Transactions of the Royal Society of Tropical Medicine and Hygiene (2007) **101**, 691–698

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevierhealth.com/journals/trst

ESAT-6 and CFP-10 can be combined to reduce the cost of testing for *Mycobacterium tuberculosis* infection, but CFP-10 responses associate with active disease

Annette Fox*, David J. Jeffries, Philip C. Hill, Abdulrahman S. Hammond, Moses D. Lugos, Dolly Jackson-Sillah, Simon A. Donkor, Patrick K. Owiafe, Keith P.W.J. McAdam [1], Roger H. Brookes [2]

Tuberculosis Division, Bacterial Diseases Programme, Medical Research Council (UK) Laboratories, Banjul, The Gambia

Received 4 November 2006; received in revised form 28 February 2007; accepted 1 March 2007
Available online 16 April 2007

KEYWORDS
Tuberculosis;
Infection;
Disease;
ESAT-6;
CFP-10;
ELISPOT

Summary Commercial tests measuring IFN-γ responses to ESAT-6 and CFP-10 are available for diagnosing *Mycobacterium tuberculosis* infection. Measures that minimize cost and complexity will facilitate their application in less-developed countries. We investigated whether overlapping peptides representing both ESAT-6 and CFP-10 are required to detect *M. tuberculosis* infection in a high TB-burden country, and whether they can be combined in a single pool. ESAT-6 and CFP-10 peptides were compared in IFN-γ enzyme-linked immunospot (ELISPOT) in 183 HIV-negative smear-positive TB cases and 1673 HIV-negative household contacts. Separate peptide pools for each antigen were compared with a combined pool in 498 contacts. Forty per cent of responsive contacts recognized both antigens, 51% only ESAT-6 and 10% only CFP-10, whereas 56% of responsive cases recognized both antigens, 30% only ESAT-6 and 13% only CFP-10. Accordingly, CFP-10 response rates were higher for TB cases (odds ratio 2.409, $P < 0.001$). Low purified protein derivative response rates indicated that responses to CFP-10 only were non-specific in contacts. Agreement between peptides in separate versus combined pools was good ($\kappa = 0.758$, $r = 0.840$). Therefore a combined ESAT-6/CFP-10 peptide pool provided maximum sensitivity and efficiency, but CFP-10 was mainly required to detect active disease.

* Corresponding author. Present address: 20 Alley 1/54 Au Co Road, Tay Ho, Ha Noi, Viet Nam. Tel.: +84 4 7197473.
E-mail address: afox@pacific.net.au (A. Fox).
[1] Present address: Infectious Diseases Institute, Faculty of Medicine, Makerere University, Mulago Hospital Complex, P.O. Box 22418, Kampala, Uganda.
[2] Present address: Sanofi Pasteur, Immunology Research (Sunnybrook S5 44), Connaught Campus, 1755 Steeles Avenue West, Toronto, Ontario, M2R 3T4, Canada.

0035-9203/$ – see front matter
doi:10.1016/j.trstmh.2007.03.001

1. Introduction

The transmission of *Mycobacterium tuberculosis* before tuberculosis (TB) diagnosis is a key factor maintaining the epidemic (Whalen, 2005). The tuberculin/purified protein derivative (PPD) skin test may lack specificity for *M. tuberculosis* infection because it can be induced by exposure to Bacillus Calmette-Guerin (BCG) and non-tuberculous environmental mycobacteria (Black et al., 2002; Huebner et al., 1993). *Mycobacterium tuberculosis* antigens ESAT-6 and CFP-10 (Berthet et al., 1998; Sorensen et al., 1995) are not expressed by BCG or environmental mycobacteria, except *M. gastrii*, *M. kansasii*, *M. marinum*, *M. flavescens* and *M. szulgai* (Behr et al., 1999; Colangeli et al., 2000; Gordon et al., 1999; Harboe et al., 1996). They are highly immunogenic (Dillon et al., 1999; Mustafa et al., 1998; Ulrichs et al., 2000), and studies in low and intermediate TB-prevalence settings have found that tests utilizing them are more specific for *M. tuberculosis* infection than the PPD skin test (Arend et al., 2000a, 2001; Ferrara et al., 2006; Kang et al., 2005; Lalvani et al., 2001b; Lein et al., 1999; van Pinxteren et al., 2000).

Commercial tests using ESAT-6 and CFP-10 may well be more cost-effective than the PPD skin test for contact screening in low TB-prevalence settings (Diel et al., 2006; Wrighton-Smith and Zellweger, 2006) but remain prohibitively expensive in developing countries. Furthermore, the current commercial tests assess ESAT-6 and CFP-10 responses separately and require at least 4 ml blood, which may be prohibitive for paediatric use. In this study we investigated whether it is necessary to include both ESAT-6 and CFP-10 and to test them separately. We assessed ex-vivo IFN-γ enzyme-linked immunospot (ELISPOT) responses to separate and combined pools of overlapping 15mer peptides representing ESAT-6 and CFP-10 in smear-positive pulmonary TB cases and their household contacts in a high-TB-burdened developing country.

2. Materials and methods

2.1. General protocol

Participants were part of a TB case and household contact cohort study described in detail previously (Hill et al., 2004). Briefly, smear-positive pulmonary TB cases aged 15 years and over were recruited upon diagnosis in local Gambian clinics. The average time between index case and contact recruitment was 3 weeks. Contacts were at least 6 months of age and lived the majority of the time in the house of the case or in a separate house on the same compound with the head of the case's household. All participants were offered HIV testing after appropriate counselling. Only HIV-negative subjects were included in the analysis. Tuberculin skin test was performed using 2 TU, PPD (RT23, SSI, Copenhagen, Denmark). Contacts with a positive skin test (defined as a mean diameter of induration of 10 mm or more) underwent further assessment. Co-prevalent and secondary TB cases were referred to the Gambian National TB Control Programme for treatment and were excluded from analysis.

Blood samples were taken from TB cases at recruitment and 12 months later, and from contacts at recruitment and 3 months later. Blood samples from up to 12 participants from two households per recruitment day were processed within 3 hours. Subjects were randomly selected if more than 12 samples were received.

Free and informed consent was obtained from the study subjects or their legal guardians and recruitment was performed according to approved protocols.

2.2. Antigens

Fifteen amino acid peptides overlapping by 10 representing ESAT-6 and CFP-10 were synthesised by ABC, Imperial College, London, UK. Peptides representing each antigen were split into two pools of eight to nine adjacent peptides or tested as single pools in which the concentrations for individual peptides were 5 and 2.5 μg/ml, respectively. Peptides representing both antigens were also tested as a single pool of 33 peptides with individual peptides at 1.25 and 5 μg/ml. Recombinant ESAT-6 produced in *Escherichia coli* (Rv3875, generous gift from Statens Serum Institute, Copenhagen, Denmark) and CFP-10 (Rv3874, provided by Corixa Corp., Seattle, WA, USA) were used at 10 μg/ml. PPD (RT49, SSI, Copenhagen, Denmark) was used at 20 μg/ml. Phytohaemaglutinin (PHA; Sigma-Aldrich, UK) was used at 5 μg/ml.

2.3. ELISPOT

Peripheral blood mononuclear cells (PBMCs) from 1275 successively taken samples were incubated in duplicate with media only, PPD, PHA, and two pools each for ESAT-6 and CFP-10 peptides. For a further 2000 samples, ESAT-6 and CFP-10 peptides were tested as single pools each. PBMC preparation and ELISPOT were performed on the day of sampling by laboratory staff blinded to subject details, and according to published protocols (Lalvani et al., 1997). PBMC, 200 000 per well, were incubated with antigens or media only overnight. Spot-forming unit (SFU) counts were automatically transferred from an ELISPOT counter (AID-GmbH, Strasbourg, Germany) to an ACCESS-based relational database (Jeffries et al., 2004). Positive test wells were predefined as containing at least 10 SFUs more than, and at least twice as many as, negative control wells. For analysis, PHA-positive control wells were required to have at least 150 SFUs more than negative controls, and negative control wells were required to have fewer than 30 SFUs.

2.4. Analysis

Approximately 50% of subjects were tested with overlapping peptides representing ESAT-6 and CFP-10 split into two pools each and the remainder with peptides in one pool for each antigen. Results were consistent for each subset and for all subjects combined, and only combined results are presented. Chi-square test and odds ratios were used to compare ESAT-6 and CFP-10 response rates for ESAT-6/CFP-10-responsive TB cases versus contacts. The significance of discordance between ESAT-6 and CFP-10 was assessed by McNemar's test. SFU counts were compared using Student's *t* test with square-root-transformed data.

The degree to which contrasted antigen formulations concur in their sorting of each subject as positive or negative by ELISPOT was assessed using the kappa statistic. Kappa values of 0.4—0.5, 0.6—0.8 and 0.8—1.0 indicate moderate, good and very good agreement, respectively. SFU counts for contrasted antigen formulations were compared using Spearman's correlation. Differential responsiveness to antigen formulations will be focused among responders and could be masked by a high non-responder rate. We therefore compared SFU counts only for subjects responding positively to at least one antigen using a paired Student's *t* test of the square-root-transformed counts.

All other data were compared using Kruskal-Wallis and the χ^2 test for group-wise contrasts and Mann-Whitney *U* test and Fisher's exact test for pair-wise contrasts of continuous and categorical data, respectively.

3. Results

3.1. Relative response rates for overlapping peptides representing CFP-10 and ESAT-6 in contacts versus TB cases

Three hundred and eighty-four TB cases and 2381 contacts were recruited between June 2002 and August 2004. ELISPOT was performed at recruitment for 243 TB cases and 1963 contacts. Thirty-two TB cases and 123 contacts were excluded from analysis because they were HIV-positive or serology was not available. The results from 28 TB cases and 167 contacts did not meet the predefined inclusion criteria for analysis. TB cases and contacts and the subset that responded to ESAT-6 or CFP-10 in ELISPOT differed with respect to sex, age, percentage with a BCG scar (Table 1) and ethnicity (group-wise χ^2 test $P = 0.026$ for all subjects and $P = 0.003$ for the ELISPOT-positive subset), with a significantly greater proportion of Mandinka among TB cases (Table 1).

There was significant discordance between ESAT-6 and CFP-10, most notably in contacts ($P < 0.001$), because the majority of positive responders recognized only ESAT-6 (Table 2). In contrast, although discordance remained significant in TB cases ($P = 0.002$), the majority of responders recognized both antigens (Table 2). The proportion of responders that recognized ESAT-6 was high and did not differ significantly between TB cases and contacts, whereas the proportion recognizing CFP-10 was significantly higher for TB cases (Table 2). The proportion of responders recognizing only CFP-10 was low and similar for both groups.

For 45 TB cases tested with ESAT-6 and CFP-10 peptides at both recruitment and 12 months post-recruitment, the proportion responding to either antigen dropped from 78% to 51%. While the proportion of responders that recognized ESAT-6 was high at both times (90 and 89%, respectively), the proportion that recognized CFP-10 dropped from 79% at recruitment to 47% 12 months later.

Subjects that recognized CFP-10 did not differ significantly from other responders with respect to sex ($P = 0.427$), percentage with a BCG scar ($P = 0.488$), ethnic distribution ($P = 0.234$) or age ($P = 0.811$). Furthermore, high CFP-10 response rates in TB cases remained significant when controlling for age, gender and ethnic group.

SFU counts were higher for positive ESAT-6 responses than for positive CFP-10 responses in both TB cases ($P < 0.001$) and contacts ($P < 0.001$). We therefore hypothesized that CFP-10 recognition rates are higher for TB cases because the burden of *M. tuberculosis* infection required to generate CFP-10 responses is greater than for ESAT-6 responses. To explore this hypothesis we compared ESAT-6 SFU counts and PPD skin test diameter for subjects that do and do not recognize

Table 1 Characteristics of tuberculosis (TB) cases and contacts included in enzyme-linked immunospot (ELISPOT) analysis and of the ESAT-6/CFP-10 ELISPOT-positive subsets

	All contacts ($n = 1673$)	All TB cases ($n = 183$)	*P*-value	ELISPOT$^+$ contacts ($n = 498$)	ELISPOT$^+$ TB cases ($n = 145$)	*P*-value
Age (years) [median (IQR)]	16 (8—26)	27 (22—36)	<0.001	18 (9—29)	27 (21—37)	<0.001
Female (%)	52	25	<0.001	54	26	<0.001
Male (%)	48	75		46	74	
Ethnicity						
Mandinka (%)	32	41	0.026	25	42	<0.001
Wolof (%)	14	12	0.612	13	11	0.463
Fula (%)	9	14	0.051	12	16	0.320
Serahuli (%)	2	1	1.000	1	0	
Jola (%)	26	21	0.318	29	22	0.165
Aku (%)	1	2	0.207	2	2	0.860
Serere (%)	6	4	0.572	7	4	0.402
Manjago (%)	6	1	0.010	6	0	0.003
Other (%)	5	3	0.312	5	3	0.446
ELISPOT$^+$ (%)	30	79	<0.001	100	100	
PPD skin test (mm) [median (IQR)]	0 (0—15)	16 (11—18)	<0.001	15 (10—18)	16 (12—19)	0.174
With BCG scar (%)	48	26	<0.001	43	29	0.007

IQR: interquartile range; PPD: purified protein derivative; BCG: Bacillus Calmette-Guerin.

Table 2 Proportion of tuberculosis (TB) cases versus healthy household contacts responding to overlapping peptides representing ESAT-6 and CFP-10[a]

Antigen[b]	Cases ($n=183$)	Contacts ($n=1673$)	Odds ratio (95% CI)	P-value
ESAT-6$^+$	126 (87)	448 (89)	0.740 (0.421—1.301)	0.804
CFP-10$^+$	101 (70)	243 (46)	2.409 (1.622—3.576)	<0.001
ESAT-6$^+$CFP-10$^+$	82 (56)	197 (40)	1.989 (1.368—2.891)	<0.001
ESAT-6$^+$CFP-10$^-$	44 (30)	253 (51)	0.422 (0.284—0.626)	<0.001
ESAT-6$^-$CFP-10$^+$	19 (13)	48 (10)	1.414 (0.802—2.492)	0.295
ESAT-6$^-$CFP-10$^-$	38	1178		

[a] Values are expressed as total number, with percentage of positive responders in parentheses.
[b] Each antigen was represented by either a single pool or two pools of eight to nine peptides with the concentration for individual peptides at 2.5 and 5 mg/ml, respectively.

CFP-10, and compared ESAT-6 and CFP-10 response rates for contacts across a gradient of exposure to the TB index case. Both ESAT-6 SFU counts (Figure 1A) and PPD skin test induration diameter (contacts $P=0.013$, TB cases $P=0.083$) were significantly higher for ESAT-6/CFP-10-responsive subjects that recognized CFP-10. The proportion of contacts that responded to both ESAT-6 and CFP-10 peptides increased dramatically with increasing exposure to the index TB case in terms of sleeping proximity (Figure 1B). The proportion of contacts that responded to ESAT-6 only was higher in the low exposure categories but did not increase as dramatically with increasing sleeping proximity to the index case because of the increasing co-recognition of CFP-10 (Figure 1B).

There was little association between recognition of CFP-10 alone in contacts and the level of exposure to the index case (Figure 1B), indicating that such responses may be non-specific. To investigate this possibility, we compared PPD skin and ELISPOT response rates and magnitudes for ESAT-6$^+$CFP-10$^+$, ESAT-6$^+$CFP-10$^-$ and ESAT-6$^-$CFP-10$^+$ contacts. Contacts that recognized only CFP-10 had low PPD ELISPOT and skin test response rates (Table 3), and the diameter of PPD skin test induration was also significantly smaller (Figure 1C). For 163 ESAT-6- or CFP-10-responsive contacts also tested with ESAT-6 and CFP-10 peptides in a single combined pool, response rates were strikingly lower among contacts that recognized CFP-10 only (Table 3). PPD and combined peptide pool responses (Table 3) and PPD skin test induration diameter (data not shown) were not significantly different for cases that recognized only CFP-10 compared to cases that recognized ESAT-6. Consistent with these findings, CFP-10 SFU counts for contacts that recognized only CFP-10 were close to the positive cut-off and significantly lower than for TB cases that recognized CFP-10 only ($P=0.007$). CFP-10 SFU counts were also significantly lower for contacts but not TB cases that recognized CFP-10 only compared to those recognizing both ESAT-6 and CFP-10 (Figure 1D).

3.2. Comparison of ELISPOT responses to ESAT-6 and CFP-10 peptides tested as separate and combined pools

To address the concern that peptide pooling may diminish responses to individual epitopes, we assessed, in contacts, peptides in two pools of eight to nine for each antigen against their respective proteins, and then against increasingly larger pools. Peptides representing ESAT-6 ($n=82$, 88% concordance, $\kappa=0.698$, $r=0.847$, $P<0.001$) and CFP-10 ($n=75$, 91% concordance, $\kappa=0.728$, $r=0.641$, $P<0.001$) performed at least as well as their respective recombinant proteins, and ESAT-6 peptides in a single pool of 16 performed as well as two pools of eight peptides (Figure 2A). Responses to ESAT-6 and CFP-10 peptides in a single combined pool of 33 peptides versus one or two pools each for ESAT-6 and CFP-10 peptides were also similar

Table 3 Characteristics of concordant versus discordant ESAT-6/CFP-10-responsive contacts and cases[a]

	ESAT-6$^+$ CFP-10$^+$	ESAT-6$^+$ CFP-10$^-$	ESAT-6$^-$ CFP-10$^+$	P-value
Contacts				
PPD ELISPOT$^+$	191 (97)[b]	238 (93)[b]	36 (77)	<0.001
Combined ESAT-6/CFP-10 peptide pool$^+$	58 (95)[b]	67 (84)[b]	10 (45)	<0.001
PPD skin test$^+$	163 (86)[b]	178 (76)[b]	24 (56)	<0.001
Cases				
PPD ELISPOT$^+$	77 (94)	40 (91)	17 (89)	0.728
Combined ESAT-6/CFP-10 peptide pool$^+$	18 (90)	12 (92)	7 (78)	0.543
PPD skin test$^+$	47 (80)	25 (89)	9 (75)	0.447

PPD: purified protein derivative.
[a] The number of subjects for which each test was performed varied, and results are presented as number, with percentage of each group positive in parentheses.
[b] Fisher's exact test $P<0.01$ for contrasts with ESAT-6$^-$CFP-10$^+$ subset.

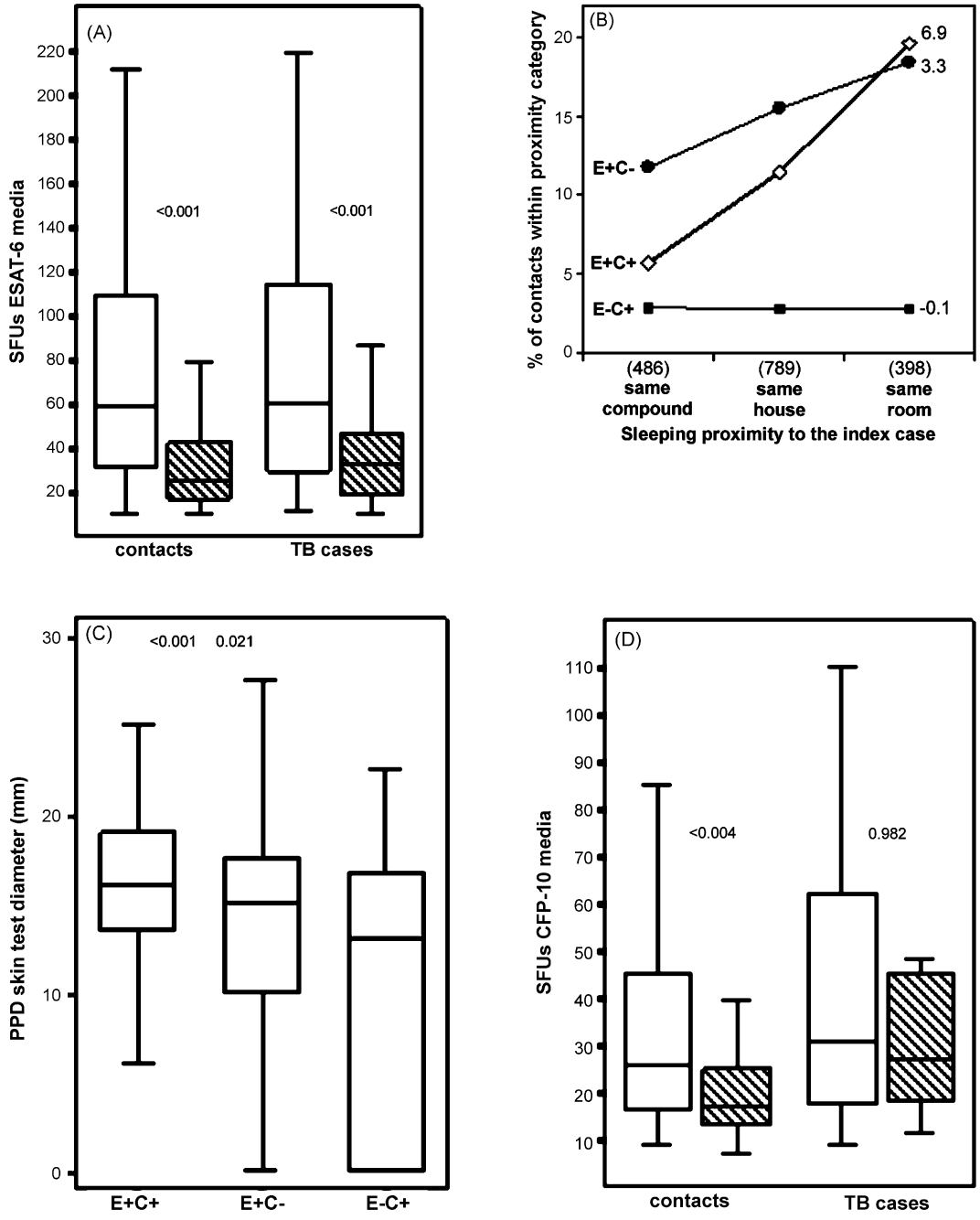

Figure 1 Relationship between discordant and concordant recognition of ESAT-6 and CFP-10 and select variables. (A) Spot-forming unit (SFU) counts for ESAT-6 overlapping peptides are compared in tuberculosis (TB) cases and healthy contacts that do (empty bars) and do not (hatched bars) recognize CFP-10. (B) Proportion of contacts categorized according to sleeping proximity to the index case that respond to both ESAT-6 and CFP-10, to ESAT-6 only or CFP-10 only. The number of contacts in each category is indicated in parenthesis and the number to the right of each line is the slope. (C) Purified protein derivative (PPD) skin test induration for contacts categorized according to concordant or discordant recognition of ESAT-6 and CFP-10 peptides. (D) SFU counts for CFP-10 overlapping peptides are compared in TB cases and healthy contacts that do (empty bars) and do not (hatched bars) recognize ESAT-6. Data in panels A,C and D are presented as median, interquartile range and range, and *P*-values are shown for Student's *t* test on square-root-transformed data comparing concordant versus adjacent discordant responders (A and D) or Mann-Whitney *U* test comparing E^+C^+ and E^+C^- subsets with the E^-C^+ subset (C).

(Figure 2B,C). Concordance was high and SFU counts for discordant responses were focused around the positive cut-off (Figure 2B,C). The proportion of contacts responding to a single pool (33.7%) was high when contrasted with two pools (24.7%) but low (33.8%) when contrasted with four pools (36.7%). However, summed SFU counts for four (Figure 2B, $P < 0.001$) but not two pools (Figure 2C, $P = 0.492$) were significantly higher than for a single combined pool.

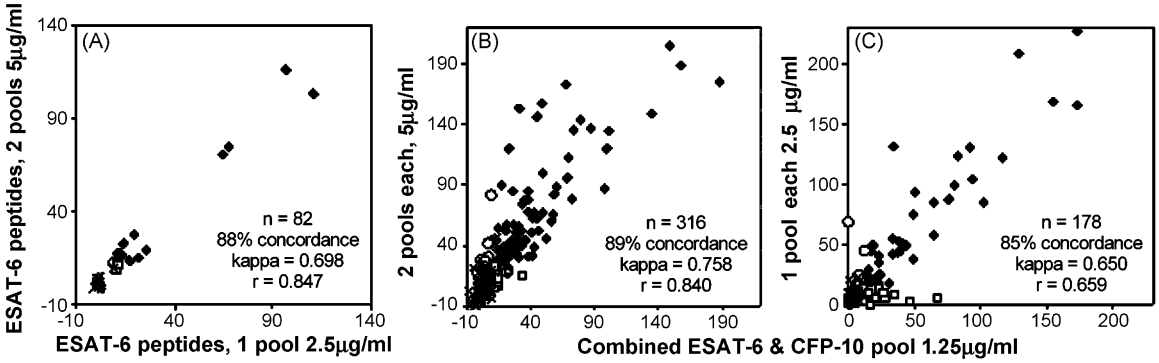

Figure 2 Comparison of responses to CFP-10 and ESAT-6 peptides pools of varying peptide number. Results are presented as average spot-forming unit (SFU) counts for duplicate wells stimulated with pools of 8—33 peptides minus the average of duplicate wells incubated with media only. Peptides were either tested as single pools or as multiple pools and SFU counts summed. Responses for all subjects are shown and categorized dual positive ♦, positive for *y*-axis variable only ◯, positive for *x*-axis variable only □, or dual negative ×. Responses were classified positive if the SFU count was at least 10 higher than and twice the media control. Data for all subjects were used for Spearman's correlations and kappa statistics.

As per common practice, peptide concentrations were not adjusted when peptide pool sizes were increased. To examine whether SFU counts were lower for a single pool compared with four pools because the concentration for individual peptides was lower, we compared the single pool with peptides at 1.25 versus 5 μg/ml in 44 contacts. SFU counts correlated well ($r = 0.824$, $P < 0.001$) and did not differ significantly ($P = 0.174$).

4. Discussion

Results from this large-scale comparison of ESAT-6 and CFP-10 responses demonstrate that, although ESAT-6 was the dominant antigen, CFP-10 contributed to the detection of an additional 13 and 10% of responsive TB cases and contacts, respectively. However, a number of factors indicate that responses to CFP-10 alone have poor specificity for *M. tuberculosis* infection in healthy contacts. First, there was no association with the level of exposure to TB cases. Second, the proportion that were PPD skin test or ELISPOT positive, and the diameter of PPD skin test induration was low for contacts recognizing only CFP-10 compared with contacts that recognized both antigens or ESAT-6 only. Finally, less than half of the contacts that recognized CFP-10 alone recognized a combined pool of ESAT-6 and CFP-10 peptides. These trends indicative of a high false positive rate were probably observed for contacts that recognized CFP-10 only but not for TB cases or contacts that also recognized ESAT-6 because CFP-10 SFU counts were significantly lower. In particular, this was the only responder group in which the majority of responses were less than 20 SFU counts, and we have previously found that below this level specificity declines with SFU count (Jeffries et al., 2006). Thus, while CFP-10 may be of little benefit for detecting *M. tuberculosis* infection in healthy contacts, it may prove to have an important role in the detection of infection in patients with active TB. Although TB cases were approximately twice as likely to recognize CFP-10, we suggest that the difference in CFP-10 response rates between TB cases and contacts is not sufficient to support the use of CFP-10 recognition as a marker for active disease and thus separate testing of ESAT-6 and CFP-10. However, further understanding of the factors underlying this difference in CFP-10 response rates may be useful in the development of tests to differentiate between latent *M. tuberculosis* infection and active TB.

Interestingly, CFP-10 recognition was not inherently associated with a propensity to develop active TB, because 6 months after completing anti-tuberculous chemotherapy CFP-10 recognition rates in TB cases were similar to those in healthy contacts. Both response rates and SFU counts indicate that ESAT-6 is more immunogenic than CFP-10 in the Gambian population. Thus, CFP-10 recognition may be associated with active disease because higher levels of *M. tuberculosis* infection are required to generate CFP-10 compared with ESAT-6 responses. In support of this, ESAT-6 SFU counts were higher in participants that responded to both ESAT-6 and CFP-10 than in participants responding to ESAT-6 alone. Furthermore, compared with ESAT-6, CFP-10 responses in contacts were more dependent upon the level of exposure to *M. tuberculosis* infection.

The differing immunogenicity of ESAT-6 and CFP-10 probably reflects the number and nature of T cell epitopes. In another study we found that CFP-10 responses are more reliant than ESAT-6 responses on CD8 T-cells and that ex-vivo CD8-IFN-γ responses were frequently detected in TB cases but not contacts (A. Fox et al., unpublished). Therefore, it is possible that CFP-10 response rates may differ in TB cases versus contacts because CFP-10 responses are more reliant on CD8 T-cells. CFP-10 is also recognized more frequently than ESAT-6 in HIV-positive but not HIV-negative tuberculous meningitis patients (Simmons et al., 2006). As CD4 counts tend to be low in HIV-positive TB cases, these findings provide further indication that, relative to ESAT-6, CFP-10 responses may be more reliant on CD8 T-cells.

Others have found that CFP-10 is recognized more frequently than ESAT-6 among community members in India ($n = 100$) (Lalvani et al., 2001a), Zambia ($n = 54$) (Chapman et al., 2002) and Holland ($n = 44$) (Arend et al., 2001) but not Viet Nam ($n = 56$) (Simmons et al., 2006). The proportion of Indian and Zambian community members that recognize

ESAT-6 or CFP-10 is far higher than found in the current and previous studies assessing healthy Gambians (Hill et al., 2004; Vekemans et al., 2001), and 80% of Zambians were PPD skin test positive (Chapman et al., 2002) compared with only 41% of contacts tested here. CFP-10 responsiveness has also been associated with the HLA-DR15 (Arend et al., 2000b) haplotype and with *M. africanum* infection, which accounts for one-third of smear-positive TB cases in The Gambia but is infrequently encountered outside West Africa (de Jong et al., 2006). Studies assessing TB cases recruited in Denmark (van Pinxteren et al., 2000), Holland (Arend et al., 2000a), Zambia (Chapman et al., 2002) and Japan (Mori et al., 2004) are consistent with the current study in that ESAT-6 response rates are equivalent or higher than those for CFP-10.

Overlapping 15mer peptides representing ESAT-6 and CFP-10 could be used in a single pool. SFU counts tended to be higher when multiple pools were used. This is likely to reflect a loss of precision when multiple wells are summed. However, we cannot rule out competition for antigen presenting or co-stimulatory factors when single pools are used. Our results also concur with our recent study showing good agreement between ESAT-6 and CFP-10 peptides tested separately versus a fusion protein of the two antigens (Hill et al., 2005).

5. Conclusions

Peptides representing both ESAT-6 and CFP-10 provide maximum sensitivity for detecting *M. tuberculosis* infection in healthy subjects and TB cases, and can be used as a single pool. We have documented that there is differential responsiveness to CFP-10 between TB cases and contacts. This requires further investigation as to possible mechanisms and application. If CFP-10 response rates prove to be higher in contacts that progress to active disease compared with those that remain healthy, such a finding could have important implications for TB control.

Authors' contributions: AF, PCH, RHB and KPWJM designed the study protocol; PCH, KPWJM and DJS carried out the clinical assessment; ASH, PKO and MDL carried out the immunoassays; AF, DJJ, SAD and RHB carried out data analysis; AF, PCH and DJJ interpreted these data; AF, PCH and DJJ drafted the manuscript. All authors read and approved the final manuscript. AF is guarantor of the paper.

Acknowledgements: We thank the study subjects for their participation and the field workers and laboratory assistants of the Medical Research Council for their hard work. We thank the staff of the National Leprosy and Tuberculosis Control Programme in The Gambia for their ongoing collaboration.

Funding: This study was funded by the Medical Research Council and the GlaxoSmithKline Action TB programme and shared some staff and consumables with a study funded by the European Commission (ICFP599A4PR01).

Conflicts of interest: Roger Brookes has a patent relating to the ex-vivo ELISPOT licensed through Oxford University. All other authors have no conflicts of interest concerning the work reported in this paper.

Ethical approval: The combined Gambia Government/Medical Research Council national ethics committee of The Gambia (L2002.5: TB case contact study).

References

Arend, S.M., Andersen, P., van Meijgaarden, K.E., Skjot, R.L., Subronto, Y.W., van Dissel, J.T., Ottenhoff, T.H., 2000a. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. J. Infect. Dis. 181, 1850–1854.

Arend, S.M., Geluk, A., van Meijgaarden, K.E., van Dissel, J.T., Theisen, M., Andersen, P., Ottenhoff, T.H., 2000b. Antigenic equivalence of human T-cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. Infect. Immun. 68, 3314–3321.

Arend, S.M., Engelhard, A.C., Groot, G., de Boer, K., Andersen, P., Ottenhoff, T.H., van Dissel, J.T., 2001. Tuberculin skin testing compared with T-cell responses to *Mycobacterium tuberculosis*-specific and nonspecific antigens for detection of latent infection in persons with recent tuberculosis contact. Clin. Diagn. Lab. Immunol. 8, 1089–1096.

Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S., Small, P.M., 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284, 1520–1523.

Berthet, F.X., Rasmussen, P.B., Rosenkrands, I., Andersen, P., Gicquel, B., 1998. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 144, 3195–3203.

Black, G.F., Weir, R.E., Floyd, S., Bliss, L., Warndorff, D.K., Crampin, A.C., Ngwira, B., Sichali, L., Nazareth, B., Blackwell, J.M., Branson, K., Chaguluka, S.D., Donovan, L., Jarman, E., King, E., Fine, P.E., Dockrell, H.M., 2002. BCG-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. Lancet 359, 1393–1401.

Chapman, A.L., Munkanta, M., Wilkinson, K.A., Pathan, A.A., Ewer, K., Ayles, H., Reece, W.H., Mwinga, A., Godfrey-Faussett, P., Lalvani, A., 2002. Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. AIDS 16, 2285–2293.

Colangeli, R., Spencer, J.S., Bifani, P., Williams, A., Lyashchenko, K., Keen, M.A., Hill, P.J., Belisle, J., Gennaro, M.L., 2000. MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis*, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. Infect. Immun. 68, 990–993.

de Jong, B.C., Hill, P.C., Brookes, R.H., Gagneux, S., Jeffries, D.J., Otu, J.K., Donkor, S.A., Fox, A., McAdam, K.P., Small, P.M., Adegbola, R.A., 2006. *Mycobacterium africanum* elicits an attenuated T cell response to early secreted antigenic target, 6 kDa, in patients with tuberculosis and their household contacts. J. Infect. Dis. 193, 1279–1286.

Diel, R., Nienhaus, A., Lange, C., Schaberg, T., 2006. Cost-optimisation of screening for latent tuberculosis in close contacts. Eur. Respir. J. 28, 35–44.

Dillon, D.C., Alderson, M.R., Day, C.H., Lewinsohn, D.M., Coler, R., Bement, T., Campos-Neto, A., Skeiky, Y.A., Orme, I.M., Roberts, A., Steen, S., Dalemans, W., Badaro, R., Reed, S.G., 1999. Molecular characterization and human T-cell responses to a member of a novel *Mycobacterium tuberculosis* mtb39 gene family. Infect. Immun. 67, 2941–2950.

Ferrara, G., Losi, M., D'Amico, R., Roversi, P., Piro, R., Meacci, M., Meccugni, B., Dori, I.M., Andreani, A., Bergamini, B.M., Mussini, C., Rumpianesi, F., Fabbri, L.M., Richeldi, L., 2006. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. Lancet 367, 1328—1334.

Gordon, S.V., Brosch, R., Billault, A., Garnier, T., Eiglmeier, K., Cole, S.T., 1999. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol. Microbiol. 32, 643—655.

Harboe, M., Oettinger, T., Wiker, H.G., Rosenkrands, I., Andersen, P., 1996. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infect. Immun. 64, 16—22.

Hill, P.C., Brookes, R.H., Fox, A., Fielding, K., Jeffries, D.J., Jackson-Sillah, D., Lugos, M.D., Owiafe, P.K., Donkor, S.A., Hammond, A.S., Otu, J.K., Corrah, T., Adegbola, R.A., McAdam, K.P., 2004. Large-scale evaluation of enzyme-linked immunospot assay and skin test for diagnosis of *Mycobacterium tuberculosis* infection against a gradient of exposure in The Gambia. Clin. Infect. Dis. 38, 966—973.

Hill, P.C., Jackson-Sillah, D., Fox, A., Franken, K.L., Lugos, M.D., Jeffries, D.J., Donkor, S.A., Hammond, A.S., Adegbola, R.A., Ottenhoff, T.H., Klein, M.R., Brookes, R.H., 2005. ESAT-6/CFP-10 fusion protein and peptides for optimal diagnosis of mycobacterium tuberculosis infection by ex vivo enzyme-linked immunospot assay in the Gambia. J. Clin. Microbiol. 43, 2070—2074.

Huebner, R.E., Schein, M.F., Bass Jr., J.B., 1993. The tuberculin skin test. Clin. Infect. Dis. 17, 968—975.

Jeffries, D.J., Donkor, S., Brookes, R.H., Fox, A., Hill, P.C., 2004. Design and implementation of relational databases relevant to the diverse needs of a tuberculosis case contact study in the Gambia. Int. J. Tuberc. Lung Dis. 8, 1095—1099.

Jeffries, D.J., Hill, P.C., Fox, A., Lugos, M., Jackson-Sillah, D.J., Adegbola, R.A., Brookes, R.H., 2006. Identifying ELISPOT and skin test cut-offs for diagnosis of *Mycobacterium tuberculosis* infection in The Gambia. Int. J. Tuberc. Lung Dis. 10, 192—198.

Kang, Y.A., Lee, H.W., Yoon, H.I., Cho, B., Han, S.K., Shim, Y.-S., Yim, J.-J., 2005. Discrepancy between the tuberculin skin test and the whole-blood interferon γ assay for the diagnosis of latent tuberculosis infection in an intermediate tuberculosis-burden country. JAMA 293, 2756—2761.

Lalvani, A., Brookes, R., Hambleton, S., Britton, W.J., Hill, A.V., McMichael, A.J., 1997. Rapid effector function in CD8+ memory T cells. J. Exp. Med. 186, 859—865.

Lalvani, A., Nagvenkar, P., Udwadia, Z., Pathan, A.A., Wilkinson, K.A., Shastri, J.S., Ewer, K., Hill, A.V., Mehta, A., Rodrigues, C., 2001a. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. J. Infect. Dis. 183, 469—477.

Lalvani, A., Pathan, A.A., Durkan, H., Wilkinson, K.A., Whelan, A., Deeks, J.J., Reece, W.H., Latif, M., Pasvol, G., Hill, A.V., 2001b. Enhanced contact tracing and spatial tracking of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Lancet 357, 2017—2021.

Lein, A.D., von Reyn, C.F., Ravn, P., Horsburgh Jr., C.R., Alexander, L.N., Andersen, P., 1999. Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *Mycobacterium avium* complex and those with pulmonary disease due to *Mycobacterium tuberculosis*. Clin. Diagn. Lab. Immunol. 6, 606—609.

Mori, T., Sakatani, M., Yamagishi, F., Takashima, T., Kawabe, Y., Nagao, K., Shigeto, E., Harada, N., Mitarai, S., Okada, M., Suzuki, K., Inoue, Y., Tsuyuguchi, K., Sasaki, Y., Mazurek, G.H., Tsuyuguchi, I., 2004. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am. J. Respir. Crit. Care Med. 170, 59—64.

Mustafa, A.S., Amoudy, H.A., Wiker, H.G., Abal, A.T., Ravn, P., Oftung, F., Andersen, P., 1998. Comparison of antigen-specific T-cell responses of tuberculosis patients using complex or single antigens of *Mycobacterium tuberculosis*. Scand. J. Immunol. 48, 535—543.

Simmons, C.P., Thwaites, G.E., Quyen, N.T., Torok, E., Hoang, D.M., Chau, T.T., Mai, P.P., Lan, N.T., Dung, N.H., Quy, H.T., Bang, N.D., Hien, T.T., Farrar, J., 2006. Pretreatment intracerebral and peripheral blood immune responses in Vietnamese adults with tuberculous meningitis: diagnostic value and relationship to disease severity and outcome. J. Immunol. 176, 2007—2014.

Sorensen, A.L., Nagai, S., Houen, G., Andersen, P., Andersen, A.B., 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infect. Immun. 63, 1710—1717.

Ulrichs, T., Anding, P., Porcelli, S., Kaufmann, S.H., Munk, M.E., 2000. Increased numbers of ESAT-6- and purified protein derivative-specific gamma interferon-producing cells in subclinical and active tuberculosis infection. Infect. Immun. 68, 6073—6076.

van Pinxteren, L.A., Ravn, P., Agger, E.M., Pollock, J., Andersen, P., 2000. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. Clin. Diagn. Lab. Immunol. 7, 155—160.

Vekemans, J., Lienhardt, C., Sillah, J.S., Wheeler, J.G., Lahai, G.P., Doherty, M.T., Corrah, T., Andersen, P., McAdam, K.P., Marchant, A., 2001. Tuberculosis contacts but not patients have higher gamma interferon responses to ESAT-6 than do community controls in The Gambia. Infect. Immun. 69, 6554—6557.

Whalen, C.C., 2005. Diagnosis of latent tuberculosis infection: measure for measure. JAMA 293, 2785—2787.

Wrighton-Smith, P., Zellweger, J.P., 2006. Direct costs of three models for the screening of latent tuberculosis infection. Eur. Respir. J. 28, 45—50.