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# Prognostic variables for high titres in a fluorescent antibody test to diagnose tuberculosis ☆

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**KEYWORDS**
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**Summary** *Setting*: The four hospitals and a tuberculosis clinic in the province of Zeeland, The Netherlands.

*Objective*: To assess the usefulness of PPD antibody measurement in the diagnosis of tuberculosis in patients admitted to hospital.

*Patients and methods*: Sixty-one patients presenting with active tuberculosis, and 215 control patients were included in the study. Initial serum PPD antibody titres were determined with a macrophage uptake Fluorescent antibody test (MuFat) to construct a discrimination model between Tuberculosis (TB) and non-TB. We also retrospectively collected clinical parameters of the TB patients at presentation. Univariate and multivariate logistic regression are used to identify variables predicting high antibody titres.

*Results*: In TB patients, the presence of clinical symptoms (OR = 10.63) and the presence of at least two concurrent non-lymph node disease localizations outside thorax and abdomen (OR = 13.94) are necessary and sufficient to predict high titres.

The logistic model shows a significant contribution of the $^{2}$log (titre) to the discrimination between TB and non-TB patients. At a cut-off value of 128, a specificity, sensitivity, and positive predictive and negative predictive values of 97%, 39%, 80% and 85%, respectively, are calculated in the study cohort.

*Abbreviations:* BSE, blood sedimentation rate of erythrocytes; CI, confidence interval; Hb, haemoglobin concentration; MuFat, macrophage uptake of fluorescent antibody test; LN, lymph nodes; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value; ROC, receiver operating characteristic; TB, tuberculosis; ZN, Ziehl-Neelsen

☆This study was performed at the Department of Tuberculosis Control of the Zeeland Public Health Office, Goes, The Netherlands.

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*Conclusion*: Our data suggest an application of the test at high cut-off values for timely diagnosis of difficult-to-diagnose TB patients. The results of this retrospective study will have to be confirmed in further prospective studies.


## Introduction

Timely diagnosis is crucial in confining tuberculosis (TB) morbidity and disease transmission. In several low-incidence countries, diagnostic delay constitutes a risk to TB morbidity and mortality.[1,2]

Diagnosis, especially of sputum smear-negative TB, can be cumbersome because of the variety of symptoms and localizations, and the time lapse between initial suspicion and confirmation by culture.[3] Serodiagnosis has been widely explored in search for a rapid alternative to the standard of mycobacterium identification and as a way to circumvent the need to collect proper bacteriological specimens.[4] Numerous serological tests using various antigens and a variety of detection system have been developed.[5] Still, even the modern ELISA-based tests give insufficient results for clinical application as a useful screening tool.

Although until now disappointing as a screening instrument, surprisingly little satisfactory effort is given to serology as a diagnostic tool in clinically ill TB patients.[6] With this background, the aims of the present study were:

1. to identify variables predicting high TB antibody titres from historical data;
2. to construct a discrimination model and cut-off values between TB and non-TB patients in a case control design.

By using a PPD antibody assay in a fixed clinical setting, applied at the moment of presentation of the patient, we determined the appropriate sensitivity and specificity levels of the test in the study cohort. Based on the results, we discuss the potential use of this test in clinically ill patients sent to hospital for diagnosis and treatment.

## Patients and methods

### Patients

For this study patients were recruited from the Zeeland provincial reference laboratory database during the period 1992–1998. The reference laboratory covers the four hospitals and the TB clinic in the province. The database contains personal data (age, sex, ordering department), laboratory data (TB antibody titre), sample type, results of culture and smear, clinical chemistry) and one or two clinical symptoms as stated on the order form by the ordering physician. TB antibody tests were requested by clinicians in the provincial hospitals and by Public Health Officers from the Department of Tuberculosis Control, when TB was suspected or considered. The retrospective study was done in the cohort of patients investigated for TB in the sequence they showed up.

The availability of the first test result pro patient (i.e. within two days of presentation) and the availability of the results of simultaneous bacteriological cultures (Löwenstein or Coletsos cultures) were a prerequisite for inclusion. Patients with hampered immune response (HIV positive, concurrent tumour or immune disease, treatment with corticosteroids or cytostatics[7,8]), under six years of age,[9] or with a medical history of TB within the last 15 years were excluded.

Patients in the database with TB were regarded as study subjects. Diagnosis of TB was applicable if the patients were registered by the Dutch Public Health TB Index, i.e. met registration protocol criteria.

From the 68 TB patients found, we excluded 7 TB patients because of HIV positivity ($n = 1$), concurrent tumour or immune disease ($n = 2$), treatment with corticosteroids or cytostatics ($n = 1$) or medical history of TB in the last 15 years ($n = 3$). Of the 61 TB patients included in the study, TB was confirmed by culture of *Mycobacterium Tuberculosis* (58 study subjects), or the patients met the criteria otherwise (two study subjects as traced contacts with a radiological diagnosis of primo TB, and one study subject with a radiological diagnosis and a confirmation of *M. Tuberculosis* presence by polymerase chain reaction).

All TB patients gave informed consent. Clinical data of the TB patients were obtained from hospital records and the Public Health Office.

In the same period 1992–1998 the Dutch Public Health Authority registered 217 new TB patients in the province of Zeeland, less than 2% of them younger than six years. All registered TB patients were treated with antituberculous drugs.[10] Seventy-five percent of them (75%) were registered

as confirmed TB patients, forty-five percent (45%) as only culture positive.[10,11]

As we excluded seven TB patients because of HIV positivity ($n = 1$), concurrent tumour or immune disease ($n = 2$), treatment with corticosteroids or cytostatics ($n = 1$) or medical history of TB in the last 15 years ($n = 3$), there are 149 officially registered TB patients not included in the study. Either they did not meet our prerequisite criteria (test results available within 2 days of presentation), or they were not in the database at all (e.g. traced contacts with primo infection).

Non-TB patients were selected by taking every fourth TB antibody test result from the database and constituted the control group for comparison. Diagnosis of TB in these patients was excluded by concomitant negative TB culture results and by cross-checking with the Dutch Public Health TB Index. Control patients were also not registered in the Index in the two years following the entry date in our database. Other diagnoses were confirmed by bacteriological culture, virological testing or histological examination. The resulting group of 215 (control group) presented with respiratory (pneumonia, recurrent bronchitis, interstitial lung disease, mediastinal mass) or non-respiratory clinical signs and symptoms (lymphadenopathy, arthritis, febris e.c.i., malaise, anaemia, osteitis, non-specific abdominal pains).

Microbiological samples were obtained by spontaneous expectoration, sputum induction, bronchial washing, broncho-alveolar lavage, aspiration or open-tissue biopsy. Samples were processed for acid-fast bacilli smear examination (Ziehl-Neelsen staining (ZN)) or for culture as usual.[10] Blood samples drawn within 48 h after the patients' first presentation were used to determine haemoglobin level (Hb), blood sedimentation of erythrocytes (BSE), and TB antibody titre.

## Macrophage uptake of fluorescent antibody test (MuFat)

At arrival in the laboratory, sera were tested for antibodies against TB the MuFat, an non-commercially available fluorescence test with purified protein derivate (PPD; RIVM, Bilthoven; The Netherlands) as antigen as described before.[12] PPD-presenting macrophages were obtained by irrigating (phosphate-buffered saline PBS, pH=7.2) the peritoneal cavity of euthanized C57 black mice inoculated intraperoneally 24 h earlier with 2 mg of PPD. The resulting macrophage suspension was washed and diluted to a turbidity of McFarland 3. Four drops of the suspension were transferred by means of a platinum loop to each microscope slide and these were allowed to dry in the air. After fixation (acetone, 10 min, 4 °C), the slides can be stored at −20 °C. Before use the slides need to be washed in cold PBS for 10 min. The MuFat was carried out on serial dilutions of patient sera according to standard procedure. One series of serum dilutions, from dilution 1:16 onwards, was tested per slide. The serum dilutions were transferred to the dried spots of antigen, and the slides were incubated in a humid environment (30 min, room temperature). After a washing step (cold PBS, 10 min), a titrated conjugate solution was added as a second layer, and the slides were again incubated in a humid environment (30 min, room temperature).

The conjugate solution (rabbit antihuman Ig/FITC (Fluorescein Isothiocyanate, Nordic Pharmaceuticals, Tilburg, The Netherlands) was pre-absorbed with *M. Avium* suspension and was titrated against a standard set of positive and negative controls.

After a second washing step (cold PBS, 10 min), the slides were covered with cover slips and read with a fluorescence microscope.

The highest dilution with a typical fluorescence of the macrophages was interpreted as the titre (1:16–1:512 or more). Results were recorded as the (inverse) titre, e.g. 16 for a 1:16 diluted serum and 512 for a 1:512 or higher serum dilution. The resulting titre was used as outcome variable in a prediction model.

## Risk factors

Chest radiographs of TB patients were reviewed. The extension of pulmonary features on each patient's X-ray was described as the number of diseased lung quadrants or involvement of other intrathoracic tissues (mediastinal lymph nodes (LN), pleura fluid).

Further categories of clinical localization were intra-abdominal (organs, mesenterium, LN, psoas abscess); palpable peripheral lymph node localizations; other structures (superficial, e.g. skin, fistula, pharynx wall or deep, such as bone, muscle, intra-articular (other localization)). An individual patient could at the same time fall into several categories. The information on localizations was obtained by the combination of accurate physical and technical examination. Data for TB patients on clinical signs and symptoms such as cough, fever, sweating, fatigue or malaise, weight loss and other signs and symptoms were collected as described before[10] and used as categorical parameters.

Disease localizations, laboratory data, clinical signs and symptoms, the actual disease history (i.e. duration of the main clinical symptom in months) and epidemiological data were all used as parameters for statistical analysis.

## Statistical analysis

Comparisons between TB patients and controls were made with the Mann–Whitney test in case of quantitative variables, and the $X^2$ test was used in case of qualitative variables (i.e. Fisher's exact test for two-by-two tables). Values of $P<0.05$ were considered as statistically significant. The number of controls was based on power analysis. It was calculated that for a case control design with 60 cases, more than 200 controls were needed (power=90%, $I = 5\%$, $P_0 = 15\%$, $P_1 = 2\%$, $M = 3$).

Univariate logistic regression was used to evaluate the ability of the $^2$log (titre) to discriminate between TB patients and controls. Because in particular the $^2$log (titre) is expected to be non-linearly related in the logistic regression model, reasonable cut-off values to discriminate between TB and non-TB are constructed.

At this point, the condition of weighted 'costs' of misclassification of cases and non-cases was used. In other words, the optimal cut-off value for a particular titre was chosen so that the weighted sum of sensitivity and the specificity to discriminate between TB patients and controls was maximal (that is, loss in specificity costs 5 times as much as gain in sensitivity).

Multivariate logistic regression with forward selection procedures was used to identify variables that contributed independently to discriminate high titre (dilution of 128 times or more) from low titre (dilution of less than 128 times). Because forward selection procedures did not identify other important variables, *P*-values for entry in the model were considered in order to find close alternatives to the variables selected.[13]

Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) were calculated and crude or adjusted odds ratios (OR) with 95% confidence intervals (95% CI) were presented.

# Results

## Patient characteristics

The 61 TB patients and 215 control patients varied in age from 6 to 88 years, the mean age of the TB and control patients being 36.3 years and 37.8 years, respectively (Table 1). TB and control patients were admitted to the hospitals or the provincial TB Public Health clinic. Table 2 shows the characteristics of all TB patients. The relative number of TB patients from the TB clinic, acquired by passive case finding, was significantly higher than the number of TB patients admitted to the hospitals ($P<0.01$). There was no statistically significant difference between the numbers of TB and control patients who were admitted to the chest, internal or other hospital departments.

**Table 1** Characteristics of TB and control patients in the province of Zeeland.

| | TB patients ($n = 61$) | Controls ($n = 215$) | *P* |
|---|---|---|---|
| Age* | | | |
| Mean (SD) | 36.3 (19.6) | 37.8 (18.6) | 0.57* |
| Nationality | | | |
| Dutch | 24 (39%) | 115 (53%) | 0.06** |
| Gender | | | |
| Male | 39 (64%) | 129 (60%) | 0.66** |
| Origin | | | |
| Hospital dept | | | |
| Chest | 22 (36%) | 92 (43%) | |
| Internal | 10 (16%) | 67 (31%) | |
| Other | 9 (13%) | 27 (12%) | 0.27*** |
| TB clinic | 20 (33%) | 29 (14%) | |
| All hospital depts | 41 (67%) | 186 (87%) | <0.01** |
| TB titre | | | |
| 16 | 25 (41%) | 188 (88%) | |
| 32 | 7 (11%) | 14 (7%) | |
| 64 | 5 (8%) | 7 (3%) | |
| 128 | 9 (15%) | 3 (1%) | |
| 256 | 9 (15%) | 2 (1%) | |
| 512 | 6 (10%) | 1 (0%) | |

*Age in years; SD=Standard deviation; dept.=department; *n*=number; %=percentage; *P*=*P*-values for statistically significant differences between TB and control patients (*Mann-Whitney, **Fischer exact, ***$X^2$ test).

Of the TB patients, 39 (64%) were male and 24 (39%) had the Dutch nationality. Twenty-five TB patients (41%) were from Africa, three from Asian countries and four from different European countries. The foreign TB patients were in The Netherlands as asylum seekers, immigrants or contract workers. The control group had comparable percentages of patients from countries with high or low TB incidence. There were no statistically significant differences in age, gender or nationality between the TB and control patients (Table 1).

Of the TB patients, 48 (79%) presented with at least one intrathoracic TB localization, of which three had a purely mediastinal LN TB, six had pleural TB, and six had a miliary TB. Ten TB patients

**Table 2** Clinical characteristics of TB patients ($n = 61$).

| TB Localization | |
|---|---|
| Intra-thoracic | 48 (79%) |
| Intra-abdominal | 10 (16%) |
| Peripheral lymph nodes | 7 (12%) |
| Other* | 13 (22%) |
| Laboratory | |
| Smear-negative | 46 (75%) |
| Hb | |
| Median (range) | 8.3 (5.3–10.3) |
| BSE | |
| Median (range) | 34 (2–112) |
| Clinical symptoms | |
| Cough | 19 (31%) |
| Fever | 11 (18%) |
| Weight loss | 18 (30%) |
| Night sweat | 8 (13%) |
| Fatigue | 14 (23%) |
| Other symptom† | 20 (33%) |
| History‡ | 19 (31%) |

*Other=e.g. skin, fistula, pharynx wall, bone, muscle, intraarticular (multiple localization categories possible); Hb=haemoglobin concentration (mmol/l); BSE=blood sedimentation rate of erythrocytes (mm/h).

†Other symptoms=e.g. localized pain, diarrhoea, haemoptysis, dyspnoea.

‡History=history of the main symptom more than one month preceding presentation.

had at least one intra-abdominal localization, seven had at least one peripheral LN localization and 13 patients (22%) had at least another localization of the process (Table 2).

At the time of presentation, 46 of the TB patients (75%) had negative sputum ZN smears and two patients had no smear performed. The TB patients had a mean BSE of 43 (range 2–112) mm/h and a mean Hb of 8.0 (range 5.3–10.3) mmol/l.

Cough was a symptom in 19 TB patients (31%), 18 patients had weight loss, and 37 patients (61%) had at least one of the five categories of clinical symptoms. Twenty patient (33%) had other symptoms (localized pains, diarrhoea, etc.) and 19 patients had a history of the symptoms lasting more than one month.

All TB patients showed a favourable response to antituberculous treatment except one patient who died within one month after start of treatment.

Diagnosis of the six non-TB patients with titres of at least 128 were:

- unknown (lost to follow-up-solitary pulmonary nodule, pleural cap),
- sarcoidosis,
- death by cardiac insufficiency-cardiomegaly. (Post-mortem examination showed no infection focus),
- rheumatoid arthritis,
- pan-sinusitis and non-allergic bronchial asthma,
- non-specified, atypical pneumonia and diabetes mellitus.

## Titres to predict TB

The distribution of TB titres of TB and non-TB patients is shown in Table 1. The results of the logistic model shows that $^2$log (titre) statistically significantly contributes to discriminate between TB and controls (Fig. 1). The crude OR for the variable is 2.456 (95% CI: 1.889–3.194).

Fig. 1 shows the ROC curve of the titre to discriminate between TB and control patients. The area under the curve is 78%. Furthermore, the optimal cut-off value using the weighted cost principle was found at titre=128. At this threshold of 128, a specificity, sensitivity, PPV and NPV of 97% and 39%, 80% and 85%, respectively, was attained. A prior disease probability of 0.1–0.2 for the clinical subpopulation was estimated.

At the threshold of 128 for the ZN sputum negative patients a PPV of 74% was calculated.

At a cut-off value of 512, TB can be predicted with a specificity of 99.5%, a PPV of 86% and a sensitivity of 9.8%.

## Prognostic variables to predict high titres in TB patients

Table 3 shows the crude OR to discriminate between high ($\geqslant$128) titre and low ($<$128) TB antibody titre in TB patients. Note that in this section, in contrast to the previous one, titres are studied in TB patients only.

The results of the univariate logistic regression showed that several parameters were identified as potential covariates. Only the localization parameters 'lung quadrants' (number of lung quadrants involved), 'extended' (patients with 1 intra-abdominal localization or at least 2 peripheral LN localizations or other localizations, or involvement of at least 3 lung quadrants) and 'neither pulmonary nor intra-abdominal' (patients with two concurrent other localizations) could predict high titres in TB patients, based on the level of significance.

Of the laboratory parameters, BSE was a potential covariate of low-to-intermediate significance

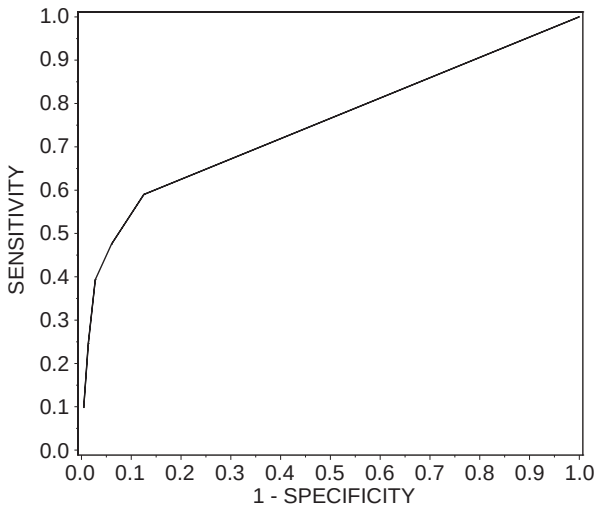


**Figure 1** ROC curve of the $^{2}$log-TB antibody titres to discriminate between TB and non-TB patients.

**Table 3** Crude-odds ratios (95% confidence intervals) of clinical and demographical patient characteristics to predict high titre ($\geqslant$128) TB using univariate logistic regression.

| Variable* | OR |
|---|---|
| Age | 1.01 (0.98; 1.04) |
| Dutch | 0.88 (0.33; 2.53) |
| Male | 1.22 (0.55; 4.83) |
| Lung quadrants | 1.79 (1.04; 3.11) |
| Pulmonary | 7.20 (0.75; 68.89) |
| Extended | 7.23 (2.19; 23.84) |
| Neither pulmonary nor intra-abdominal | 5.83 (1.07; 31.88) |
| BSE | 1.02 (1.00; 1.04) |
| Hb | 0.89 (0.56; 1.40) |
| Clinical symptoms | 1.81 (1.07; 3.06) |
| Clinical presence | 7.09 (2.22; 22.67) |

*Lung quadrants=number of lung quadrants involved; Pulmonary=presence of pulmonary localization(s); Extended=one abdominal localization, or at least two peripheral lymph node or other localizations, or at least three lung quadrant localizations; Neither pulmonary nor intra-abdominal=two concurrent other localizations; BSE=blood sedimentation rate of erythrocytes; Hb=haemoglobin concentration; Clinical symptoms=number of clinical symptoms; Clinical presence=presence of clinical symptom(s).

level. The parameters 'clinical symptoms' (total number of clinical symptoms) and 'clinical presence' (presence of clinical symptoms) were found to be potential covariates (intermediate- and high-significance level, respectively). The actual disease history did not give a potentially significant contribution to a disease model. Demographical parameters such as nationality (Dutch or not Dutch), gender or age were also not significantly related to high titre.

Table 4 shows the adjusted OR to predict high titre and low titre using multivariate logistic regression. All variables with statistically significant crude OR were entered at the start of the selection procedure. This selection procedure shows that 'neither pulmonary nor intra-abdominal' and 'clinical presence' are the only independent variables sufficient and necessary to predict a high titre, explaining 37% of the total variance ($R^2 = 37\%$).

Clinical variables as the total number of the clinical symptoms or demographic variables did not give an additional contribution to the prediction of high titres (i.e. $\geqslant$128).

## Discussion

The present report studies the characteristics of a TB antibody test in patients, when the diagnosis TB was suspected or considered. It differs from other studies in that it compares only TB and non-TB patients from one adherence area[14] who were admitted to either a general hospital or specialist clinic. A major finding in this study is the identification of two independent factors, sufficient for the prediction of high TB antibody titres. To our knowledge, no other diagnostic serological studies aimed at a subpopulation of sick patients that present adjusted OR with confidence intervals, have been published until today. In contrast to our results, most models that find a clinical and pulmonary factor as necessary and sufficient determinants predicting TB only deal with patients with pulmonary disease, and are aimed at infection prevention.[15,16] The presence of two concurrent non-lymph node localizations outside thorax and abdomen as predicting factor sustains the assumption that not the (radiological) extensiveness of the disease process but the total bacillary load at sites far from the entry site explains the activation status of the humeral response at the moment of presentation. All patients with a low bacillary load (pleuritis tuberculosa, primo TB, mediastinal TB), in spite of the impressive radiological deviations and sometimes considerable compression dyspnoea in the last group, had very low to low titres.[17,18]

The only other predictor for high TB antibody titres found in this study was the presence of clinical symptoms. In The Netherlands most TB patients found by contract tracing and without

**Table 4** Adjusted-odds ratios (95% confidence intervals) of patient characteristics to predict high titre (⩾128) TB using multivariate logistic regression with forward selection procedures.

| Variable* | OR | $R^2$ | AUC |
|---|---|---|---|
| Neither pulmonary nor intra-abdominal | 13.94 (1.85; 105.01) | | |
| Clinical presence | 10.63 (2.59; 43.67) | 37% | 78% |

*Neither pulmonary nor intra-abdominal=two concurrent other localizations; Clinical presence=presence of clinical symptom(s): $R^2$=maximum rescaled $R$-squares indicating the % explained variance; AUC=area under the ROC curve (all variables with statistically significant crude odds ratios were entered into the model).

major clinical symptoms are not admitted to hospital but are generally treated at home by the Public Health Service and the study is not aimed at this group of patients.[19]

Obviously, the study was restricted in that only the clinical data set of TB patients was available. Also no patients with cerebral or urinary TB were present in the study. Further analysis with clinical data of TB and non-TB patients could reveal other contributing factors as the history of clinical symptoms, and can give a more precise estimation of the weight of contributing risk factors.

Unlike the results of others,[18,20] our study reflects a test of an acceptable discriminating power (area under the ROC curve is 78%) and a specificity, sensitivity and PPV of 97%, 39% and 80%. Recalculation with only the use of the culture confirmed TB patients gave similar results, not surprisingly because of the limited number (3) of otherwise defined TB patients.

In line with others who report lower rates of seropositivity for extrapulmonary TB compared to that for sputum smear-positive TB,[21,22] there is no reason to believe that the use of only sputum smear-negative TB patients should alter the principal conclusions of the study.

Obviously, the results of the study cannot be generalized to an open population. First, we chose to limit this study of a serological test to immunologically non-impaired patients. Second, the study aims at the description of risk factors for high antibody titre TB patients admitted to hospital. The unexpectedly high TB prevalence in the study cohort should not be confused with the TB prevalence in contact groups (up to 5%) or otherwise defined groups. Because PPV is a function of the prevalence of TB, the described PPV applies only to a hospital population of sick and difficult-to-diagnose patients, i.e. *without* an obviously pulmonary TB and *with* symptoms of extrapulmonary or general disease. Clearly, the found cut-off value has to be validated in future prospective studies.

As in many older tests, the MuFat test was developed in veterinary studies and uses PPD tuberculin as an antigen.[23] The macrophage system and PPD can mimic the in vivo antigenic capacity that evokes a broad humoral response.[24] The visually assessed test is liable to improvement as standardization of flow cytometry is in progress.[25]

Recent reports use highly purified or recombinant antigens that reach sensitivities of 60–80% and specificities up to 97%,[26–28] and it would be interesting to study a set of these tests in a similar clinical setting.

We think that the results of our study can be useful in clinical practice. Clearly, this serological test does not discriminate between mediastinal TB and intrathoracic sarcoidosis. In our region, we further estimate that less than 5% of the cumulative number of incident TB patients could theoretically be identified with a high specificity. Nevertheless, in at least seven smear-negative patients out of the tested 61 TB patients (two patients categorized as miliary TB, a pregnant TB patient,[10] three patients with abdominal TB) with titres of at least 256, the test results made TB probable and influenced the decision towards a more active attitude, e.g. surgical exploration or the start of a trial therapy, at least one month before culture results were known.

We think that, if a proper selection of ZN smear-negative, clinically ill patients is made and false negative results are accepted, limited serological TB testing can be useful in clinical decision-making in low-incidence countries. This requires future prospective studies with serological TB tests, utilising complete and comprehensive databases.

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