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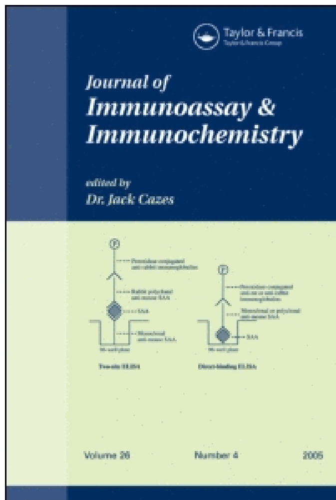

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A Rapid Latex Agglutination Test for Detection of Antibodies in Tuberculosis and Hansen's Disease

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A RAPID LATEX AGGLUTINATION TEST FOR DETECTION OF ANTIBODIES IN TUBERCULOSIS AND HANSEN'S DISEASE

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ABSTRACT

Antigens of Mycobacterium w, a saprophytic fast growing organism having antigenic epitopes cross-reactive with Mycobacterium leprae and Mycobacterium tuberculosis, were coated on to latex beads (o.33 µm in size), and the reactivity tested with sera of tuberculosis and Hansen's disease (HD) patients. Seventy nine percent of lepromatous leprosy (LL) and eighty five percent of pulmonary tuberculosis (TB) patients sera showed an agglutination rection easily read by naked eye. Specificity of the test was further checked by testing sera of non-mycobacterial infection cases and all of them were found negative. Among apparently healthy controls, 4.3% were found positive from non-endemic and 8.8% from endemic area. The sensitivity of the assay is further enhanced from 78.7% to 90.4% and 85.7% to 91.6% in both LL HD and pulmonary tuberculosis respectively, by using immune complexes extracted from the patients sera. Potential of these antigen coated beads to detect the two major human mycobacterial diseases,LL HD and pulmonary TB was also put evidence in a double blind study on coded sera samples obtained from various hospitals in India. The antigen coated beads are stable for upto 6 months at 4°C. The latex slide agglutination test reported here, is simple, rapid, easy to perform and can be used even in rural areas of developing countries.

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Key Words : Hansen's disease, Tuberculosis, Latex Agglutination, Mycobacterium w, Antibodies

INTRODUCTION

Mycobacteria, both pathogenic and non-pathogenic are widespread in the soil and environment. Two of the well known pathogens, Mycobacterium leprae (M. leprae) and Mycobacterium tuberculosis (M. tuberculosis) are of major interest as these are the causative agents of Hansen's disease (HD) and tuberculosis. HD, a chronic infectious disease of man is one of the six major diseases identified as priority global health problems by the World Health Organization. It affects 10-12 million people in the world (1). The prevalence rate in India is about one third of the HD cases in the world. Total number of HD patients in the country estimated in 1981 was around four million (1). Tuberculosis is a major killer disease in the developing countries. It affects 30-40 million people every year (2), with an estimated 10 million new cases developing annually, resulting in three million deaths per year. In India alone, there are 12 million people suffering from tuberculosis at any given time, two and a half million new cases add up per year.

Humoral immune response during the mycobacterial infection has been studied extensively with the purpose of developing serological tests (3). Several tests have been reported for the diagnosis of HD and tuberculosis (4,5,6,7,8,9). These have varying degrees of sensitivity, specificity and ease of performance. The sensitivity ranges between 31 to 80% (10,11,12). But none of these tests is simple enough to be used in rural field areas. We describe in this report a simple and rapid

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agglutination test for detection of both tuberculosis and LL HD. The test utilises soluble antigens extracted from a cultivable, new species of non-pathogenic mycobacteria, Mycobacterium w (M. w),which shares antigenic determinants with M. leprae and/or M. tuberculosis (13).

MATERIALS AND METHODS

Antigen preparation

Mycobacterium w (M. w) was identified from amongst several coded strains for its desirable properties of immunological reactivity with cells of HD patients (14). It is a fast grower, its metabolic and growth properties have been described earlier (15,16). The organism was grown in Middlebrooke broth (7H9) (Difco Laboratories, Detroit, Michigan, USA), at 37°C for 10 days. Bacteria were harvested by centrifugation at 5000 g for 20 minutes at 4°C in a Sorvall centrifuge (RC5C, Du Pont, USA). The pellet was washed thrice with double distilled water and finally suspended in 0.01 M phosphate buffer saline (PBS), pH 7.2 at a concentration of $1x10^9$ bacilli/ml. Phenylmethylsulfonylfluoride (PMSF) (Sigma Chem. Co. MO. USA) and ethylenediaminetetraacetic acid (EDTA) (Glaxo Laboratories, Bombay, India) were added as the proteolytic enzyme inhibitor and chelating agent respectively, to the bacterial suspension. The bacilli were sonicated in an ultrasonic disintegrator (MSE Co., U.K.). The sonicate was centrifuged at 20,000 g for 60 minutes at 4°C. The supernatant was dialyzed against 0.01 M phosphate buffer pH 7.2 for 24 hours at 4°C with constant stirring and then lyophilized to minimum volume. The sonicate was stored in aliquotes at -20°C. The protein content was estimated by Lowry's method (17).

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Sensitization of beads

The polystyrene beads 0.33 μm size (Sigma Chem. Co., Mo., USA) were coated with M. w supernates (312 ng protein/ml) at room temperature overnight.

Agglutination procedure

One drop (30 μl) of antigen coated beads was put on a glass plate and to this, equal volume of diluted test serum was added. This was mixed thoroughly with a plastic stick and the plate was gently rocked thereafter. Agglutination in 2-10 minutes was an indication of positivity. The agglutination of the test sample was compared with that of standard negative (normal human serum) and a positive LL (LL HD patient pooled serum) sera. The degree of agglutination was rated according to the following criteria : 4+ = Large scale agglutination with clear surrounding fluid (100%); 3+ = Medium sized clumps in a slightely turbid fluid (75%); 2+ = Small clumps in a turbid liquid (50%); 1+ = Very small clumps and fluid (25%); ± = Trace; - = No agglutination. ELISA procedure followed was essentially the same as described earlier by Moudgil et al (18).

Immune complex extraction

Immune complexes were extracted from patient sera by polyethylene glycol (PEG) method (19). PEG was adjusted to a working concentration by mixing 6 ml of 20% PEG with 3 ml 0.2 M EDTA and 1 ml veronal buffered saline. 30 μl of working solution of PEG was added to 150 μl of each test serum (in duplicates). The mixture was incubated overnight at 4°C, followed by centrifugation at 2000 rpm for 20 minutes at 4°C. The tubes were placed in ice and the supernatant was discarded. The precipitates were resuspended in 2 ml of 2% PEG in 0.01 M EDTA in veronal buffered

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saline. The mixture was again centrifuged at 2000 rpm for 20 minutes at 4°C. The supernatant was removed and the volume was made upto 150 μl. Incubation was done at 37 C for 1 hour to redissolve the precipitates.

Bacteriological Status

The bacteriological status of HD patients was established by measurement of Bacterial Index (BI). Slit skin smears were taken from six sites, the right and left ear lobes, the right and left eye brows and two representative skin patches. The smears were stained by the Ziehl- Neelsen method and average bacterial density calculated according to the Ridley's logarithmic scale (20).

Sera samples

Sera of clinically and bacteriologically confirmed HD patients were collected from Safdarjung and Ram Manohar Lohia hospitals, New Delhi and from Central Leprosy Training Research Institute, Chingalpattu, Madras. The patients were clinically and histopathologically classified according to the Ridley and Jopling scale (20). Sera from clinically active patients of pulmonary tuberculosis admitted in wards of Mehrauli Tuberculosis Hospital, New Delhi, and District Tuberculosis Hospital, Hisar were also tested by this assay. Diagnosis of tuberculosis was based on clinical, radiological and bacteriological criteria. All the patients included in this study suffered from pulmonary tuberculosis as confirmed by the radiological and bacteriological findings. Their PPD reports were not available. Serum samples from individuals who belonged to high endemic areas were obtained from Chingalpattu, Madras. Normal sera were collected from New Delhi, a low endemic area of HD.

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RESULTS

Initial investigations showed that the sera of HD patients reacted with sonicate of M. w (13,18). An ELISA was employed to determine whether M. w sonicate used as antigen was bound by antibodies present in tuberculosis and HD patients and whether such ELISA could provide high enough sensitivity and specificity. Sera from 120 HD patients belonging to different categories of clinical spectrum of HD were examined. In view of the fact that M. w has the advantage of sharing antigens with M. tuberculosis, sera from 40 confirmed cases of pulmonary tuberculosis were also tested. As controls, sera from 10 patients of amoebiasis, 10 patients of typhoid and 35 apparently healthy controls and 5 controls from an endemic area of HD Madras were also taken. The antibody response to M. w antigens and the percent positivity was derived on the basis of cut off limits set at mean ± 2 S.D. value of control healthy subject samples (Fig. 1). Most of the LL HD patients gave positive reaction. The range of positivity in other forms of HD is given in Table 2. A large percentage of tuberculosis patients (87.5%) also gave a positive reaction with M. w sonicate. On the other hand, antibodies reacting with M. w were not present in sera of typhoid and amoebiasis patients and in controls (Fig.1).

Results of the plate ELISA in the present study showed that the antigen in use was quite sensitive to pick up the multibacillary HD and active pulmonary tuberculosis cases (Table 1). In the next set of experiments, an attempt was made to simplify the test from ELISA to agglutination test modality. M. w antigens were bound to polystyrene beads. Pooled sera samples from HD and tuberculosis

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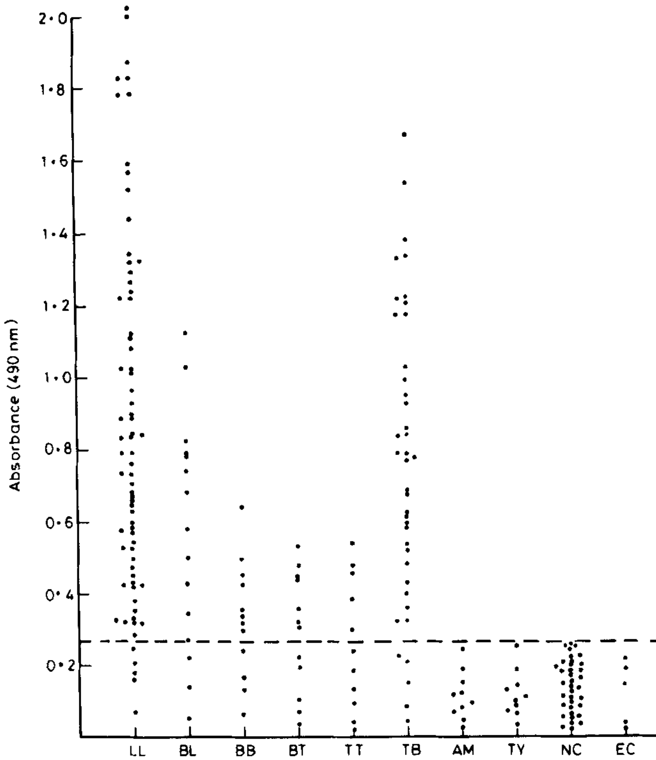

FIGURE 1.

Absorbance at 490 nm of sera from patients with HD,tuberculosis and controls in microtiter plate ELISA quantitating antibodies to soluble antigens of M. w sonicate. The cut off point (0.256 O.D.) was based on immunoreactivity (Mean value + 2 S.D.) of normal sera. LL= Lepromatous Leprosy; BL= Borderline lepromatous; BB= Mid borderline; BT= Borderline tuberculoid; TT= Tuberculoid leprosy; TB= Tuberculosis; AM= Amoebiasis; TY= Typhoid; NC= Normal control; EC= Endemic controls.

patients and that of controls were tested with M. w sensitized beads at neat, 1:2, 1:5, 1:10 and 1:20 dilutions made in glycine buffer saline to determine the optimum serum dilution required for the test. Dilution of 1:5 was found to give the best descriminating agglutination reaction in patients as compared to

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TABLE 1. ELISA results with M. w sonicate antigens in sera of various categories of HD patients and in patients suffering from active pulmonary tuberculosis.

Group	Number of sera examined	Number positive by ELISA	Sensitivity
I Hansen's Disease			
LL	70	65	92.8
BL	15	12	80.0
BB	12	8	66.6
BT	12	7	58.3
TT	12	6	50.5
II Tuberculosis	40	35	87.5

LL = Lepromatous Leprosy; BL = Borderline lepromatous; BB = Mid borderline; BT = Borderline tuberculloid; TT = Tuberculoid leprosy.

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controls. This dilution was therefore employed in further analysis of individual sera from patients of HD and tuberculosis. The agglutination test gave comparable results to ELISA. The sensitivity of assay for both HD and tuberculosis was found to be 78.7% and 85.7% respectively. Amonst the apparently healthy individuals and controls from endemic area showed the positivity of 4.3% and 8.8% respectively (Table 2). These 12 controls from both the groups were later confirmed positive also by radiodiagnosis. The test could also be correlated with the bacillary load of the patients (Fig.2). The coefficient of correlation between the two tests was $r=0.992$, which is statistically highly significant.

It was seen that the gradation of positivity goes down as the patient starts improving. This was confirmed by doing the follow

TABLE 2. Investigations on sera from patients of HD and tuberculosis employing the standardized agglutination assay.

Group	Number of sera	Number positive	Sensitivity
I. Hansen's Disease Patients			
LL	108	85	78.7
BL	48	16	33.3
BB	5	-	-
BT	5	-	-
TT	8	-	-
II. Pulmonary Tuberculosis Patients	84	72	85.7
III. Healthy Controls from Teaching Institutes (Delhi)	137	6	4.3
IV. Endemic Controls from Endemic area (Madras)	68	6	8.8

LL = Lepromatous leprosy; BL = Borderline lepromatous; BB = Mid borderline; BT = Borderline tuberculoid; TT = Tuberculoid leprosy.

up studies of six leprosy patients for six to eight months, who were undergoing the M. w vaccine trials at three different hospitals in India (Data not shown). Similar results were seen with tuberculosis patients who were undergoing the routine treatment in the hospitals.

Reproducibility and evaluation of the test

Reproducibility of the test was checked at the Institute by workers from other laboratories. The samples were decoded after

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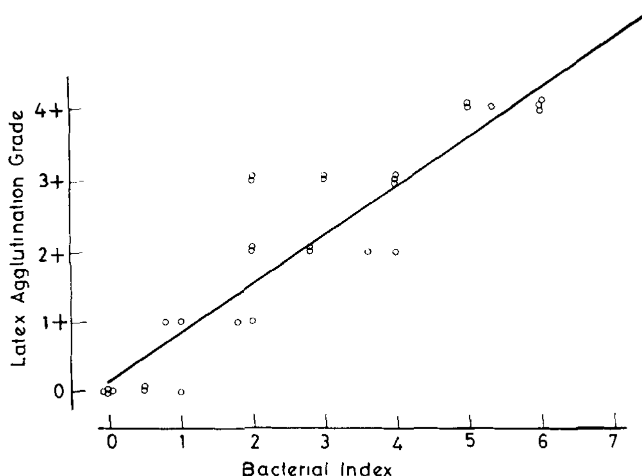

FIGURE 2.

Correlation between Bacterial index and degree of agglutination.

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the results were obtained. Another evaluation was done at an outside Institute, Central Leprosy Training Research Institute, Chingalpattu, Madras, where all the cases were coded and the code was broken after obtaining the results. The test was also validated on coded samples at different centres in Delhi (Table 3). The results showed that the assay worked with equal sensitivity in different hands.

Immune complex extraction in sera

The agglutination assay was performed on the same serum samples of HD, tuberculosis and normal healthy individuals after precipitating out the immune complexes by PEG. The assay determined the presence of anti-mycobacterial antibodies in PEG-extracted precipitates of patient sera. Of the 94 LL HD sera tested, 74 were positive in the direct serum agglutination assay.

TABLE 3. Validation of agglutination assay based on M. w antigens coated particles with coded sera.

Group	Number detected positive/total tested			
	CLTRI Madras	LHMC New Dellhi	LNJP New Delhi	SFJ New Delhi
I	Hansen's			Disease
LL	35/44	22/30	-	57/74
BL	3/10	1/3	-	4/13
BB	-/1	-/6	-	-/7
BT	-/2	-	-	-
TT	-/3	-/1	-	-
Histoid	1/1	-	-	-
polyneuritic	-/1	-	-	-
Indeterminate	-/1	-	-	-
II Pulmonary tuberculosis	-	-	53/60	43/48
III Controls	2/3	-/15	3/39	-/26

CLTRI = Central Leprosy Training Research Institute; SFJ = Sufdarjung Hospital; LNJP = Lok Nayak Jay Prakash Hospital; LHMC = Lady Hardinge Medical College.

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All these 74 cases remained positive even after PEG precipitation. The remaining 20 undetected LL HD patients sera when subjected to PEG precipitation and screening by agglutination test, showed 14 of them as positive. PEG precipitation, therfore, enhanced the sensitivity of agglutination assay from 78.7% to 90.4% in case of HD patients (Table 4). Similarly, 84 cases of tuberculosis were screened by direct serum agglutination assay. Of these, 71 were detected as positive and all of them again remained positive after PEG precipitation. Six out of the remaining 13 undetected cases when

Table 4. Detection of antimycobacterial antibodies extracted from immune complexes (IC) by agglutination assay.

Group	Number of sera	Number of positive and (%) positivity	
		Direct agglutination assay	Agglutination after extraction of ICs.
Hansen's Disease	94	74 (78.7)	85 (90.4)
Tuberculosis	84	71 (85.0)	77 (91.6)
Apparently Healthy Individuals	35	2 (5.7)	2 (5.7)

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tested after precipitation turned positive by agglutination, thereby increasing the percentage positivity from 85.7% to 91.6%. Of the normal controls, 33 out of 35 were negative and 2 were positive by direct serum agglutination as well as after PEG precipitation.

DISCUSSION

Sharing of several specific and cross-reactive B-cell epitopes of M. leprae and M. tuberculosis with M. w has been reported earlier by us (13). Experimental laboratory animal studies and delayed hypersensitivity testing in human subjects have also shown the capability of M. w antigens in inducing cell mediated immune responses against M. leprae (21,22,23). The selected shared cross-reactivity of M. w with M. leprae and M. tuberculosis is further reflected by ELISA and agglutination tests, where antibody reactivity to M. w antigen is detected in majority of HD and tuberculosis patients. Among the different categories of

HD patients the ELISA showed 92.8% positivity in the LL HD category and 50% in TT (Tuberculoid leprosy) and the agglutination test showed positivity of 78.7% for LL HD but could not detect TT patients. Though, it is practically difficult to compare the results of the different studies reported on the use of various antigens on sera from different geographical areas, the low readability for TT patients remains to be a major limitation of most of the immunodiagnostic tests developed for HD. Reactivity of M. w antigens for active tuberculosis patients with agglutination test (85.7%) was nearly the same as was with ELISA (85.5%). For tuberculosis, the sensitivity attained by these tests is higher than the tests reported earlier. A sensitivity range of 31% to 80% has been reported by sero-agglutination tests using phenolized suspension of M. tuberculosis H37Ra or its antigens coated onto latex particles (10,11,12). This latex test appears to be quite sensitive and more specific as it was negative in patients with typhoid and amoebiasis and only 4.3% of the apparently healthy individuals were positive. Among the endemic controls, 8.8% apparently healthy individuals were also found positive. The positivity of these on reconfirming by radiodiagnosis further proved the specificity of the assay. The positivity of these controls could be due to the close contact and exposure to open pulmonary tuberculosis cases. It remains to be seen whether these individuals were infected by M.leprae or M. tuberculosis and were able to ward off infection or were in latent stage of the diseases.

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This latex agglutination test does not distinguish HD and tuberculosis, the clinical distinction between these two diseases are not difficult as their signs and symptoms manifested are

quite different. HD is primarily a disease of skin and nerve. The disease manifest itself as a hypopigmented anesthetic patch (24,25,26,27). While pulmonary tuberculosis is mostly accompanied by respiratory symptoms, fever and general weakness.

The latex agglutination assay is simple to perform owing to the one step antigen antibody reaction and requires only five minutes for completion of a large number of samples. The studies are in progress to simplify the procedure of extraction of imune complex in order to retain the simplicity and enhance the sensitivity of the agglutination in both HD and tuberculosis respectively.

As regards HD, the importance of the test lies in its ability to identify the most infectious form i.e. LL HD. These are the bacteriologically positive LL and are potential transmitters of the disease. Early detection of such patients for introduction of proper chemotherapy can curtail spread of infection in the community.

It is hard to explain the basis for the efficiency of antigens derived from non-Mycobacterium leprae and non-Mycobacterium tuberculosis organisms as diagnostic reagents. The antigens used in this test contain epitopes which reacted with both specific and cross-reactive monoclonal antibodies of M. leprae and M. tuberculosis (13). This study defies to some extent the present thinking that for development for immunodiagnostics the antigen and antibodies have to be specific only to the pathogenic organisms concerned. Experience in several labs revealed that such concepts have been tenable only for viral infections but not mycobacterial and parasitic infections, specifically in a situation where the antigenic load in the host is low. Our

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experience has been that antigens with limited cross reaction are good diagnostic tools. One explaination for this could be the cross reactive antigens of the pathogen get easily identified by the immune system because of the previous priming with a non-pahtogenic organism. Hence, even with low antigenic load the detectable level of antibody response is generated against it as compared to antigens specific to the pathogens.

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