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

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ABSTRACT: Interferon-γ release assays for the diagnosis of tuberculosis (TB) can give indeterminate results. The prevalence of indeterminate test results (ITRs) among T-SPOT™.*TB* tests was assessed.

A retrospective analysis of samples processed in 2005 was performed. ITRs were assessed by age, sex, 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 analysed separately.

Of a total of 1,429 tests, 49 (3.4%) were indeterminate. ITRs were significantly associated with old age (>75 *versus* 5–75 yrs; odds ratio (OR) 7.97, 95% confidence interval (CI) 3.968–15.438) and the season during which samples were transported (autumn and winter *versus* spring and summer; OR 3.47, 95% CI 1.753–7.514). The incidence of ITR was 302 (2.0%) among TB contacts, 75 (1.6%) among immigrants, 156 (3.0%) in TB suspects and 32 (3.0%) among immunosuppressed patients. Sex, young age and distance to the laboratory were not associated with the rate of ITR. Of the 13 tests with ITR that were repeated, 10 gave a clear positive or negative result.

Indeterminate test results with T-SPOT™.*TB* under routine conditions were infrequent and more common in individuals aged >75 yrs than in children and younger adults. The incidence of indeterminate test results was low and similar among healthy tuberculosis contacts, immigrants with a positive tuberculin skin test, tuberculosis suspects and the immunosuppressed. The conditions of transportation may influence the incidence of indeterminate test results.

KEYWORDS: Interferon-γ release assays, latent tuberculosis infection, T-SPOT™.*TB*, tuberculosis

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

Two new interferon-γ release assays (IGRAs), based on *in vitro* detection of interferon-γ released by T-cells in response to antigens specific to *Mycobacterium tuberculosis* and encoded by the RD1 region, are available for the diagnosis of TB infection: T-SPOT™.*TB* (Oxford Immunotec, Abingdon, Oxfordshire, UK) and QuantiFERON®-TB Gold (Cellestis, Carnegie, Victoria, Australia) [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 the T-SPOT™.*TB* test [6–11] and <40% for the QuantiFERON®-TB Gold test [12–17]. Their occurrence seems to be associated with immunosuppression [10, 18] and very young or very old age (patients aged <5 or >80 yrs).

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

METHODS

Retrospective analysis

A retrospective analysis of all T-SPOT™.*TB* tests performed in 2005 was conducted. The tests were requested by the regional Office of Public Health, public and private hospitals in Lausanne, Geneva and other remote hospitals in Switzerland, private physicians and organisations caring for immigrants. The indications were: 1) contact

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tracing for TB after contact with an index case; 2) surveillance of exposed healthcare workers; 3) assessment of immigrants with positive TSTs discovered at entry in Switzerland; 4) suspicion of active TB; and 5) 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, peripheral blood mononuclear cells (PBMC) were resuspended and standardised to 250,000 cells·well^{-1} and incubated overnight with phytohaemagglutinin, early secretory antigenic target-6 or culture filtrate protein-10. Spot-forming units (SFUs) were read manually with a magnifying glass.

Results were classified as positive, negative or indeterminate. Indeterminate test results (ITRs) were defined as the presence of >10 SFUs in the nil-control wells (*i.e.* high background) and/or <20 SFUs in the mitogen-positive control wells. Each plate was re-checked in order to determine the technical classification of the ITR.

For all ITRs, the referring physician was contacted in order to obtain further information about any comorbidity, 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, sex, distance from sampling location to the laboratory, season of the year, comorbidity, immune status, drug treatment, indication for the test), the ITR was calculated as a percentage with 95% confidence intervals (CIs). As children aged <5 yrs have unreliable test results in some studies and a higher risk of disease if infected, they were analysed separately. A multivariate analysis was used in order to assess the true underlying relationships between ITR and age, sex, distance to laboratory and season. CIs were calculated using the iterative profile likelihood method and the model was chosen using stepwise regression, starting from the null model, in order to choose parameters.

RESULTS

Of the 1,468 requests, 26 tests could not be performed for technical reasons (broken test tube, insufficient blood sample) and were excluded from further analyses. A total of 13 indeterminate tests were repeated (only the first test was considered in the analysis). Of the remaining 1,429 results, 407 (28%) were positive, 973 (67.8%) were negative and 49 (3.4%) were indeterminate. Of these, 37 (2.6%) were attributed to the absence of sufficient response to mitogen control, 10 (0.7%) were due to a high background in the wells preventing the counting of spots, and two (0.1%) had >10 spots in the nil-control well. Of the 49 individuals with ITRs, clinical information was obtained for 37 patients; the 12 cases without information were excluded from the calculation of the association with comorbidity, immune status and medication.

A total of 13 of the 49 ITRs were re-tested later by clinicians. The re-testing period ranged 1–16 weeks after the initial test. Valid results were obtained (*i.e.* either positive or negative) in 10 (77%) of these.

Among the 49 ITRs, 31 were females and 18 males representing 3.9% (31 out of 795) and 2.8% (18 out of 634) of the total female and male populations, respectively.

ITRs were observed more frequently in autumn and winter (34 (5.2%) of 642 tests performed in autumn and winter) than in spring and summer (15 (1.9%) of 787 performed in spring and summer).

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

ITRs were more frequent in children aged <5 yrs and in elderly patients. The frequency of ITRs was significantly higher for patients aged 75–84 yrs (8 (14%) of 57 test performed in this age-range) and for patients aged >85 yrs (7 (33%) of 21 tests performed in this age-range; fig. 1). Of the 15 samples with ITR from patients aged >75 yrs, 11 were sent to the laboratory during the autumn and winter.

In multivariate analysis (table 1), the two parameters found to significantly affect the incidence of ITR were old age (>75 *versus* 5–75 yrs; odds ratio (OR) 7.97, 95% CI 3.97–15.44; p=0.006) and the season during which samples were transported (autumn and winter *versus* spring and 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 (0–4 *versus* 5–75 yrs) were found not to significantly affect the ITR.

Among the 37 ITRs with clinical information, rheumatologic disease was reported in 11 (29.7%), cardiovascular disease in seven (18.9%), the subject was underweight in three (8.0%), HIV positive in two (5.4%), haematological disorder in two (5.4%), oncologic disease in one (2.7%) and chronic renal

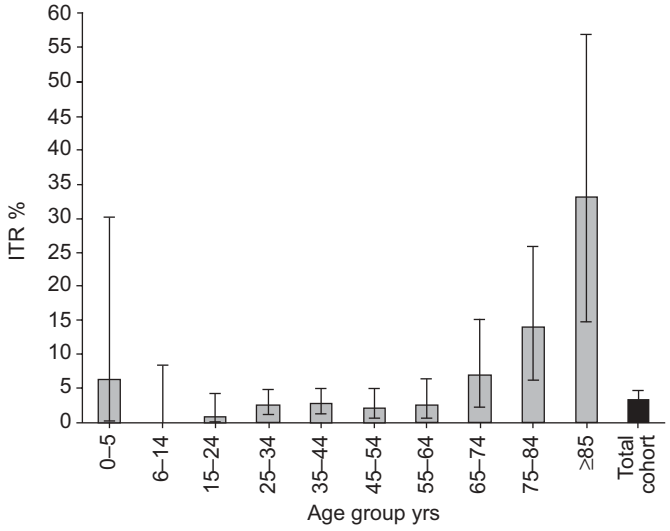

FIGURE 1. Proportion of indeterminate test results (ITR) stratified by age. Whiskers represent 95% confidence intervals.

TABLE 1 Relationship of indeterminate incidence with certain parameters

Parameter	T-SPOT™.*TB*	
	OR (95% CI)	p-value
Sex		
Female *versus* male	1.26 (0.68–2.36)	0.47
Age group		
>75 *versus* 5–75 yrs	7.97 (3.97–15.44)	0.006
0–4 *versus* 5–75 yrs	2.27 (0.12–12.40)	0.84
Sample transport conditions		
Autumn and winter *versus* spring and summer[#]	3.47 (1.75–7.51)	0.0007
Transported from far *versus* from near[¶]	1.29 (0.70–2.35)	0.41

Data are expressed as odds ratios (OR) of indeterminates in group 1 *versus* group 2 for each variable. CI: confidence interval. [#]: autumn and winter are defined as September 21–March 20, inclusive, and spring and summer are defined as March 21–September 20, inclusive; [¶]: far is defined as >15 km from the laboratory.

failure in one (2.7%). The mean age of patients with rheumatologic disorders was 66 yrs. Nine (24.3%) patients had no known comorbidity or drug treatment.

Drug treatment was received by 17 of the 37 patients with ITRs: six were treated with steroids; nine with immunosuppressive therapy (methotrexate, anti-tumour necrosis factor (TNF)-α); two with antiretroviral therapy; and one patient was on haemodialysis. The other patients did not receive any drug treatment with possible influence on the immune system. As sufficiently detailed information could not be obtained about the possible comorbidities present in all patients (*i.e.* including those with positive or negative results), the affect of comorbidity or drug treatment on the incidence of ITR could not be statistically determined.

Of 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 healthcare workers), 75 were immigrants from countries with a high incidence of TB and had a positive TST, 156 had clinical or radiological suspicion of TB, and 32 were immunosuppressed or were tested before the prescription of anti-TNF-α therapy. Of this group of 565 patients, 12 test results were indeterminate; six (2.0%) of the 302 tested were among contacts; one (1.3%) among the 75 immigrants; five (3.2%) among the 156 TB suspects; and one (3.1%) among the 32 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, sex, 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 yrs) and those of very young age (<5 yrs) showing a higher ITR incidence. However, the number of ITRs (and the number of samples) was small in the very young and very old groups, which leads to large CIs. Only the association with old age was statistically significant.

Among patients with ITRs, the majority had an associated comorbidity, particularly rheumatologic disorders (most of them were being treated with steroids or methotrexate). As information was not collected with regard to the possible comorbidities present in all patients with positive or negative results, it cannot be demonstrated from these data whether comorbidity or drug treatment alone is a risk factor for ITR or that this observation was merely coincident with other factors, such as the age of the patients and sample transport conditions. However, the published evidence on T-SPOT™.*TB* [9, 12, 14, 20–22] supports the conclusion that morbidity and drug treatment are not a significant cause of indeterminate results.

Upon re-testing, >75% of initially indeterminate results gave clear positive or negative results. In similar re-testing of indeterminate samples in 2006 it was found that 19 (79%) of the 24 initially indeterminate results gave clear positive or negative results. If these results are characteristic of the entire indeterminate population, re-testing would have reduced the overall indeterminate population from 3.4% to <1%. This is, however, speculative, because not all the ITRs were re-tested. Thus, all indeterminate results should be re-tested within 4 weeks.

Indeterminate results arise from three test observations: 1) insufficient response to the mitogen positive control; 2) unspecific background staining in the wells; and/or 3) nonspecific interferon-γ 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 may cause a weak response 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 transport, affecting the viability of the T-cells within the sample (and, thus, the mitogen response) [24]; and 3) technical errors in the performance of the test by laboratory personnel, which may result in all three indeterminate reactions.

The fact that the indeterminate responses were quickly resolved in the majority of re-tested cases suggests that the latter two causes were likely to occur in most indeterminate reactions (*i.e.* either technical errors while processing the sample or inappropriate storage conditions during transport). It could be speculated that the remaining persistent indeterminates were due to persistent inability of the patients' samples to respond, consistent with either old age or chronic drug treatment, both of which do not change over the 2-week re-testing period. Of the four persistent ITRs, two were from patients aged ⩾85 yrs and one from a patient aged 75–84 yrs.

The higher rates of ITR observed in cold seasons could potentially have been a consequence of exceeding the recommended limits for temperature and duration of transport [8, 24] However, ITR was not significantly associated with the distance travelled by the sample (<15 h) and so the T-SPOT™.*TB* assay, therefore, appears robust even if the 8-h 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 (*e.g.* children, the elderly and the immunosuppressed) due to the high rate of indeterminate results [25–27]. The present results show that T-SPOT™.*TB* performed well in all patient groups and the indeterminate rates of 3.4% overall and 3.1% among 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, in fact, 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 the present results, the use of T-SPOT™.*TB* should not be restricted on account of the potential for indeterminate results, as these occur relatively rarely 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 [23].

The main limitation of the present study is the fact that, due to its retrospective character, the immune status (CD4 cell count and TST reaction) could not be assessed for 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 small number of patients were very young or very old. Finally, although re-testing seemed to decrease the proportion of ITRs, not all samples were re-tested and a firm conclusion cannot be made 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 and conditions of transportation. Careful attention to the pre-analytical conditions should minimise this proportion.

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