Seroagglutination Test for Identification of *Mycobacterium paratuberculosis*

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Received for publication 2 May 1975

Twenty-nine of 30 strains of *Mycobacterium paratuberculosis* isolated from clinical specimens were serologically identified. No cross-reactions were observed with *Mycobacterium avium* serotypes 1, 2, 4, and 8.

Precise laboratory identification of *Mycobacterium paratuberculosis* is characterized by slow growth and mycobactin dependence. The latter feature may be lost on repeated subculture; consequently, there is need for other definitive tests. The purpose of this investigation was twofold: (i) to determine the specificity of a seroagglutination procedure in identifying *M. paratuberculosis* and (ii) to compare the validity and efficiency of a new microagglutination procedure with the routine tube test of Schaefer (4). A micromodification of this technique was recently described for identifying *Mycobacterium avium* (5).

Thirty isolates of *M. paratuberculosis* were investigated. Because *M. avium* (*M. avium*-*M. intracellulare*) (2) serotypes 1, 2, 4, and 8 constitute 90% of the *M. avium* strains received at this laboratory, 15 strains each of *M. avium* serotypes 1 and 2 and 13 strains each of *M. avium* serotypes 4 and 8 were included in the study. The cultures of *M. paratuberculosis* and *M. avium* isolates were from routine diagnostic specimens. The *M. paratuberculosis* isolates were identified by slow growth and by their mycobactin dependency (3) whereas the *M. avium* isolations were identified as described previously (6).

All cultures were grown for 6 weeks at 37 C on Herrold egg yolk medium containing mycobactin. Antigens were prepared for both the tube and microagglutination tests by suspending two 3-mm loopfuls of growth in 2.0 ml of sterile Middlebrook 7H9 broth (7H9 broth base [Difco Laboratories, Detroit, Mich.], 4.7 g; distilled water, 900 ml. Dispense in 100-ml amounts and autoclave 15 lbs. for 15 min; no enrichments are added). The antigens were heat killed by immersion in a 76 C water bath for 1 h. The tubes were allowed to cool, and 5.0 ml of sterile Middlebrook 7H9 broth was added. The tubes were kept at 20 C for 18 h. The turbid supernatant fluid was removed with a Pasteur pipette and adjusted with 7H9 broth to 0.50

optical density at 525 nm using a Bausch & Lomb Spectronic 20 spectrophotometer. All antigens were coded.

The antisera used in the tube and microagglutination tests were produced in rabbits according to the method of Schaefer (4) from two known strains of *M. paratuberculosis* isolated from cattle and from each of two strains of *M. avium* serotypes 1, 2, 4, and 8.

The standard tube test was performed in tubes (11 by 100 mm) as described by Schaefer (4). For the micromethod, equal amounts (0.08 ml) of antigen and antiserum were added to wells (6 mm in diameter) of a flat-bottom well plate (IS-FB-96 TC Dispo Plate, Linbro Chemical Co., New Haven, Conn.). A control was prepared by adding 0.08 ml of antigen and 0.08 ml of Middlebrook 7H9 broth to a well. Optimal concentrations of antiserum were determined by each of the procedures using homologous strains with serum titers ranging from 1:20 to 1:160 (5). The optimal dilution of *M. paratuberculosis* antisera used in the tests was 1:160.

The microagglutination plates were agitated for 45 s on a Thomas-Boerner shaking apparatus, covered with a plastic lid (model 53, Linbro Chemical Co.), and placed in a moist chamber at 37 C; readings were made after incubating for 3 and 6 h. The presence of agglutinated cells was recorded on a scale of 1+ to 4+ as determined by the degree of clumping. These results indicate that the percentage of agreement between the two tests is at least 96.5% (Table 1). Antigens prepared from 29 of 30 strains of *M. paratuberculosis* were agglutinated by both of the *M. paratuberculosis* antisera, using the tube and microagglutination tests. No cross-reactions were observed on either test when the antigens prepared from *M. paratuberculosis* isolates were tested against *M. avium* antisera. Antigen prepared from one strain of *M. paratuberculosis* was not agglutinated by any of the test antisera. No agglutination was observed in the control test.

TABLE 1. Results of tube and microagglutination tests on 30 strains of *M. paratuberculosis* and 56 strains of *M. avium*^a

Antigen	No. of isolates tested	Test ^b	Antiserum ^c				
			<i>M. avium</i> serotypes				<i>M. paratuberculosis</i>
			1	2	4	8	
<i>M. paratuberculosis</i>	30	T	0	0	0	0	29
	30	M	0	0	0	0	29
<i>M. avium</i> serotype 1	15	T	15	0	0	0	0
	15	M	15	0	0	0	0
<i>M. avium</i> serotype 2	15	T	0	15	0	0	0
	15	M	0	15	0	0	0
<i>M. avium</i> serotype 4	13	T	0	0	13	0	0
	13	M	0	0	13	0	0
<i>M. avium</i> serotype 8	13	T	0	0	0	13	0
	13	M	0	0	0	13	0

^a The lower limit of confidence according to statistical analysis by binomial theory is 96.5%.

^b T, Tube test; M, microagglutination.

^c Number of strains showing reaction.

Johne's disease appears to be widespread in cattle in the United States (1). Serological identification of *M. paratuberculosis* is advantageous since it makes possible the detection of various serotypes within this species. The identification of serotypes of *M. paratuberculosis* would be of value in tracing infection sources.

Serological tests for the diagnosis of Johne's disease have been known and used for many years, but no reports are available on the serological identification of *M. paratuberculosis*. This may be due in part to the difficulty in the past of culturing the organism and the tendency of some strains to produce unstable cell suspensions which autoagglutinate. Serological identification will provide an additional laboratory tool in combination with growth rate and mycobactin dependency in confirming an isolate as *M. paratuberculosis*.

Since *M. avium* serotypes 1, 2, 4, and 8 are so commonly found in specimens of veterinary interest, only antisera against these organisms were included in this study. Additional investigations should be made using antisera of *M.*

avium serotypes other than 1, 2, 4, and 8.

We thank George P. Kubica and W. B. Schaefer for their very helpful suggestions in reviewing the manuscript.

LITERATURE CITED

1. Kopecky, K. E. 1973. Distribution of bovine paratuberculosis in the U.S. J. Am. Vet. Med. Assoc. 162:787-788.
2. Meissner, G., K. H. Schroder, G. E. Amadio, W. Anz, S. Chaparas, H. W. B. Engel, P. A. Jenkins, W. Kappler, H. H. Kleeberg, E. Kubala, M. Kubin, D. Lauterbach, A. Lind, M. Magnusson, ZD. Mikova, S. R. Pattyn, W. B. Schaefer, J. L. Stanford, M. Tsukamura, L. G. Wayne, I. Willers, E. Wolinsky. 1974. A co-operative numerical analysis of nonscoto- and non-photochromogenic slowly growing mycobacteria. J. Gen. Microbiol. 83:207-235.
3. Merkai, R. S. 1970. Diagnostic methods for detection of paratuberculosis (Johne's disease), p. 620. In Proc. 74th Annu. Meet. U.S. Anim. Health Assoc.
4. Schaefer, W. B. 1965. Serologic identification and classification of the atypical mycobacteria by their agglutination. Am. Rev. Resp. Dis. 92:85-93.
5. Thoen, C. O., J. L. Jarnagin, and M. L. Champion. 1975. Micromethod for serotyping strains of *Mycobacterium avium*. J. Clin. Microbiol. 1:469-471.
6. U.S. Department of Agriculture. 1974. Laboratory methods in veterinary mycobacteriology, revised ed. U.S. Department of Agriculture, Ames, Iowa.