Intrinsic Catalase Dot Blot Immunoassay for Identification of *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare*

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The heat-labile T class of mycobacterial catalase exhibits peroxidase activity with some substrates. Most species of mycobacteria produce T-catalase, which is serologically characterized by a combination of shared epitopes and unique, species-specific epitopes. Antibodies to T-catalases from *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare* have been cross absorbed with T-catalases from heterologous species and applied as dots to nitrocellulose membranes. When these membranes were incubated with crude sonic extracts of 93 strains of mycobacteria that produce sufficient T-catalase, and were then exposed to 3,3′-diaminobenzidine peroxidase substrate, only those extracts made from one of the three species represented yielded a discrete brown dot at the site of the corresponding globulin. The sensitivity of the test was at least 96.5%, and the specificity was in excess of 99.5%.

Solid-phase immunoassay techniques are rapidly becoming the methods of choice for recognizing a wide range of biological products because of their sensitivity and simplicity of performance. The idea of an intrinsic-enzyme-based solid-phase immunoassay for purposes of bacterial identification is especially attractive because it would eliminate the need for second antibody which is required for many solid-phase immunoassays, relying instead on the serologic binding of an indicator enzyme produced by the organism itself.

An optimal system for identifying members of a given genus would require that the desired enzyme retain its activity after reaction with antibody and that the desired enzyme be present in most members of the genus, but that antigenic regions (i.e., epitopes) occur on the enzyme molecule that are unique to the members of each species within that genus. In the genus *Mycobacterium*, the catalase system meets these criteria. Two classes of catalase have been recognized among the mycobacteria. M-catalase retains its activity after 1 min of exposure to 68°C, but is inactivated by aminotriazole; T-catalase is inactivated by heat, but resists aminotriazole (10). Both classes retain their activity after precipitation by specific antibody, and many species of mycobacteria produce both catalases. Seroprecipitation methods have been used to estimate evolutionary structural divergence of these catalases for taxonomic purposes (5, 8–10). Cross absorption of antisera to both M- and T-catalases have been effective in amplifying the serologic distinctions between these enzymes from different mycobacterial species (11, 12). Recent observations on the utility of a 3,3′-diaminobenzidine stain for visualizing mycobacterial T-catalases in polyacrylamide gels, by virtue of the peroxidase-like activity of this class of catalase (13), suggested that a simplified technique could be developed for detecting serologic binding of this enzyme by cross-absorbed antibody. Use of T-catalase was especially attractive because it is produced by virtually all pathogenic, slowly growing mycobacteria (10). We elected to develop a dot-blot method, since the large surface area afforded by the porous structure of nitrocellulose membranes permits adsorption of relatively large amounts of crude immunoglobulin, thus eliminating the need for the immunoaffinity purification of the antibody that was required in the previously described enzyme-linked immunoassay (ELISA) method (12).

The present study was restricted to identification of members of the *Mycobacterium tuberculosis* complex (*M. tuberculosis* and *M. bovis*) and of *Mycobacterium avium* and *Mycobacterium intracellulare*, since these species, in the aggregate, account for over 86% of the pathogenic, slowly growing mycobacteria encountered by state health laboratories in the United States (3).

**MATERIALS AND METHODS**

**Bacterial strains.** Reference strains of 21 serovars of *M. avium* and *M. intracellulare*, as well as a number of strains from the Trudeau Mycobacterial Collection (TMC designations), were provided by Anna Tsang, National Jewish Hospital and Research Center, Denver, Colo., under a contract from the United States-Japan Cooperative Medical Sciences Program. In keeping with current knowledge of DNA homologies (2) and their high correlation with catalase serology (12), serovars 1, 2, 3, 4, 5, 6, 8, 9, and 10 are considered *M. avium* and serovars 7 and 12 through 25 are *M. intracellulare*. Clinical isolates of *M. tuberculosis* and *Mycobacterium xenopi* were provided by Wendy M. Gross, VA Medical Center, West Haven, Conn. The other cultures used in this study were from the collection of this laboratory; their identities were confirmed by use of a diagnostic probability matrix as described previously (16).

**Preparation and standardization of catalase.** The large batches of partially purified T-catalases that were required for immunization of rabbits and cross absorption of antibody were prepared from *M. tuberculosis* H37Rv, *M. avium* SJB 2 (serovar 8), and *M. intracellulare* Boone (serovar 14) by methods described previously (10). They were confirmed to be free of M-catalase by the zero-order kinetic assay in the presence of 3-amino-1,2,4-triazole (10).

For preparing the smaller samples of crude catalase...
needed for serologic testing, the test strains were grown in single tubes containing 10 ml of a medium prepared from Dubos Broth Base (Difco) enriched with Dubos Oleic Albumin Complex (Difco) and 1% (wt/vol) glycerol. The cells were harvested, washed, and sonicated in the original screw-capped culture tube, using the cup-horn adapter to a model 185D sonifier (Heat Systems-Ultrasonics, Inc.) as described previously (11), and the activity of the extracts was determined by assaying for T-catalase in the presence of amnotiazole.

Preparation and cross absorption of antibody. New Zealand rabbits were immunized with partially purified T-catalases isolated from \textit{M. avium} SJB-2, \textit{M. intracellulare} Boone, and \textit{M. tuberculosis} H$_3$Rv, and the sera were titrated against homologous antigen by the seroprecipitation supernatant assay as described previously (10). The sera were treated with 50% saturated ammonium sulfate to precipitate the immunoglobulins, which were recovered by centrifugation and dissolved in a small volume of 0.01 M phosphate-buffered saline (pH 7.5) preserved with 0.1 mg of thimerosal per ml (PBSM); residual ammonium sulfate was removed by passage over Sephadex G-25 (Pharmacia) equilibrated with PBSM. Based on the homologous antigen titers of the globulin preparations and previously reported immunologic distances of T-catalases from heterologous species (10), a portion of each globulin solution was cross absorbed with a twofold excess of PBSM solution of T-catalase from a species with the lowest immunologic distance score to the homologous catalase (i.e., antibodies to T-catalases from \textit{M. tuberculosis} H$_3$Rv, \textit{M. avium} SJB-2, and \textit{M. intracellulare} Boone were cross absorbed by T-catalases from \textit{M. avium} SJB-2, \textit{M. intracellulare} Boone, and \textit{M. avium} SJB-2, respectively). After overnight incubation, the preparations were centrifuged, and excess, unpurified catalase was removed from the supernatant fluids by absorption onto DEAE-Sephadex (Pharmacia) as described previously (11, 12).

Dot-blot serologic assay. The desired dilutions of cross-absorbed and unabsorbed immunoglobulins were made in PBSM. Samples of 1 µl were spotted to nitrocellulose membranes (BioRad) and allowed to dry at room temperature. The sheets were soaked in 0.05 M bicarbonate buffer (pH 9.4) for 18 h at 3°C. An equal volume of a solution of 2 mg of bovine albumin fraction V per ml in 0.1 M phosphate buffer (pH 7.5), preserved with 0.1 mg of thimerosal per ml, was then added to the bicarbonate buffer and allowed to block the membrane at 37°C for 60 min. The blocking fluid was aspirated, the membrane was washed once with PBSM, and strips of membrane containing the desired immunoglobulin dots were cut and transferred to individual test tubes for reaction with selected catalase solutions.

In a typical assay, a nitrocellulose strip (6 by 45 mm), bearing four immunoglobulin spots and a pencil mark for orientation, was soaked for 2 h at 37°C in 2 ml of PBSM containing the T-catalase to be tested in a screw-cap tube (13 by 100 mm), lying flat. After incubation and aspiration of unreacted catalase, the strips were washed twice with PBSM. The membrane was then transferred to a clean tube and flooded with 2 ml of a freshly prepared solution containing 500 µg of 3,3'-diaminobenzidine (Sigma) and 30 µg of H$_2$O$_2$ per ml of 0.01 M phosphate buffer in saline (pH 7.2) (7). After 30 min of incubation at room temperature, the diaminobenzidine solution was aspirated and the strip was soaked in H$_2$O for 10 min and placed on blotting paper to dry. The appearance of a brown spot at the site of a globulin dot indicated specific binding of the T-catalase.

RESULTS

Each of the test immunoglobulin products was evaluated, before and after cross absorption, by spotting 1-µl dots of a twofold dilution series corresponding to a range of undiluted through 1:32 dilution equivalents of the original sera from which the globulins were derived. Each product was tested against homologous antigen and the heterologous T-catalase used to cross absorb it. Cross absorption diminished the homologous reaction to varying degrees, depending on the immunologic relatedness of the catalase, and effectively eliminated the heterologous reactions (Fig. 1).

A survey of crude mycobacterial sonic extracts was performed, using nitrocellulose strips primed with a 1-µl “monitor” dot and 1-µl dots of indicated dilutions of cross-absorbed immunoglobulin G to T-catalase from \textit{M. tuberculosis} H$_3$Rv (1:10), \textit{M. avium} SJB-2 (1:16), and \textit{M. intracellularare} TMC 1403 (1:4). The monitor dot was made with a mixture of unabsorbed immunoglobulins against T-catalase from \textit{M. tuberculosis} (1:32), \textit{M. avium} (1:16), and \textit{Mycobacterium kansasii} (TMC 1201) (1:32) and was used as a positive, nonspecific control to confirm the presence of an adequate amount of mycobacterial T-catalase. A positive reaction was demonstrated by the appearance of a discrete solid brown dot at the site of a reference globulin, which persisted after the test strip had completely dried. After a preliminary series had been run, it became evident that a challenge load of at least 0.3 U of T-catalase was required for a test strip to yield consistent results. This amount is usually available in the sonic extract of a 10-ml liquid culture that had reached an $A_{590}$ in excess of 0.50 at harvest (12).

The results of testing extracts of 93 strains, representing 11 species or complexes of slow-growing mycobacteria, are recorded in Table 1. Of 5 strains of \textit{M. bovis} and 15 of \textit{M. tuberculosis}, all reacted with the monitor dot and with the anti-\textit{M. tuberculosis} dot, but with no other immunoglobulin. Sixteen strains corresponding to serovars that represent \textit{M.}}
avium (12) were tested; all gave a positive monitor reaction and reacted with the anti-M. avium immunoglobulin dot. Twenty-one strains that corresponded to serovars that represent M. intracellulare gave positive monitor reactions, but only 19 reacted with the anti-M. intracellulare dot. One of the samples that failed to react was derived from a serovar 7 strain (P-49), and the other was from a serovar 18 strain (Melnick); both strains produced more M-catalase than T-catalase and were urease positive. The M-catalase from both failed to react with specific antibody to M-catalase from Mycobacterium scrofulaceum, M. simiae, M. gordoniae, M. szulgai, or M. asiaticum by the seroprecipitation method described previously (10).

Extracts of 36 strains, representing Mycobacterium gastri, M. kansasi, M. malmoense, M. scrofulaceum, M. simiae, M. szulgai, and M. xenopi exhibited positive reactions for T-catalase on the monitor dot, but not on any of the three species-specific dots. Extracts of four strains of M. gordoniae, two of M. terrae, two of M. nonchroemogenicus, and three of M. triviale failed to react with the T-catalase monitor dot and are not included in Table 1. The latter three species comprise the “terrace” complex and have previously been reported to produce only M-catalase (10). While M. gordoniae does produce traces of T-catalase, the M-catalase is present in great excess (10), and extracts prepared as described for this test did not yield sufficient T-catalase to provide a satisfactory control dot response.

**DISCUSSION**

Cross-absorbed sera to M-catalases were used in our earlier seroprecipitation method to permit precise and sensitive identification of five selected species (11). Although useful at a research level, the centrifugations, washing steps, and precise assays required for that study placed some limit on its practical utility in the routine diagnostic laboratory. Cross-absorbed sera to T-catalases were used in an ELISA procedure for distinguishing between extracts of M. avium and of M. intracellulare (12). Among the practical disadvantages of this method were the need for quantitative assay and for very high-titered antibody, because of the limited protein-binding capacity of the surface of polystyrene micro-wells. The intrinsic T-catalase dot immunoassay described here has several practical advantages over the earlier methods. T-catalase is a better enzyme for identification purposes than is M-catalase, since it is found in all pathogenic species of cultivable, slowly growing mycobacteria (10), with the occasional exception among some strains of M. tuberculosis that have mutated to high isoniazid resistance (6). The seroprecipitation assay for M-catalase and the ELISA for T-catalase both depend on quantitative measurement of destruction of H2O2 substrate by the enzyme-antibody complex and thus require precise standardization and volumetric measurement of reactants. The dot-blot method depends on the positive color reaction associated with the peroxidase function of the enzyme and is essentially qualitative and thus more forgiving of minor quantitative variations in reagents. The use of a small antibody dot in a larger membrane field reduces the uncertainties in interpreting the contribution of nonspecific background reactions that are inherent in ELISAs; the clear zone around the dot is the control, and the contrast between dot and background is readily observed. The porous structure of the membrane permits immobilization of a much larger amount of immunoglobulin in a small zone than does the solid polystyrene surface of an ELISA well, eliminating a need for immunofluorescence purification of antibody. Finally, the use of multiple dots in a single membrane strip permits the test to be formed as a unit, instead of as a series of subtests as in seroprecipitation or ELISA wells.

The data in Table 1 represent exposure of three species-specific antibody dots to each of 93 mycobacterial extracts. Of the 57 antibody dots exposed to an extract of homologous species, 55 gave positive reactions, for a sensitivity of 96.5%. This figure may be too conservative, since the two strains (P-49 and Melnick) representing serovars 7 and 18, respectively, which are nominally M. intracellulare serovars but failed to react with the corresponding anti-T-catalase dot, gave reactions in tests for urease and M-catalase that would be sufficient to cause their rejection in a phentic taxonomic probability matrix (15). Serovars can cross species lines; an example is the demonstration of serovar 18 reactivity in many strains of M. simiae (14). In the present case, neither of the M-catalases from the two peculiar strains reacted with antibody to M-catalase from M. simiae or four other species, so their identities remain unresolved.

Of the 222 antibody dots exposed to extracts of heterologous species, none gave a positive reaction, for a specificity score greater than 99.5%. It must be pointed out that the reaction of T-catalase from nominal M. bovis with the antibody to T-catalase from M. tuberculosis is consistent with current recognition of the synonymy of these two species, as reflected in a high order of DNA-DNA homology between them (1, 4).

The three species represented in the test as evaluated here account for over 86% of the pathogenic or potentially pathogenic mycobacteria reported by state laboratories. M. kansasi, M. scrofulaceum, and members of the M. fortuitum complex in the aggregate account for another 12.3% of the opportunistic pathogens, and the remaining 1% are distributed among at least six other species (3). Most of these species have been shown to produce T-catalases that exhibit some degree of evolutionary divergence from one another (10), so it seems likely that a dot-blot system can be developed that might permit identification of almost all clinically significant cultivable mycobacteria.

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| Table 1. Responses of extracts of strains of mycobacterial species in dot-blot immunoassay on cross-absorbed antibody to selected T-catalases |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Extract          | M. tuberculosis | M. avium | M. intracellulare |
| M. avium       | 0               | 16          | 0               | 0               |
| M. bovis       | 5               | 0           | 0               | 0               |
| M. gastri      | 0               | 0           | 1               | 0               |
| M. intracellulare | 0            | 0           | 19              | 2               |
| M. kansasi     | 0               | 0           | 0               | 17              |
| M. malmoense   | 0               | 0           | 0               | 2               |
| M. scrofulaceum| 0               | 0           | 0               | 6               |
| M. simiae      | 0               | 0           | 0               | 3               |
| M. szulgai     | 0               | 0           | 0               | 3               |
| M. tuberculosis| 15              | 0           | 0               | 4               |
| M. xenopi      | 0               | 0           | 0               | 4               |
LITERATURE CITED


