Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak

Katie Ewer, Jonathan Deeks, Lydia Alvarez, Gerry Bryant, Sue Waller, Peter Andersen, Philip Monk, Ajit Lalvani

Summary

Background The diagnosis of latent tuberculosis infection relies on the tuberculin skin test (TST), which has many drawbacks. However, to find out whether new tests are better than TST is difficult because of the lack of a gold standard test for latent infection. We developed and assessed a sensitive enzyme-linked immunospot (ELISPOT) assay to detect T cells specific for Mycobacterium tuberculosis antigens that are absent from Mycobacterium bovis BCG and most environmental mycobacteria. We postulated that if the ELISPOT is a more accurate test of latent infection than TST, it should correlate better with degree of exposure to M tuberculosis.

Methods A large tuberculosis outbreak in a UK school resulted from one infectious index case. We tested 535 students for M tuberculosis infection with TST and ELISPOT. We compared the correlation of these tests with degree of exposure to the index case and BCG vaccination.

Findings Although agreement between the tests was high (89% concordance, κ=0.72, p<0.0001), ELISPOT correlated significantly more closely with M tuberculosis exposure than did TST on the basis of measures of proximity (p=0.03) and duration of exposure (p=0.007) to the index case. TST was significantly more likely to be positive in BCG-vaccinated than in non-vaccinated students (p=0.002), whereas ELISPOT results were not associated with BCG vaccination (p=0.44).

Interpretation ELISPOT offers a more accurate approach than TST for identification of individuals who have latent tuberculosis infection and could improve tuberculosis control by more precise targeting of preventive treatment.

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Introduction

Identification and treatment of people who have latent tuberculosis infection by targeted tuberculin skin testing and preventive therapy is a cornerstone of tuberculosis control in developed countries. The main drawback of the tuberculin skin test (TST) is poor specificity, since previous Mycobacterium bovis BCG vaccination and environmental mycobacterial exposure can lead to false-positive results. More than half the burden of tuberculosis in developed countries is carried by foreign-born immigrants from high-prevalence countries, among whom BCG vaccination and environmental mycobacterial exposure are common. The TST also has several operational drawbacks, including the need for a return visit and operator-dependent variability in placement and reading of the test. A more accurate rapid test for latent infection is a major priority for improved tuberculosis control.

The identification of genes in the M tuberculosis genome that are absent from M bovis BCG and most environmental mycobacteria offers an opportunity to develop more specific tests for M tuberculosis infection. Early secretory antigen target-6 (ESAT-6) and culture filtrate protein 10 (CFP10) are two such gene products that are strong targets of the cellular immune response in tuberculosis patients and contacts. The presence of ESAT-6-specific T cells, detected by the rapid ex-vivo enzyme-linked immunospot (ELISPOT) assay for interferon-gamma, is a highly sensitive and specific marker of M tuberculosis infection in patients who have culture-confirmed tuberculosis; its sensitivity is substantially higher than that for the TST. In a UK pilot study of 50 contacts at risk of latent tuberculosis infection, we noted a correlation between ESAT-6 ELISPOT results and the extent of exposure to tuberculosis cases, whereas unexposed people were uniformly ELISPOT-negative.

In February, 2001, a secondary school student who had had a chronic cough for 9 months was diagnosed with sputum-smear-positive cavitatory pulmonary tuberculosis. The health authority screened 1128 of 1208 students at the school with TST and diagnosed 69 secondary cases of active tuberculosis and 254 cases of latent infection. This outbreak presented a unique opportunity to compare the effectiveness of the ELISPOT assay with the TST.

In the absence of a gold standard reference test, direct assessment of the sensitivity and specificity of a new test for latent tuberculosis infection is impossible. However, since airborne transmission of M tuberculosis is promoted by increasing duration and proximity of contact with an infectious case, a key determinant of infection is the amount of time spent sharing room air with the source case. We formed the hypothesis that if the ELISPOT assay is a more sensitive and specific test than the TST, it should correlate more closely than the TST with degree of exposure to M tuberculosis and should be independent...
Samples were processed and scored by two scientists who from 545 of the 550 students, 2–4 h after venepuncture.

Tuberculosis were classified as having active tuberculosis positive radiological or clinical findings suggestive of disease. These students were treated with standard short-course chemotherapy for 6 months, including pyrazinamide and ethambutol for the first 8 weeks.

Risk factors for tuberculosis exposure outside school

History of household contact 36 (6-7%)  
Born in high-prevalence country* 76 (14-2%)  
Ethnic origin  
Indian 436 (81.5%)  
Pakistani 35 (6.5%)  
White 19 (3.6%)  
Mixed race 19 (3.6%)  
Black African 12 (2.2%)  
Black Caribbean 7 (1.3%)  
Other 7 (1.3%)  

Clinical

BCG vaccinated 467 (87-3%)  
Heaf grade (equivalent induration after 10 TU Mantoux)  
Negative 0 (<5 mm) 141 (26-4%)  
1 (<5 mm) 129 (24-1%)  
2 (5-14 mm) and BCG vaccinated 110 (20-6%)  
Positive 2 (5-14 mm) and BCG unvaccinated 10 (1.9%)  
3 (>15 mm) 83 (15.5%)  
4 (>15 mm) 62 (11-5%)  
Diagnosis based on conventional criteria  
Latent tuberculosis infection 128 (23-9%)  
Active tuberculosis disease 27 (5-0%)  

*India (44), Pakistan (nine), Bangladesh (three), Africa (Malawi, Kenya, and Tanzania, 14), Portugal (two), and Greece, Malaysia, Sri Lanka, and Turkey (one each).

Table 1: Characteristics of students tested by TST and ELISPOT

Participants

The study was approved by the Leicestershire ethics committee. We invited 963 students, aged 11–15 years, from the same school as the index case to participate. We obtained written informed consent from 594 (62%) children and their parents. In May and June, 2001, the school nurses interviewed 550 (57% of the total invited) of these children about place of birth and history of tuberculosis exposure outside school. At the same time they drew 10 mL blood samples that were stored in sequentially numbered heparinised containers.

TST and ELISPOT testing

Leicestershire Health Authority screened 1128 children with the Heaf test, in accordance with UK guidelines for tuberculosis contacts (table 1).25,26 535 of whom were in our sample of 550. Screening was done over 2 weeks, from March 26 to April 11, 2001, 2 months after the index case was admitted to hospital for treatment.

Tuberculin skin testing was done by standard multiple-needle Heaf test with a six-needle disposable-head Heaf gun (Bignall Surgical Instruments, Littlehampton, UK)26 and concentrated purified protein derivative 100 000 tuberculin units per mL (Evans Medical, Liverpool, UK), in accordance with national guidelines.26 Heaf tests were administered and read by the medical and nursing staff of the outbreak management team. Cutaneous induration was scored 1 week later, in accordance with standard guidelines, from grade 0 to 4.25 Generally, although the read-out of the automated Heaf test is quantified less precisely than the Mantoux test—ie, grades 0–4 instead of mm of induration, a continuous variable—the two tests generally correlate well with each other,27–29

Students who reported symptoms underwent chest radiography and clinical assessment for possible active tuberculosis, irrespective of skin test results. Asymptomatic students with Heaf grades 0 or 1 or Heaf grade 2 and a BCG scar or documented history of BCG vaccination were deemed uninfected30,29 and no action was taken; students with Heaf grades 3 or 4 (irrespective of BCG vaccination history) or grade 2 with no evidence of previous BCG vaccination were deemed infected.24 All underwent chest radiography and those with normal radiographs were deemed to have latent tuberculosis infection and received 3 months’ chemoprophylaxis with rifampicin and isoniazid. Students with abnormal radiographic findings or with symptoms were further assessed in hospital for active tuberculosis; those with positive cultures for M tuberculosis from clinical samples or positive radiological or clinical findings suggestive of tuberculosis were classified as having active tuberculosis disease. These students were treated with standard short-course chemotherapy for 6 months, including pyrazinamide and ethambutol for the first 8 weeks.

We did ELISPOT assays in Oxford on blood samples from 545 of the 550 students, 2–4 h after venepuncture. Samples were processed and scored by two scientists who had no access to personal identifiers or TST results. How ELISPOT assays are done has been previously described,14 for this study we used a simplified, faster protocol incorporating ELISPOT plates precoated with monoclonal antibody to interferon-gamma (Mabtech AB, Stockholm, Sweden), and a detector monoclonal antibody to interferon-gamma precoated to alkaline-phosphatase (Mabtech). Plates were seeded with 2·5×105 peripheral blood mononuclear cells per well; duplicate wells contained no antigen (negative control), phytohaemagglutinin (positive control; ICN Biomedicals, OH, USA), recombinant dimeric ESAT-6 (dimESAT-6), or one of 12 different peptide pools derived from ESAT-6 and CFP10. After overnight incubation at 37°C, 5% carbon dioxide2,4 different peptide pools, no antigen (negative control wells). This cut-off14 was predefined before the results were revealed. Assays were deemed positive if they contained a mean of at least five more spot-forming cells than the mean of the negative control wells. This cut-off14 was predefined before the results were revealed. Assays were deemed positive if there was a positive response to one or more pools of the ESAT-6-derived or CFP10-derived peptides, or to dimESAT-6.

As previously described,14 we used peptides spanning the length of ESAT-6 and CFP10 (ResGen, Huntsville, AL, USA). Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues; purity was more than 70%. Peptides were arranged into 12 pools comprising two arrays of six pools each, where each array contained all 35 peptides from both molecules in equivalents of induration after 10 TU Mantoux)
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stratification of TST-positive students with presumed latent tuberculosis infection by ELISPOT result

128 TST-positive students with no clinical or radiographical signs of active tuberculosis disease. Each circle represents one student: white=students with direct classroom exposure to index case; grey=students with no direct exposure.

(p<0.002), with substantially more Heaf grade 3 (81 of 467 vs 2 of 68, p=0.001), and grade 2 results in BCG-vaccinated individuals (table 3).

Of the 128 participants presumed to have latent tuberculosis infection on the basis of a positive TST with no evidence of active tuberculosis, 97 (76%) tested positive with ELISPOT. This ELISPOT-positive subgroup had significantly higher Heaf grades and significantly more exposure to *M. tuberculosis* than did the ELISPOT-negative students. Heaf grades were significantly higher among ELISPOT-positive students than among ELISPOT-negative students (p<0.0001, figure 2). In the ELISPOT-positive group there were significantly more students with direct exposure to the index case than in the ELISPOT-negative group (35 of 97 vs one of 31, p<0.0001; figure 2).

Agreement between TST and ELISPOT was high (κ=0.72 [95% CI 0.64-0.80], p<0.0001), with concordant results in 475 (89%) students (table 4). For students in whom test results were discordant, it is impossible to know for certain which test was correct because there is no reference test. However, table 4 shows that an isolated positive ELISPOT result (ie, one associated with a negative TST) was a strong predictor of *M. tuberculosis* exposure, whereas an isolated positive TST result was not. This finding suggests that isolated positive ELISPOT results are more likely to be true positives than are isolated positive TST results. For students with positive TST and ELISPOT results, the relative risk of direct exposure to the index case, compared with that for students with negative TST and ELISPOT, was 17.6

### Table 3: Effect of previous BCG vaccination on ELISPOT and TST results

<table>
<thead>
<tr>
<th>Vaccinated (n=467)</th>
<th>Unvaccinated (n=68)</th>
<th>p for vaccinated vs unvaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISPOT Positive</td>
<td>131 (28.1%)</td>
<td>16 (23.5%)</td>
</tr>
<tr>
<td>ELISPOT Negative</td>
<td>336 (71.9%)</td>
<td>52 (76.5%)</td>
</tr>
<tr>
<td>Heaf grade 1</td>
<td>52 (11.1%)</td>
<td>10 (14.7%)</td>
</tr>
<tr>
<td>Heaf grade 2</td>
<td>81 (17.6%)</td>
<td>2 (2.9%)</td>
</tr>
<tr>
<td>Heaf grade 3</td>
<td>110 (23.6%)</td>
<td>10 (14.7%)</td>
</tr>
<tr>
<td>Heaf grade 4</td>
<td>116 (24.8%)</td>
<td>13 (19.1%)</td>
</tr>
<tr>
<td>Heaf grade 5</td>
<td>108 (23.1%)</td>
<td>33 (48.5%)</td>
</tr>
</tbody>
</table>

*p* test for trend across all five Heaf grades.

Table 4: Analysis of concordant and discordant test results

<table>
<thead>
<tr>
<th>Students’ exposure to <em>M. tuberculosis</em></th>
<th>Mean (range) duration of direct exposure (weeks)</th>
<th>Possible causes of false-positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct exposure (n [%])</td>
<td>In index case’s class (n [%])</td>
<td>In year 9 (n [%])</td>
</tr>
<tr>
<td>TST+ ELISPOT+ (n=121)</td>
<td>42 (34-7)</td>
<td>18 (14-9)</td>
</tr>
<tr>
<td>TST- ELISPOT+ (n=26)</td>
<td>6 (23-1)</td>
<td>18 (7-7)</td>
</tr>
<tr>
<td>TST+ ELISPOT- (n=34)</td>
<td>2 (5-9)</td>
<td>0</td>
</tr>
<tr>
<td>TST- ELISPOT- (n=354)</td>
<td>7 (2-0)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test results</th>
<th>BCG vaccinated (n [%])</th>
<th>Foreign born (n [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>TST+/ELISPOT+ (n=121)</td>
<td>42 (34-7)</td>
<td>18 (14-9)</td>
</tr>
<tr>
<td>TST+/ELISPOT- (n=354)</td>
<td>7 (2-0)</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 4: Analysis of concordant and discordant test results

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tuberculosis infection is determined by exposure to mycobacterial exposure. Unlike TST, it is not confounded by environmental exposure, its independence from foreign birth suggests strongly with all other measures of positive. Given that the ELISPOT assay correlates exposure) only the TST was significantly more likely to be differing by one and three bands each.

Molecular strain typing by variable-number tandem repeat, mycobacterial interspersed repetitive unit, and spoligotyping showed that all nine secondary isolates of M tuberculosis from students at the school were identical to that of the index case. IS6110-based restriction fragment length polymorphism (figure 3) showed that seven of the nine secondary isolates were identical to that of the index case, whereas two were very similar, differing by one and three bands each.

Discussion
In the absence of a gold standard test for latent tuberculosis infection, the sensitivity and specificity of the ELISPOT assay or the TST cannot be directly quantified. However, given that the likelihood of latent tuberculosis infection is determined by exposure to M tuberculosis, we were able to rank the tests according to their diagnostic accuracy. Agreement between TST and ELISPOT results was high, but discordance in 11% of students shows that the tests are not equivalent. Our results indicate that ELISPOT probably has higher sensitivity and specificity than TST. First, the significantly closer correlation of ELISPOT than TST with degree of exposure to M tuberculosis suggests a higher sensitivity for detection of latent infection. Second, TST, but not ELISPOT, was confounded by BCG vaccination, despite 11–15 years having elapsed since vaccination, which suggests a higher specificity for the ELISPOT assay. TST and ELISPOT were more likely to be positive in students who had a history of household tuberculosis contact, a marker of M tuberculosis exposure outside school, than in students without such a history. By contrast, for the students born in high-prevalence countries, mainly Africa and Asia (a risk factor for environmental mycobacterial exposure) and M tuberculosis exposure) only the TST was significantly more likely to be positive. Given that the ELISPOT assay correlates strongly with all other measures of M tuberculosis exposure, its independence from foreign birth suggests that, unlike TST, it is not confounded by environmental mycobacterial exposure.

The high specificity of ELISPOT might explain the strong relation between positive ELISPOT results and TST induration size in individuals who have positive TST results. The size of the TST response is positively associated with higher tuberculosis case rates during follow-up; thus, the ELISPOT assay may have identified the subgroup of TST-positive individuals who actually have latent tuberculosis infection. These individuals are distinct from those whose weakly positive TST responses represent false-positive results due to antigenic cross reactivity of purified protein derivative. Moreover, the ELISPOT-negative group had substantially more exposure to M tuberculosis than did the ELISPOT-negative group. This improved specificity of the ELISPOT could help to avoid unnecessary chemoprophylaxis in uninfected individuals; this ability to screen out false-positive TST results will become increasingly important as the prevalence of latent infection falls in low-prevalence countries. The cross-reactivity of purified protein derivative may explain why a new whole-blood interferon-gamma ELISA based on purified protein derivative seemed to be confounded by BCG.

There is compelling evidence that the outbreak we studied was due to one index case, who was the first symptomatic case of pulmonary tuberculosis in the school. The molecular epidemiology also suggests that this was a point-source outbreak. Only two other children were potentially infectious. Both children were symptomatic for less than 2 weeks before admission to hospital and both were in year 11, which did not participate in the study. Moreover, their M tuberculosis isolates were identical to that of the index case by all four typing methods.

The high rates of tuberculosis infection and disease at the school are unlikely to merely reflect the epidemiology of tuberculosis in the local community. First, this outbreak accounted for a third of all tuberculosis cases in Leicester in 2001. Second, all 1226 household contacts of the 69 tuberculosis cases and 254 cases of latent tuberculosis infection were screened by the health authority and no cases of infectious pulmonary tuberculosis were identified. Third, four other Leicester schools were screened by TST, and the rates of positive skin tests were 1–4%. Fourth, when year 8 students at this school underwent Heaf testing in 1997–98 only 2·7% were positive. The minimum exposure to an infectious person that is required for M tuberculosis transmission is unknown, but must be low, since many well-documented cases of infection result from brief exposure and many students who did not share lessons with the source case must have acquired infection in this way. The amount of exposure required before transmission of M tuberculosis becomes inevitable is also unknown. Since all students with 5 or more school-weeks of exposure had positive results on the ELISPOT assay, however, our findings suggest that 130 h sharing room air with a person with sputum smear-positive cavitary tuberculosis is certain to result in infection.

Longitudinal assessment of the positive predictive value of this assay for subsequent development of active tuberculosis will be necessary. In one report workers suggest that T-cell responses to ESAT-6 in healthy contacts are associated with subsequent active disease. Students in our study who had positive ELISPOT but negative TST results, who have not had chemoprophylaxis, are receiving close clinical and radiographic follow-up. We used the Heaf test, because it is used for tuberculosis testing in contact investigations in the UK, and is stipulated in national guidelines. Since the Mantoux
method is more widely used internationally, ELISPOT could be compared in the future with this method; we have recently started such studies in several countries. ELISPOT gives quantitative results the morning after taking a blood sample and is more convenient, objective, and rapid than the TST. Although TST is cheap, related indirect costs are associated with return visits and the trained staff required to administer and read the test. Introduction of ELISPOT might initially increase the cost of tuberculin control, but the savings that would follow from improved diagnosis of latent tuberculosis infection could make it very cost effective in the long term. Better detection of latent infection would lessen the number of cases of active tuberculosis and, therefore, the attendant cost of diagnosis, hospital admission and contact tracing. Fewer false-positive results in uninfected contacts would avoid the costs associated with unnecessary chemoprophylaxis and its associated toxic effects.

Contributors
Ajit Lalvani, Katie Ewer, Jonathan Deeks, Gerry Bryant, and Philip Monk designed the study. Ajit Lalvani coordinated the study. Katie Ewer and Lydia Alvarez did the ELISPOT assays. Sue Waller interviewed and enrolled all the participants. Demographic information was obtained and recorded by Sue Waller, Gerry Bryant, and Philip Monk. Jonathan Deeks compared the duration of exposure and did the statistical analysis. Peter Andersen synthesised the recombinant ESAT-6 and provided technical advice and support. Ajit Lalvani wrote the paper with help from Katie Ewer and Jonathan Deeks, and all researchers reviewed the final report.

Conflict of interest statement
AL is the named inventor on several patents related to T-cell-based diagnosis filed by the University of Oxford since 1996. Regulatory approval and commercialisation of the ELISPOT assay will be undertaken by a spin-out company of the University of Oxford (Oxford Immunotec), in which AL has an equity stake. PA is the named inventor of several patents filed by Statens Serum Institute relating to the discovery of M tuberculosis-specific antigens.

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