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Indeterminate test results of T-SPOT.*TB* performed under routine field conditions

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Abstract:

Background: Interferon Gamma Release Assays can give indeterminate results. We assessed the prevalence of indeterminate test results (ITR) among T-SPOT.*TB* tests.

Method: A retrospective analysis of samples processed in 2005. ITR were assessed by age, gender, immunosuppression, distance to the laboratory, and season. A subgroup of tests performed for specific indications (contact tracing, migrants with positive tuberculin skin test, TB suspects, and immunosuppression) were analyzed separately.

Results: Among 1429 tests, 49 (3.4%) were indeterminate. ITR were significantly associated with old age (>75 year old vs. 5-75 years old, OR=7.97 95%CI 3.968-15.438, p=0.006) and the season during which samples were transported (autumn & winter vs. spring & summer, OR=3.47 95%CI 1.753-7.514, p=0.0007). The rate of ITR was 2.0% among TB contacts (n=302), 1.6% among immigrants (n=75), 3.0% in TB suspects (n=156) and 3.0% among immunosuppressed patients (n=32). Gender, young age and the distance were not associated with the rate of ITR. Among 13 ITR tests that were repeated, 10 gave a clear positive or negative answer.

Conclusions: ITR with T-SPOT.*TB* under routine conditions are infrequent. ITR were more common among elderly persons >75 years than among children and adults. The rate of ITR is low and similar among healthy TB contacts, immigrants with a positive TST, TB suspects and immunosuppressed. The conditions of transportation may influence the rate of ITR

The tuberculin skin test (TST) has been used for decades for the detection of latent tuberculosis infection but is not entirely reliable due to its low specificity (influence of prior vaccination by BCG and contact with environmental mycobacteria) and sensitivity (influence of the immune state of the patient) [1].

Two new Interferon-Gamma Release Assays (IGRAs), based on *in vitro* detection of interferon-gamma released by T cells in response to antigens specific to *M. tuberculosis* and encoded by the RD1 region, are available for the diagnosis of TB infection (T-SPOT.*TB* and Quantiferon-TB Gold) [2]. These tests with positive and nil internal controls are more specific than TST in diagnosing TB infection [3-5] and equally or more sensitive in patients with immune deficiencies . Nevertheless, indeterminate results have been reported for both tests with a frequency of 0-5.4% for T-SPOT.*TB* test [6-11] and up to 40% for Quantiferon-TB Gold test [12-17]. Their occurrence seems to be associated with immunosuppression [10, 18] and very young or very old age (children younger than 5 years and patients older than 80 years).

T-SPOT.*TB* was introduced as a routine test for the detection of tuberculosis infection in Lausanne in 2004 [19]. Although the experience demonstrated that the blood test was more specific than the TST, some results were indeterminate. Therefore, we wanted to assess retrospectively the possible internal (test-related) and external factors that could explain the indeterminate results when using a T-SPOT.*TB* test under routine conditions.

Methods

We conducted a retrospective analysis of all T-SPOT.*TB* tests performed in 2005. The tests were requested by the regional Office of Public Health, public and private hospitals in Lausanne, Geneva and some more remote hospitals in Switzerland, private physicians and organizations caring for immigrants. The indications were contact tracing for tuberculosis after contact with an index case, surveillance of exposed health-care workers, assessment of immigrants with positive TSTs discovered at entry in Switzerland, suspicion of active tuberculosis and screening for latent TB prior to initiation of immunosuppressive therapies. The tests were all performed and interpreted in a private medical analysis laboratory (BBR-LTC Laboratory, Lausanne) by trained technicians and according to the manufacturer's instructions. After centrifugation of the samples, PBMC were resuspended and standardized to 250,000 cells/well and incubated overnight with phytohaemagglutinin, ESAT-6 or CFP-10. Spot-forming units were read manually with a magnifying glass.

Results were classified by the laboratory as either positive, negative or indeterminate. Indeterminate test results (ITR) were defined as the presence of more than 10 spot-forming units (SFUs) in the nil-control wells (high background) and/or less than 20 SFUs in the mitogen positive control wells. We also re-checked each plate to determine the technical classification of the ITR.

For all ITR, we contacted the referring physician to obtain further information about the co-morbidity, immunosuppression and drug treatment with possible influence on the immune system (cancer chemotherapy, antiretroviral therapy, steroids). The indications for performing the test (contact tracing, positive TST among immigrants, suspicion of TB or immunosuppression) were known only for the patients from the local University hospitals in Lausanne and Geneva.

Statistical analysis: in all categories (age, gender, distance from sampling place to the laboratory, season of the year, co-morbidity, immune status, drug treatment, indication for the test), we calculated the ITR as a percentage with 95% confidence intervals. As children under 5 years have unreliable test results in some studies and a higher risk of disease if infected, they were analyzed separately. We used multivariate analysis to assess the true underlying relationships between ITR and age, gender, distance and season. Statistical analysis was performed using PROC LOGISTIC in SAS, version 9.1.3. Confidence intervals were calculated using the iterative profile likelihood method and the model was chosen using stepwise regression, starting from the null model, to choose parameters.

Results

Of 1468 requests, 26 tests could not be performed for technical reasons (broken test tube, insufficient blood sample) and were excluded from further analysis. Thirteen indeterminate tests were repeated in the same subject (only the first test was considered in the analysis). Of the remaining 1429 results, 407 (28%) were positive, 973 (67.8%) were negative and 49 (3.4%) were indeterminate. 37 (2.6% of 1429) were attributed to the absence of sufficient response to the mitogen control, while 10 (0.7%) were due to a high background in the wells, preventing the counting of spots, and 2 (0.1%) had >10 spots in the nil control well. Among the 49 ITR results we were able to obtain clinical information for 37 patients. The 12 cases without information were excluded from the calculation of the association with co-morbidity, immune status and medication.

Thirteen of the 49 indeterminate results were retested later by the requesting clinicians. The retesting period varied between 1-16 weeks after the initial test. We found that in 77% (10/13) of these, valid results were obtained (i.e. either positive or negative).

Among the 49 ITR, 31 were females and 18 males representing 3.9% (31 of 795) and 2.8% (18 of 634) of the total population.

ITR were observed more frequently in autumn and winter (34/642=5.2%) than in spring and summer (15/787=1.9%).

32 of 909 (3.5%) of tests requested by the University Hospital and private doctors in Lausanne were indeterminate, compared to 17 of 520 (3.3%) among tests sent from hospitals or physicians outside Lausanne, including the University outpatient dept of Geneva.

ITR were more frequent in children less than 5 years and in elderly patients. The frequency of ITR is significantly higher for elderly patients 75-84 years (8/57=14%) and for patients >85 years (7/21=33%) (Fig). 11/15 samples with ITR from patients aged >75 were sent to the laboratory during the cold season.

In multivariate analysis (Table), the two parameters found to significantly affect the rate of ITR were old age (>75 year old vs. 5-75 years old, OR=7.97 95%CI 3.97-15.44, p=0.006) and the season during which samples were transported (autumn & winter vs. spring & summer, OR=3.47 95%CI 1.75-7.51, p=0.0007). The sex of the subjects, the distance that the sample travelled and young age were found not to significantly affect the ITR.

Among 37 ITR with clinical information, rheumatologic disease was reported in 11 (29.7 %), cardiovascular disease in 7 (18.9%), underweight in 3 (8.0%), HIV in 2 (5.4%), haematological disorder in 2 (5.4%), oncologic disease in 1 (2.7%), chronic renal failure in 1 (2.7%). The mean age of patients with rheumatologic disorders was 66 years. Nine patients (24.3%) had no known co-morbidity or drug treatment.

Drug treatment was mentioned in 17/37 patients: 6 were treated with steroids, 9 with immunosuppressive therapy (methotrexate, anti TNF-α), 2 with antiretroviral therapy. One patient was in hemodialysis. The other patients did not receive any drug treatment with possible influence on the immune system. As we could not gather sufficiently detailed information about the possible co-morbidities present in all patients (i.e. including those with positive or negative results) we could not statistically determine the affect of co-morbidity or drug treatment on the rate ITR.

Among the 565 patients from the university hospitals in Lausanne and Geneva for whom the indication of the test was known, 302 were tested for contact after TB exposure (including health-care workers), 75 were immigrants from countries with a high incidence of TB and a positive tuberculin skin test, 156 had clinical or radiological suspicion of TB and 32 were immunosuppressed or were tested before the prescription of anti-TNFα therapy. Twelve results were indeterminate, 6/302 (2.0%) among contacts, 1/75 (1.3%) among immigrants, 5/156 (3.2%) among TB suspects and 1/32 (3.1%) among immunosuppressed patients (no statistically significant differences).

Discussion

The vast majority of T-SPOT.*TB* tests performed was clearly positive or negative. Only a very small proportion of tests (3.4%) were indeterminate.

Among the factors considered, gender, distance to the laboratory, young age and indications for the test do not appear to be associated with ITR. The season of sampling was statistically associated with an increased ITR rate. In addition, there appears to be a relationship between ITR and age – with those of very old age (>75 years) and those of young age (<5 years) showing higher ITR rates. However, the number of ITR results (and the number of samples) were small in the very young and very old groups which leads to large confidence intervals. Only the association with old age was statistically significant.

Among patients with ITR, a majority had an associated co-morbidity, particularly rheumatologic disorders (most of them being treated with steroids or methotrexate). As we were unable to collect information about the possible co-morbidities present in all patients with positive or negative results, we cannot demonstrate from these data that co-morbidity or drug treatment alone is a risk factor for ITR; or whether this observation was merely coincident with other factors such as the age of the patients and sample transport conditions. The published evidence on T-SPOT support the conclusion, however, that morbidity and drug treatment are not a significant cause of indeterminate results [9, 12, 14, 20-22]

Over 75% of initially indeterminate results gave clear positive or negative results upon retesting. In similar retesting of indeterminate samples in 2006 we found that 79% (19/24) initially indeterminate results retested later gave clear positive or negative results. If these results are characteristic of our entire indeterminate population, retesting would have reduced the overall indeterminate from 3.4% to less than 1%. This is, however, speculative as only a part of the ITR were retested. Our advice, based upon these results would be to retest all indeterminate results within 4 weeks.

Indeterminate results arise from three test observations: insufficient response to the mitogen positive control, unspecific background staining in the wells and/or non-specific IFN-gamma release by the PBMCs in the well (resulting in a high nil control spot count). These effects may, in principle, be caused by three broad categories of effects:
1) Drug and disease effects on the patient's immune system. These may cause a weak response of the patient's immune system to the mitogen positive control yielding a response insufficiently strong to measure (e.g lymphopenia)[23]
2) Degradation of the patient's sample due to the effect of transport, affecting the viability of the T cells within the sample (and the mitogen response)[24].
3) Technical errors in the performance of the test by laboratory personnel – which may cause all three indeterminate reactions.

The fact that the indeterminate responses resolved quickly in the majority of retested cases suggests that, the second two reasons were the cause of most indeterminate reactions (namely either technical errors in processing the sample, or inappropriate storage conditions during transport). We might speculate that the remaining persistent indeterminates were due to persistent inability of the patient's samples to respond, consistent with either old age or chronic drug treatment, both of which do not change over the 2 week retesting period. (Among the four persistent ITR, two were from patients ≥85 years of age, and a further one from a patient >75 years of age).

The higher rates of ITR we observed in cold seasons may have been a consequence of exceeding the recommended limits for temperature and duration of transport [8, 24] Actually, ITR was not significantly associated with the distance travelled by the sample (up to 15 hours) and the T-SPOT.*TB* assay therefore appears robust even if the 8 hour time limit is exceeded. The strong association with season suggests that the temperature at which the sample is stored during shipment is perhaps more important than the distance or duration of transport.

Concern has been raised about the use of the new IGRAs in certain populations (for example: children, the elderly and the immunosuppressed) due to the high rate of indeterminate results[25-27]. Our results show that T-SPOT.*TB* performed well in all patient groups and the indeterminate rates of 3.4% overall and 3.1% amongst the immunosuppressed are consistent with the published literature on this test. As the two IGRA tests use different methodologies, differences in the ITR between them would be expected and have been observed in head to head studies [17,22]. Therefore, the reservations over the use of the new IGRAs should not necessarily apply equally to both tests. Based on our results, we feel that the use of T-SPOT.*TB* should not be restricted on account of the potential for indeterminate results as this is a relatively rare occurrence in all patients except the very old. In addition, an indeterminate result may give useful information about the functional status of the lymphocytes of the patient and may point to a condition associated with immunosuppression [31].

The main limitation of this study is the fact that, due to its retrospective character, the immune status (CD4 cell count and tuberculin skin test reaction) could not be assessed for the patients with an ITR. The presence of immunodeficiency or immunosuppressive treatment was known for a subgroup of patients from the hospital and did not seem to influence the results. Another potential limitation is the fact that only a minority of patients were very young or very old. Finally, although retesting seems to decrease the proportion of ITR, not all samples were retested, so that we cannot take firm conclusions on this point.

In conclusion, only a very small proportion of T-SPOT.*TB* tests performed under routine field conditions in a private laboratory gave indeterminate results and these were mainly associated with old age. Careful attention to the pre-analytical conditions should minimize this proportion.

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		T SPOT-TB		
Parameter	*Variable*	*OR*	*P value*	*95% CI*
Sex of subject	Female vs. Male	1.26	0.47	0.68-2.36
Age Group	>75 vs. 5-75	7.97	0.006	3.97-15.44
Age Group	0-4 vs. 5-75	2.27	0.84	0.12-12.40
Sample transport conditions	Autumn/Winter vs. Spring/Summer[1]	3.47	0.0007	1.75-7.51
Sample transport conditions	Transported from far vs. near[2]	1.29	0.41	0.70-2.35

Table: Relationship of indeterminate rates with certain parameters. Results expressed as Odds Ratio (OR) of indeterminates in group 1 vs. group 2 for each variable.

[1] Autumn/Winter =21 Sept-20 March inclusive. Spring/Summer = 21 March-20 September inclusive

[2] Far is defined as a location >15km from the laboratory.

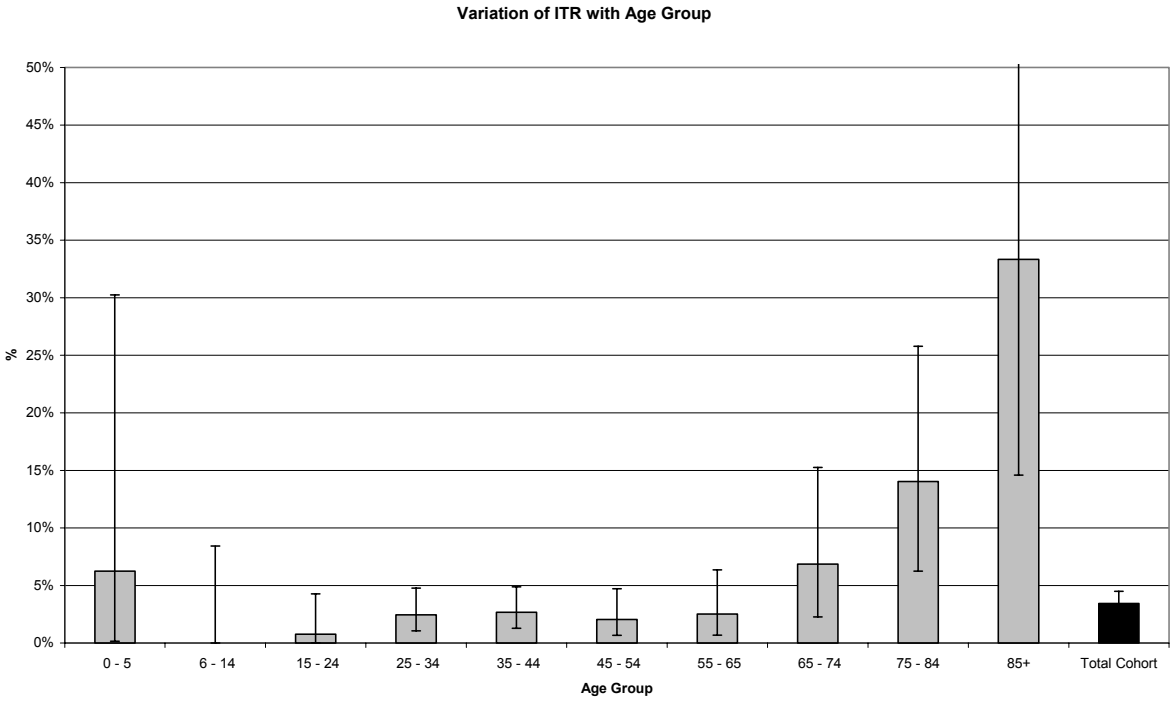

Figure: Proportion of indeterminate test results, by age. Bars show ITR (%) and error bars show 95% Confidence Intervals.

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