Mycobacterium avium-M.intracellulare complexの血清学的同定法,特に螢光抗体法に関する研究

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Studies on Serological Identification of the *Mycobacterium avium-M. intracellulare* Complex, with Special Reference to the Fluorescent Antibody Test

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Abstract. The fluorescent antibody (FA) test was applied to the serological identification of the *Mycobacterium avium-M. intracellulare* complex in comparison with the agglutination and complement fixation (CF) test. The results are summarized as follows: (1) As a serological method of identification, CF test was a little less effective than the agglutination test. The agglutinin adsorption was necessary for about half the number of strains of the serotypes examined. A few cross reactions were eliminated by the use of CF antigen purified with chloroform. Complement-fixing substances were detected as an anthron-positive spot by thin-layer chromatography. (2) The indirect fluorescent antibody test (IFAT) was successfully applied to the identification of organisms of the known serotypes, as well as the unknown primary cultures of swine origin. (3) Such cross reactions as observed sometimes in the agglutination and CF tests did not appear in the IFAT, even when unabsorbed immune sera were employed in this test.

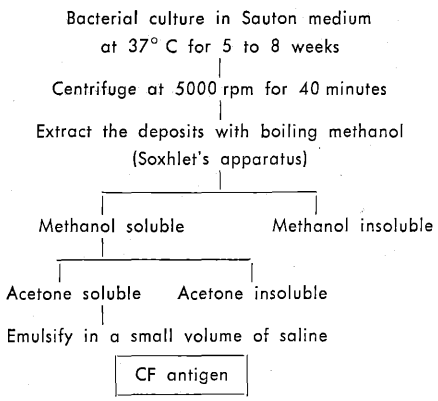
Mammalian tuberculosis has gradually been decreasing in incidence due to the extensive application of the "test and slaughter" method employing the tuberculin test. On the other hand, outbreaks of atypical mycobacteriosis have been detected increasingly in the recent years [5, 10–13].

Atypical mycobacteria are widely distributed in nature. The clarification of their etiological correlations between man and animals is attracting the researchers' interest. These organisms are composed of many known species and also presumably some other species not yet determined. There seems to be some confusions concerning their identification. Under such circumstances, rapid, simple and accurate methods for identification are eagerly desired by many workers.

In 1959, Runyon [7] briefly classified atypical mycobacteria into four groups on the basis of their biological and biochemical characteristics, such as pigmentation and growth rate. On the other hand, Schaefer [8, 9] carried out the serological identification of these organisms by the agglutination test. At present, Schaefer's method is accepted as the most reliable one. There are, however, several deterrents for its practical use because of the laborious procedures for the preparation of antigens. All the strains, except some, of the *Mycobacterium avium-M. intracellulare* complex generally provide stable cell suspensions for the agglutination reaction. For these autoagglutinable strains, the complement-fixation (CF) test and fluorescent antibody (FA) test seem to be effective as diagnostic tools, because they are

Mycobacterium avium-M. intracellulare complex の血清学的同定法，特に螢光抗体法に関する研究：石古博昭・清水亀平次（帯広畜産大学獣医学科家畜微生物学教室）

Fig. 1. Extraction of type specific CF antigen



well-known valuable methods for the serological identification of microorganisms.

The present report deals with the evaluation of the FA test as a method of identification of atypical mycobacteria, in comparison with that of the agglutination and CF tests.

Materials and Methods

Agglutination test: Rabbit immune sera were prepared against 15 reference strains of each serotype which had been supplied by Dr. Schaefer. The bacterial suspension for immunization was prepared by the method of Yoder and Schaefer [14]. The test was performed by Schaefer's method [8].

The results of the test were recorded as codes from 0 to 4 plus. The agglutination more than 2 plus was regarded as positive.

It was regarded as a cross reaction when antisera showed positive reactions with the heterologous antigen in serum dilutions within 4 times concentration of their homologous final positive titers.

CF test: The antisera used for the test were the same as those in the preceding test. As indicated in Fig. 1, antigens were prepared in almost the same manner as reported by Schaefer [8] and Yachida et al. [13].

Some purification of antigen was tried by the following method: The original CF antigen was freeze-dried and then thawed in a small volume of chloroform-methanol (2:1). Thus, the solution was partitioned into a water and a chloroform layer. The complement-fixing activity was proved in the chloroform layer. Then the dried material from the chloroform layer was dissolved in a small volume of saline and used as purified CF antigen.

Chemical nature of the antigenic substance was examined by thin-layer chromatography. A silica gel GB-5 (Wako) layer on the glass plate was dried by heating at 120°C for 30 minutes. The running of the material was made with the solvent system of (1) chloroform-methanol-water (65:25:4 by volume) and of (2) petroleum ether-hexan-acetic acid (90:10:1 by volume). Spots were detected by spraying 50% sulfuric acid and 0.2% ninhydrin in water saturated with butanol for the amino group, anthron reagent for sugar, and dittmer reagent for phosphates.

The CF test was carried out on the plastic plate by a microvolume system (antigen 0.025 m*l* + antiserum 0.025 m*l* + complement 0.05 m*l*—at 5°C overnight, + hemolytic system 0.05 m*l*—at 37°C for 2 hours). The inhibition of hemolysis more than 75% was recorded as positive.

FA test:

1. Cultures: Mycobacterial strains which showed cross reactions in the agglutination and CF tests were examined by this method.

2. Antisera: Organisms from Löwenstein-Jensen medium were subcultured in TB Broth Base-Bacto containing 10% bovine serum albumin and 5% glycerin for 7 to 10 days. The concentration of the bacterial cultures were adjusted to an optical density of 1.0 at 525 mμ wavelength with the spectrophotometer, and 1 m*l* of each culture was injected into rabbits by the intravenous route. Sera which showed a titer of more than 1:320 with FA test were used for experiments.

3. Preparation of antirabbit γ-globulin serum: A goat was inoculated subcutaneously with 20 mg/4 m*l* or rabbit γ-globulin plus an equal volume of incomplete Freund's adjuvant 4 times at 2 weeks' intervals. Moreover, it was given a booster dose of 5 mg of antigen 1 week after the final inoculation and bled 8 days later. It showed a precipitin titer of 1:128 (by the antigen constant-serum dilution method).

Crude γ-globulin was collected from goat serum by salting out with the saturated ammonium sulfate solution. Then it was conjugated with fluorescein isothiocyanate and purified by the method described by Kawamura [3].

4. Performance of the test: The titers of the antisera used for the first reaction were 4 times as high as the final serum dilution which gave a bright fluorescence against homologous antigens. The optimal dilution of the labeled antibody was determined by the box titration, in which 4 units of serum dilution was used.

Bacterial smear preparations on the glass slide were fixed at 80°C for 2 hours in an oven and then covered with a drop of the diluted antiserum at

Table 1. Agglutination test of the *M. avium-M. intracellulare* complex

Antiserum \ Antigen	*M. avium* 1	*M. avium* 2	*M. avium* 3	IIIa	IIIb	IV	V	VI	VII	Chance	Howell	Altmann	Watson	Dent	Davis
M. avium 1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. avium 2	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
M. avium 3	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
IIIa	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
IIIb	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
IV	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
V	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
VI	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
VII	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Chance	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Howell	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Altmann	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Watson	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Dent	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Davis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

Table 2. Complement fixation test of the *M. avium-M. intracellulare* complex

Antiserum \ Antigen	*M. avium* 1	*M. avium* 2	*M. avium* 3	IIIa	IIIb	IV	V	VI	VII	Chance	Howell	Altmann	Watson	Dent	Davis
M. avium 1	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
M. avium 2	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
M. avium 3	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
IIIa	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
IIIb	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
IV	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−
V	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
VI	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−
VII	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Chance	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Howell	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Altmann	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Watson	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Dent	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Davis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

Table 3. Strains and sera used for absorption

Antiserum	Strains for absorption
M. avium 1	*M. avium* 2
M. avium 2	*M. avium* 3
M. avium 3	*M. avium* 2
IIIa	IIIb
IV	VI
VI	IV
VII	Chance
Chance	VII
Howell	Chance

room temperature for 30 minutes. After washing with phosphate buffered saline, they were again stained with the labeled antibody in the same manner as in the first reaction.

5. Observation of samples: The stained bacterial smear preparations were examined with the UV system using the Chiyoda fluorescence microscope. The results were recorded with codes 0 to 4 plus. Code 0 means no fluorescence at all, code 1 plus a pale bluish autofluorescence, code 2 plus a pale greenish fluorescence not exceeding that of the background, and code 3 or 4 plus a bright green or yellowish green fluorescence. Codes 3 and 4 plus were regarded as positive.

Results

Experiments on agglutination and CF tests

For the serological identification of the *M. avium-M. intracellulare* complex, the CF test was examined for effectiveness, in comparison with the results of the agglutination test. Tables 1 and 2 give the results of the agglutination and CF tests on the 15 strains of different serotypes of this complex.

1. Agglutination test: As can be seen from Table 1, cross reactions were observed in the following serotypes: each serotype of *M. avium* and serotypes IIIa, IIIb, VII, Chance and Howell. Of the serotypes of *M. avium*, *M. avium* 1 and 2 antigens reacted with their homologous serotype antisera. The antiserum of *M. avium* 2, however, cross-reacted with the *M. avium* 3 antigen, and the *M. avium* 3 antigen reacted with both *M. avium* 2 and 3 antisera.

Of serotypes VII, Chance and Howell, antigen Howell reacted only with the homologous antiserum, but serotype Chance revealed cross reactions with the antisera of serotypes Howell and VII, and serotype VII

Table 4. Agglutination and CF test with absorbed and unabsorbed sera (1)

Antigen	Unabsorbed serum					
	CF test			Agglutination test		
	M. avium 1	*M. avium* 2	*M. avium* 3	*M. avium* 1	*M. avium* 2	*M. avium* 3
M. avium 1	1:128	0*	0	1:1280	1: 80	1: 40
M. avium 2	1:128	1: 512	0	1: 20	1:640	0
M. avium 3	1:256	1: 256	1:512	0	1:320	1:1280

Antigen	Absorbed serum					
	CF test			Agglutination test		
	#702 *M. avium* 1	#700 *M. avium* 2	#765 *M. avium* 3	#702 *M. avium* 1	#700 *M. avium* 3	#765 *M. avium* 3
M. avium 1	1:128	0	0	1: 320	0	0
M. avium 2	0	1:1024	0	0	1:320	0
M. avium 3	0	0	1: 64	0	0	1:320

Remarks. *: <1:16

Serum #702: Absorbed with *M. avium* 2. Serum #700: Absorbed with M. avium 3.

Serum #765: Absorbed with *M. avium* 2.

Table 5. Agglutination and CF tests with absorbed and unabsorbed sera (2)

Antigen	Unabsorbed serum			
	CF test		Agglutination test	
	IIIa	IIIb	IIIa	IIIb
IIIa	1:1024	0	1:1280	0
IIIb	1: 256	1:256	1: 160	1:640

Antigen	Absorbed serum			
	CF test		Agglutination test	
	IIIa	IIIb	IIIa	IIIb
IIIa	1: 512	0	1: 320	0
IIIb	0	1:256	0	1:640

Remarks.
Serum IIIa: Absorbed with IIIb.
Serum IIIb: Absorbed with IIIa.

Table 6. Agglutination and CF tests with absorbed and unabsorbed sera (3)

Antigen	Unabsorbed serum			
	CF test		Agglutination test	
	Type IV	Type VI	Type IV	Type VI
Type IV	1:128	1: 64	1:160	1: 40
Type VI	1: 64	1:128	0	1:320

Antigen	Absorbed serum			
	CF test		Agglutination test	
	Type IV	Type VI	Type IV	Type VI
Type IV	1: 64	0	1:160	0
Type VI	0	1: 64	0	1:160

Remarks.
Serum type IV: Absorbed with type VI.
Serum type VI: Absorbed with type IV.

Table 7. Agglutination and CF tests with absorbed and unabsorbed sera (4)

Antigen	Unabsorbed serum					
	CF test			Agglutination test		
	Type VII	Chance	Howell	Type VII	Chance	Howell
Type VII	1:128	1:128	0	1:160	1:160	1: 40
Chance	1: 32	1:256	1:128	1: 40	1:320	1:320
Howell	0	0	1:512	0	0	1:640

Antigen	Absorbed serum					
	CF test			Agglutination test		
	Type VII	Chance	Howell	Type VII	Chance	Howell
Type VII	1: 64	0	0	1: 80	0	0
Chance	0	1: 64	0	0	1: 80	0
Howell	0	0	1:128	0	0	1:320

Remarks.
Serum type VII: Absorbed with Chance.
Serum Chance: Absorbed with type VII.
Serum Howell: Absorbed with Chance.

antigen also reacted with the antiserum of serotype Chance.

In the case of serotypes IIIa and IIIb, the IIIa antiserum reacted with the serotype IIIb antigen. The other strains of the serotypes only reacted with their homologous antisera.

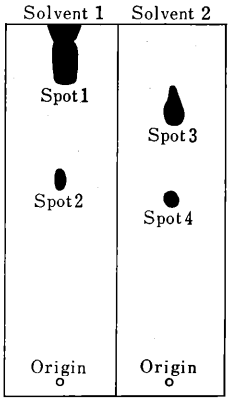
2. CF test: The occurrence of cross reactions in the CF test was nearly equal to that in the agglutination test, except the additional one between serotypes IV and VI, as described in Table 2.

In the serotypes of *M. avium*, the patterns of cross reaction were a little different from those in the agglutination test. *M. avium* 1 antigen reacted only with the homologous

Table 8. Fluorescent antibody test of the *M. avium-M. intracellulare* complex by the indirect method

Antiserum \ Antigen	*M. avium* 1	*M. avium* 2	*M. avium* 3	IIIa	IIIb	VII	Chance	Howell	Davis	Wilson	Yandle	New type
M. avium 1	+	−	−	−	−	−	−	−	−	−	−	−
M. avium 2	−	+	−	−	−	−	−	−	−	−	−	−
M. avium 3	−	−	+	−	−	−	−	−	−	−	−	−
IIIa	−	−	−	+	−	−	−	−	−	−	−	−
IIIb	−	−	−	−	+	−	−	−	−	−	−	−
VII	−	−	−	−	−	+	−	−	−	−	−	−
Chance	−	−	−	−	−	−	+	−	−	−	−	−
Howell	−	−	−	−	−	−	−	+	−	−	−	−
Davis	−	−	−	−	−	−	−	−	+	−	−	−
Wilson	−	−	−	−	−	−	−	−	−	+	−	−
Yandle	−	−	−	−	−	−	−	−	−	−	+	−
New type	−	−	−	−	−	−	−	−	−	−	−	+

Fig. 2. Chromatograph of CF antigen of serotype Howell



Remarks.
Spot 1: Positive for the anthron reagent and negative for the ninhydrin and dittmer reagent.
Spots 2, 3, and 4: Negative for all the reagents.

antisera, but *M. avium* 2 antigen reacted with *M. avium* 1 and 2 antisera, and *M. avium* 3 antigen with the antisera of all the serotypes of *M. avium*.

As for serotypes IV and VI, reciprocal cross reactions were observed. In the other serotypes, the patterns of cross reaction were the same as those in the agglutination test.

3. Agglutination and CF tests with absorbed sera: Mycobacterial strains with which the absorbed sera were prepared are shown in Table 3. The results of the agglutination and CF tests with these absorbed sera are seen in Tables 4 to 7, in comparison with those obtained with unabsorbed sera. As is clear from these tables, all the absorbed sera showed specific reactions only with the homologous antigen both in the agglutination and CF tests.

4. Purification of CF antigen: In the CF test, the strains of some serotypes presented cross reactions which were rather difficult to differentiate from one another, as described above. Then some experiments were carried out to remove nonspecific components disappeared from the absorbed antisera. The method used for purification was stated in the chapter Materials and Methods.

From the results of the experiments, it became clear that the cross reactions between serotypes IV and VI disappeared by employing the purified CF antigen (chloro-

Table 9. Comparison of serotyping between agglutination and fluorescent antibody tests

Agglutination test with unabsorbed serum		Positive fluorescence antibody test with test serum	
No. of strains examined	Serotype determined	No. of strains examined	Serotype determined
2	*M. avium* 1	2	*M. avium* 1
2	*M. avium* 2	2	*M. avium* 2
1	*M. avium* 1 or 2	1	*M. avium* 2
1	*M. avium* 2 or 3	1	*M. avium* 3
2	IIIa	2	IIIa
1	IIIa or IIIb	1	IIIb
1	VII or Chance	1	VII
1	VII, Chance or Howell	1	Chance
1	Howell	1	Howell
3	Davis	3	Davis
1	Wilson	1	Wilson
1	Yandle	1	Yandle
1	New type or Davis	1	New type
2	Rough form	2	*M. avium* 2
2	Unclassified	2	Unclassified

form-soluble antigen), though the other cross reactions remained as in the test with the orignal antigen.

The antigenic substances contained in the chloroform layer were examined by thin-layer chromatography. As shown in the chromatogram of Fig. 2, four components were differentiated. Of them, spot 1 was positive for the anthron reagent, but negative for ninhydrin and dittmer reagents. By comparing with the pure standard materials, spot 3 was identified as triglyceride and spot 4 as fatty acid, but spot 2 remained unknown.

From the experiments with these substances, CF antigenicity was demonstrated in the substances of spot 1. Similar spots were detected in the CF antigens of the 15 reference strains of the different serotypes examined. Each spot reacted with its homologous serotype antiserum.

Experiments on FA test

At first, each antiserum was titrated with its homologous antigen. Table 8 indicates the results of the reciprocal indirect FA (IFA) test on 12 strains which showed cross

Table 10. Fluorescent antibody test of strains of primary culture from swine

No. of strains examined		Reaction with polyvalent sera*				No. of strains examined		Reaction with each serotype serum				
		A	B	C	D			AV 1	AV 2	AV 3	Davis	New type
19	10	—	—	—	###	19	14	—	—	—	###	—
	3	##	—	—	###		5	—	—	—	###	##
	6	—	—	—	##							
								IIIa	IIIb	Wilson	Yandle	
5	4	—	###	—	—	5		###	—	—	—	
	1	—	###	—	##							
7	1	—	—	##	##	7		Unclassified				
	6	—	—	—	—							

Remarks.

* Polyvalent sera are composed of the following serotypes.

A: *M. avium* 1, 2 and 3.

B: IIIa, IIIb, Wilson and Yandle.

C: VII, Chance and Howell.

D: Davis and New type.

reactions in the agglutination or CF test. The positive sign (+) means code 3 or 4 and the negative sign (−) code 2, 1 or 0.

From Table 8, it is clear that all the immune sera of each serotype reacted specifically with each corresponding serotype antigen. Then further experiments were carried out on the other serotype strains, several isolates from the lesion of swine mycobacteriosis, strains which could not be differentiated by the agglutination test with unabsorbed sera, and autoagglutinable strains. The results obtained are shown in Table 9.

So far as these experiments are concerned, the IFA test seems to be a very excellent method for the identification of the serotypes of the *M. avium-M. intracellulare* complex.

For the identification of the unknown strains, it is convenient to perform preliminary test with several kinds of polyvalent sera. After these tests, a positively reacted strain should be tested against individual antiserum which is contained in this polyvalent sera.

By the IFA test, 31 strains of the primary cultures originated from swine were examined. The results obtained are listed in Table 10. Twenty-four strains were clearly identified. The remaining 7 strains showed no strongly positive reactions against any polyvalent serum employed.

Discussion

Reznikov and Leggo [6] used a modification of Schaefer's agglutination test and indicated the patterns of cross reaction with the unabsorbed sera of the following groups: each serotype of *M. avium*, serotypes VII, Chance and Howell, and serotypes Yandle and Wilson. The same cross reaction was observed in the strains belonging to the same serotype.

The present authors performed the agglutination test on the 15 reference strains of the *M. avium-M. intracellulare* complex, except serotypes Yandle and Wilson. Comparison of the present results with those by the previous authors showed that the cross reactions between serotypes IIIa and IIIb were different from each other, while the other patterns of cross reaction mostly coincided with one another. This discrepancy might be due to the difference in the strains employed for the preparation of rabbit immune serum.

Schaefer [8] reported the CF test with the antigen of acetone-soluble substances on some strains of serotypes, and showed that the immune serum of *M. avium* 1 did not cross-reacted with the type Davis antigen, but that the serotype *M. avium* 2 antigen cross-reacted with *M. avium* 1 serum. The present authors also lend support to these findings. Research has been conducted very infrequently on the CF test for the identification of atypical mycobacteria. Then an experiment was carried out on the specificity of this test was studied in comparison with that of the agglutination test.

As a result, it became clear that cross reactions were demonstrated more frequently in the CF test than in the agglutination test against unabsorbed antisera. But with absorbed sera, however, each strain could readily be identified. Thus, the specificity of the CF test was distinctly demonstrated.

The original CF antigen was further purified with chloroform. It was ascertained that the substances from the chloroform layer contained more specific CF antigen. From studies by thin-layer chromatography, 4 components were differentiated. Of them, an anthron-positive component showed CF antigenicity. It seems probable that this antigenic substance may be common to both CF and agglutination reactions.

At the beginning of the experiment, the

antisera to be used for the IFA test were prepared by immunizing rabbits with phenolized organisms, but good results were not obtained. Thereafter, antisera from the early phase of infection were employed according to the recommendation made by Bennedsen [1, 2]. In the use of these antisera, the strains which had shown cross reactions with heterologous antisera in the agglutination and CF tests were clearly identified without difficulty even with unabsorbed sera. Very recently, the present authors noticed that Martins et al. [4] had reported the production of type-specific fluorescent antibody in rabbits injecting with cells killed by ultraviolet ray for the identification of mycobacteria. They have, however, not yet performed any experiment by this method.

How to fix the bacterial smear preparation is also one of the important factors. The authors tried various methods and found that the fixation in an oven at 80°C for 2 hours provided the best results.

There are some problems on the identification of atypical mycobacteria by the agglutination test. The procedure of the test is very timeconsuming. There are a few strains which will show autoagglutination.

The CF test is a useful method of identification. Moreover, it may be applied successfully to the identification of autoagglutinable strains, although its procedure is much complicated and it needs many organisms to prepare antigen.

On the contrary, one loopful suspension of mycobacteria is enough for the identification by FA test. It is possible to identify a single colony of the primary culture by this test. Such preliminary grouping with several polyvalent sera as described in Table 13, for example, may be convenient for simplifying the procedure to such extent as in the serological identification of Salmonella. Then, individual antiserum which is included in the corresponding group of polyvalent sera should be applied to the final determination of the serotype.

The authors are confident that the FA test must be widely accepted as most reliable, simple method for the identification of the *M. avium-M. intracellulare* complex.

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