JOURNAL OF CLINICAL MICROBIOLOGY, July 1991, p. 1372–1375
0095-1137/91/071372-04$02.00/0

Vol. 29, No. 7

Enzyme-Linked Immunosorbent Assay Using Monoclonal Antibodies for Identification of Mycobacteria from Early Cultures

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Received 13 November 1990/Accepted 9 April 1991

A simple enzyme-linked immunosorbent assay (ELISA) for the identification of cultured mycobacteria belonging to the *Mycobacterium tuberculosis* complex, the *Mycobacterium avium* complex, and *Mycobacterium kansasii* has been developed (R. Schöningh, C. P. H. J. Verstijnen, S. Kuijper, and A. H. J. Kolk. J. Clin. Microbiol. 28:708–713, 1990). The test for the routine identification of cultured mycobacteria was introduced in five clinical laboratories located in Tanzania, Thailand, Vietnam, and The Netherlands. The ELISA can be conducted without an ELISA reader since the test can be read visually. The results of identification of 255 strains of the *M. tuberculosis* complex by microbiological means and by ELISA were compared; the specificity and the sensitivity were 100%. For the *M. avium* complex, the specificity was 100% and the sensitivity was 64%. All 26 *M. kansasii* strains tested could be identified as *M. kansasii*. The ELISA described here proved to be useful in both well- and modestly equipped laboratories and may replace the microbiological method of identification of *M. tuberculosis* and *M. kansasii*.

Tuberculosis (TB) remains one of the major health problems in the world, with about 10 million new cases each year. Between 2 million and 3 million people die from TB each year. Over three-quarters of the TB cases occur in the tropics, where mortality is highest. The TB problem has been compounded by the development of the AIDS epidemic (5, 15).

The definitive diagnosis of TB depends on either direct observation of the causative agent by the Ziehl-Neelsen procedure or culture of the organism on a variety of different media.

In developing countries, the laboratory diagnosis of TB is mainly based on the presence of acid-fast bacilli in the sputum. This technique identifies pulmonary TB, which is important since patients with pulmonary TB infection may spread the disease. The diagnosis of sputum-negative patients (for instance, young children) and patients with nonpulmonary TB is more difficult. In these cases, culture of mycobacteria and identification are necessary for the definitive diagnosis.

Various methods for the identification of isolated mycobacteria have been used in the past decade (19). These conventional methods are time-consuming and laborious.

Radiometric methods with ^{14}C-labeled palmitic acid in a liquid medium have been developed (10, 14). Hybridization of ^{125}I-labeled DNA probes with rRNA have been used (3). More recently, gene amplification techniques by the polymerase chain reaction have been developed (4, 6–8). These three methods have the disadvantage that they use radioactively labeled substrates or probes and, in the case of gene amplification, are expensive and need well-trained staff.

Detection of mycobacterial antigens in sputum (22), in cerebrospinal fluid (21), and following culture (2) has been described. These tests lack specificity because polyclonal antibodies are used. We have developed a Western blot (immunoblot) and immunofluorescence test for the identification of mycobacteria with monoclonal antibodies (MAbs) (20).

There is clearly a need for an easy-to-use test which can be widely used even in laboratories with modest facilities. We have developed a simple enzyme-linked immunosorbent assay (ELISA) using MAbs for the identification of cultivated heat-killed mycobacteria (18). This test can shorten the time required for the identification of cultured mycobacteria.

In this report we describe the results obtained from five microbiological laboratories with this ELISA for the identification of mycobacteria which belong to the *M. tuberculosis* complex, the *M. avium* complex, and *M. kansasii* and which were derived from early cultures.

MATERIALS AND METHODS

MAbs used in the ELISA. The optimal dilution of the MAbs and antigen concentration needed were established by checkerboard titration (18). In Table 1 the optimal dilution of the MAbs is given for a concentration range from 2.5×10^6 to 2.5×10^7 bacteria per well. Two MAbs, F23-49 and F24-2, are specific for the *M. tuberculosis* complex; both reacted with a 16-kDa protein (12). MAbs F85-2 and F85-10, which are specific for the *M. avium-M. intracellulare-M. scrofulaceum* complex, reacted with an epitope on glycopeptidolipids on the surface of the cell wall (11). MAb F126-22 was selected for the identification of *M. kansasii* and is directed against an *M. kansasii*-specific glycolipid. MAb F141-3 reacts with a common mycobacterial epitope on a 30-kDa protein, and MAb F30-5 (12) is directed against lipoarabinomannan, which is present in high concentrations in the cell walls of all mycobacteria (9). These two MAbs can distinguish mycobacteria from other bacteria.

ELISA procedure. The ELISA was performed as de-

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TABLE 1. MAbs used in the ELISA

MAb	MAb directed against:	Antigen nature[a]	Reference	Dilution
F23-49	*M. tuberculosis*	16-kDa prot.	12	1:1,600
F24-2	*M. tuberculosis*	16 kD prot.	12	1:1,600
F85-2	*M. avium*	GPL	12	1:400
F85-10	*M. avium*	GPL	12	1:400
F126-22	*M. kansasii*	Glycolipid	20	1:400
F141-3	*M. avium*	30-kDa protein	18	1:1,000
F30-5	*M. leprae*	LAM	12	1:1,000

[a] GPL, glycopeptidolipid; LAM, lipoarabinomannan.

scribed by Schöningh et al. (18), with minor modifications. Mycobacteria were cultured on Löwenstein-Jensen (LJ) medium until enough bacteria (>6 × 10^7) could be harvested. A loop full of the cultivated strain was transferred to an Eppendorf biovial of 1.5 ml with 350 μl of phosphate-buffered saline (PBS). The vials were then heated in a water bath for 5 to 10 min at 80°C and then homogenized manually in the Eppendorf vial with a polypropylene pestle. The thickness of the suspensions was adjusted to a McFarland standard no. 3, or the A_{420} was adjusted to 0.300, which corresponds to 2 × 10^8 bacteria per ml. ELISA plates (Greiner, Nürtingen, Germany) were filled with 25 μl of suspension per well; tests were performed in duplicate.

Plates were dried in 10 min on a heating block (50 to 60°C) and with a hair dryer or were incubated overnight at 37°C without a cover in a dry incubator. Plates were blocked for 1 h at 37°C with 100 μl of 1% bovine serum albumin (BSA) per ml dissolved in PBS. The PBS-BSA was removed, and the plates were incubated for 30 min at 37°C with 50 μl of MAb solution diluted in 1% BSA in PBS.

The plates were washed three times with PBS and incubated for 30 min at 37°C with peroxidase-labeled sheep anti-mouse immunoglobulin heavy and light chains (Pasteur Institute, Paris, France) diluted in 1% BSA in PBS. After washing the plates three times, 100 μl of tetramethylbenzidine substrate solution was added to each well. The A_{405} was measured, or plates were scored visually. The results were compared with known strains of *M. tuberculosis*, *M. avium* serotype 4, and *M. kansasii*. An A_{405} of >0.8 was considered positive.

Laboratories involved in the study. The ELISA for the identification of cultured mycobacteria was performed at the following laboratories: (i) Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands (hereafter named Amsterdam); (ii) Muhimbili Medical Centre, Dar es Salaam, Tanzania (hereafter named Dar es Salaam); (iii) National Institute of Hygiene and Epidemiology, Hanoi, Vietnam (hereafter named Hanoi); (iv) Tuberculosis Division, Bangkok, Thailand (hereafter named Bangkok); and (v) Municipal Health Service Amsterdam, Amsterdam, The Netherlands (hereafter named Municipal Health Service).

Routine culture and identification. In Amsterdam, sputum samples obtained from Tanzania, Thailand, and South Korea were the source of mycobacteria. The sputum samples were kept at 4°C, transported by air, and processed within 1 week of production. The samples were decontaminated by the *N*-acetyl-L-cysteine–NaOH treatment method (13, 16) and were cultivated on LJ medium. Cultures were tested as soon as possible after a visible colony could be seen on LJ medium or if enough, >6 × 10^7, bacteria could be harvested. Strains were identified as belonging to the *M. tuberculosis* complex or mycobacteria other than *M. tuberculosis* (MOTT) strains by examining pigmentation, the niacin test result, the *para*-nitrobenzoic acid test result, and growth at 25°C. The same procedures for the identification of the isolates were used in Dar es Salaam, Bangkok, and Hanoi.

In Dar es Salaam, tests were performed on all mycobacterial strains isolated over a period of 4 months from patients presenting with pulmonary infection. Sputum samples were decontaminated by the addition of an equal volume of 4% NaOH and were cultured on LJ medium.

In Bangkok, sputum samples from patients with suspected pulmonary TB were decontaminated by oxalic acid treatment (1, 17) and cultured on LJ medium.

In Hanoi, mycobacterial strains were cultured from patients with TB who did not respond to first-line therapy. Sputum samples were decontaminated by the NaOH method and were cultured on LJ medium. The strains tested by ELISA were subcultured at least once.

The mycobacterial strains from patients attending the Municipal Health Service clinics were isolated by the Municipal Health Service laboratory, subcultured at least once, and then tested by ELISA. The strains were identified by the Reference Laboratory for Mycobacteria of the National Institute of Public Health and Environmental Protection (RIVM) in Bilthoven, The Netherlands, by conventional microbiological identification methods.

In all five laboratories, strains or isolates were tested by ELISA without knowledge of the results of the microbiological identification.

Other mycobacterial strains used. Twenty *M. kansasii* strains isolated from patients were kindly provided by M. Kubin, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia, and were tested at the Royal Tropical Institute in Amsterdam.

RESULTS

The results of comparisons between ELISA and routine microbiological identification obtained in Amsterdam, Dar es Salaam, Hanoi, and Bangkok are presented in Table 2. All cultures of *M. tuberculosis* were correctly identified as *M. tuberculosis* complex strains by ELISA. Table 3 shows the results from the laboratory of the Municipal Health Service. All *M. tuberculosis*, *M. bovis*, and *M. africanum* strains were identified by the ELISA as belonging to the *M. tuberculosis* complex. Of the MOTT strains, the six *M. kansasii* strains were correctly identified by ELISA. The *M. gordonae* strain identified by the ELISA as *M. kansasii* was mistyped.

The 20 *M. kansasii* strains isolated from patients from Czechoslovakia were all identified as such by the ELISA (data not shown).

The results of the ELISAs performed in the different laboratories are summarized in Table 4. One strain initially identified in Dar es Salaam as *M. tuberculosis* by culture and as a mycobacterium (not *M. tuberculosis*) by the ELISA was identified as *M. fortuitum* by the Reference Laboratory for Mycobacteria of the RIVM. By the ELISA, 255 *M. tuberculosis* cultures were identified, giving a sensitivity of 100% for the ELISA.

DISCUSSION

Once mycobacteria have been cultured for 2 to 3 weeks after isolation (when 6 × 10^7 bacteria can be harvested), they can be used in the ELISA described here. This is much

TABLE 2. Results of ELISA compared with those of the microbiological identification method for mycobacteria isolated from sputum and identified in Amsterdam, Dar es Salaam, Hanoi, and Bangkok

Location	ELISA identification		Microbiological identification	
	No. of strains	Organism	No. of strains	Organism
Amsterdam[a]	31	*M. tuberculosis* complex	31	*M. tuberculosis* complex
Dar es Salaam[b]	130	*M. tuberculosis* complex	130	*M. tuberculosis* complex[c]
	1[d]	*Mycobacterium* sp.	1[d]	*M. tuberculosis* complex
	2	*Mycobacterium* spp.	2	MOTT
	1	Not a *Mycobacterium* sp.	1	Not a mycobacterium
Hanoi[e]	53	*M. tuberculosis* complex	53	*M. tuberculosis* complex[c]
	5	MAIS complex[f]	5	MOTT
	2	*Mycobacterium* spp.	2	MOTT
Bangkok[g]	14	*M. tuberculosis* complex	14	*M. tuberculosis* complex[c]
	2	*M. kansasii*	2	MOTT
	2	MAIS complex	2	MOTT
	23	*Mycobacterium* spp.	23	MOTT

[a] Primary isolates; mean cultivation time, 25 days.
[b] Primary isolates; mean cultivation time, 3 weeks.
[c] Only *M. tuberculosis* strains were identified.
[d] One strain was initially identified as *M. tuberculosis* by culture and as a mycobacterium (not *M. tuberculosis*) by the ELISA; it was retested as *M. fortuitum* by the Reference Laboratory for Mycobacteria of the RIVM.
[e] Identification of mycobacteria.
[f] MAIS complex, *M. avium-M. intracellulare-M. scrofulaceum* complex.
[g] Primary isolates; mean cultivation time, 3 weeks.

shorter than the time needed for microbiological identification of mycobacteria. Compared with radiometric cultures and hybridization tests, the ELISA described here is cheap and easy to perform. The use of a polypropylene pestle and an Eppendorf vial instead of a sonicator for the homogenization of the mycobacteria is an advantage for modestly equipped laboratories. All five laboratories agreed that the test can be conducted without an ELISA reader, since the test result can be read visually.

All of the early cultures of *M. tuberculosis* were correctly identified by the ELISA as *M. tuberculosis* complex strains (Table 2). One culture was identified as *M. tuberculosis* in Dar Es Salaam, but it was identified as a mycobacterium by the ELISA, a finding confirmed by the reference laboratory in The Netherlands.

We (Amsterdam) found that some 2-week-old primary cultures showed no reaction with MAbs F24-2 and F23-49. When retested after a total of 4 weeks of cultivation, the cultures showed a positive reaction with these MAbs, which are directed against the 16-kDa protein. It seems that the expression of these epitopes on the surface of the mycobacteria is variable and depends on the strain and age of the culture. Experiments are in progress to increase the reactions with MAbs F24-2 and F23-49 in order to identify all cultures of *M. tuberculosis* as soon as a sufficient number of bacteria can be harvested.

Because the laboratories in Dar es Salaam, Hanoi, and Bangkok did not identify MOTT strains, we could not determine the sensitivity of the test for *M. avium*. From the known *M. avium* strains from the Municipal Health Service, 9 of 14 were identified by ELISA as belonging to the *M. avium-M. intracellulare-M. scrofulaceum* complex. We were not able to identify by the ELISA all the *M. avium* strains, even after repeated blind testing. The sensitivity of the ELISA for the identification of the *M. avium* complex

TABLE 3. Identification of early cultures of mycobacteria by ELISA compared with microbiological identification determined by the Municipal Health Service

ELISA identification		Microbiological identification	
No. of strains	Organism	No. of strains	Organism
23	*M. tuberculosis* complex	23	*M. tuberculosis* complex
1	*M. tuberculosis* complex	1	*M. africanum*
2	*M. tuberculosis* complex	2	*M. bovis*
1	*M. tuberculosis* complex	1	*M. bovis* BCG
9	*M. avium* complex	9	*M. avium* complex
1	*M. avium* complex	1	*M. chelonei*
6	*M. kansasii*	6	*M. kansasii*
1	*M. kansasii*	1	*M. gordonae*
5	*Mycobacterium* spp.	5	*M. avium* complex
12	*Mycobacterium* spp.	12	*M. fortuitum*
8	*Mycobacterium* spp.	8	*M. gordonae*
8	*Mycobacterium* spp.	8	*M. perigrinum*
9	*Mycobacterium* spp.	9	*M. terrae*
1	*Mycobacterium* sp.	1	*M. chelonei*

TABLE 4. Summary of the results of the ELISA and microbiological identification obtained from the five different laboratories

Organism	No. of strains identified by:	
	ELISA	Culture
M. tuberculosis	255[a]	256
MOTT	96	96
Not mycobacteria	1	1

[a] One strain initially identified as *M. tuberculosis* by culture and as mycobacterium (not *M. tuberculosis*) in the ELISA was retested as *M. fortuitum* by the Reference Laboratory for Mycobacteria of the RIVM.

was 64%. Possible explanations for this finding are that the glycopeptidolipid identified by these MAbs is either absent or produced in low quantities or that the epitopes are not exposed on the cell wall surface as we have described previously (11, 18).

All of the early cultures of MOTT strains could be identified by ELISA as belonging to *Mycobacterium* species (Table 2). Some of the MOTT strains tested by the Municipal Health Service were only weakly positive with MAb F141-3, but they were strongly positive with MAb F30-5. An explanation could be that these strains express only small amounts of the epitope recognized by MAb F141-3 on the surface of the cell wall. Lipoarabinomannan proved to be better expressed on the surface of mycobacteria, since the number of mycobacteria needed for a positive ELISA with MAb F30-5 was 10 times lower than that required for MAb F141-3 (data not shown). The MAb against lipoarbinomannan, MAb F30-5, was not available at the start of the study and was used only in Amsterdam. For future study, we recommend the use of MAb F30-5 instead of MAb F141-3 for the identification of mycobacteria.

If the results of the identification of the 256 strains of the *M. tuberculosis* complex by microbiological means and by ELISA are compared, the specificity and the sensitivity are 100%. For the *M. avium* complex, the specificity was 100% and the sensitivity was 64%. The 26 *M. kansasii* strains tested could all be identified as *M. kansasii*.

The ELISA described here has proved to be useful in modestly equipped laboratories, since only microtiter plates and reagents were required for this simple test.

ACKNOWLEDGMENTS

We thank D. G. Groothuis from the National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands, for the typing of the mycobacterial strains and Min Ja Kim from the College of Medicine, Korea University, Seoul, Korea, for the sputum specimens.

This study was supported by a grant from DiaTech, under cooperative agreement DPE-5935-A-00-5065-00 between the U.S. Agency for International Development and the Program for Appropriate Technology in Health, subagreement 860700087; by the Commission of the European Communities Directorate General for Science Research and Development, project TS2-0111; and by the UNDP/World Bank/WHO special program for Research and Training in Tropical Diseases.

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