

Diagnostic Performance of Commercially Available Enzyme-Linked Immunosorbent Assay Kit in the Diagnosis of Extrapulmonary Tuberculosis

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ABSTRACT

Objectives: Antibody based serodiagnosis tests for tuberculosis (TB) was used widely in developed and developing countries. Pathozyme Myco[®] immunoglobulin (Ig) M, IgA, and IgG were evaluated in pulmonary TB in many studies.

Materials and Methods: In this study we assessed this commercially available kit in detecting extrapulmonary TB (EPTB).

Results: A total of 354 subjects were recruited for the study, of which 217 (61.2%) were EPTB patients and 137 (38.7%) were subjects with no suggestive TB. The mean age was 29.7 ± 13.7 and 31.2 ± 15.2 years, respectively for two groups. Serum samples were tested for IgM, IgA, and IgG using Pathozyme Myco[®] IgM, IgA, and IgG kit. The individual specificity rates of IgM, IgA, and IgG were 70.8% (95% confidence interval (CI): 62.7-77.7), 77.3% (95% CI: 68.6-83.5), and 68.6%. (95% CI: 60.4-75.7); while their sensitivity was 29% (95% CI: 23.4-35.4), 24.4% (95% CI: 19.1-30.5), and 34.5% (95% CI: 28.5-41.1); respectively.

Conclusion: The serological tests either singly or in combination failed or performed poorly to diagnose EPTB.

Key words: Diagnosis, extrapulmonary tuberculosis, serology

INTRODUCTION

The World Health Organization (WHO) declared tuberculosis (TB) a global public health emergency in 1993. TB causes ill-health among millions of people each year and ranks as the second leading infectious disease causing death worldwide. Human immunodeficiency virus (HIV) coinfection and the development of multidrug resistant (MDR) and extensively drug resistant (XDR) strains cause a major hurdle in treatment and containment of TB.^[1] The latest estimates of 2011 reports that there are almost 9 million new cases and 1.4 million TB deaths of which 990,000 are among HIV negative people and 430,000 HIV-associated TB deaths.^[2]

TB affects the lung which results in symptoms of respiratory system known as pulmonary TB. However, other organs, such as the pleura, lymph node, kidney, and meninges, may be involved in TB under certain circumstances and this is known as extrapulmonary TB (EPTB). Patients with EPTB present with organ-related symptoms which would develop into serious complications that threaten patients' lives and cause morbidity. Reports of recent years infer that the prevalence of EPTB is getting worse, which has drawn more public attention. In 2011, 19% of new EPTB cases were notified in India.^[2] One critical and early aspect of managing the TB epidemic is early diagnosis, so that the appropriate treatment can be started at the appropriate time. Diagnosis of EPTB is challenging. Routine methods for TB diagnosis such as smear for acid-fast bacilli (AFB) and culture of *Mycobacterium tuberculosis* on solid media, and X-rays are poorly sensitive worldwide, nucleic acid amplification test, and liquid culture methods, such as BACTEC MGIT 960 are costly and required sophisticated infrastructure.

Due to these diagnostic limitations antibody based immunodiagnostic tests have been widely used

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which do not require a specimen of the affected organ for microbiological or histological examinations. There are numerous *M. tuberculosis* antigens, including native, semisynthetic, and recombinant ones that have been used in these diagnostic kits. Some of the antigens such as lipoarabinomannan (LAM) were commonly used and evaluated in areas with low and high burden of TB. These antibody detection methods have evolved into various formats, includes microtiter well enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assays. The latter being the most widely adopted, because these do not require sophisticated instrumentation. Based on these serological methods many studies are carried out on pulmonary TB patients, while only few studies are done on EPTB patients. A meta-analysis conducted in 2007^[3] on nine published articles (25 studies) provide glimpse of variability in sensitivity (0-100%) and specificity (59-100%) of these tests. However, only one study was included from India^[4] in this meta-analysis.

In 2011, World Health Organization (WHO)^[5] issued strong policy recommendations against the use these antibody-based commercialized serological tests. An expert group^[3,6] that reviewed the evidence on use of commercial, antibody-based serodiagnostic tests found that these tests provide inconsistent and imprecise results with highly variable values for sensitivity and specificity. No evidence was found that existing commercial serological assays improve outcomes that are important to patients.

We evaluated Pathozyne Myco[®] immunoglobulin (Ig G, IgA, and IgM (Omega Diagnostic Limited, Scotland, UK) on pulmonary TB patients from Delhi, India^[7] and the findings were also highly discouraging. Simultaneously we also evaluated Pathozyne Myco[®] IgG, IgA, and IgM in EPTB patients and the results are presented here.

MATERIALS AND METHODS

Setting

The study was conducted between 2007 and 2011 at the Tuberculosis Research Laboratory, Division of Clinical Microbiology and Molecular Medicine, All India Institute of Medical Sciences, New Delhi. Ethical committee of the All India Institute of Medical Sciences (AIIMS), New Delhi approved the study protocol in accordance with National Guidelines by Indian Council of Medical Research, New Delhi.

All patients with suspected EPTB were prospectively enrolled. All the demographic details and relevant

clinical symptoms, signs, and duration were documented in predesigned subject information form. No subjects identified as having human immunodeficiency virus infection was included in the study.

Case definition

Enrolled patients were classified as “confirmed tuberculosis” if his sample was positive for smear/culture for *M. tuberculosis*; or had positive *M. tuberculosis*-specific DNA amplification from biological specimens; or histo-pathological findings consistent with TB or was highly suggestive radiological findings of TB (having excluded other disease) including appropriate response to antituberculosis therapy.

We defined non-TB patients as those who were bacteriologically negative for *M. tuberculosis* with either a resolution of clinical symptoms after an antibiotic therapy, or confirmed to have alternative diagnosis on histopathology.

Sample collection and processing

Following inclusion, a 2-4 mL venous blood sample was collected in 4 mL BD vacutainers (Becton-Dickinson, Sparks, USA) without anticoagulation and allowed it to clot at room temperature for 1 h, centrifuged at 4°C for 10 min at 3000 × g and clear serum was collected, aliquoted, and stored at -80°C till further use. No sample underwent more than one freeze-thaw cycle before analysis.

All the presumably contaminated samples were processed using modified Petroff's methods (NALC-NaOH), but samples from sterile sites such as cerebrospinal fluid (CSF), synovial fluid, bone marrow, etc., were inoculated directly in BACTEC MGIT[™]960 (Becton-Dickinson, Sparks, USA) and Lowenstein Jensen (LJ) medium as published elsewhere.^[8] Before culture inoculation all samples were examined microscopically after Zeihl-Neelsen stain. All the isolates were confirmed as *M. tuberculosis* by species specific in-house multiplex polymerase chain reaction (PCR) and phenotypic methods.^[9]

PATHOZYME[®] MYCO IgG, IgA, and IgM test

These three assays/kits are based on two highly purified immunodominant antigens. One is cell wall lipoarabinomannan (LAM) antigen which has been proved to elicit early stage antimycobacterial immune response in some studies, and second is 38-kDa mycobacterial recombinant antigen expressed and purified from *E. coli*. The kits claimed to be having 91% specificity and 72% sensitivity.^[10] Three immunoglobulin based ELISA

kits (PATHOZYME® MYCO IgG, IgA, and IgM) were used to check levels of antimycobacterial antibodies against two antigens in the serum of diseased and controls. The ELISA tests were performed according to the instructions in kits' manual (Omega Diagnostics Limited, Scotland, UK) and repeated three times as published earlier.^[7]

Statistical analysis

For proper analysis of performance, ELISA tests were evaluated on confirmed EPTB cases and non-disease group. Sensitivity, specificity, positive predictive values (PPV), negative predictive value (NPV), and likelihood ratio for positive (LRP) test were calculated with 95% confidence intervals (CI) to determine the correct diagnosis potential of the tests. STATA SE.9 (StataCorp LP, Texas, USA) software was used for all statistical analysis.

RESULTS

Subjects and clinical characteristics

During the study period a total of 217 (61.2%) confirmed EPTB (as per the criteria mentioned in

materials and methods) and 137 (38.7%) non-TB subjects were recruited. The mean age of patients with confirmed EPTB and non-TB patients was 29.7 ± 13.7 and 31.2 ± 15.2 years, respectively. Among the confirmed EPTB patients, disseminated TB was the most common ($n = 75$), followed by genitourinary ($n = 57$), lymph node ($n = 37$), pleural ($n = 20$), central nervous system ($n = 13$), gastrointestinal tract ($n = 9$), and skeletal system TB ($n = 6$). All subjects were examined for Bacillus Calmette–Guérin (BCG) vaccination, and 181 (83.4%) were found positive in confirmed EPTB patients and 117 (85.4%) positive in non-TB groups.

Performance of PATHOZYME® MYCO IgG, IgA, and IgM

ELISAs were performed in all 354 subjects. The performance of all the three ELISAs were analyzed [Table 1]. The individual and overall sensitivity rates of IgM, IgA, and IgG were 29, 24.4, and 34.5%, respectively; while their specificities were 70.8, 77.3, and 68.6%, respectively. When we analyzed these in combination of two or more ELISAs, the sensitivity further decreased and ranged from 7.8 to 11.9%; but their specificities improved significantly and ranged from 83.9 to 96.3% [Table 1]. The PPV for IgM, IgA, and IgG were 61.7,

Table 1: Performance of Pathozyme Myco® IgM, IgA, and IgG on extrapulmonary tuberculosis cases and non-EPTB subjects

ELISA (n=354)	Type		Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	LRP (95% CI)
	EPTB (n=217)	Without EPTB (n=137)					
IgM							
Pos	63 (29)	40 (29)	29	70.8	61.7	38.6	0.9
Neg	154 (71)	97 (71)	(23.4-35.4)	(62.7-77.7)	(51.1-70)	(32.8-44.8)	(0.8-1.2)
IgA							
Pos	53 (24.4)	31 (22.7)	24.4	77.3	63.1	39.2	1.0
Neg	164 (75.6)	106 (77.3)	(19.1-30.5)	(68.6-83.5)	(54.4-72.6)	(33.6-45.2)	(0.9-1.2)
IgG							
Pos	75 (34.6)	43 (32)	34.5	68.6	63.56	39.8	1.1
Neg	142 (65.4)	94 (68.6)	(28.5-41.1)	(60.4-75.7)	(54.5-71.6)	(33.8-46.1)	(1.0-1.2)
IgM, IgA							
Pos	26 (12)	12 (8.7)	11.9	91.2	68.4	39.5	1.3
Neg	191 (88)	125 (91.2)	(8.3-16.9)	(85.3-94.9)	(52.2-80.9)	(34.3-45)	(0.6-2.8)
IgM, IgG							
Pos	35 (16.1)	22 (16)	16.1	83.9	61.4	38.7	1
Neg	182 (83.8)	115 (84)	(11.8-21.6)	(76.8-89.1)	(48.4-72.9)	(33.3-44.3)	(0.6-1.4)
IgA, IgG							
Pos	34 (15.7)	15 (11)	15.6	89	69.3	40	1.4
Neg	183 (84.3)	122 (89)	(11.4-21.1)	(82.7-93.2)	(55.4-80.4)	(34.6-45.5)	(0.9-2.2)
IgM, IgA and IgG							
Pos	17 (7.8)	5 (3.6)	7.8	96.3	77.2	39.7	2.1
Neg	200 (92.2)	132 (96.3)	(4.9-12.1)	(91.7-98.4)	(56.5-89.8)	(34.6-45.1)	(0.3-12.3)
Any ELISA*							
Pos	113 (52)	70 (51.1)	52	48.9	61.7	39.1	1
Neg	104 (48)	67 (48.9)	(45.4-58.6)	(40.6-57.1)	(54.5-68.4)	(32.1-46.6)	(0.9-1)

ELISA: Enzyme-linked immunosorbent assay, Pos: Positive, Neg: negative, CI: Confidence interval, PPV: Positive predictive value, NPV: Negative predictive value, LRP: Likelihood ratio for positive test, Ig: Immunoglobulin, EPTB: Extrapulmonary tuberculosis; *Any ELISA represents subjects detected positive by at least one of the three (IgM/IgA/IgG) ELISA tests

63.1, and 63.5%, respectively; whereas their NPV were 38.6, 39.2, and 39.8%, respectively. The LRP test for IgM, IgA, and IgG were 0.9, 1, and 1.1; respectively. The mean optical density (OD) values of IgM, IgA, and IgG for confirmed EPTB patients were 2.1 ± 0.7 , 1.8 ± 0.8 , and 1.6 ± 0.4 OD; respectively and for without TB subjects were 1.78 ± 0.6 , 1.8 ± 0.9 , and 1.6 ± 0.5 OD; respectively [Figure 1].

Performance of serology in various organ-specific disease forms

The performance of ELISA among different disease categories is shown in Table 2. The highest degree of detection for IgM was found best in central nervous system (CNS) TB (odds ratio of 2.67, 95% CI: 0.69-11.06),

for IgA performed best in pleural TB (odds ratio of 2.55, 95% CI: 0.71-9.08), and IgG positive lymph node TB (odds ratio of 3.69, 95% CI: 1.02-13.34) as shown in Table 2. While comparing the detection rate among the three kits, IgG kit detected the most number of cases among lymph node TB (51.3%), gastrointestinal TB (44.4%), disseminated TB (37.3%), and bone/joint TB (33%) cases.

Effect of BCG vaccination on detection rate of ELISA kit

When compared the performance of ELISA on BCG vaccination status, no statistical significance was observed neither in confirmed EPTB patients nor in non-TB groups [Table 3].

Table 2: Correlation between different sites of TB infection and ELISA results

Site of infection	No. of EPTB cases	IgM+ (%)	IgA+ (%)	IgG+ (%)	Any ELISA+* (%)
Bone/joint	6	1 (16.6)	1 (16.6)	2 (33.3)	2 (33.3)
Disseminated	75	15 (20)	14 (18.6)	28 (37.3)	36 (48)
Gastrointestinal	9	3 (33.3)	3 (33.3)	4 (44.4)	6 (66.6)
Lymph node	37	15 (40.5)	12 (32.4)	19 (51.3)	25 (67.5)
Pleura	20	6 (30)	8 (40)	6 (30)	12 (60)
CNS	13	5 (38.4)	4 (30.7)	4 (30.7)	6 (46.1)
Genitourinary	57	18 (31.5)	11 (19.2)	12 (21)	26 (45.6)

+: Positive, ELISA: Enzyme-linked immunosorbent assay, OR: Odds ratio, CI: Confidence interval, CNS: Central nervous system, Ig: Immunoglobulin, TB: Tuberculosis, *Any ELISA represents subjects detected positive by at least one of the three (IgM/IgA/IgG) ELISA tests

Table 3: Effect of BCG vaccination on positivity of Pathozyme Myco® ELISA

ELISA	EPTB patients (n=217) BCG scar			Non-disease group (n=137) BCG scar		
	Pos (n=181)	Neg (n=36)	P* value	Pos (n=117)	Neg (n=20)	P value*
IgM						
Pos	51 (28.1)	12 (33.3)	0.5336	34 (29)	6 (30)	0.9319
Neg	130 (71.8)	24 (66.6)		83 (70.9)	14 (70)	
IgA						
Pos	43 (23.7)	10 (27.7)	0.6081	26 (22.2)	5 (25)	0.7838
Neg	138 (76.2)	26 (72.2)		91 (77.7)	15 (75)	
IgG						
Pos	63 (34.8)	12 (33.3)	0.8652	36 (30.7)	7 (35)	0.7063
Neg	118 (65.1)	24 (66.6)		81 (69.2)	13 (65)	
IgM, IgA						
Pos	22 (12.1)	4 (11.1)	0.8602	9 (7.6)	3 (15)	0.2868
Neg	159 (87.8)	32 (88.8)		108 (92.3)	17 (85)	
IgM, IgG						
Pos	28 (15.4)	7 (17.2)	0.5537	18 (15.3)	4 (17)	0.6034
Neg	153 (84.5)	29 (80.5)		99 (84.6)	16 (80)	
IgA, IgG						
Pos	29 (16)	5 (13.8)	0.7478	13 (11.1)	2 (10)	0.8831
Neg	152 (83.9)	31 (86.1)		104 (88.8)	18 (90)	
IgM, IgA, and IgG						
Pos	14 (7.7)	3 (8.3)	0.9029	4 (3.4)	1 (5)	0.7275
Neg	167 (92.2)	33 (91.6)		113 (96.5)	19 (95)	
Any ELISA#						
Pos	92 (50.8)	21 (58.3)	0.4104	60 (51.2)	10 (50)	0.9156
Neg	89 (49.1)	15 (41.6)		57 (48.7)	10 (50)	

*Two-tailed P value (Pearson Chi-square test); #any ELISA represents subjects detected positive by at least one of the three (IgM/IgA/IgG) ELISA tests; Pos: Positive, Neg: Negative, ELISA: Enzyme-linked immunosorbent assay, BCG: Bacillus Calmette-Guérin, EPTB: Extrapulmonary tuberculosis, Ig: Immunoglobulin

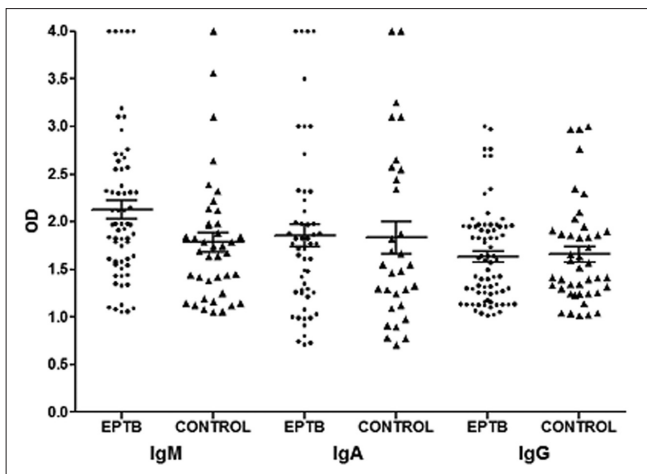


Figure 1: Optical density values of IgM, IgA, and IgG positive among extrapulmonary tuberculosis (EPTB) patients without EPTB subjects

DISCUSSION

The diagnosis of active TB largely depends upon initial clinical suspicion and radiographic findings, with subsequent laboratory confirmation by bacteriologic studies. Because appropriate specimen might be difficult to obtain from extrapulmonary sites, and the number of bacilli is generally low, bacteriological confirmation of EPTB is often more difficult than pulmonary TB.^[11] Even if the appropriate sample (e.g., CSF in suspected meningitis) is obtained, the sensitivity of conventional bacteriological methods are dismally poor. Because of these limitations, number of investigators relied on serology as a diagnostic tool in all settings.

With the technical advances, sets of *M. tuberculosis* specific antigens were first identified using either hybridoma or recombinant DNA technologies.^[12] However, the findings that antibody levels are considerably higher and more frequent in the multibacillary than in paucibacillary forms of the disease^[13] was noted as an obstacle to clinical application of these antigens in many studies, and thus combinations of multiple antigens were used.^[14]

Here, we report poor utility of ELISA test in which the sensitivity of Pathozyme® Myco IgM was 29%, IgA was 24.4%, and IgG was 34.5% in our patients of EPTB. These findings were by-and-large similar to our findings in pulmonary TB patients, where the sensitivity rates of the same kit were 48.7, 25.7, and 24.4%; for IgM, IgA, and IgG, respectively.^[7] However, the specificity of serology was better in EPTB than pulmonary TB [Table 1]. But, the sensitivity and specificity of these kits varied among various disease forms, for example, among bone/joint TB and lymph node TB cases IgG had the highest OR of 0.83 (95% CI: 0.11-6.01),

and 3.69 (95% CI: 1.02-13.34); IgA had OR of 1.6 (95% CI: 0.18-14.13), 1.25 (95% CI: 0.24-6.25), and 2.55 (95% CI: 0.71-9.08) for disseminated, gastrointestinal and pleural TB respectively, while CNS TB had the maximum OR of 2.67 (95% CI: 0.69-11.06) for IgM.

Not many authentic studies are published, but reported sensitivity of ANDA IgG detection kit used for the diagnosis of EPTB was 64% (95% CI: 28-92) for lymph node TB and 46% (95% CI: 29-63) for pleural TB, and its specificity was reported to be 90% and 87%, respectively for these patients.^[3] The alternative serology method MycoDot™ is reported to have higher sensitivity of 80% and 66.7% in TB lymphadenitis and in pleural TB patients, respectively.^[15] However, all studies have reported highly variable results, mainly because of difference in patient selection, criteria of diagnosis and cut-offs used. In one study, sensitivity of microwell ELISA based Pathozyme® Myco IgM in EPTB has been reported as low as 9%^[16] but in another study based on 38 kDa antigen immunochromatographic-TB test the sensitivity of 46% and specificity of 59.6% were reported in pleural TB patients.^[17] Senol *et al.*,^[18] also reported sensitivity of 22.2% and 25% in pleural TB and lymph node TB, respectively; and had similar specificity of 93.3% using Pathozyme-TB Complex Plus test.

This explains the fact that not all TB patients produce antibodies against all antigenic epitopes in the cell walls of the tubercle bacilli which infers on the inconsistency in specificity of antibody based assays among different patient groups like gender, age, ethnicity, and geographical distribution. It is also well known^[19] that person-to-person variation in antigen recognition is the key feature of human humoral immunity against TB.^[20]

Despite the inconsistency and imprecise sensitivity and specificity of the commercial serological tests, these were being marketed liberally and used widely in major endemic regions including India until recently when WHO banned commercialization of these kits.^[5] As a result of this WHO guideline, in June 2012 the Government of India stopped the import, manufacture, distribution, and sale of commercial serodiagnostic tests for TB. This bold action is expected to greatly reduce the frequency of false diagnoses of TB and facilitate the introduction of WHO approved diagnostics into the market. Therefore, in conclusion even though the specificity of the Pathozyme Myco IgA, IgM, and IgG was significantly better and acceptable as compared to pulmonary TB, the sensitivity of these kits was dismally poor and their use cannot be recommended for diagnosis of EPTB.

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