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Identification and Partial Characterization of *Mycobacterium avium* and *Mycobacterium intracellulare* by Using DNA Probes

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We attempted to identify the *Mycobacterium avium* complex (MAC) isolated in Japan by using DNA probes specific for *M. avium* or *Mycobacterium intracellulare* (Gen-Probe Rapid Diagnostic System for MAC; Gen-Probe, Inc., San Diego, Calif.). The source and drug susceptibility distributions were examined. This assay system proved to be rapid, sensitive, specific, and reliable for identification of MAC and of the species as either *M. avium* or *M. intracellulare*. The DNA probe test showed that of the generally accepted MAC serovars, serovars 1 to 6, 8 to 11, and 21 belonged to *M. avium* and 7 and 12 to 20 belonged to *M. intracellulare*. Moreover, with the DNA probe test we found that the distribution patterns of *M. avium* and *M. intracellulare* isolates in Japan differed depending on the district in which MAC was isolated. The ratio of *M. avium* was much higher in eastern Japan. In Tokai and Shimane districts, the ratio of *M. avium* and *M. intracellulare* isolates significant in human disease was related to that of isolates from soil and house dust (natural sources). In *M. avium*, human disease-associated isolates were more resistant to rifampin, streptomycin, and kanamycin than were isolates from natural sources. However, this source dependence was not evident for *M. intracellulare*. In human disease-associated MAC, *M. avium* isolates were more resistant to most agents, except for quinolones, than were *M. intracellulare* isolates.

Mycobacterium avium and *Mycobacterium intracellulare* are virtually indistinguishable phenotypically and are referred to as the *M. avium* complex (MAC). Conventional culture and biochemical tests do not provide a clear differentiation between the two. The known biochemical differences between the two species lie in the activities of arylsulfatase and nitrite reduction (11). However, it is difficult to differentiate *M. avium* and *M. intracellulare* on the basis of enzyme activities because the features are not always consistent.

DNA probe technology facilitates a rapid and specific identification of microorganisms. A commercial kit (Gen-Probe Rapid Diagnostic System for MAC; Gen-Probe, Inc., San Diego, Calif.) is available for confirmation and identification of species of organisms belonging to MAC (4). This system is based on the hybridization of ^{125}I-labeled DNA probes for *M. avium* and for *M. intracellulare* with rRNA from a test organism. Using the DNA probe test, we attempted to identify MAC freshly isolated in Japan. The source and drug susceptibility distributions of MAC isolates were examined.

MATERIALS AND METHODS

Organisms. We used 293 strains of MAC isolated from humans, natural sources, and swine. Human disease-associated MAC (231 strains) consisted of 8 strains provided by A. Kuze, National Sanatorium Sapporo Minami Hospital, Hokkaido, Japan; 50 by H. Shimoide, National Sanatorium Tokyo Hospital, Tokyo; 3 by H. Masai, Kanto Central Hospital, Tokyo; 32 by C. Abe, Research Institute of Tuberculosis, Tokyo; 52 by M. Tsukamura, National Sanatorium Chubu Hospital, Aichi; 23 by N. Kita, National Sanatorium Kinki Central Hospital, Osaka; 4 by S. Yoshimoto, National Sanatorium Minami Okayama Hospital, Okayama; 20 by T. Kamata, National Sanatorium Hiroshima Hospital, Hiroshima; 6 by R. Kanayama, National Sanatorium Matsue Hospital, Shimane; 6 by T. Mitsui, National Sanatorium Sanyoso Hospital, Yamaguchi; 2 by Y. Yamamoto, National Sanatorium Ehime Hospital, Ehime; 3 by J. Tani, National Sanatorium Higashi Kochi Hospital, Kochi; 10 by H. Arakawa, National Sanatorium Higashi Fukuoka Hospital, Fukuoka; and 12 by T. Ishibashi, National Sanatorium Ohmuta Hospital, Fukuoka. Natural source-derived MAC (55 strains; 25 from soil, 2 from ditch mud, 13 from house dust, and 15 from pigpen sawdust) included 30 strains provided by M. Tsukamura; 10 by H. Saito, Shimane Medical University, Shimane; and 15 by A. Sato, Public Health Research Institute of Kobe City, Hyogo. Swine-associated MAC strains (seven strains) were provided by A. Sato. In addition, 45 mycobacterial strains, including MAC strains with code numbers, were provided by F. Kuze, Chest Disease Research Institute, Kyoto University, Kyoto, and were used only for the blind test.

DNA probe test. The DNA probe test was performed according to the manual for the Gen-Probe Rapid Diagnostic System for MAC. Briefly, 0.1 ml of bacterial suspension prepared from cultures grown on 1% Ogawa egg medium (7) for about 3 weeks was thoroughly sonicated in tubes containing glass beads and lysing reagents in a sonicator bath at 59°C. One milliliter each of ^{125}I-labeled DNA-probe solution for *M. avium* or *M. intracellulare* was added to the resultant cell lysate containing rRNA and heated at 72°C for 1 h for annealing. The incubation mixture was mixed with 4 ml of separation suspension consisting of hydroxyapatite and buffer solution, incubated at 72°C for 5 min, and centrifuged. The precipitate was washed once with wash solution, and

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radioactivity was counted with a gamma counter. Percent hybridization was calculated as [(sample counts per minute − background counts per minute)/(total counts per minute − background counts per minute)] × 100. Hybridization greater than or equal to 10% was regarded as positive.

α-Antigen analysis. α-Antigen of test mycobacteria was analyzed by agar gel precipitation with anti-α-antigen serum, as described elsewhere (10).

Drug susceptibility testing. MICs of rifampin, rifabutin, lamprene, streptomycin, kanamycin, ethambutol, ofloxacin, and ciprofloxacin against MAC were determined by the usual agar dilution method with Middlebrook 7H11 medium, as described previously (8).

RESULTS

Comparison of DNA probe test with conventional identification for MAC. The specificity and sensitivity of the Gen-Probe Rapid Diagnostic System for MAC were evaluated by blind testing of the 45 clinical isolates of mycobacteria provided by F. Kuze and identified by conventional culture and biochemical methods. The DNA probe test revealed that the 45 strains contained 20 strains of MAC, 13 of *M. avium*, and 7 of *M. intracellulare*. The DNA probe test results agreed 100% with conventional culture and biochemical identification of MAC. Of the 45 strains tested by DNA probing, 20 were positive and 0 were negative for MAC; 0 were positive and 25 were negative for other organisms. The diagnostic specificity and sensitivity of the DNA probe test were 100%.

Similarly, there was good correlation between the DNA probe test and conventional culture and biochemical identification results for the 232 mycobacterial strains (231 of them MAC strains) obtained mainly from 13 national sanatoriums in various districts of Japan as human disease-associated MAC. Of the 232 strains tested by DNA probing, 229 were positive and 2 were negative for MAC; 0 were positive and 1 was negative for other organisms (see below). The diagnostic specificity and sensitivity of the DNA probe test were 100 and 99%, respectively. The one strain with a different result proved to be *Mycobacterium gordonae*, as determined by M. Tsukamura.

The correlation between the DNA probe test and conventional culture and biochemical identification results for the 64 mycobacterial strains (62 of them MAC strains) isolated from natural sources (55 MAC) and swine (7 MAC) (see Materials and Methods) was also good. Of the 64 strains tested by DNA probing, 62 were positive and 0 were negative for MAC; 0 were positive and 2 were negative for other organisms (see below). The DNA probe test identified MAC with 100% specificity and 100% sensitivity. The two strains negative for MAC in the DNA probe test were proved to be mycobacteria other than MAC. These were originally misidentified, and the present study showed that they were lacking in MAC-specific α-antigen.

These results show the excellent reliability of the DNA probe test in distinguishing MAC from the other mycobacterial species (3–5, 9).

In separate experiments, reference serovar strains (72 strains, including 2 to 4 strains for each serovar) of MAC obtained from A. Tsang, National Jewish Center, Denver, Colo., and G. P. Kubica, Centers for Disease Control, Atlanta, Ga., were identified as such by reaction with either the *M. avium* or *M. intracellulare* probe. One MAC strain belonging to serovar 1 gave percent hybridization values of 43.3 and 1.0% in response to *M. avium* and *M. intracellulare* DNA probes, respectively. Conversely, another MAC strain belonging to serovar 7 gave percent hybridization values of 1.4 and 42.2% in response to *M. avium* and *M. intracellulare* DNA probes, respectively. Results of the DNA probe test clearly identified MAC strains belonging to serovars 1 to 21. With DNA probe testing, MAC belonging to serovars 1 to 6, 8 to 11, and 21 was identified as *M. avium* and MAC belonging to serovars 7, 12 to 20, and 25 was identified as *M. intracellulare* (9; manuscript in preparation). In the latter part of this work, we attempted to identify MAC strains isolated from various sources, such as humans, swine, and the environment in Japan.

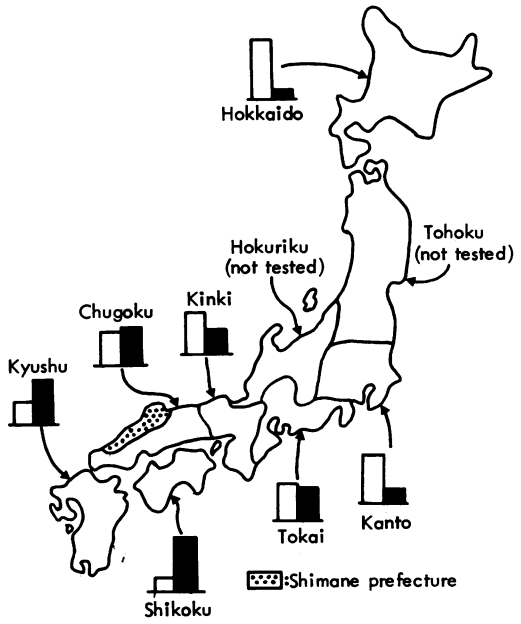

FIG. 1. Map of Japan and distribution by district of *M. avium* (□) and *M. intracellulare* (■) isolates. See Table 1 for further details.

Distribution of *M. avium* and *M. intracellulare* isolated in various areas of Japan. Figure 1 and Table 1 show the distribution of *M. avium* and *M. intracellulare* significant in human disease and isolated in various districts of Japan. Two hundred thirty-one MAC strains isolated mainly at 13 national sanatoriums were tested for their reactivity with *M. avium* and *M. intracellulare* probes. In Hokkaido, Kanto,

TABLE 1. Distribution of *M. avium* and *M. intracellulare* among MAC isolates in various districts of Japan

District	No. of test strains	No. of strains (%)		
		M. avium	*M. intracellulare*	Unidentified species
Hokkaido	8	7 (88)	1 (12)	0 (0)
Kanto	85	70 (82)	15 (18)	0 (0)
Tokai	52	29 (56)	23 (44)	0 (0)
Kinki	23	14 (61)	8 (35)	1 (4)
Chugoku	36	16 (44)	20 (56)	0 (0)
Shikoku	5	1 (20)	4 (80)	0 (0)
Kyushu	22	6 (27)	15 (68)	1 (5)
Total	231	143 (62)[a]	86 (37)[b]	2 (1)

[a] The mean distribution ratio of *M. avium* among the indicated districts was 54%.

[b] The mean distribution ratio of *M. intracellulare* among the indicated districts was 44%.

TABLE 2. Source distribution of *M. avium* and *M. intracellulare* isolated in Tokai and Shimane districts

Source	No. of strains (%)			
	Tokai District		Shimane Prefecture[a]	
	M. avium	*M. intracellulare*	*M. avium*	*M. intracellulare*
Human	29 (56)	23 (44)	1 (17)	5 (83)
Natural	24 (80)	6 (20)	3 (43)	4 (57)

[a] Shimane Prefecture lies in Shimane District (Fig. 1).

Tokai, and Kinki districts located in the eastern and central parts of Japan, the ratio of *M. avium* isolates was larger than that of *M. intracellulare* isolates. The opposite was true in Chugoku, Shikoku, and Kyushu districts located in the western part of Japan; that is, the ratio of *M. avium* isolates was smaller than that of *M. intracellulare* isolates. A χ^2 test showed a statistically significant difference ($P < 0.01$) in MAC distribution between the eastern and central parts (Hokkaido, Kanto, Tokai, and Kinki) and the western part (Chugoku, Shikoku, and Kyushu) of Japan. There were statistically significant differences in MAC distribution between Kanto and Chugoku ($P < 0.01$), Kanto and Kyushu ($P < 0.01$), and Tokai and Kyushu ($P < 0.05$).

Source distribution of *M. avium* and *M. intracellulare* isolated in Tokai and Shimane districts. Table 2 shows the distribution of *M. avium* and *M. intracellulare* isolates from humans and from natural sources (soil, house dust, and water) in Tokai and Shimane districts. In Tokai District, disease-associated MAC had a higher ratio of *M. avium* (56%) than of *M. intracellulare* (44%). It is noteworthy that in this district, the ratio of *M. avium* isolates from natural sources (80%) was also higher than that of *M. intracellulare* (20%) (Table 2). Therefore, there may be some correlation between the distribution of *M. avium* and *M. intracellulare* isolates of human origin and that of isolates from natural sources, at least in Tokai District, as discussed below. In Shimane District, although the number of test isolates was too small for a conclusion to be reached, the ratio of *M. avium* isolates was lower than that of *M. intracellulare* isolates from both human and natural sources.

Growth of MAC strains at 45°C. Table 3 shows the growth at 45°C of *M. avium* and *M. intracellulare* isolated from various sources. In the case of *M. avium*, many isolates from humans and all the isolates from swine grew at that temperature. All the *M. avium* isolates from pigpen sawdust also grew at 45°C, implying contamination of sawdust with *M. avium* of swine origin. On the other hand, all the *M. avium* isolates from house dust and soil did not grow at 45°C. The ratio of 45°C growth-positive isolates from humans was significantly different from that of natural source-derived isolates ($P < 0.01$, χ^2 test). All the test strains of *M. intracellulare* were negative for growth at 45°C, regardless of their origin.

Susceptibilities of MAC strains to various antimicrobial agents. Table 4 lists the susceptibilities of human disease-associated and natural source-derived *M. avium* and *M. intracellulare* isolates identified by the DNA probe test to various antimicrobial agents. Human disease-associated isolates of *M. avium* were more resistant to rifampin, streptomycin, and kanamycin than were *M. avium* isolates from natural sources. However, irrespective of the source, *M. intracellulare* was equally susceptible to all the test agents. In the case of human-derived MAC, *M. avium* was fourfold more resistant to rifampin than was *M. intracellulare*.

TABLE 3. Growth of *M. avium* and *M. intracellulare* isolates from various sources at 45°C[a]

Organism and source	No. of strains	% Strains showing:	
		Growth	No growth
M. avium			
Human	48	77	23
Swine	4	100	0
Sawdust	6	100	0
House dust	8	0	100
Soil	18	0	100
M. intracellulare			
Human	57	0	100
Swine	3	0	100
Sawdust	9	0	100
House dust	5	0	100
Soil	7	0	100

[a] Test organisms were grown on Ogawa egg medium at 45°C.

DISCUSSION

The present study revealed that the DNA probe test can identify MAC from various mycobacterial species with high levels of specificity and sensitivity and in a reproducible and accurate manner. In separate experiments with reference strains of MAC belonging to serovars 1 to 28, we found that MAC strains of serovars 1 to 6, 8 to 11, and 21 showed positive hybridization with *M. avium* probe but not with *M. intracellulare* probe, indicating that these serovars belong to *M. avium*. In contrast, MAC strains of serovars 7, 12 to 20, and 25 showed positive hybridization with *M. intracellulare* probe but not with *M. avium* probe, indicating that these serovars are *M. intracellulare* (9; manuscript in preparation). This is in agreement with the results of DNA-DNA hybridization tests by Baess (1, 2) and sensitin tests on guinea pigs by Magnusson (6), which suggested that strains belonging to serovars 4 to 6 and 8 were more closely related to *M. avium* than to *M. intracellulare*. However, complicated results were obtained for the remaining serovars; i.e., most organisms belonging to these serovars were reactive neither to *M. avium* nor to *M. intracellulare* probe, although the organisms of serovars 23 and 28 had MAC-specific α-antigen. Moreover, the organisms of serovars 22 and 27 had α-antigen specific for *M. scrofulaceum* (9; manuscript in preparation).

MAC strains with α-antigen specific for MAC did not always give a clear-cut value for positive hybridization with either *M. avium* or *M. intracellulare* probe. Two of 231 MAC strains isolated from patients showed low percent hybridization values to *M. avium* and *M. intracellulare* probes (strain N-294, 7.7 to 17.3 and 2.6 to 9.3% for *M. avium* probe and *M. intracellulare* probe, respectively; strain N-297, 1.1 and 1.1%, respectively). Thus, it is difficult or impossible to be certain that these are MAC strains. However, both strains possessed MAC-specific α-antigen. Similar exceptions have been described by Enns (4). He reported that 8 of 60 strains identified as MAC by conventional culture and biochemical identification had no positive hybridization values (≥10%) with either the *M. avium* or *M. intracellulare* probe and that 1 in 53 strains identified as MAC by the DNA probe test was a mycobacterium other than MAC, as determined by conventional culture and biochemical tests. Therefore, it is important to develop additional DNA probes which can cover or eliminate these exceptional strains.

As for the results presented in Table 2, the χ^2 test indicated a statistically significant difference ($P < 0.05$)

TABLE 4. Susceptibilities of *M. avium* and *M. intracellulare* isolated from humans and natural sources to various antimicrobial agents[a]

Source and organism	No. of strains[b]	MIC (μg/ml)[c]															
		50%								90%							
		RFP	RFB	LMP	SM	KM	EB	OFLX	CPFX	RFP	RFB	LMP	SM	KM	EB	OFLX	CPFX
Human																	
M. avium	40	6.3	0.8	1.6	25	25	25	6.25	1.6	25	1.6	3.2	50	100	50	50	12.5
M. intracellulare	58	1.6	0.4	0.8	12.5	12.5	12.5	25	6.3	3.2	0.8	1.6	25	25	25	50	12.5
Natural																	
M. avium	30	0.8	0.4	0.8	6.3	6.3	6.3	12.5	3.2	1.6	0.8	1.6	12.5	12.5	25	25	6.3
M. intracellulare	22	1.6	0.2	0.8	6.3	6.3	12.5	12.5	3.2	3.2	0.4	1.6	25	12.5	25	50	12.5

[a] RFP, Rifampin; RFB, rifabutin; LMP, lamprene; SM, streptomycin; KM, kanamycin; EB, ethambutol; OFLX, ofloxacin; CPFX, ciprofloxacin.
[b] Test strains were randomly selected from 229 disease-associated MAC strains and 62 natural-source-derived strains.
[c] 50% and 90%, Mean MICs at which the growth of 50 and 90% of test strains was inhibited, respectively.

between the human disease-associated *M. avium*/*M. intracellulare* ratio and the environment-originated *M. avium*/*M. intracellulare* ratio in Tokai District. This may suggest that there is not a strict epidemiologic relation between disease-associated MAC and MAC associated with natural sources. However, the ratio for house-dust-derived MAC (13 strains; 62% *M. avium* and 38% *M. intracellulare*) closely resembled that for disease-associated MAC (56% *M. avium* and 44% *M. intracellulare*), and the difference was not significant ($\chi^2 = 0.141$, $P > 0.1$). On the other hand, 15 strains from soil showed a markedly different ratio, 93% *M. avium* and 7% *M. intracellulare*. The difference between this ratio and that for human disease isolates was significant ($P < 0.01$), indicating the close epidemiologic relation of disease-associated MAC with house-dust-derived MAC but not with soil-derived MAC.

The DNA probe test for MAC described in this article is most useful for identifying MAC strains at the species level. Using this system, we elucidated the precise distribution of *M. avium* and *M. intracellulare* in various districts of Japan (Fig. 1 and Table 1). It is interesting to note that *M. avium* and *M. intracellulare* are distributed predominately in the eastern and western parts of Japan, respectively. Because there is no marked difference between the two regions in such epidemiologic factors as population, race, age, sanitary conditions, mode of life, and climate (the eastern region is somewhat colder than the western), further studies of other epidemiologic factors are required.

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