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SUSCEPTIBILITY

Antibiotic Susceptibility and Genotyping of *Mycobacterium avium* Strains That Cause Pulmonary and Disseminated Infection

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ABSTRACT *Mycobacterium avium* subsp. *hominissuis* mainly causes disseminated infection in immunocompromised hosts, such as individuals with human immunodeficiency virus (HIV) infection, and pulmonary infection in immunocompetent hosts. However, many aspects of the different types of *M. avium* subsp. *hominissuis* infection remain unclear. We examined the antibiotic susceptibilities and genotypes of *M. avium* subsp. *hominissuis* isolates from different hosts by performing drug susceptibility testing using eight antibiotics (clarithromycin, rifampin, ethambutol, streptomycin, kanamycin, amikacin, ethionamide, and levofloxacin) and variable-number tandem-repeat (VNTR) typing analysis for 46 isolates from the sputa of HIV-negative patients with pulmonary *M. avium* subsp. *hominissuis* disease without previous antibiotic treatment and 30 isolates from the blood of HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease. Interestingly, isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients were more resistant to seven of the eight drugs, with the exception being rifampin, than isolates from HIV-positive patients. Moreover, VNTR typing analysis showed that the strains examined in this study were roughly classified into three clusters, and the genetic distance from reference strain 104 for isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients was statistically significantly different from that for isolates from HIV-positive patients ($P = 0.0018$), suggesting that *M. avium* subsp. *hominissuis* strains that cause pulmonary and disseminated disease have genetically distinct features. Significant differences in susceptibility to seven of the eight drugs, with the exception being ethambutol, were noted among the three clusters. Collectively, these results suggest that an association between the type of *M. avium* subsp. *hominissuis* infection, drug susceptibility, and the VNTR genotype and the properties of *M. avium* subsp. *hominissuis* strains associated with the development of pulmonary disease are involved in higher levels of antibiotic resistance.

KEYWORDS *Mycobacterium avium* subsp. *hominissuis*, pulmonary disease, disseminated disease, antibiotic susceptibility, variable-number tandem repeats

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment, including natural water, soil, and household dust (1, 2), and can cause significant disease in humans and animals (3). NTM infection was thought to be caused by NTM residing in the environment (2, 4, 5). However, person-to-person transmission has recently been reported among cystic fibrosis patients infected with *Mycobacterium abscessus* (6). The

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incidence of NTM pulmonary infection is increasing annually in many countries, including the United States and Japan (7–10). In Japan, the causative NTM strain for pulmonary disease with the highest incidence is *M. avium* (approximately 60%), followed by *M. intracellulare*, *M. kansasii*, and *M. abscessus*, and the incidence per 100,000 population increased remarkably from 5.7 in 2007 to 14.7 in 2014 (9, 11).

M. avium is the most clinically significant NTM species in humans and animals and consists of four subspecies (*M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *paratuberculosis*), each of which has specific pathogenic and host range characteristics (12–14). Among the *M. avium* subspecies, *M. avium* subsp. *hominissuis* has been isolated from patients with respiratory disease, human immunodeficiency virus (HIV) infection, and lymphadenitis (3), as well as from asymptomatic pigs with granulomatous lesions (15). *M. avium* subsp. *hominissuis* mainly causes disseminated infection via the gastrointestinal route in immunocompromised hosts or pigs and pulmonary infection via the respiratory route in immunocompetent hosts. Recent studies have reported the isolation of genetically different *M. avium* subsp. *hominissuis* strains from different hosts or different countries and regions (16, 17), showing the genetic diversity of *M. avium* subsp. *hominissuis*. We have previously reported genetic differences between strain TH135, isolated from a patient with pulmonary *M. avium* subsp. *hominissuis* disease, and strain 104, obtained from an HIV-positive patient, by comparing the genomes of the strains (18). Such genetic differences may affect not only the pathological manifestation of *M. avium* subsp. *hominissuis* infection but also produce various phenotypes of *M. avium* subsp. *hominissuis*, such as various antibiotic susceptibility phenotypes. Elucidation of the phenotypic differences would facilitate the understanding of *M. avium* subsp. *hominissuis* infections and provide a valuable insight into antibiotic treatments.

The guidelines for antibiotic treatment of pulmonary *M. avium* subsp. *hominissuis* disease recommend macrolide-based multidrug therapy, comprising macrolides, such as clarithromycin or azithromycin, in combination with rifampin and ethambutol. In addition, aminoglycosides, such as streptomycin or amikacin, are recommended for patients with severe disease (19). However, the therapeutic efficacy of the drug above certain levels is unknown (19–21). Moreover, the clinical course of patients with pulmonary *M. avium* subsp. *hominissuis* disease is diverse, and some patients remain stable without treatment, while the symptoms cause deterioration in others, despite long-term multidrug therapy, leading to severe lung damage (3, 22, 23). This is possibly the result of host factors as well as bacterial factors. In our previous study, comparative genome analysis revealed the presence of potential genetic determinants, including a pMAH135 plasmid (24), associated with the progression of pulmonary *M. avium* subsp. *hominissuis* disease (25). These results suggest the involvement of bacterial factors in the progression of pulmonary *M. avium* subsp. *hominissuis* disease.

This study sought to examine the features of antibiotic susceptibility and the genotype in *M. avium* subsp. *hominissuis* isolates from hosts with different types of *M. avium* subsp. *hominissuis* infection. Thus, we performed drug susceptibility testing and variable-number tandem-repeat (VNTR) typing analysis of 46 isolates from HIV-negative patients with pulmonary *M. avium* subsp. *hominissuis* disease without previous antibiotic treatment and 30 isolates from HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease.

RESULTS

Drug susceptibility of *M. avium* subsp. *hominissuis* isolates from different origins. We examined the characteristics of antibiotic susceptibility of *M. avium* subsp. *hominissuis* isolates from different hosts by measuring the MICs of eight drugs (clarithromycin, rifampin, ethambutol, streptomycin, kanamycin, amikacin, ethionamide, and levofloxacin) for 46 isolates from the sputa of HIV-negative patients who were diagnosed with pulmonary *M. avium* subsp. *hominissuis* disease but received no antibiotic treatment, as well as 30 isolates from the blood of HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease, by the broth dilution method (see Tables

TABLE 1 Comparison of drug resistance and susceptibility in isolates from different hosts

Antimicrobial agent	No. (%) of isolates[a]				*P* value[b] (pMAH vs HIV)
	pMAH (n = 46)		HIV (n = 30)		
	R	S	R	S	
Clarithromycin	1 (2.2)	45 (97.8)	3 (10.0)	27 (90.0)	0.294
Rifampin	1 (2.2)	45 (97.8)	0 (0)	30 (100)	1
Ethambutol	39 (84.8)	7 (15.2)	13 (43.3)	17 (56.7)	≤0.001
Streptomycin	14 (30.4)	32 (69.6)	5 (16.7)	25 (83.3)	0.278
Kanamycin	15 (32.6)	31 (67.4)	4 (13.3)	26 (86.7)	0.065
Amikacin	7 (15.2)	39 (84.8)	3 (10.0)	27 (90.0)	0.731
Ethionamide	30 (65.2)	16 (34.8)	10 (33.3)	20 (66.7)	0.009
Levofloxacin	0 (0)	46 (100)	1 (3.3)	29 (96.7)	0.395

[a]The breakpoints of the antimicrobial agents were determined according to the criteria described in the BrothMIC NTM system manual and Materials and Methods. pMAH, isolates from the sputa of patients with pulmonary *M. avium* subsp. *hominissuis* disease; HIV, isolates from the blood of HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease; R, resistant; S, susceptible.
[b]*P* values were calculated using Fisher's exact test.

S1 and S2 in the supplemental material). Interestingly, ethambutol and ethionamide resistance was observed in 84.8% (39/46) and 65.2% (30/46) of the isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients, respectively, and 43.3% (13/30) and 33.3% (10/30) of the isolates from HIV-positive patients, respectively (Table 1). A significantly higher percentage of strains with resistance to both drugs was found among the isolates from patients with pulmonary *M. avium* subsp. *hominissuis* disease than among the isolates from HIV-positive patients. Resistance to streptomycin, kanamycin, and amikacin was observed in 30.4% (14/46), 32.6% (15/46), and 15.2% (7/46) of the isolates from patients with pulmonary *M. avium* subsp. *hominissuis* disease, respectively, and 16.7% (5/30), 13.3% (4/30), and 10.0% (3/30) of the isolates from HIV-positive patients, respectively; the former group of isolates showed stronger resistance to all three drugs. In contrast, clarithromycin resistance was observed in 10.0% (3/30) of the isolates from HIV-positive patients and in only 1 strain (2.2%) among the isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients. Regarding rifampin and levofloxacin, almost all isolates in both groups showed susceptibility.

Next, the Mann-Whitney U test was performed to analyze the difference in drug susceptibility among the isolates from the two different groups of hosts. Table 2 shows the mean $\log_2$ values ± standard deviations of the MIC of individual drugs for isolates from each group. There was a significant difference in the $\log_2$ MICs of seven of the eight drugs, with the exception being rifampin, among the isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients and HIV-positive patients; the former group of isolates had higher MIC values of all seven drugs. These findings reveal clear differences in susceptibility between the isolates from both groups and an overall higher rate of antibiotic resistance among the isolates from patients with pulmonary *M.*

TABLE 2 Mean $\log_2$ values of MICs of test drugs for isolates from different hosts

Antimicrobial agent	$\log_2$ MIC (mean ± SD)		*P* value[a] (pMAH vs HIV)
	pMAH[b]	HIV[c]	
Clarithromycin	−0.83 ± 1.44	−1.75 ± 2.74	0.004
Rifampin	−2.22 ± 2.42	−2.56 ± 1.97	0.710
Ethambutol	3.33 ± 0.97	2.37 ± 1.54	≤0.001
Streptomycin	1.93 ± 1.12	0.63 ± 1.83	≤0.001
Kanamycin	2.93 ± 1.14	1.27 ± 1.86	≤0.001
Amikacin	2.33 ± 1.10	1.33 ± 1.63	0.008
Ethionamide	2.76 ± 0.77	2.10 ± 0.99	0.003
Levofloxacin	0.63 ± 1.02	−0.27 ± 1.88	0.037

[a]*P* values for $\log_2$ MIC values between two different groups were calculated using the Mann–Whitney U test.
[b]pMAH, isolates from the sputa of patients with pulmonary *M. avium* subsp. *hominissuis* disease.
[c]HIV, isolates from the blood of HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease.

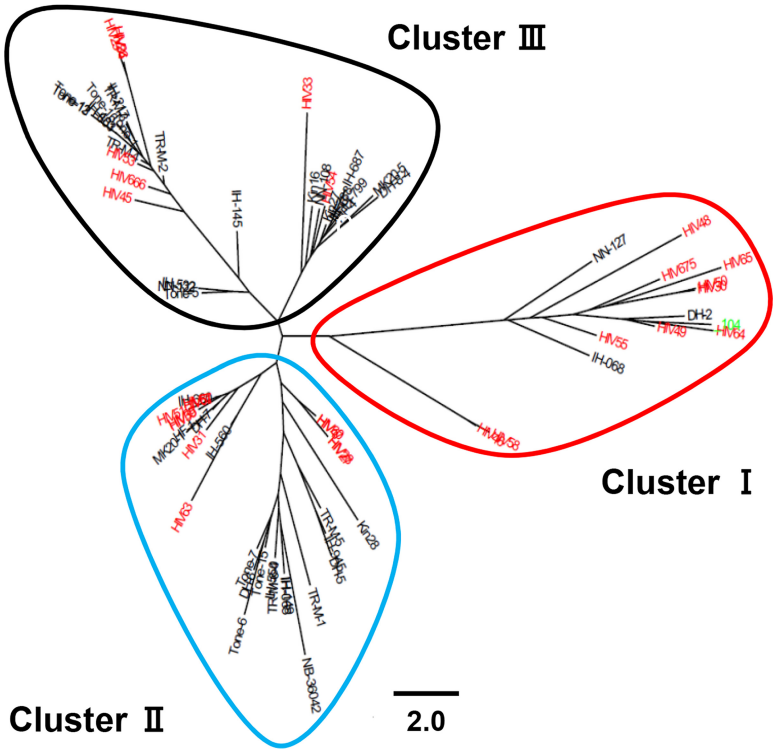

FIG 1 Cluster analysis of *M. avium* subsp. *hominissuis* isolates from different hosts based on MATR-VNTR profiles. The *M. avium* subsp. *hominissuis* isolates comprised 46 strains (black) from pulmonary *M. avium* subsp. *hominissuis* disease patients and 30 strains (red) from HIV-positive patients, including strain 104 (green) as a reference. The phylogenetic distribution was created from distance matrix files by use of the Fitch-Margoliash algorithm according to the MATR-VNTR markers. The scale bar indicates the Manhattan distance. *M. avium* subsp. *hominissuis* isolates were classified into clusters I to III by MATR-VNTR typing analysis.

avium subsp. *hominissuis* disease. This suggests an association between drug susceptibility and the type of *M. avium* subsp. *hominissuis* infection.

VNTR genotypes of *M. avium* subsp. *hominissuis* isolates from different origins. In a recent study conducting VNTR typing analysis using 13 *M. avium* tandem repeat (MATR) loci (MATR-VNTR typing analysis), Adachi et al. reported that *M. avium* subsp. *hominissuis* isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients at the National Hospital Organization (NHO) Higashinagoya National Hospital in the Aichi Prefecture of Japan, HIV-positive patients, and pigs have different VNTR genotypes (26). The isolates from HIV-positive patients used in the present study were almost identical to the strains used by Adachi et al. (26). In this study, we used different strains isolated from pulmonary *M. avium* subsp. *hominissuis* disease patients without previous antibiotic treatment at 9 NHO hospitals throughout Japan and performed VNTR typing analysis using 15 MATR loci to examine the VNTR genotypes of the isolates from hosts with different types of *M. avium* subsp. *hominissuis* infection. As shown in Fig. 1, 76 strains examined in this study were roughly classified into three clusters: cluster I, cluster II, and cluster III. The proportion of isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients and that of isolates from HIV-positive patients in each cluster were 6.5% (3/46) and 36.7% (11/30), respectively, for cluster I, 41.3% (19/46) and 36.7% (11/30), respectively, for cluster II, and 52.2% (24/46) and 26.7% (8/30), respectively, for cluster III (Table S3). The ratio of isolates from HIV-positive patients to those from pulmonary *M. avium* subsp. *hominissuis* disease patients was significantly higher in cluster I than in the other two clusters ($P = 0.0017$ by Fisher's exact test) (Table S3), indicating that a group of isolates from HIV-positive patients has a unique VNTR genotype. Furthermore, to compare the genetic distances of the isolates from pulmo-

TABLE 3 Mean $\log_2$ values of MICs of test drugs for isolates within the three VNTR clusters

Antimicrobial agent	$\log_2$ MIC (mean ± SD)			*P* value[a]			
	Cluster[b] I	Cluster II	Cluster III	Clusters I vs II vs III	Clusters I vs II	Clusters I vs III	Clusters II vs III
Clarithromycin	−1.94 ± 1.95	−1.44 ± 2.21	−0.63 ± 1.94	0.011	0.398	0.010	0.020
Rifampin	−2.95 ± 1.35	−3.03 ± 1.73	−1.46 ± 2.69	0.046	0.758	0.106	0.020
Ethambutol	2.57 ± 1.02	2.97 ± 1.59	3.09 ± 1.12	0.156	0.449	0.058	0.224
Streptomycin	0.64 ± 0.84	1.16 ± 1.71	2.0 ± 1.5	0.003	0.137	≤0.001	0.038
Kanamycin	1.36 ± 1.01	2.03 ± 1.94	2.91 ± 1.4	0.002	0.076	≤0.001	0.058
Amikacin	1.07 ± 0.92	1.73 ± 1.46	2.50 ± 1.32	0.001	0.093	≤0.001	0.028
Ethionamide	1.79 ± 0.80	3.0 ± 0.69	2.34 ± 0.9	≤0.001	≤0.001	0.044	0.004
Levofloxacin	−0.64 ± 1.22	0.73 ± 1.46	0.25 ± 1.44	0.003	0.001	0.022	0.119

[a]*P* values for the $\log_2$ MIC values between the indicated clusters were calculated using the Kruskal-Wallis test for the differences between three clusters and the Mann-Whitney U test for the differences between two clusters.
[b]Each cluster was classified by phylogenetic analysis, as shown in Fig. 1.

nary *M. avium* subsp. *hominissuis* disease patients and HIV-positive patients, we performed a multiple-comparison analysis using strain 104 as a clinically unbiased standard to estimate the Manhattan distance of the individual isolates of each cluster. The genetic distance from strain 104 for isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients was statistically significantly different from that for isolates from HIV-positive patients ($P = 0.0018$) (Fig. S1). These results suggest that *M. avium* subsp. *hominissuis* strains that cause pulmonary and disseminated disease have genetically distinct features.

Next, we analyzed the association between the VNTR genotype and drug susceptibility in strains within each cluster by the Kruskal-Wallis test, revealing a significant difference in the $\log_2$ MICs of seven of the eight drugs, with the exception being ethambutol (Table 3). The Mann-Whitney U test was then used to further compare two clusters. The strains in cluster I were more susceptible to ethionamide and levofloxacin than the strains in cluster II and were more susceptible to clarithromycin, streptomycin, kanamycin, amikacin, ethionamide, and levofloxacin than the strains in cluster III. The strains in cluster II were more susceptible to clarithromycin, rifampin, streptomycin, and amikacin but more resistant to ethionamide than the strains in cluster III. A comparison of the presence of resistant and susceptible strains among the clusters revealed that cluster III had the highest percentage of strains resistant to ethambutol, streptomycin, kanamycin, and amikacin and cluster I had the lowest (Table S4). Furthermore, a significant difference in ethionamide resistance was found among the clusters, with the highest percentage of resistant strains being found in cluster II. Accordingly, intergroup comparisons revealed that strains in cluster I had the lowest MIC values and those in cluster III tended to have the highest values. These results are related to the proportions of isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients and those of isolates from HIV-positive patients in each cluster. In good agreement with the findings presented above, the proportion of isolates from HIV-positive patients was the highest in cluster I and the lowest in cluster III (Table S3).

Association between the presence of IS*Mav6* and drug susceptibility. Based on a previous report on the association between the presence of IS*Mav6* and drug susceptibility (27), we investigated such possible associations for *M. avium* subsp. *hominissuis* isolates from different hosts in the present study. We previously reported the presence of IS*Mav6*, which is a novel insertion sequence, with 60 point mutations compared with the nucleotide sequence of the original IS*901* in Japanese human clinical *M. avium* subsp. *hominissuis* isolates (28). IS*Mav6* was present in 41.3% of the isolates from patients with pulmonary *M. avium* subsp. *hominissuis* disease and 40.0% of those from HIV-positive patients, with no significant intergroup difference. Comparisons of the isolates from the different hosts revealed that isolates harboring Is*Mav6* were significantly more resistant to clarithromycin, rifampin, streptomycin, kanamycin, and amikacin than isolates not harboring Is*Mav6* (Table 4), showing the association between the presence of IS*Mav6* and drug susceptibility. Intriguingly, a comparison of

TABLE 4 Association between presence of IS*Mav6* and drug susceptibility

Antimicrobial agent	Log_2 MIC (mean ± SD)		*P* value[a] (IS*Mav6*-positive vs -negative isolates)
	IS*Mav6*-positive isolates	IS*Mav6*-negative isolates	
Clarithromycin	−0.46 ± 2.55	−1.70 ± 1.53	0.009
Rifampin	−1.31 ± 2.64	−3.07 ± 1.59	0.003
Ethambutol	2.87 ± 1.36	3.0 ± 1.28	0.488
Streptomycin	1.74 ± 1.92	1.20 ± 1.25	0.034
Kanamycin	2.68 ± 1.85	2.0 ± 1.49	0.017
Amikacin	2.39 ± 1.65	1.62 ± 1.13	0.005
Ethionamide	2.45 ± 1.03	2.53 ± 0.84	0.698
Levofloxacin	0.32 ± 1.45	0.24 ± 1.51	0.888

[a]*P* values were calculated using the Mann-Whitney U test.

the presence of resistant and susceptible strains between the two groups revealed that higher percentages of strains possessing IS*Mav6* than isolates not possessing IS*Mav6* were resistant to clarithromycin, streptomycin, kanamycin, and amikacin (Table S5). No such trend could be found for rifampin or levofloxacin because there was only one strain resistant to these antibiotics. The result was consistent with that shown in Table 4, once these drugs were removed from the analysis. We then investigated the association between the IS*Mav6* and VNTR genotypes. The highest prevalence (22/31, 71%) of IS*Mav6* was observed among strains in cluster III, which had the highest MIC values toward individual drugs, whereas no strains harboring IS*Mav6* were observed among the isolates in cluster I, which overall had the lowest MIC values (Table S6). These findings suggest the involvement of IS*Mav6* in the association between the VNTR genotype and drug susceptibility.

DISCUSSION

In this study, we compared the MICs of eight drugs for 46 isolates from patients with pulmonary *M. avium* subsp. *hominissuis* disease without previous antibiotic treatment and 30 isolates from HIV-positive patients with disseminated *M. avium* subsp. *hominissuis* disease. Interestingly, isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients were more resistant to all drugs except rifampin than isolates from HIV-positive patients. Thus, isolates from different hosts showed distinct differences in drug susceptibility, and isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients had higher levels of drug resistance. Such differences in drug susceptibility are thought to be caused by differences in genetic characteristics. In our previous study, comparative genome analysis of strain TH135, isolated from a patient with pulmonary *M. avium* subsp. *hominissuis* disease, and strain 104, derived from an HIV-positive patient, showed that many strain-specific regions including virulence-associated genes were present in the genomes of both strains (18). These results suggest that *M. avium* subsp. *hominissuis* strains that cause pulmonary and disseminated disease possess genetically distinct features, and these differences possibly reflect different drug susceptibilities among the isolates from both groups. These findings suggest an association between drug susceptibility and the types of *M. avium* subsp. *hominissuis* infection, and the genetic characteristics of *M. avium* subsp. *hominissuis* strains associated with the development of pulmonary disease may be involved in higher levels of antibiotic resistance.

We performed MATR-VNTR typing analysis to compare the genotypes of *M. avium* subsp. *hominissuis* isolates from different hosts. MATR-VNTR typing analysis showed that the strains examined in this study were roughly classified into three clusters. Cluster I contained 3 isolates (DH-2, IH-068, and NN-127) from pulmonary *M. avium* subsp. *hominissuis* disease patients and 11 isolates from HIV-positive patients. The ratio of isolates from HIV-positive patients to those from pulmonary *M. avium* subsp. *hominissuis* disease patients was significantly higher in cluster I than in the other two clusters, indicating that a group of isolates from HIV-positive patients exhibited a unique VNTR genotype. We previously performed phylogenetic analysis based on

single nucleotide variants using the genome information for isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients, the same isolates used in the present study, and *M. avium* subsp. *hominissuis* strains isolated abroad, including the United States, Belgium, and Germany (25). Most isolates in Japan were grouped into clusters different from those containing strains isolated abroad. However, 3 strains, DH-2, IH-068, and NN-127, were grouped into the clusters containing strains isolated abroad, suggesting that the strains in cluster I are genetically similar to those isolated abroad. Previous studies reported the association between genotype and drug susceptibility in NTM (29, 30). In the present study, significant differences in the $\log_2$ MICs of seven of eight of the drugs tested, with the exception being ethambutol, were observed among the isolates in the three clusters grouped by VNTR typing of *M. avium* subsp. *hominissuis* isolates. Moreover, intergroup comparisons revealed that the strains in cluster I had the lowest MIC values and those in cluster III tended to have the highest values. This result correlated with the proportion of isolates from HIV-positive patients in each cluster.

Given that a report on the association between IS*Mav6* and drug susceptibility has been previously published (27), we investigated the association between the presence of Is*Mav6* and differences in drug susceptibilities among *M. avium* subsp. *hominissuis* isolates from different hosts. The presence of IS*Mav6* did not differ among the isolates, indicating that differences in drug susceptibility and the presence of IS*Mav6* are unrelated. However, isolates possessing Is*Mav6* were significantly more resistant to five of the drugs tested than those not possessing Is*Mav6*, indicating an association between Is*Mav6* and drug susceptibility. Furthermore, isolates with IS*Mav6* had higher percentages of strains resistant to clarithromycin, streptomycin, kanamycin, and amikacin than isolates without IS*Mav6*. Because resistance to macrolides and aminoglycosides is caused by mutations in the genes encoding 23S rRNA and 16S rRNA (31, 32), respectively, this result suggests a possible correlation between mutations of these genes and the presence of IS*Mav6*. The proportion of isolates possessing Is*Mav6* relative to all *M. avium* subsp. *hominissuis* isolates is higher in Japan and South Korea than in the United States, with almost no report of Is*Mav6* being made for *M. avium* subsp. *hominissuis* isolates recovered in Germany or the Netherlands (17, 27, 33), suggesting that drug susceptibility varies among different geographical regions and that *M. avium* subsp. *hominissuis* isolates in Japan and South Korea are more resistant to antibiotics than *M. avium* subsp. *hominissuis* isolates in other countries with a low rate of possession of Is*Mav6*. This also suggests that Is*Mav6* serves as a predictor of drug susceptibility, and to clarify this, further study is needed to compare the findings of drug susceptibility in the present study with those obtained from *M. avium* subsp. *hominissuis* strains isolated abroad.

This study has some limitations. Isolates from patients with pulmonary *M. avium* subsp. *hominissuis* disease were obtained before the administration of antibiotics. However, it is not known whether multidrug therapy with antibiotics was performed in HIV-positive patients, and if so, the effect of antibiotics on the drug susceptibility of the isolates from these hosts cannot be disregarded. However, in such cases, isolates would have higher MIC values than isolates from patients not treated with antibiotics. In other words, the difference in drug susceptibility between the two groups would be greater if the isolates were collected from HIV-positive patients not treated with multidrug therapy using antibiotics. Therefore, we think that this does not alter the conclusions of this study. Also, we did not investigate the mechanisms underlying the resistance of *M. avium* subsp. *hominissuis* isolates to individual antibiotics. Thus, this investigation can be regarded as a preliminary study.

In conclusion, we observed a difference in drug susceptibility and VNTR genotypes between *M. avium* subsp. *hominissuis* isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients and those from HIV-positive patients. These differences indicate the association with types of *M. avium* subsp. *hominissuis* infection, and the genetic characteristics of *M. avium* subsp. *hominissuis* strains that are involved in the establishment of pulmonary disease in immunocompetent hosts or disseminated disease in

immunocompromised hosts are thought to influence these differences. Additionally, the enhanced antibiotic resistance of the isolates from pulmonary *M. avium* subsp. *hominissuis* disease patients might potentially make treatment of pulmonary *M. avium* subsp. *hominissuis* disease patients difficult. Further study is needed to examine the genes involved in drug resistance by comparing the genome of each *M. avium* subsp. *hominissuis* isolate with the genomes of other isolates and to examine *M. avium* subsp. *hominissuis* strains isolated abroad to clarify whether the results obtained in the present study are specific to strains prevalent in Japan.

MATERIALS AND METHODS

Bacterial strains. As reported previously (34), 46 *M. avium* subsp. *hominissuis* isolates from HIV-negative patients with pulmonary *M. avium* subsp. *hominissuis* disease were provided by nine National Hospital Organization (NHO) hospitals across Japan. These clinical isolates were obtained from the sputa of 46 patients who did not undergo treatment immediately after a diagnosis of pulmonary *M. avium* subsp. *hominissuis* disease (corresponding to the diagnostic criteria of the American Thoracic Society and the Infectious Diseases Society of America [19]) between July 2008 and September 2009. Also, 30 *M. avium* subsp. *hominissuis* isolates from the blood of HIV-positive patients, including strain 104, derived from an AIDS patient and used as a standard (35), with disseminated *M. avium* subsp. *hominissuis* disease were provided by the National Center for Global Health and Medicine, formerly the International Medical Center of Japan. However, it is not known whether antibiotic therapy was provided to the HIV-positive patients. Moreover, the age, sex, and location of the patients with pulmonary *M. avium* subsp. *hominissuis* disease were known, while those of the HIV-positive patients were not. Only one strain per patient was analyzed in this study.

Identification of subspecies of *M. avium*, growth condition, and DNA isolation. The subspecies of the *M. avium* isolates was identified to be *M. avium* subsp. *hominissuis* by sequence analysis of the 3′ fragment of the *hsp65* gene (36). The organism was grown in Middlebrook 7H9 liquid medium supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco, Sparks, MD) at 37°C. DNA was extracted using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Drug susceptibility testing. The BrothMIC NTM system (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) was used to determine the susceptibility of the *M. avium* subsp. *hominissuis* strains to clarithromycin, rifampin, ethambutol, streptomycin, kanamycin, amikacin, ethionamide, and levofloxacin, according to the manufacturer's instructions for each antimicrobial agent; this procedure is in compliance with the standard protocol of the Clinical and Laboratory Standards Institute, formerly called the National Committee for Clinical Laboratory Standards (37, 38). Serial dilutions of each antimicrobial and Middlebrook 7H9 broth (Difco) were reconstituted by inoculation of 0.1 ml of a cell suspension prepared in distilled water by 1:100 dilution of a 0.5 McFarland suspension in air-dried microplates. After inoculation, the isolates were incubated at 37°C at pH 7.4 for clarithromycin and at pH 6.6 for the remaining antimicrobials. Growth endpoints were read visually after incubation for 7 days. The MIC breakpoints of the drugs indicating resistance were determined according to the criteria described in the BrothMIC NTM system manual, as follows: clarithromycin, ≥32 μg/ml; rifampin, ≥8 μg/ml; ethambutol, ≥8 μg/ml; streptomycin, ≥8 μg/ml; kanamycin, ≥16 μg/ml; amikacin, ≥16 μg/ml; ethionamide, ≥8 μg/ml; and levofloxacin, ≥8 μg/ml.

VNTR genotyping. Variable-number tandem-repeat (VNTR) typing analysis using *M. avium* tandem repeats (MATR) was carried out using 15 VNTR loci (MATR-1 to MATR-16, except for MATR-10) and the corresponding primer sets, as described previously (39). After the number of base pairs in the target VNTR loci was estimated according to their relationship to molecular weight markers by agarose gel electrophoresis, the number of repetitions of various VNTR loci in each strain was determined and regarded as an allele profile. The amplification product of *M. avium* subsp. *paratuberculosis* ATCC 19698, for which the number of repetitions of each VNTR locus had been determined by sequence analysis, was used as a positive control. The Manhattan distance was determined on the basis of each obtained allele profile, and the genotypic diversity of the *M. avium* subsp. *hominissuis* isolates was analyzed with a Fitch-Margoliash algorithm using PHYLIP software (version 3.68). Branches were supported by the bootstrap value as 1,000 replicates of a randomly assembled data set. The phylogenetic distribution was generated according to the genotypic diversity of the isolates using FigTree software (version 1.3.1).

Detection of IS*Mav6*. The presence of the IS*Mav6* gene in *M. avium* subsp. *hominissuis* isolates was determined by using specific PCR primers, as described previously (28). The resulting PCR products were purified using a GenElute PCR DNA purification kit (Sigma-Aldrich, St. Louis, MO), and direct sequencing analysis was performed using the same primers used for PCR. The resulting nucleotide sequences were compared with the sequence data for IS*Mav6*. The suitability of the present DNA samples for screening clinical isolates by PCR was determined by amplification of the *hsp65* gene, the gene used to identify the subspecies of *M. avium* isolates.

Statistical analysis. The Mann-Whitney U test and the Kruskal-Wallis test were used for comparison of the mean $\log_2$ values of the MICs of the test drugs for *M. avium* subsp. *hominissuis* strains derived from two different hosts and the three VNTR clusters, respectively. The genetic distances estimated from the Manhattan distance matrix data for the *M. avium* subsp. *hominissuis* isolates were analyzed using the Mann-Whitney U test. Fisher's exact test was used for categorical variables. All statistical analyses were

performed using GraphPad Prism (version 5.0) software (GraphPad Software, San Diego, CA). *P* values of <0.05 were considered significant.

Ethics. This study was approved by the Ethics Review Committee for Human Research of the NHO Higashinagoya National Hospital, and written informed consent was obtained from all patients with pulmonary *M. avium* subsp. *hominissuis* disease.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02035-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare no conflicts of interest.

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