Characterization of *Mycobacterium avium* clinical isolates in Japan using subspecies-specific insertion sequences, and identification of a new insertion sequence, ISMav6

Kazuya Ichikawa,1,2 Tetsuya Yagi,3 Makoto Moriyama,1,2,4 Takayuki Inagaki,1,2 Taku Nakagawa,5 Kei-Ichi Uchiya,1 Toshiaki Nikai1 and Kenji Ogawa2,5

1Department of Microbiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya, Aichi 468-8503, Japan
2Department of Clinical Research, National Hospital Organization, Higashinagoya National Hospital, 5-101 Umemorizaka, Meito-ku, Nagoya, Aichi 468-8620, Japan
3Department of Infectious Diseases, Center of National University Hospital for Infection Control, Nagoya University Hospital, 65 Tsurumai, Showa-ku, Nagoya, Aichi 466-8560, Japan
4Department of Pharmacy, National Hospital Organization, Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya, Aichi 460-0001, Japan
5Department of Pulmonary Medicine, National Hospital Organization, Higashinagoya National Hospital, 5-101 Umemorizaka, Meito-ku, Nagoya, Aichi 468-8620, Japan

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Clinical isolates of *Mycobacterium avium* (n=81) from patients with pulmonary infections who were HIV-negative and isolates (n=33) from HIV-positive patients were subjected to genetic analysis by PCR detection of three *M. avium*-specific insertion sequences (IS901, IS1245 and IS1311), and nucleotide sequencing of the heat-shock protein 65 (*hsp65*) gene. All clinical isolates were identified as ‘*M. avium* subspecies hominisuis’ by sequence analysis of hsp65.

Compared with clinical isolates of *M. avium* reported elsewhere, IS1245 was found less frequently in Japanese isolates (96/114 isolates, 84 %) and IS901 was detected more frequently (76/114 isolates, 67 %). One isolate was found to lack IS1311, which has not been reported previously for ‘*M. avium* subsp. hominisuis’. Nucleotide sequence analysis of the PCR products for IS901 revealed that all clinical isolates had the same new insertion sequence, designated ISMav6, which had 60 point mutations compared with the nucleotide sequence of the original IS901. These results suggest that ‘*M. avium* subsp. hominisuis’ with ISMav6 is prevalent in Japan. ISMav6 may have implications for the virulence of *M. avium* and contribute to an increase of *M. avium* infections in this country.

**INTRODUCTION**

Infection with the *Mycobacterium avium* complex has increased in recent years (Marras & Daley, 2002). In Japan, the prevalence of *M. avium* complex disease has increased from 0.82 per 100 000 persons in the 1970s to 3.52 per 100 000, as shown by a recent epidemiological study (Sakatani, 1999), which is considerably higher than the reported rate of 0.2 per 100 000 in Europe (Henry et al., 2004). Pulmonary *M. avium* infection has been reported frequently among HIV-negative middle-aged women without any organic lung diseases. The human or bacterial factors contributing to the pathophysiology of *M. avium* infection and unresponsiveness to therapy remain to be elucidated.

*M. avium* comprises four subspecies that infect specific hosts and are differentiated by the presence or absence of specific insertion sequences. *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*, isolated from birds, possess IS901 and low copy numbers of IS1245 (Dvorska et al., 2003; Kunze et al., 1992; Ritacco et al., 1998). ‘*M. avium* subsp. *hominisuis’,
from humans and swine, usually harbours high copy numbers of IS1245 and IS1311, but none of IS901 (Mijls et al., 2002). M. avium subsp. paratuberculosis, from ruminant livestock and wildlife, has IS900 but not IS1245 or IS901 (Chacon et al., 2004; Harris & Barletta, 2001). However, it is difficult to determine definitively the type of subspecies by using insertion sequences, because some strains lack some specific insertion sequences (Turenne et al., 2007).

Accordingly, classification based on the nucleotide sequence of hsp65 has been proposed (Turenne et al., 2006).

The purpose of this study was to characterize M. avium pathogenic clinical isolates recovered from patients with or without HIV infection in Japan using different molecular methods.

**METHODS**

**Strains.** M. avium subsp. avium (GTC00603, derived from M. avium ATCC 25291 and ATCC 35718), M. avium subsp. silvaticum (ATCC 49884) and ‘M. avium subsp. hominisuis’ (104 and ATCC 19978) were used as standard strains. The clinical isolates were 81 strains recovered from sputum, blood and lymph node, respectively.

**Identification of strains.** The standard strains GTC00603 and ATCC 25291 and ATCC 35718, M. avium subsp. avium, from HIV-negative patients with pulmonary diseases, and 33 strains from HIV-positive patients, including 5, 27 and 1 recovered from sputum, blood and lymph node, respectively.

**PCR analysis.** The identified strains were cultured in 5 ml 7H9 liquid medium supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment at 37 °C for 1–2 weeks, and then transferred to 25 ml of the same medium for further culture. The culture was centrifuged and then DNA was extracted with Isoplant II according to the manufacturer’s recommendations (Nippon Gene). The extracted DNA was dissolved in 50 μl Tris EDTA buffer (Sigma) and used as a template. Mixtures were prepared containing template DNA (5–50 ng), dNTP mixture (5 μl), 10× PCR buffer (5 μl), each primer set (both 12.5 μM), 2.5 U AmpliTaqGold (Applied Biosystems) and DMSO (Wako) to a final concentration of 4%. Sterilized purified water was added to bring the total volumes up to 50 μl and the mixtures were subjected to PCR. The PCR primers for IS901-1, IS1245, IS1311, IS901-flanking region 300 (FR300) and the hsp65 gene are shown in Table 1. The PCR program used consisted of an initial denaturing step at 95 °C for 10 min, followed by 35 cycles of denaturing at 94 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s, and then a final extension step at 72 °C for 7 min.

The amplified DNA products were subjected to electrophoresis on a 2% agarose gel (Invitrogen) to determine their size (Table 1). Judgment was done by comparison with the size of the products of the standard strains as positive controls.

**Sequence analysis.** The PCR products of IS901 and the hsp65 gene were subjected to sequence analysis by the method of Turenne et al. (2006) with the same forward and reverse primers as used for PCR. The nucleotide sequences of the hsp65 gene thus obtained were compared with the reported data by BLAST analysis using the NCBI server (http://www.ncbi.nlm.nih.gov/). Alignment was performed with CLC Sequence Viewer 4.6.2 (CLC Bio). In order to determine the nucleotide sequence upstream and downstream of the PCR products of IS901, two primer sets (Table 1) were designed for IS901-2 and IS901-3 using Primer3 (http://frodo.wi.mit.edu/). DNA was amplified under the same PCR conditions as above, and sequence analysis of each product was performed.

**RFLP analysis.** Probes were prepared by using a PCR DIG Probe Synthesis kit with the corresponding primers shown in Table 1, according to the manufacturer’s recommendations (Roche). Four nanograms of template DNA extracted from each strain was digested

**Table 1. Primers used for identification and characterization of the M. avium isolates**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Product size (bp)</th>
<th>Nucleotide sequence</th>
<th>Reference</th>
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| IS901-1     | 1108             | F: 5’-GCAACGTTGCTGTGCTTGGAA-3’  
                    R: 5’-TGATACGGCGCAATCCGGT-3’  
                    | This work |
| IS901-2     | 532              | F: 5’-TTCCCTAGGTTGAAAGGTTG-3’  
                    R: 5’-GTCGGTGCTTGCCTTACCT-3’  
                    | This work |
| IS901-3     | 502              | F: 5’-GGATCCGAGCAATGAAAGGATTG-3’  
                    R: 5’-TCTCAATAGGCTGTCAC-3’  
                    | This work |
| FR300       | 300, 1776        | F: 5’-CCAGCCGCCGAATTTGATCC-3’  
                    R: 5’-CAATACGGAGCACCTTCACC-3’  
                    | Nishimori et al. (1995) |
| IS1245      | 427              | F: 5’-GCAACGTTGCTGTGCTTGGAA-3’  
                    R: 5’-AGGTGGCGTCGAGGAAGA-3’  
                    | Johansen et al. (2005) |
| IS1311      | 198              | F: 5’-GGTCTGAGGCTCCTGTGTTGGA-3’  
                    R: 5’-ATGAGGCGAGTCGAGGAAGA-3’  
                    | Johansen et al. (2005) |
| hsp65 3’    | 1059             | F: 5’-ATGAGGCGAGTCGAGGAAGA-3’  
                    R: 5’-ATGAGGCGAGTCGAGGAAGA-3’  
                    | Turenne et al. (2006) |
| DT-6        | 187              | F: 5’-ATGAGGCGAGTCGAGGAAGA-3’  
                    R: 5’-ATGAGGCGAGTCGAGGAAGA-3’  
                    | Thiery et al. (1993) |
with the restriction endonuclease PvuII and then subjected to electrophoresis on a 1% agarose gel (Takara), transferred to Hybond-N+ (GE Healthcare), and detected with a DIG Luminescent Detection kit (Roche). DIG-labelled marker II (Roche) was used as an internal DNA size marker. Finally, the membrane was exposed to X-ray film (GE Healthcare), and RFLP analysis was performed with Fingerprinting II (Bio-Rad Laboratories). After stripping of the DIG probe, we used the same membrane for probing for the DT-6 gene, which was reported (Thierry et al., 1993) as a M. avium-specific gene, to verify the performance of the assay.

**Statistical analysis.** Categorical data for the detection rate of insertion sequences were analysed statistically by the chi-square test.

### RESULTS AND DISCUSSION

#### Identification of clinical isolates

All of the 114 clinical strains were identified as M. avium by using the COBAS AMPLICOR Mycobacterium Test. As a result of nucleotide sequence analysis of the 3’ fragment of the hsp65 gene, all strains were identified as ‘M. avium subsp. hominisuis’ (Table 2), and two new sequevars of code 16 (GenBank accession no. AB453830) and code 17 (GenBank accession no. AB453831) were found. These codes, 16 and 17, differed from those of bird-type M. avium subsp. avium, because base no. 645 was substituted to C from T which was reported to be specific to M. avium subsp. avium.

#### Distribution of insertion sequences

PCR analysis for ‘M. avium subsp. hominisuis’-specific insertion sequences showed that a relatively low detection rate of IS1245 was one of the characteristics of clinical isolates in Japan, as reported previously (Kuwabara et al., 2004; Moriyama et al., 2006), and one IS1311-negative strain was newly identified (Table 3). Confirmation by Southern hybridization and reblotting with the same membrane for DT-6 showed that this isolate was truly IS1311-negative (Fig. 1a, b).

Three sputum isolates and one blood isolate that were negative for IS1245 had hsp65 sequevar code 2. It has been suggested that isolates lacking IS1245 with hsp65 sequevar code 16 (GenBank accession no. AB453830) and code 17 (GenBank accession no. AB453831) were found. These codes, 16 and 17, differed from those of bird-type M. avium subsp. avium, because base no. 645 was substituted to C from T which was reported to be specific to M. avium subsp. avium.

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code 2 might represent environmental contamination (Alvarez et al., 2008). However, taking into account the fact that all the patients from whom such strains were detected in the present study had symptoms of infection, and that specimens were handled carefully according to laboratory quality control standards, contamination was unlikely. Moreover, no strains with the same genotype were identified during the same period when *M. avium* isolates lacking IS1245 were detected.

In the clinical environment, we found that the *hsp65* gene analysis was useful to identify the subspecies of *M. avium*, especially when there were substantial numbers of isolates with no subspecies-specific insertion sequences.

PCR analysis for IS901 showed a unique distribution among Japanese *M. avium* isolates (Table 3). Although Pavlik et al. (2000) reported that strains possessing IS901 were isolated from 12 (8 %) out of 152 human isolates, IS901 has either been detected at a very low frequency in human isolates or not detected at all (Alvarez et al., 2008; Möbius et al., 2006; Ritacco et al., 1998). Therefore the detection rate of IS901 in Japan was significantly higher than in other countries.

**Characterization of a newly detected insertion sequence**

The high frequency of detection of IS901 by PCR in *M. avium* clinical isolates prompted us to conduct nucleotide sequence analysis on this element. Compared with the nucleotide sequence of the original bird-type IS901, 60 point mutations were found in the sequence of the insertion sequence detected from the clinical isolates (Supplementary Fig. S1 in JMM Online). These mutations were identical in all PCR-positive clinical isolates. The
sequence identity with the original IS901 was 95%. Therefore, this new insertion sequence was designated ISMav6 (GenBank accession no. AB447556). The presence of ISMav6 in the sequenced M. avium 104 strain was examined by PCR with three sets of primers (primers IS901-1F and IS901-1R, IS901-2F and IS901-2R, and IS901-3F and IS901-3R) (Table 1). No PCR amplifications for ISMav6 were found with these primers sets, which was consistent with the fact that there is no gene corresponding to ISMav6 in the genome database of M. avium 104 (accession no. CP000479, data not shown). ISMav6 encoded a putative protein of 403 amino acids, which showed 96% identity with that encoded by IS901. The PCR for FR300, which was supposed to amplify IS901 together with the flanking regions of the insertion sites, gave a 1776 bp band for bird-type standard strains containing IS901. However, a 300 bp band was detected for M. avium 104, ATCC 19978 and all clinical strains, indicating no insertion sequence at the insertion sites for IS901 (data not shown).

The hybridization profile with the probe derived from IS901 appeared to be quite similar to that with the probe prepared from ISMav6 of the clinical isolates, indicating cross-reactivity of the IS901-type probe with ISMav6 in clinical isolates. RFLP analysis with the IS901 probe revealed that its copy number in the bird-type strains was 10–13, while that of ISMav6 in the clinical isolates was 0–6 (Fig. 2), showing relatively infrequent insertions at sites away from FR300. This new insertion sequence, possibly specific to ‘M. avium subsp. hominissuis’, was detected at a significantly higher frequency in the clinical isolates from man, pig, and cattle.

As IS901 is reported to have an influence on the pathogenicity of M. avium subsp. avium (Kunze et al., 1991; Pavlik et al., 2000), ISMav6 may also be related to the pathogenicity of ‘M. avium subsp. hominissuis’, especially in strains causing pulmonary disease.

In conclusion, clinical M. avium infections in Japan are caused by ‘M. avium subsp. hominissuis’, as is the case in other countries. Sixteen per cent of the Japanese ‘M. avium subsp. hominissuis’ isolates lacked IS1245 and one isolate lacked IS1311. A new insertion sequence, ISMav6, which is homologous to the original bird-type IS901, was found frequently in the Japanese clinical isolates, especially those recovered from sputum. The presence of ISMav6 may be one of the bacterial factors that enhances pathogenicity and contributes to the increase of M. avium pulmonary infections in Japan.

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primers that discriminate between \textit{M. avium} and \textit{Mycobacterium intracellulare}. J Clin Microbiol 33, 2102–2106.


