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Identifying ELISPOT and skin test cut-offs for diagnosis of *Mycobacterium tuberculosis* infection in The Gambia

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SUMMARY

SETTING: An urban area, The Gambia.

OBJECTIVE: To identify ELISPOT and PPD skin test cut-offs, targeting sensitivity and specificity equivalence.

DESIGN: Tuberculosis cases >15 years of age and their household contacts underwent ELISPOT, HIV and PPD skin tests. Cases and contacts sleeping in a different house were used to estimate sensitivity and specificity, providing two planes for estimating cut-offs. Specificity was adjusted for infection from previous exposure using a multivariate discrimination algorithm.

RESULTS: The point on the line of intersection of the planes that maximised sensitivity and specificity equivalence occurred at 4 spots (95% confidence interval [CI] 3.5–5, multiplier = 0) for CFP-10 and 5.5 spots (4.5–8, multiplier = 0 for ESAT-6), yielding a sensitivity and specificity of 76% for both antigens. Combining ESAT-6 and CFP-10 using an 'or' statement yielded a maximum equivalence sensitivity and specificity of 76.5% at 6 spots for ESAT-6 and 11.5 spots for CFP-10. For the PPD skin test sensitivity and specificity, an equivalence of 78% occurred at 11 mm induration (9–13 mm).

CONCLUSION: An ELISPOT cut-off for ESAT-6 or CFP-10 could be set at 4–8 spot forming units (20–40 spots per million), with little benefit from combining the results. A cut-off of 9–13 mm for the PPD skin test is reasonable when comparing with the ELISPOT.

KEY WORDS: ELISPOT; PPD skin test; cut-off; sensitivity; specificity

THE DIAGNOSIS of *Mycobacterium tuberculosis* (TB) infection in humans in the absence of disease is inherently problematic in TB endemic tropical settings. The ability of a test to diagnose disease could be regarded as an indicator of sensitivity to infection. However, the inability to be certain that any individual is not infected poses major problems for the calculation of specificity. Furthermore, the traditional purified protein derivative (PPD) skin test antigens are not specific for *M. tuberculosis* and the test is subject to waning: an induced response is negative within 2 years in over 80% of individuals in tropical settings.[1]

Immunoassays for gamma-interferon (IFN-γ) production in response to early secreted antigenic target 6 (ESAT-6)/culture filtrate protein 10 (CFP-10) have been evaluated in TB cases, their contacts and the general community. They have been shown to be relatively sensitive in TB cases (>80%), approaching 100% specificity in non-endemic general communities, and are not influenced by previous bacille Calmette-Guérin (BCG) vaccination.[2–4] Using a gradient of increasing likelihood of *M. tuberculosis* infection, we have shown that the enzyme-linked immunospot (ELISPOT) assay probably improves specificity in the diagnosis of *M. tuberculosis* in The Gambia, but at the cost of some sensitivity.[5] There are several possibilities as to why we found evidence for a sensitivity cost, including that two *M. tuberculosis*-specific antigens may not be sufficient for the diagnosis of *M. tuberculosis* infection.[6] One possible reason is that the cut-off used for a positive result might be wrong.

Published studies have utilised different criteria for positivity for the ELISPOT test and different cut-offs for protein vs. peptide responses.[4,7] None of these criteria have been justified or subjected to scrutiny. In a phenotypically well characterised population of TB cases and their household contacts in The Gambia, we used mathematical tools to search for appropriate cut-offs for the two tests, assuming a target of equivalence for sensitivity and specificity.

METHODS

Participants

Sputum smear-positive TB index cases over 15 years of age and their household contacts were recruited in Greater Banjul, as previously described.[8] Blood samples were taken for ELISPOT and human immuno-

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deficiency virus (HIV) testing after informed consent. TB contacts were categorised according to where they slept: in the same bedroom as the case, in a different bedroom in the same house, or in a different house.

Cases and contacts underwent a PPD skin test (2 tuberculin units [TU], PPD RT23, Statens Serum Institut [SSI], Copenhagen, Denmark). Induration was recorded at 48–72 h. Subjects with a mean skin test diameter of ⩾10 mm were offered a chest X-ray and those with symptoms underwent clinical assessment. Those found to have TB disease were referred to the National TB Programme for free treatment.

This study was approved by the Gambia Government/Medical Research Council joint ethics committee.

Laboratory procedures

Sputum smears were prepared and stained with auramine-phenol[9] and confirmed by Ziehl-Neelsen (ZN). Decontaminated specimens were inoculated and mycobacterial cultures identified and confirmed using standard procedures, as previously described.[5]

The ex-vivo ELISPOT assays for IFN-γ were performed as previously described.[10] For this study, synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (Advanced Biotechnology Centre, Imperial College, London, UK) were used. Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. ESAT-6 and CFP-10 peptides, used at 5 μg/ml, were each divided equally and sequentially into pools of peptides. PPD (*M. tuberculosis*, RT49, SSI) was used at 10 μg/ml. The positive control was phytohaemagglutinin (PHA, Sigma-Aldrich UK, Poole, UK). All antigens were tested in duplicate, with the spot forming units (SFUs) averaged over the duplicate wells.

Assays were scored by an ELISPOT counter (AID-GmbH, Strassberg, Germany). PHA-positive control wells were set to at least 150 SFUs above negative control wells. For this study, negative control wells were required to have fewer than 20 SFUs. Quantitative counts were represented as SFUs above the negative control well.

Testing for HIV-1 or HIV-2 infection was by competitive enzyme-linked immunosorbent assay (ELISA) (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France). HIV-infected subjects were referred to our specialist HIV clinic and excluded from the analysis.

Data management

The numbers of SFUs/well were automatically entered into a relational ACCESS database (Microsoft Corp, Redmond, WA, USA).[11] All other data were added by double data entry and checked for errors.

Mathematical modelling

The standard method for defining a cut-off is determined by the inequalities:

$$\text{antigen SFU} \geqslant A + \text{negative control SFU and}$$
$$\text{antigen SFU} \geqslant B \times \text{negative control SFU}$$

where A (the corrected count) and B (a multiplier to account for a high negative control count) are typically taken as 5–10 and 2 SFU, respectively.[7]

Preliminary analysis revealed that the distribution of the ELISPOT SFUs showed no significant evidence of bimodality. Therefore, techniques that assume underlying mixture distributions could not be used to estimate a cut-off. Assuming that case contacts sleeping in a different house to the index case were the least likely to be infected, the proportion of subjects who were negative for particular values of A and B was used to estimate specificity. Index cases were used to estimate the sensitivity as the proportion of subjects who are positive for particular values of A and B. This gave two planes of sensitivity and specificity estimates from which appropriate cut-off points were selected.

We adjusted the specificity to allow for *M. tuberculosis* infection from previous exposure (including exposure to the index case): a multivariate discrimination algorithm was used to identify infected contacts. Each subject was classified as a case or a contact, and certain variables (SFUs from CFP-10 wells, SFUs from ESAT-6 wells and SFUs for the negative control wells) were used for the adjustment. A canonical discrimination[12] rule was then derived by taking the linear combination of the variables that maximised the difference between the cases and contacts. Each subject was scored using the linear combination of variables and contacts with 'high' scores were assumed to be infected and dropped from the specificity analysis. All statistical analyses were conducted using Stata software (version 8, Stata Corp, College Station, TX) and Matlab software (MathWorks Inc, Natick, MA, USA).

RESULTS

Eighty-five HIV-negative TB cases had ELISPOT results that met inclusion for analysis (77 with skin test results), plus 220 HIV-negative contacts who slept in a different house (215 with skin test results). For comparison across an exposure gradient, 137 (126 with skin test results) contacts sleeping in the same bedroom as a case and 313 (302 with skin test results) sleeping in a different bedroom in the same house were HIV-negative and had ELISPOT results that met the criteria for analysis.

Varying A from 0 to 20 and B from 0 to 4 gave the sensitivity and specificity planes in Figure 1 for CFP-10 (a) and ESAT-6 (b), where specificity is the upper plane. It can be seen that the standard cut-offs of A = 10 and B = 2 gave a sensitivity of 55% and specificity of 90% for CFP-10 and a sensitivity of 62% and specificity of 80% for ESAT-6. The intersection of the planes that maximised an equally weighted combina-

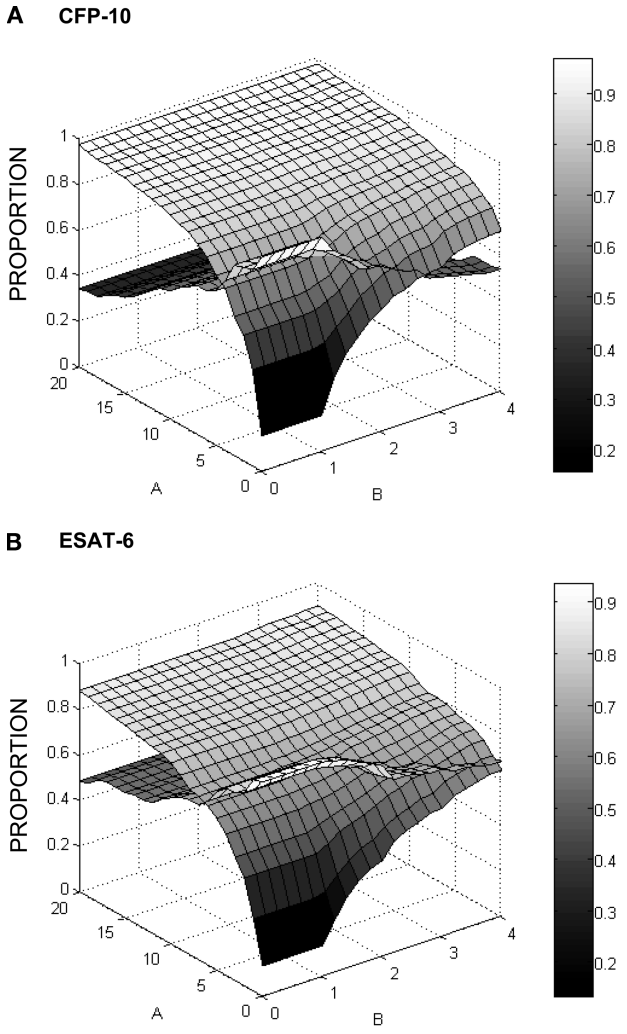

Figure 1 Sensitivity and specificity planes, expressed as a proportion, for (**A**) CFP-10 and (**B**) ESAT-6. Sensitivity is represented by the lower plane, specificity by the upper plane. A. Cut-off (SFU above the negative control well). B. Multiplier (see Methods). CFP = culture filtrate protein; ESAT = early secreted antigenic target.

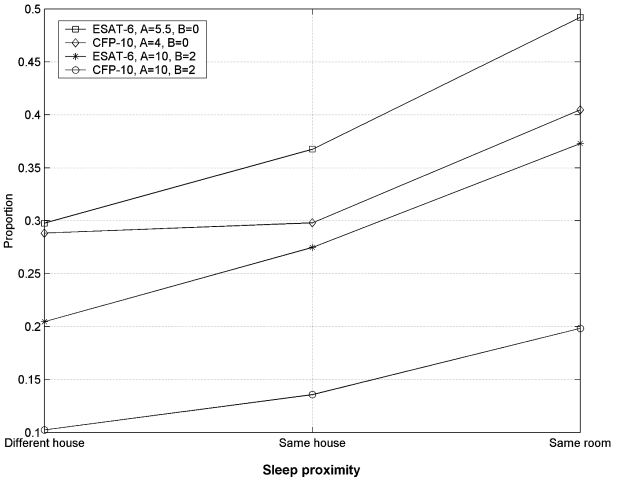

Figure 2 Proportion of TB case contacts that were ELISPOT test positive by sleeping exposure gradient for various cut-offs. A. SFU above the negative control well. B. Multiplier (see Methods). ESAT = early secreted antigenic target; CFP = culture filtrate protein; TB = tuberculosis; SFU = spot forming units.

By reducing the cut-off, estimates for the sensitivity of the ELISPOT increased and those for specificity decreased. To gain further insights into this, we assessed the new cut-offs in our case contacts against a natural exposure gradient according to sleeping proximity to a case (Figure 2).[5] It can be seen that the reduced cut-offs increased the proportions of positive subjects without compromising the slope of the line of increasing positivity across the exposure gradient. Indeed, the slope was seen to increase slightly.

We assessed the added value of combining the ESAT-6 and CFP-10 results, introducing an 'or' statement. Assuming B = 0, varying cut-offs of ESAT-6 and CFP-10 between 0 and 20 SFU were plotted against sensitivity and specificity in an adjusted analysis (Figure of sensitivity and specificity occurred at A = 4.5 (bootstrapped 95%CI 4–6), B = 0 for CFP-10 and A = 6.5 (bootstrapped 95%CI 5.5–10), B = 0 for ESAT-6. This yielded an 'equivalence' sensitivity and specificity of 73% for CFP-10 and 71% for ESAT-6.

Twenty (9.3%) of 215 case contacts were misclassified using the canonical discriminator, and the median maximum SFU above negative control for these subjects was 39. This adjustment increased the specificity and decreased the cut-offs to A = 4 (bootstrapped 95%CI 3–5), B = 0 for CFP-10 and A = 5.5 (bootstrapped 95%CI 4.5–8), B = 0 for ESAT-6, for equally weighted sensitivity and specificity. This yielded a sensitivity and specificity of 76% for both CFP-10 and ESAT-6. Therefore, the same cut-off is not necessarily appropriate for the two antigens, and the multiplication factor B had little effect on the sensitivity and specificity.

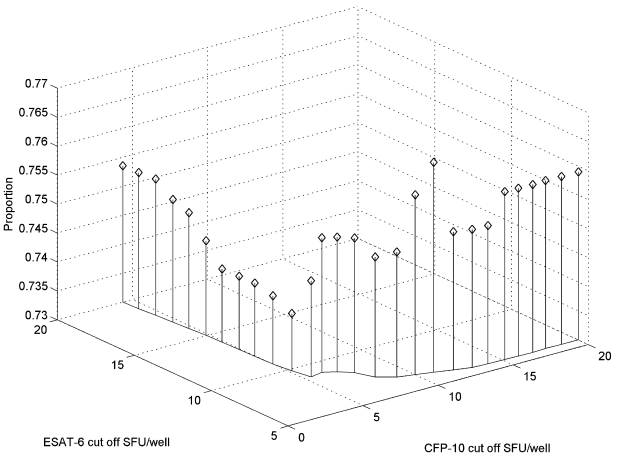

Figure 3 Equivalence sensitivity and specificity, expressed as a proportion, plot for CFP-10 and ESAT-6 when used together. ESAT = early secreted antigenic target; SFU = spot forming units; CFP = culture filtrate protein.

Table Estimates of ELISPOT sensitivity and specificity (%) according to different cut-offs for ESAT-6 and CFP-10 (SFU/well above negative control well) when used in combination

ESAT-6 cut-off, SFU/well	CFP-10 cut-off, SFU/well																			
	3		4		5		6		7		8		9		10		11		12	
	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
3	90.9	50.8	87	53.8	85.7	54.9	85.7	57.4	85.7	57.4	85.7	58.5	84.4	58.5	84.4	59	84.4	59	83.1	59
4	90.9	53.3	87	59	85.7	60.5	85.7	63.6	85.7	64.1	85.7	65.6	84.4	66.2	84.4	67.2	84.4	67.7	83.1	67.7
5	90.9	55.4	85.7	62.1	84.4	63.6	84.4	67.2	84.4	67.7	84.4	69.7	83.1	70.3	83.1	71.3	83.1	72.3	81.8	72.3
6	87	56.9	80.5	64.1	77.9	66.2	77.9	69.7	77.9	70.8	77.9	72.8	76.6	73.3	76.6	74.4	76.6	75.4	75.3	75.4
7	85.7	57.9	77.9	65.6	75.3	67.7	75.3	71.8	75.3	72.8	75.3	74.9	74	75.4	74	76.4	74	77.4	72.7	77.4
8	85.7	57.9	77.9	66.2	75.3	68.2	75.3	72.3	75.3	73.8	75.3	75.9	74	76.4	74	77.4	74	78.5	72.7	78.5
9	85.7	59	77.9	68.2	75.3	70.3	75.3	74.9	75.3	76.4	75.3	78.5	74	79	74	80.5	74	81.5	72.7	81.5
10	84.4	61.5	76.6	71.3	74	73.3	74	77.9	74	79.5	74	81.5	72.7	82.1	72.7	83.6	72.7	84.6	71.4	84.6
11	84.4	62.6	76.6	72.3	74	74.4	72.7	79	72.7	81	72.7	83.1	71.4	84.1	71.4	85.6	71.4	86.7	70.1	86.7
12	84.4	62.6	76.6	72.3	74	74.4	72.7	79	72.7	81	72.7	83.1	71.4	84.1	71.4	85.6	71.4	86.7	70.1	86.7

ESAT = early secreted antigenic target; CFP = culture filtrate proteins; SFU = spot forming units; Sens = sensitivity; Spec = specificity.

ure 3). The equivalence point of sensitivity and specificity according to various combinations of ESAT-6 and CFP-10 cut-offs all ranged from 74% to 76.5%, with the maximum at the intersection of an ESAT-6 cut-off of six spots and CFP-10 at 11.5 spots. The Table shows estimates of sensititivity and specificity of the combined analysis according to the cut-off for either antigen. It can be seen that if the individual antigen cut-offs were used with an 'or' statement, any sensitivity gain was accompanied by an equal or larger specificity loss.

Figure 4 shows the sensitivity and specificity lines by mm of induration for the PPD skin test, together with the adjusted specificity by the multivariate discrimination algorithm. The median reaction dimension of the discarded contacts was 14.5 mm. It can be seen that equivalence of sensitivity and specificity for the unadjusted analysis was 75% at 12 mm induration (bootstrapped 95%CI 9.5–14 mm) and for the adjusted analysis, it was 78% at 11 mm induration (bootstrapped 95%CI 9–13 mm). Figure 5 shows PPD skin test positivity across the exposure gradient for various cut-offs. There was very little difference across the exposure gradient in test performance for 10, 11 or 12 mm cut-offs, but a 15 mm cut-off showed a less dramatic change across the gradient, implying a disproportionate sensitivity cost per unit gain in specificity.

DISCUSSION

This analysis has taken advantage of a large case contact study to estimate test sensitivity and specificity of the ELISPOT and PPD skin test using mathematical tools and a proven gradient of exposure to a TB case. We have shown that the cut-off for a positive ELISPOT result should be approximately 4–8 SFU (20–40 SFU per million) above the negative control well, depending on the antigen concerned, and that the requirement for the result to be at least twice the negative control well is not necessary. The use of the two antigens ESAT-6 and CFP-10 together did not increase the performance of the test. We also showed that the

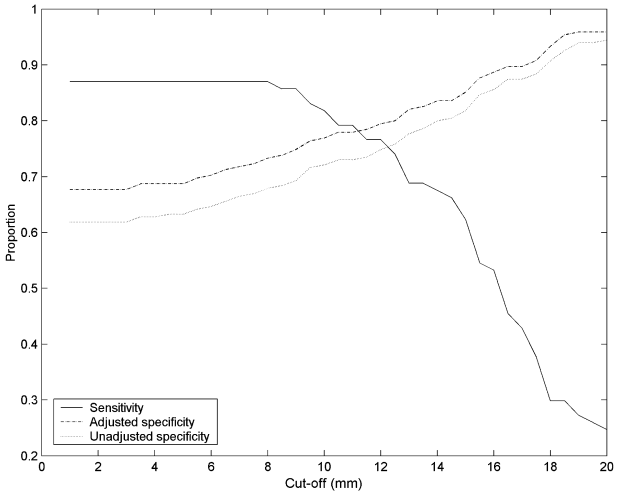

Figure 4 Sensitivity and specificity, expressed as a proportion, for the PPD skin test according to mm of induration. PPD = purified protein derivative.

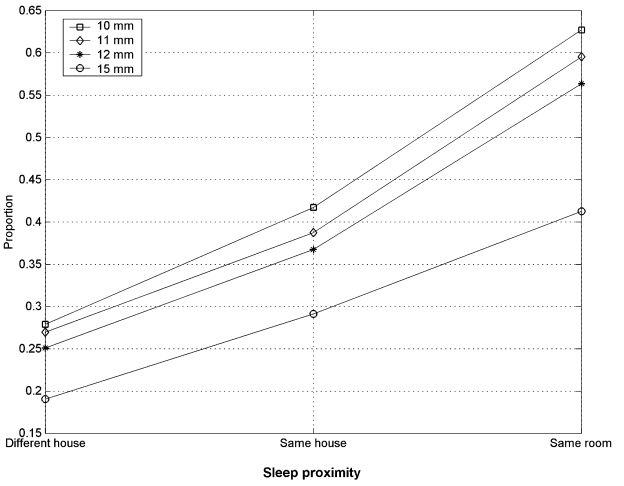

Figure 5 Proportion of TB case contacts that were PPD skin test positive by sleeping exposure gradient for various cut-offs (mm induration). TB = tuberculosis; PPD = purified protein derivative.

PPD skin test, in our setting, reaches equivalent sensitivity and specificity at approximately 11 mm of induration, with little loss of performance at a standard 10 mm cut-off. These results explain some, but not all of the poor sensitivity performance of the ELISPOT in our previous publication and assist in understanding how to make a fair comparison of the ELISPOT and the PPD skin tests in our endemic tropical setting.

The technique used in this study can be used to determine appropriate cut-offs for any prescribed weighted average of both sensitivity and specificity. A gradient of exposure such as the one used here can be used to provide a type of validation of the results. It can reveal whether a certain cut-off maintains or improves on the slope of the change in positivity according to exposure, indicating whether it yields acceptable false-positive or false-negative results.

Interestingly, we were unable to improve upon the estimated sensitivity and specificity of single antigens by using them together. Any sensitivity gain that might appear was accompanied by an equal or greater specificity loss, presumably because of the imprecision associated with the technology currently available to determine the spot count. Scientifically, this is not surprising, as ESAT-6 and CFP-10 antigens are similar in size, are in one operon and are expressed simultaneously in a similar ratio.[13,14] Our finding should be interpreted, however, with a degree of caution: we have previously shown that the response to CFP-10 peptide antigen is significantly lower than that to ESAT-6 in The Gambia,[15] while in other locations (Zambia,[16] India,[17] Denmark[18] and the Netherlands)[19] the converse was found. Therefore, the issue of whether there is added benefit from using more than one antigen should be addressed by studies in other locations and by assessing new antigens that are not closely related to ESAT-6 or CFP-10.

It is impossible to recruit a TB naïve population in The Gambia and other endemic settings, but using populations from other locations is likely to introduce confounding factors, such as ethnicity. The techniques used for adjustment cannot be fully verified without an experimental design that allows the TB status of both cases and contacts to be determined. However, further research on existing data could compare other approaches, such as mixture models to identify infected case contacts,[20] with the multivariate discrimination method used here. Assuming a certain level of endemic infection, bounds on the specificity errors can be obtained using a combinatorial probabilistic approach.

While cases have been utilised by others to provide sensitivity estimates for diagnostic tests for *M. tuberculosis* infection,[20,21] the results could be biased by the inclusion of anergic subjects. Of the TB cases in this study, three of 77 failed to respond at all to both CFP-10 and ESAT-6. We minimised the effect of anergy by excluding HIV-positive individuals, as others have done,[20] but it is an unavoidable weakness in this study design.

The approach we have described can be used to assess, comparing two very different tests with an unknown correct cut-off, what would be a fair comparison between them. This goes some way to eliminating differences seen that are simply related to the cut-off used. For example a 10 mm cut-off for the PPD skin test compared to 10 spots/well cut-off for the ELISPOT is probably an unfair comparison—the 10 mm of skin test induration may more appropriately be compared to a 4–8 spots/well cut-off. Secondly, as is well practised with the skin test, the decision about cut-off for the ELISPOT should be one that takes into account the balance of need for sensitivity and specificity in a particular setting. When it is important not to miss any infected individual, a cut-off of approximately four spots/well is reasonable. When specificity, while maintaining reasonable sensitivity, is a priority, a cut-off of 10 spots/well is appropriate. Above 10 spots, the sensitivity reduces dramatically per unit gain in specificity. A similar dramatic loss of sensitivity per unit gain in specificity occurs with respect to the PPD skin test >15 mm induration. We recommend that 4–8 SFU/well (20–40 SFU/million cells) be utilised to compare the performance of the ELISPOT with a 9–13 mm cut-off for the PPD skin test, at least in tropical TB-endemic settings.

Acknowledgements

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RÉSUMÉ

CONTEXTE : Une zone urbaine en Gambie.
OBJECTIF : Identifier les limites de positivité du test ELISPOT et du test cutané à la PPD en visant une équivalence en matière de sensibilité et de spécificité.
SCHÉMA : Des cas de tuberculose âgés de >15 ans ainsi que leurs contacts au domicile ont eu un test ELISPOT, un test VIH et un test cutané à la PPD. Les cas et les contacts dormant dans des maisons différentes ont été utilisés pour estimer la sensibilité et la spécificité, ce qui fournissait deux plans pour l'estimation des limites de positivité. La spécificité a été ajustée pour l'infection provenant d'une exposition antérieure en utilisant un algorithme multivarié de discrimination.
RÉSULTATS : Le point sur la ligne d'intersection des plans qui obtenait l'équivalence maximale de sensibilité et de spécificité est survenu à 4 points (IC95% 3,5–5 ; multiplicateur = 0) pour CFP-10 et 5,5 points (4,5–8 ; multiplicateur = 0) pour ESAT-6, ce qui obtenait une sensibilité et une spécificité de 76% pour les deux antigènes. La combinaison de ESAT-6 et de CFP-10 utilisant une prise de position « ou » a fourni une équivalence maximale de sensibilité et de spécificité de 76,5% à 6 points pour ESAT-6 et à 11,5 points pour CFP-10. L'équivalence de 78% pour la sensibilité et la spécificité du test cutané PPD se situe à une induration de 11 mm (9–13 mm).
CONCLUSION : Une limite de positivité dans le test ELISPOT pour ESAT-6 ou CFP-10 a pu être placée à 4–8 unités formant points (20–40 points par million), la combinaison des résultats n'ayant que peu d'avantages. Une limite de positivité de 9–13 mm au test cutané à la PPD est raisonnable en cas de comparaison avec l'ELISPOT.

RESUMEN

MARCO DE REFERENCIA : Una zona urbana en Gambia.
OBJETIVO : Determinar los valores discriminatorios del análisis inmunoenzimático ELISPOT y de la prueba cutánea de PPD, a fin de obtener su equivalencia en términos de sensibilidad y especificidad.
MÉTODOS : Se practicó el análisis inmunoenzimático de tinción ELISPOT, la prueba del VIH y la prueba PPD a los pacientes con tuberculosis >15 años y a sus contactos domiciliarios. El cálculo de la sensibilidad y la especificidad se hizo a partir de los casos iniciales y los contactos que dormían en domicilios diferentes, obteniendo así dos planos para determinar los valores discriminatorios de las pruebas. Se aplicó un algoritmo de discriminación de variables múltiples para corregir la especificidad con respecto a la infección por exposición previa.
RESULTADOS : El punto en la línea de intersección de ambos planos que proporcionaba equivalencia máxima de sensibilidad y especificidad correspondió a 4 manchas para el antígeno CFP-10 (IC95% 3,5–5 con multiplicador B = 0) y a 5,5 manchas para ESAT-6 (IC95% 4,5–8 con multiplicador B = 0), con una sensibilidad y especificidad del 76% para ambos antígenos. Cuando se combinaron ESAT-6 y CFP-10 aplicando el operador lógico 'o' se obtuvo una equivalencia máxima de sensi-

bilidad y especificidad del 76,5% para 6 manchas de ESAT-6 y 11,5 manchas de CFP-10. Para la prueba PPD, la equivalencia de sensibilidad y especificidad al 78% se obtuvo con una induración de 11 mm (9 a 13 mm).

CONCLUSIÓN: El valor discriminatorio de un análisis ELISPOT para ESAT-6 o CFP-10 puede fijarse entre 4 y 8 unidades formando manchas (20 a 40 manchas por millón de células) y se obtiene poco beneficio combinando los resultados. Un valor discriminatorio de 9 a 13 mm de induración para la prueba PPD es razonable cuando se compara con el análisis inmunoenzimático de tinción ELISPOT.