total 127 subjects studied (29 cases and 98 controls), 62.2% ($N = 79$) were considered to have had casual contact and 37.8% ($N = 48$) to have had close contact with SARS patients. The number of casual contacts (κ_1) was directly obtained ($= 0.7 \pm 0.2$ [day^{-1}]), while the mean of close contacts ($\kappa_2 = 0.4$ [day^{-1}]) was determined with the following equation:

$$\kappa_2 = \kappa_1 \ln(OR_{closed}) \qquad (1)$$

where OR_{closed} ($= 2.5$; 95% CI: 1.1–5.9) denotes the odds ratio (OR) of getting infected as a result of close contact. In other words, to quantify close contact, we assumed that the frequency of infection is mainly determined by the frequency of contact, so that the ratio of the frequency of close to casual contact becomes proportional to the logarithm of the OR of transmission. The protective effect of precautionary behavior was approximated by:

$$\beta = 1 - RR = 1 - \frac{a(c+d)}{c(a+b)} \approx 1 - \frac{ad}{bc} \approx 1 - OR \qquad (2)$$

where RR and OR denote the relative risk and odds ratio, respectively, of becoming infected while performing a protective behavior (with precaution = with exposure). Here, a is the number of exposed ill people; b, the number of exposed healthy people; c, the number of unexposed ill people; and d, the number of unexposed healthy people. If the outcome (i.e., disease investigated) is a rare event, that is, if a and c are very small compared with b and d, respectively, $(a + b)$ and $(c + d)$, respectively, would be closely similar to b and d alone. In this case, OR would approximate RR.

The lengths of the incubation and symptomatic periods were both assumed to be independently and identically distributed random variables with a probability density function of γ distribution, the mean and variance of which were defined as 3.8 [days] and 8.3 [days^2], and 16.2 [days] and 7.9 [days^2], respectively.[24,33] These distributions were applied to difference equations (as a discrete time model) by discretizing the probability density functions by day (for a detailed description of the simulation algorithm, see the Appendix).

The first simulation scenario hypothetically investigated the unchanged coverage and mean protective effects of a behavioral measure throughout the epidemic. Primary information on protective behaviors was obtained from our Stage 1 survey. Estimates for the extent of a protective effect, the associated causative behavior of which was found in forward stepwise logistic regression to be the most significantly associated with protection (as described above), were obtained through the use of further multivariate logistic regression analysis. This analysis incorporated all variables significantly associated with SARS on univariate analysis (i.e., other precautionary behavior, gender, age and occupation). To investigate the impact of the coverage of a protective measure on the trajectory of an outbreak, sensitivity of the cumulative number of SARS cases at Day 30 to the coverage of masks was investigated in the mean field equation. In the second scenario, it was assumed that coverage improved dramatically after entering Stage 2 (Day 7) due to an awareness of transmission. Further, in Stage 3 (Day 13), the hospital implemented not only stringent precautions but also strict isolations. To understand the trajectory of transmission in detail, the number of incubating as well as symptomatic individuals was investigated. As was in fact seen during Stage 3 of the outbreak, it was also assumed that all cases who became symptomatic were immediately isolated and that nobody except a limited number of healthcare workers were permitted to have contact with them. Because the greatest uncertainty applies to the time taken to increase coverage of a protective measure and to implement strict isolations, sensitivity analyses comparing the cumulative number of SARS cases up to Day 30 were performed with the time to change both protective measures set simultaneously on the same day. Finally, the basic reproduction number was estimated using the (effective) reproduction number obtained in Stage 1 (see Appendix).

RESULTS

Table 2 shows the univariate association between the precautionary behaviors taken (SARS and non-SARS [control] cases) in Stage 1 and SARS. The use of masks ($P = 0.011$) and gowns ($P = 0.012$) appeared to prevent infection, whereas handwashing and the use of gloves were less likely to provide protection. Only two subjects who performed all pro-

TABLE 2

Precautionary measures taken by all participants in Stage 1

	SARS cases (N = 25)	Non-SARS (N = 90)	P value*	Odds ratio† (95% CI)‡
All measures	2	44	0.059	0.2 (0.0–1.0)
Handwashing before§	12	51	0.937	1.0 (0.4–2.3)
Handwashing after¶	15	56	0.766	1.1 (0.5–2.8)
Masks‖	8	35	0.011	0.3 (0.1–0.7)
Gloves	8	30	0.643	0.7 (0.3–1.9)
Gowns	2	25	0.012	0.2 (0.0–0.8)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

tective measures developed symptomatic infections ($P = 0.059$). Forward stepwise logistic regression of the five protective measures (0.05 for entry and 0.10 for removal probability) showed that only the use of masks was significant in the final model (OR, 0.29, 95% CI; 0.11-0.73, $P = 0.009$). In Stages 2 and 3, the use of masks ($P = 0.001$) and gowns ($P = 0.010$) was significantly associated with non-infection among doctors and nurses still not infected after Stage 1 (Table 3). Most performed all the personal protective measures recommended, and only one individual who wore masks was infected. The comparative results of the behaviors of all participants at Stage 1 and after entering Stage 2 are shown in Figure 1a. The proportions of individuals who performed the investigated protective behaviors increased after entering Stage 2. However, these behavioral changes were not significantly different between the two phases ($P = 0.960$). The behaviors performed by the doctors and nurses ($N = 48$; Figure 1b) who had the closest contact with the SARS patients drastically and significantly improved after entering Stage 2 ($\chi^2 = 9.855, P = 0.043$).

The univariate associations between socio-demographic variables and SARS throughout the epidemic are shown in Table 4. Females were more likely to become infected than males ($P = 0.011$), and a significant association of SARS with nurses ($P = 0.008$) was observed. In HFH, infection was frequent in the 40–49 age strata ($P = 0.015$). Among all study subject, relatives of patients ($P < 0.001$) appeared to be the least frequently infected. Table 5 shows the interaction between the use of masks and other significantly associated variables in univariate analyses. Even though we saw no signifi-

TABLE 3

Precautionary measures taken by health care workers in Stages 2 and 3

	SARS cases (N = 4)	Non-SARS (N = 26)	P value*	Odds ratio† (95% CI)‡
All measures	1	25	0.001	< 0.1 (0.0–0.3)
Handwashing before§	4	25	1.000	NC
Handwashing after¶	4	25	1.000	NC
Masks‖	1	25	0.001	< 0.1 (0.0–0.3)
Gloves	4	25	1.000	NC
Gowns	3	26	0.010	NC

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

cant difference in the OR of using masks versus the use of gowns, females (OR = 0.2) and nurses (OR = 0.1) were more effectively protected by the use of masks than others in Stage 1. In Stages 2 and 3, the use of gowns showed overall reasonable OR (= 0.2), whereas most other interactions could not be calculated due to the scarcity of cases.

Figure 2a shows the mean and corresponding 95% CI of the trajectory (shown as prevalence) of an epidemic from 250 simulation runs which hypothetically assumed unchanged coverage as well as the protective effects of the precautionary measures observed in Stage 1. The precautionary measure in this simulation was based on a multivariate logistic regression which included all variables showing significant associations in univariate analyses, and focused on the impact of the use of masks, given the identification of this behavior as the most important protective measure ($\beta = 0.6$ obtained from OR $= 0.4$, $P = 0.020$). The coverage of masks was obtained as 52.0% from Table 2. If an outbreak was simply allowed to continue growing under these conditions, the results showed that approximately 50 to 90 symptomatic cases would occur by Day 30. The reproduction number (R) was estimated as 4.1 (95% CI; 1.9–6.4), and from this estimate the basic reproduction number was estimated as 6.0. Sensitivity of the cumulative number of cases to the coverage of masks, in the mean field, is shown in Figure 2b. Certain reduction in the cumulative number of cases was observed with significant improvements in coverage.

Figures 2c and 2d shows the outbreak trajectory of 250 simulations assuming improved coverage (from 52.0 to 81.5%) among susceptible individuals on Day 8 and restriction of contact with symptomatic individuals to health care workers on Day 13. The protective effect obtained from multivariage regression was 0.9 (OR = 0.1, $P = 0.955$). The reproduction number in Stage 2 was estimated as 0.7 (95% CI; 0.0–2.3). The number of incubating individuals began to show a decreasing trend after these events (Figure 2c), followed by a declining trend in the number of symptomatic cases (Figure 2d). Most of the simulated outbreaks eventually declined to extinction before Day 120. The sensitivity of the final size of an epidemic, evaluated through observations of the cumulative numbers of cases, to the timing of drastic changes in protective behaviors accompanied by strict isolation is shown in Figure 2e. When the stochastic effects are taken into account together with the effects of single precautionary measures and isolation, the rapid implementation of combined measures reduces the number of transmissions and increases the probability of extinction.

DISCUSSION

The findings of this case-control study indicate that the use of masks was significantly associated with the prevention of SARS transmission and that precautions against droplet contamination and contact were adequate in preventing transmission; this implies mainly to in-hospitals. The results are roughly consistent with those of previous reports.[18,20,22] Although a number of exceptions were seen with regard to protective effects during patient intubation, during which transmission to staff occurred even when droplet and contact precautions were taken,[7,34] one of the most important lessons from the SARS outbreak is the need to enhance infection control programs in hospitals.[13,35] Even though the use of
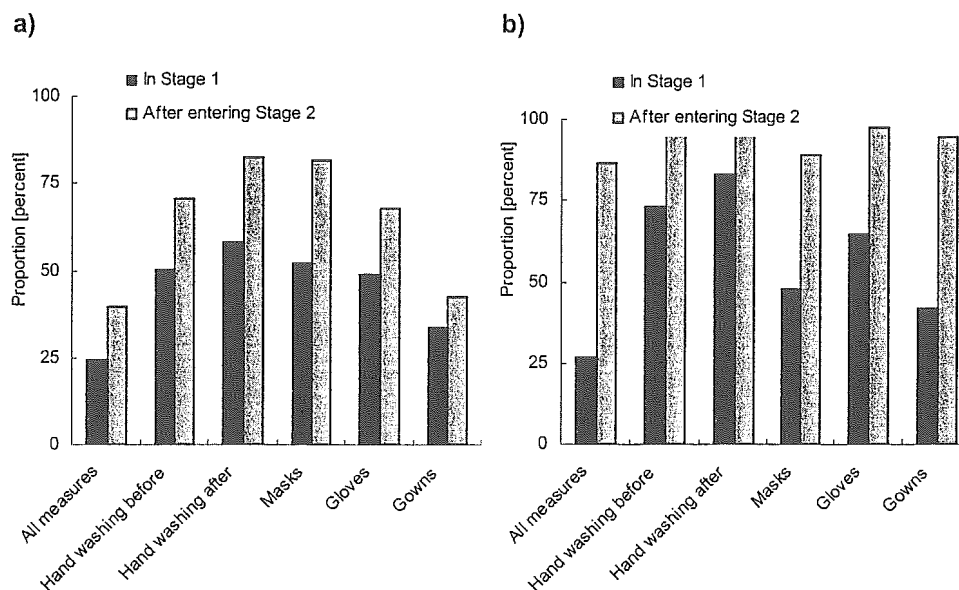
FIGURE 1. Protective behavioral changes defined by stage. a, Proportion of participants (SARS and non-SARS [control] cases) who performed each precautionary measure in Stage 1 ($N = 127$) and after entering Stage 2 ($N = 108$). Handwashing "before" and "after" denote before and after contact with a patient, respectively. b, Proportion of health care workers who performed each precautionary measure in Stage 1 ($N = 48$) and after entering Stage 2 ($N = 37$).

masks was the most effective precautionary measure, masks alone together with the observed coverage did not reduce the reproduction number below unity ($R_0 = 6.0$ and R with the protective effects of masks $= 4.1$). Put simply, the use of masks alone was shown to be insufficient to contain the epidemic. Further, it was shown that the coverage of precautionary behaviors among the study subjects increased with the progression of the outbreak, and this was especially obvious among doctors and nurses. In HFH, remarkable changes occurred in the very early phase of the outbreak before detailed information about SARS was available. According to the stochastic simulations, an increased probability of extinction would be observed if the combined measures of precaution and isolation were rapidly implemented.

With regard to sociodemographic variables, females were more frequently infected than males. Given that transmission was most frequently observed among nurses, a plausible explanation for this finding would be occupational background. Although the 40–49 age group was frequently infected, we

TABLE 4

Univariate associations between age-class/occupational categories and SARS

	Category	N	P value*	Odds ratio (95% CI)†
Sex	Male	47	0.011	0.3 (0.1–0.8)
	Female	70	0.011	3.3 (1.2–9.0)
Age class	29 y/o	29	1.000	0.9 (0.3–2.3)
	30–39 y/o	44	0.080	0.4 (0.2–1.1)
	40–49 y/o	42	0.015	2.8 (1.2–6.6)
	50 y/o	12	0.733	0.7 (0.1–3.2)
Occupation	Medical doctors	16	1.000	0.8 (0.2–2.9)
	Nurses	33	0.008	3.2 (1.3–7.7)
	Other co-medicals	36	0.076	2.2 (0.9–5.2)
	Relatives of patients	42	< 0.001	< 0.1 (0.0–0.4)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.

have no persuasive explanation for this apart from occupation: 61.9% of this stratum was medical doctors or nurses. Considering that nurses were more effectively protected from transmission by the use of masks, the control measures taken by them within HFH from early in the epidemic were admirable. The lowest frequency of infection was seen in relatives of patients, showing that our study included many relatives who remained uninfected but were nevertheless believed to have had contact. Because nonmatched case-control designs such as this are vulnerable to selection bias, we obtained estimates of the protective effect of masks by means of multivariate logistic regression analysis which entered all other variables significantly associated with infection in univariate analysis. After adjustment for internal confounding variables, the estimated reproduction number was given as 0.7 in Stages 2 and 3. Previous studies have shown that the (effective) reproduction number, defined as the average number of secondary cases generated by one index case in a susceptible population under certain restrictions and interventions, decreases with increasing awareness of the epidemic combined with several public health measures.[36,37] Using reasonable estimation procedures, another study showed that R significantly decreased after a global alert in most affected countries.[38] The current study showed that the estimated R decreased below unity after notification of a hospital outbreak, although the estimates were obtained using rough assumptions and the process of estimation was biased by various factors.

In HFH, the rapid increase in awareness, which led to not only strengthened precautionary measures and isolation but also quarantining of health care workers, seems to have been the greatest contributor to successful containment. One reason for this quick response could be attributed to the background of secondary cases that arose mainly from health care workers who had close contact with the index case. Almost all staff members working or on duty in the earliest days of the

TABLE 5
Interactions between wearing masks and other variables on the infection

	In stage 1			In stages 2 and 3		
	Odds for masks (+)	Odds for masks (−)	Odds ratio*	Odds for masks (+)	Odds for masks (−)	Odds ratio*
Gowns						
(+)	0.3	0.6	0.5	< 0.1	2.0	0.2
(−)	0.3	0.5	0.6	NC	NC	NC
Sex						
(male)	0.1	0.2	1.0	0.0	0.0	NC
(female)	0.2	0.8	0.2	0.1	NC	NC
Age class						
29 y/o	0.1	0.4	0.3	0.0	NC	NC
30–39 y/o	0.1	0.3	0.5	0.0	NC	NC
40–49 y/o	0.3	0.8	0.3	0.1	1.0	0.1
50 y/o	0.2	0.2	1.0	0.0	NC	NC
Occupation						
(Medical doctors)	NC	0.6	NC	0.0	0.0	NC
(Nurses)	0.2	1.6	0.1	0.1	0.0	NC
(Other co-medicals)	0.5	0.5	1.2			
(Relatives of patients)	NC	0.1	NC			

NC = not calculable.
* Odds ratio of being infected while taking specific precautions.

outbreak (in Stage 1) were severely infected.[39,40] Another reason might be due to the efforts led mainly by Dr. Carlo Urbani, who suggested quick improvements in the precautionary measures taken and isolation.[29] As a result, transmission leakage into the community was prevented, thus having a huge impact on the chains of transmission.[24] In HFH, those who were exposed implemented precautionary and other controlling measures quickly and efficiently, and the epidemic consequently declined to extinction.

In the interests of objective interpretation, the limitations of our study design must be addressed, as follows:

1) A study such as ours in which exposure has a strong intuitive causal link with outcome (i.e., mask usage) is vulnerable to recall bias. Even though we limited our subjects in Stages 2 and 3 to medical doctors and nurses, and cases were appropriately selected according to the probable date of infection and incubation period, our estimates are likely less accurate than would be obtained by blinded or matched case-control study. In addition to this directional bias, further bias may have been introduced by random misclassification, as our records were completed 1 year after the outbreak, and it is therefore possible that some of the precautions were uncertain exposures. The frequent use of masks among controls may have reduced the strength of the associations.
2) Model-generated results must be interpreted cautiously. Although the simulations shown here included only the effect of masks and were considered according to the results of multivariate logistic regression adjusted for internal factors, unknown external confounding factors likely exist. For example, in Stages 2 and 3, although multivariate logistic regression was performed with other variables, the P value obtained was 0.955, and overall the model was weak. Owing to the scarcity of case records, stratification in this stage failed to separate the effects of masks. Thus, the estimates of the protective effect of masks and reproduction number in this stage may include the effects of other concomitant changes, such as the reduced frequency of contacts and quarantine.

3) There are limitations concerning the simplicity of our model; for example, we neglected the possible differential susceptibility of humans to asymptomatic infections,[41,42] individual variance in severity and/or prognosis,[23,43,44] and the highly heterogenous transmission of SARS.[4,5,45] Theoretical exercises never replace reality.
4) Finally, because our model was based on a case-control study, the estimates of coverage were biased; principally, coverage in a case-control design is taken from a nonrepresentative sample. Although this study was conducted as a first attempt to incorporate the effect of behavioral factors, which change time-dependently, to model building strategies for the control of directly transmitted airborne diseases, further studies incorporating a number of methodological improvements are required.

In conclusion, given that early recognition that leads to the implementation of protective behaviors and effective control strategies is crucial in hospitals,[46] we believe our model provides intuitive results that at least partly satisfy the need to evaluate outbreak trajectories based on individual behaviors.

APPENDIX

Each simulation starts with one index case and is based on a model constructed as follows:

i) The expected number of people who used protection on each subsequent day was determined by the number of susceptible individuals (S), number of contacts per day (κ), proportion of individuals who performed the protective behavior (p), and the protective effect of the precautionary measure (β), which were obtained based on our survey. The number of infectious contacts, denoted by the product of the number of susceptible individuals (S) and the mean number of contacts (κ), was divided into two subgroups: one that represents protection due to precautionary behaviors against infection with SARS-CoV (SARS-associated coronavirus) and another that does
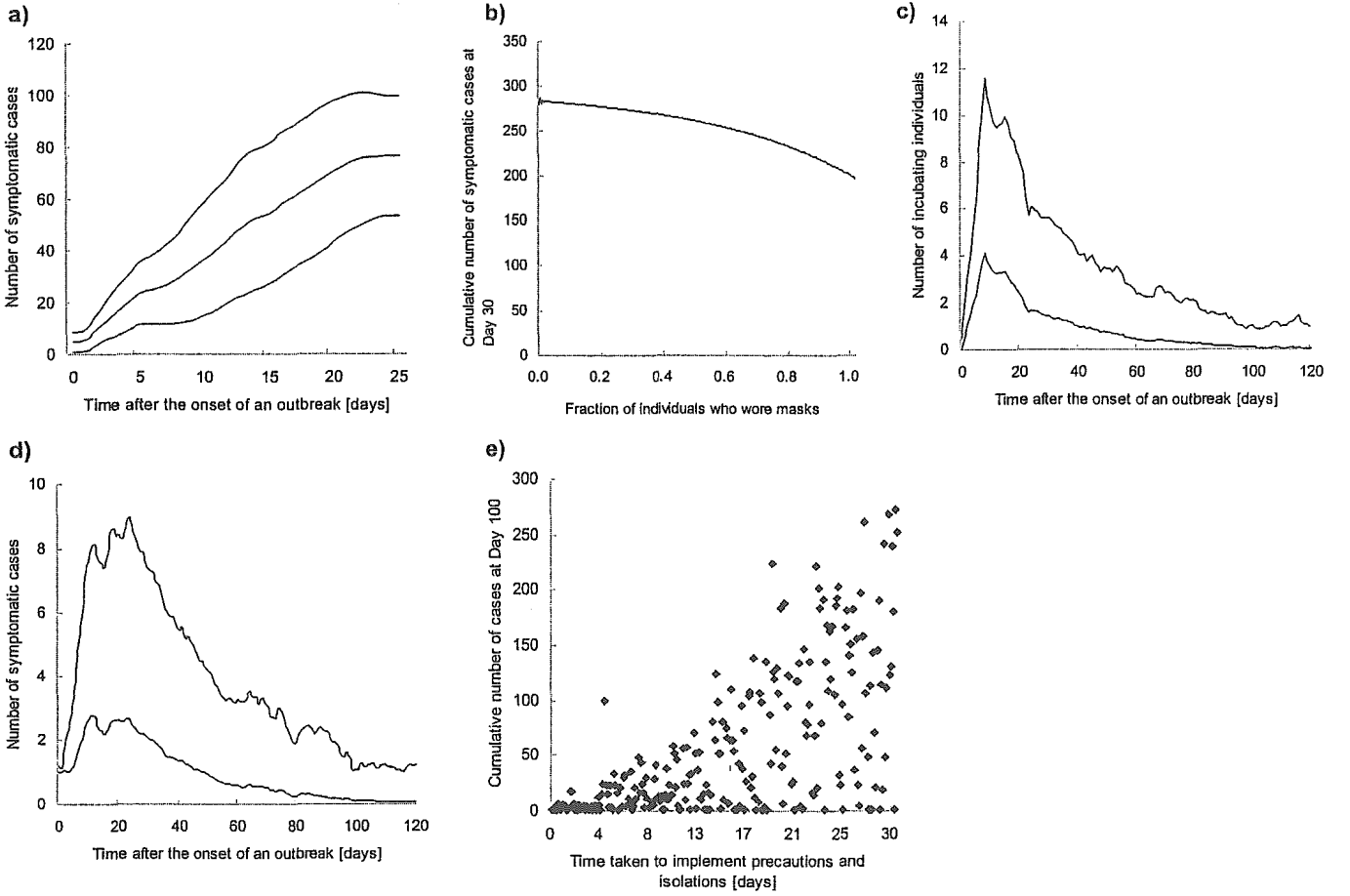
FIGURE 2. Stochastic simulations of a SARS outbreak with dependency on the coverage and protective effect of precautionary behaviors. **a,** Predicted number of symptomatic cases and corresponding 95% confidence interval (95% CI) given by 250 simulation runs assuming unchanged (stable) protective behaviors for the entire period. The reproduction number (R) was 4.1 ± 1.1. **b,** Sensitivity of the cumulative number of cases at Day 30 to the coverage of masks. The obtained line represents the simulation based on mean field (without assuming random function with binomial distribution in each transition probability). The protective effect of wearing a mask was fixed ($\beta = 0.6$). **c** and **d,** Stochastic simulations of a SARS outbreak with dependency on a combination of precautionary measures and strict isolation. **c,** The mean number of incubating individuals and corresponding 95% CI from 250 runs with changes in protective behaviors combined with strict isolation (lower 95% CI is x-axis). At Day 7, the effectiveness/coverage of precautionary measures used improved from 0.6/52.0 to 0.9/89.2, respectively. At Day 13, the number of susceptible individuals decreased from 300 to 20. The reproduction number decreased from 4.1–0.7 ± 1.1–0.8. **d,** The mean $\pm$ 95% CI of symptomatic cases given by 250 runs assuming changes in protective behaviors combined with strict isolation. The conditions were the same as those in **c. e,** Sensitivity of the size of an outbreak (represented by the cumulative number of cases) to the time taken to enhance precautionary measures and implement strict isolation; the combined measures are started at the same time and under the same conditions as in **c.**

not, according to $(1 - p\beta)$. However, these groups were not permanently fixed. The mean of the number of contacts based on our survey was approximated by:

$$\kappa = \kappa_1 \pi_1 + \kappa_2 \pi_2 = \kappa_1 \pi_1 + \kappa_1 \ln(\mathrm{OR}_{\mathrm{close}}) \pi_2 \qquad \text{(A1)}$$

where κ_1, κ_2, π_1, and π_2 denote the number of casual and close contacts and the fraction of individuals who had casual and close contacts, respectively, while the odds ratio of getting infected with close contact is represented by $\mathrm{OR}_{\mathrm{close}}$ and N, respectively.

ii) Both the incubation (E) and symptomatic (I) periods were assumed to be independently and identically distributed following an approximated probability density function with gamma distributions[33] (denoted by γ_k and c_l for the discretized stages [days] k and l, respectively). We divided the probability density functions into k ($i = 14$) and l ($j = 12$) stages; the methodology of approximation

by date was previously reported.[24] The relative measure of infectiousness for the incubation (E) period (q) was assumed to be 0.1.[12]

iii) Based on realistic settings in Vietnam, it was assumed that all individuals were isolated with the onset of early signs of clinical symptoms under the isolation measures; and for simplicity, the effect of quarantine was neglected. When considering strict isolation, the number of susceptible individuals having contact with SARS patients was limited to 20 (which is the approximate number of ward workers); the number of susceptible individuals was treated as being stable (always $S = 20$) so that S would not be exhausted thereafter; without isolation there were assumed to be 300 susceptible individuals (which is roughly the total number of people involved in possible contacts in HFH). $N = S + E + I + R$, and background mortality was neglected. The resulting simplest difference equations were formulated as follows: