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

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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 *hominissuis*' 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. *hominissuis*'. 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. *hominissuis*' 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.

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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.

The GenBank/EMBL/DDBJ accession numbers for the ISMav6, heat-shock protein 65 sequevar code 16 and heat-shock protein 65 sequevar code 17 sequences are AB447556, AB453830 and AB453831, respectively.

A figure showing the sequence alignment of ISMav6 and IS901 is available with the online version of this paper.

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. *hominissuis*',

from humans and swine, usually harbours high copy numbers of IS1245 and IS1311, but none of IS901 (Mijis *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. *hominissuis*' (104 and ATCC 19978) were used as standard strains. The clinical isolates were 81 strains recovered from the sputum of 81 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.

Identification of strains. The standard strains GTC00603 and ATCC 49884 were identified by detection of the three-band bird-type IS1245 by RFLP analysis, and sequevar code 4 was confirmed by sequence analysis of the 3' fragment of *hsp65*.

Clinical isolates were identified as *M. avium* by using the COBAS AMPLICOR *Mycobacterium* Test (Roche Diagnostic Systems). In addition, sequence analysis of the 3' fragment of the *hsp65* gene was performed to identify the subspecies of *M. avium*. The *hsp65* sequevar codes of the subspecies were code 4 for *M. avium* subsp. *avium*, codes 1, 2, 3, 7–9 and 15 for '*M. avium* subsp. *hominissuis*', and codes 5 and 6 for *M. avium* subsp. *paratuberculosis* (Alvarez *et al.*, 2008; Turenne *et al.*, 2006).

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 IS901-1 and *hsp65* or 68 °C for IS1245, IS1311 and FR300 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

Target gene	Product size (bp)	Nucleotide sequence	Reference
IS901-1	1108	F: 5'-GCAACGGTTGTTGCTTGAAA-3' R: 5'-TGATACGGCCGAATCGCGT-3'	Kunze <i>et al.</i> (1992)
IS901-2 (1–532)	532	F: 5'-TTCCTTAGGGTTGAAGGGGT-3' R: 5'-GTCGGTCTTGCCCTTACCT-3'	This work
IS901-3 (970–1472)	502	F: 5'-GGATCGCCAGATCAAGGATA-3' R: 5'-TCTCAATGAGCGTGTC AAC-3'	This work
FR300	300, 1776	F: 5'-CAGCCAGCCGAATGTCATCC-3' R: 5'-CAACTCGCGACACGTTACCC-3'	Nishimori <i>et al.</i> (1995)
IS1245	427	F: 5'-GCAACGGTTGTTGCTTGAAA-3' R: 5'-AGGTGGCGTCGAGGAAGA-3'	Johansen <i>et al.</i> (2005)
IS1311	198	F: 5'-GCGTGAGGCTCTGTGGTGAA-3' R: 5'-ATGACGACCGCTTGGGAGAC-3'	Johansen <i>et al.</i> (2005)
<i>hsp65</i> 3' fragment	1059	F: 5'-CGGTTTCGACAAGGGTTACAT-3' R: 5'-ACGGA CT CAGAAGTCCATGC-3'	Turenne <i>et al.</i> (2006)
DT-6	187	F: 5'-ATGGCCGGGAGACGATCTATGCCGGCGTAC-3' R: 5'-CGTTTCGATCGCAGTTTGTGCAGCGCGTACA-3'	Thierry <i>et al.</i> (1993)

Table 2. Distribution of *hsp65* sequevar types among *M. avium* isolates from different sources

hsp65 sequevar code 4 indicates *M. avium* subsp. *avium/silvaticum*; codes 1, 2, 3 and 15 indicate '*M. avium* subsp. *hominissuis*' (Alvarez *et al.*, 2008; Turenne *et al.*, 2006); codes 16 and 17 indicate '*M. avium* subsp. *hominissuis*' in this study.

<i>hsp65</i> code	Nucleotide at indicated base pair position (<i>hsp65</i>)							Source	
	645	928	1128	1218	1269	1277	1536	Sputum (n)*	Other (n)†
Code 1	C	C	C	A	G	C	A	5	13
Code 2	C	C	C	G	G	C	G	25	2
Code 3	C	C	G	G	C	G	G	2	1
Code 4	T	C	G	G	C	G	G	0	0
Code 15	C	T	C	A	G	C	A	37	11
Code 16	C	T	C	A	G	C	G	13	1
Code 17	C	C	C	G	G	C	A	4	0

*Sputum isolates from clinical patients with chronic obstructive pulmonary disease.

†Isolates from blood or lymph nodes.

with the restriction endonuclease *Pvu*II 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. *hominissuis*' (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. *hominissuis*'-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

Table 3. Comparison of detection rates of insertion sequences in *M. avium* isolates recovered from sputum with those from other sites

Data are presented as *n* (%) or odds ratios (95% confidence interval).

	Sputum* (n=86)	Others† (n=28)	OR (95% CI)‡	P-value
IS1245	70 (81.3)	26 (92.9)	0.34 (0.07–1.57)	0.15
IS1311	85 (98.8)	28 (100)	1.00	0.57
IS901/IS <i>Mav</i> 6§	64 (74.4)	12 (42.9)	3.88 (1.59–9.46)	0.002

*Sputum isolates from clinical patients with chronic obstructive pulmonary disease.

†Isolates from blood or lymph nodes.

‡OR, Odds ratios; CI, confidence interval.

§IS901 is likely to be uncommon and the high level detected with the IS901 probe is likely to represent primarily IS*Mav*6.

Fig. 1. Southern blot analysis for detection of IS1311. Total DNA was digested with restriction endonuclease *PvuII* and blotted with an IS1311-specific probe (a) and DT-6 probe as a positive control (b). Lanes: L, molecular mass ladder; 1, GTC00603 (*M. avium* subsp. *avium*); 2, ATCC 19978 (*M. avium* subsp. *hominissuis*); 3, lung-av-002 (IS1311-negative strain).

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

Fig. 2. RFLP analysis of IS901 after digestion with restriction endonuclease *PvuII*. Lanes: 1, molecular mass ladder; 2, GTC00603; 3, ATCC 49884; 4, ATCC 35718; 5–29, clinical isolates. Lanes 2–4 were detected by the IS901 probe and lanes 5–29 by the ISMav6 probe.

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 recovered from sputum than in those recovered from blood or lymph nodes (Table 3).

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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