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

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Summary

Introduction

Identification and treatment of people who have latent tuberculosis infection by targeted tuberculosis skin testing and preventive therapy is a cornerstone of tuberculosis control in developed countries.\(^1\) 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.\(^2\) 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.\(^4\) 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.\(^3\)

The identification of genes in the M. tuberculosis genome that are absent from M. bovis BCG and most environmental mycobacteria\(^5\) offers an opportunity to develop more specific tests for M. tuberculosis infection.\(^10\) 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.\(^11-15\) The presence of ESAT-6–specific T cells, detected by the rapid ex-vivo enzyme-linked immunospot (ELISPOT) assay for interferon-gamma,\(^13\) 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.\(^13\) 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,\(^11\) whereas unexposed people were uniformly ELISPOT-negative.\(^11\)

In February, 2001, a secondary school student who had a chronic cough for 9 months was diagnosed with sputum-smear-positive cavitary 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.\(^1\) However, since airborne transmission of M. tuberculosis is promoted by increasing duration and proximity of contact with an infectious case,\(^16-19\) a key determinant of infection is the amount of time spent sharing room air with the source case.\(^20\) 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...
of BCG vaccination status. Two measures of exposure were prespecified at the time of study design: proximity to
the index case, based on school class and year, and hours
direct classroom contact. Three features of this
outbreak made it particularly suitable for this
investigation: there was one infectious index case with
several hundred contacts; the outbreak occurred in an
enclosed environment; and school timetables permitted
precise quantification of the amount of time each child
spent sharing room air with the source case.

Patients and methods

Participants

The study was approved by the Leicestershire research
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),\(^3\)
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-
puncture Heaf test with a six-needle disposable-head Heaf
gun (Bignall Surgical Instruments, Littlehampton, UK)\(^3\)
and concentrated purified protein derivative 100 000
tuberculin units per mL (Evans Medical, Liverpool, UK),
in accordance with national guidelines.\(^4\) 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.\(^5\) 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.\(^6,7\)

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 uninfected\(^8,9\) 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.\(^10,11\) All
previous 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,\(^12,13\) 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 preconjugated to alkaline-phosphatase
(Mabtech). Plates were seeded with 2.5×10\(^5\) 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 dioxide,
plates were developed with preconjugated detector
antibody and chromogenic substrate, BCIP/NBT\(^14,15\) (Moss
Inc, Pasadena, MD, USA).

Assays were scored by automated ELISPOT counter
(AID-GmbH, Strassberg, Germany). We scored test wells
as positive if they contained a mean of at least five more
spot-forming cells than the mean of the negative control
wells and this number was at least twice the mean of the
negative control wells. This cut-off\(^16\) 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,\(^17\) 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

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contrasting combinations, so that each peptide was tested in quadruplicate.

We cloned, expressed, and purified DimESAT-6 from culture supernatant of recombinant Lactococcus lactis; purity was more than 95%.

Ascertainment of exposure
We classified school students into four groups of decreasing degrees of exposure to the index case, based on proximity and shared activities in school: the same class as the index case; students in classes in the same year (year 9) who regularly shared classes with the index case; students in classes in the same year who shared only weekly school events; and students in different years (7, 8, and 10) who shared no school events with the index case (figure 1). For students in the same school year, we used the school timetable to quantify direct exposure to the index case, taking into account the attendance record of the index case during the likely infectious period, which, on the basis of duration of cough and associated symptoms, was 9 months. Since the index case mixed with different students for each academic subject, substantial numbers of students were exposed. We classified students from other school years (years 7, 8, and 10) who had lessons in classrooms immediately after they had been vacated by the index case as indirectly exposed. Direct and indirect exposure are expressed in school-weeks (equivalent to 26 h 40 min).

Statistical methods
We focused the analysis on estimating the strength of association between degree of exposure to the index case and the ELISPOT and TST test results. Odds ratios are a function of test sensitivity and specificity,\textsuperscript{27} and increase as one or both of these measures increase. For latent tuberculosis infection we could not estimate test sensitivity and test specificity directly, but were able to estimate odds ratios relating results of each test to the likelihood of infection. We estimated the increase in odds of a positive test result for unit increase in exposure by logistic regression. We used matched-pairs logistic regression to assess the significance of the difference in the associations between the tests, taking account of the correlation between TST and ELISPOT test results. Similar analyses were undertaken to investigate whether ELISPOT and TST test results were associated with BCG vaccination, place of birth, and household tuberculosis contact. All reported p values are two sided.

Role of the funding source
The sponsors of the study had no role in the study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the paper for publication.

Results
ELISPOT and TST results were available for 535 students—44.3% of the school. Our sample was representative in terms of the proportion of non-white children (97% in our sample vs 93% in the whole school); UK-born children (86% vs 86%); children diagnosed with active tuberculosis (5% vs 6%); and participants deemed to have latent tuberculosis infection on the basis of TST result (24 vs 23%, table 1).

The odds of a test result being positive for each increase across the four stratified exposure groups increased by a factor of 2.78 (95% CI 2.22–3.48, table 2).

**Table 2:** Odds ratios (95% CI) of the relations of ELISPOT and TST with intensity of M tuberculosis exposure in school and with risk factors for exposure outside school

<table>
<thead>
<tr>
<th>Risk factors for exposure to M tuberculosis outside school</th>
<th>ELISPOT</th>
<th>p</th>
<th>TST*</th>
<th>p</th>
<th>p for TST vs ELISPOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Born in high-prevalence country (n=76)</td>
<td>1.09 (0.99–1.29)</td>
<td>0.05</td>
<td>1.79 (1.18–2.69)</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>History of household tuberculosis contact (n=36)</td>
<td>1.09 (0.99–1.31)</td>
<td>0.05</td>
<td>1.79 (1.05–2.88)</td>
<td>0.03</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Positive result defined as Hanfe grade 2 or Hanfe grade 2 without BCG.

**Figure 1:** TST and ELISPOT results for students stratified by decreasing proximity to index case based on school year and class

T+=TST positive. T-=TST negative. E+=ELISPOT positive. ELISPOT+=ELISPOT negative.
A: students in same class as index case. B: students in classes in same year who regularly shared lessons with index case. C: students in the four remaining classes in same year who shared only weekly school events but no lessons with index case. D: students in different years who shared no school events with index case.
p<0.0001) for the ELISPOT assay and 2.33 (1.88–2.88, p<0.0001) for the TST. The ELISPOT assay correlated significantly better with increasing exposure across the four groups than did the TST (p=0.03; figure 1, table 2).

Direct exposure of the 148 children in year 9 ranged from 0 to 17 school weeks; 57 students had some direct classroom exposure, with a median of 2-2 school weeks (IQR 1.4–13.4). The odds of a positive ELISPOT result increased by a factor of 2.51 (1.58–3.99, p<0.0001) with each week of direct exposure, which was significantly higher (p=0.007) than that for the TST (odds ratio 1.30 [95% CI 1.01–1.54], p=0.002; table 2).

Of the 387 children in years 7, 8, and 10, 196 pupils had indirect exposure, up to a maximum of 1.16 weeks. Although ELISPOT and TST were more likely to be positive with increasing exposure, neither showed a significant correlation (table 2).

ELISPOT assay and TST were positively correlated with a history of household tuberculosis contact (n=36, table 2). 76 children were born in countries with a high prevalence of tuberculosis and climates associated with increased exposure to environmental mycobacteria (table 1). The mean duration of residence in these countries was 7–8 years. TST results, but not ELISPOT results, were significantly associated with birth in one of these regions (table 2).

For 362 students, the date of BCG vaccination was documented in the Leicestershire Health Authority records, of whom 323 were vaccinated at birth. An additional 105 students had BCG scars but the date of vaccination was not available because they were born outside Leicester; 101 were born in countries where BCG vaccination is given at birth. Therefore 424 (91%) of 467 BCG-vaccinated students were vaccinated at birth. The ELISPOT assay showed no significant relation with BCG vaccination status (p=0.44, table 3). By contrast, BCG-vaccinated children were significantly more likely to have higher Heaf grades than unvaccinated children (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>Heaf grade</th>
<th>Vaccinated (n=467)</th>
<th>Unvaccinated (n=68)</th>
<th>p for vaccinated vs unvaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>131 (28.1%)</td>
<td>16 (23.5%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Negative</td>
<td>336 (71.9%)</td>
<td>52 (76.5%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>52 (11.1%)</td>
<td>10 (14.7%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>81 (17.6%)</td>
<td>2 (2.9%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>110 (23.6%)</td>
<td>10 (14.7%)</td>
<td>0.002*</td>
</tr>
<tr>
<td>1</td>
<td>116 (24.8%)</td>
<td>13 (19.1%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>108 (23.1%)</td>
<td>33 (48.5%)</td>
<td></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>Test results</th>
<th>Direct exposure (n [%])</th>
<th>In index case’s class (n [%])</th>
<th>In year 9 (n [%])</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>
<td>67 (55.4)</td>
<td>2-42 (0-16-9)</td>
<td>106 (87.6)</td>
</tr>
<tr>
<td>TST- ELISPOT- (n=26)</td>
<td>6 (23.1)</td>
<td>2 (7.7)</td>
<td>15 (57.7)</td>
<td>1-22 (0-16-2)</td>
<td>25 (96.2)</td>
</tr>
<tr>
<td>TST+ ELISPOT+ (n=34)</td>
<td>2 (5.9)</td>
<td>0</td>
<td>11 (32.4)</td>
<td>0-12 (0-2-8)</td>
<td>27 (79.4)</td>
</tr>
<tr>
<td>TST- ELISPOT- (n=354)</td>
<td>7 (2.0)</td>
<td>0</td>
<td>55 (15.5)</td>
<td>0-03 (0-2-21)</td>
<td>309 (87-3)</td>
</tr>
</tbody>
</table>

p

<table>
<thead>
<tr>
<th>Test results</th>
<th>0.0001</th>
<th>0.005</th>
<th>&lt;0.0001</th>
<th>NT</th>
<th>0.34</th>
<th>0.54</th>
</tr>
</thead>
<tbody>
<tr>
<td>TST+ ELISPOT+ vs TST- ELISPOT-*</td>
<td>0.18</td>
<td>1.0</td>
<td>0.03</td>
<td>NT</td>
<td>0.19</td>
<td>0.003</td>
</tr>
</tbody>
</table>

N=no statistical test undertaken due to skewed distributional shape because most students had no direct exposure. *p* values are for comparison of epidemiological characteristics of students with an isolated positive test result vs characteristics of students negative for both tests.
However, both 

For positive.

exposure (4·2–33·2, p<0·001); for those with negative TST and positive ELISPOT results it was 11:7 (4·2–33·2, p<0·001); and for those with positive TST and negative ELISPOT results, it was 2·97 (0·6–13·7, p=0·18).

Molecular strain typing by variable-number tandem repeat, mycobacterial interspersed repetitive unit, and spoligotyping showed that all nine secondary isolates of 

TST, 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 Mycobacterium 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-positive 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 Mycobacterium 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 Mycobacterium 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 Mycobacterium 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 tuberculin testing in contact investigations in the UK, and is stipulated in national guidelines. Since the Mantoux
method is more widely used internationally, ELISPOT can 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 tuberculosis 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 computed 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 Institut relating to the discovery of Mycobacterium tuberculosis-specific antigens.

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