# Evaluation of a T-cell Assay for *Mycobacterium tuberculosis* Infection in The Gambia


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**2005**

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A thesis in fulfilment of the requirements for the degree of Doctor of Medicine, the University of Auckland, 2005.

## Abstract

New generation T cell assays offer hope in the diagnosis of *Mycobacterium tuberculosis* infection and disease. We assessed the ELISPOT assay using cross-sectional and longitudinal studies and a natural gradient of *M. tuberculosis* exposure by sleeping proximity to a tuberculosis (TB) case in The Gambia. Two antigens, ESAT-6 and CFP-10 (EC), were compared to purified protein derivative (PPD) by ELISPOT and to the PPD skin test in 735 TB contacts. All three tests responded to the exposure gradient, the PPD skin test most dramatically. Inter-test comparison showed that the EC ELISPOT provided improved specificity in the diagnosis of *M. tuberculosis* infection, but at the cost of some sensitivity. Increasing discordance, particularly between PPD ELISPOT and PPD skin test results, down the exposure gradient to 105 community controls was identified. In 693 children, the EC ELISPOT was slightly less sensitive than the PPD skin test in the diagnosis of *M. tuberculosis* infection from recent exposure; neither test was confounded by prior BCG vaccination, even in the very young. A fusion protein of EC compared favourably with their respective peptides by ELISPOT assay in 488 TB contacts, a combined test result offered improved sensitivity. Quantitative ELISPOT and PPD-skin test responses were assessed in 1052 TB case contacts, according to an ELISPOT response to EC. Only the ELISPOT count was sensitive to the exposure gradient ($p=0.009$), revealing a positive dose-response relationship. In the longitudinal assessment, both ELISPOT and PPD skin test conversion occurred over time. PPD skin test reversion occurred in 10% of individuals after 18 months, ELISPOT reversion occurred in 39% at 3 months. In conclusion: the EC ELISPOT offers increased specificity in the diagnosis of *M. tuberculosis* infection in The Gambia, at the cost of some sensitivity; the PPD skin test appears to be down-regulated in the community; neither test is confounded by prior BCG vaccination; a fusion protein in combination with EC peptides offers optimal ELISPOT sensitivity; the quantitative ELISPOT response in specific-antigen-positive TB case contacts reflects the infectious load of *M. tuberculosis*; and significant early reversion of the ELISPOT test suggests it is unreliable in *M. tuberculosis* dormancy.

**I dedicate this thesis to Marian, my wife and best friend, who established our home in The Gambia and 'hung in there' so amazingly well. I could not have done this without you.**

# Acknowledgments

I have been greatly privileged to work at the MRC unit, The Gambia. I thank Professor Keith McAdam, who played a key role in my personal development and inspired the TB case contact work. When I arrived in 2001, I was able to build upon plans already in place. I thank Professor Richard Adegbola for his mentorship and his friendship, which I value so highly. I thank Dr Tumani Corrah, who took over the directorship role in the unit in 2003, for his personal support and mentorship. The studies presented here required a multi-disciplinary team of people; the table on the following page acknowledges those who have played a role in these studies to the level of authorship.

I acknowledge my very able team of field workers, supervised by Abdoulie Tunkara, including Abdoulie Cham, Salieu Barry, Masaneh, Babou Faye, Fatou Jadama, Bajun Drammeh, Aziz Bangura, Rohey Jallow, Kairaba Kanyi, and Masaneh Ceesay. Roger Brookes and Annette Fox had a dedicated team of technical laboratory assistants: Momodou Darboe, Marie Gomez, Joseph Mendy, Rohey Jallow, Leticia King and Jabou Bah. Professor Richard Adegbola and Jacob Otu were assisted by Oumie Secka, Francis Mendy and Yandeh Touray.

The research in the unit would not have been possible without ongoing collaboration with the National TB control programme. I thank Dr Kebba Manneh (coordinator until 2004) and Adama Bojang (coordinator since 2004) for the very positive relationship we have had and the opportunity to take part in improving TB control in The Gambia.

The support systems in place at the MRC laboratories were crucial to the success of the research. These have been led by Mark Radford, director of operations in the time I have been working at the MRC Labs. I also acknowledge Kathy Hill (personnel), Fanta Singhateh (travel), Simon Belcher and Abdoulie Barry (finance), Seidina Joof (transport), Cherno Fye (housing), Alison Offong (administration), Arthur Roberts (facilities) and Dembo Kanteh (Bacterial diseases administrator).

I would like to thank Prof Rod Jackson at the School of Population Health, University of Auckland for hosting me in 2004 to work on this thesis. I am also grateful to Prof Charlotte Paul, Assoc Prof Rob McGee and Prof David Skegg at the Department of Preventative and Social Medicine, University of Otago, for hosting me in 2005.

I acknowledge the love and support of my family: my wife Marian, son Nathan and daughter Rebekah; Professor Graham and Bartha Hill (my parents), Dr Andrew and Lori Hill and Drs Douglas and Jennifer Hill.

**Name and role of each person who contributed to the studies of this thesis, to the level of 'author' status. Names are listed in alphabetical order of the surnames.**

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| Roger Brookes | Immunologist. Joint coordinator of the ELISPOT work, involved in analysis and write up of all the studies. |
| Tumani Corrah | Clinician-scientist – head of clinical services, MRC Gambia. Involved in the clinical care of the TB cases and contacts. |
| Simon Donkor | Data manager. Involved in design of the databases for all the studies. Supervised data collection and verification. |
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| Annette Fox | Immunologist. Joint coordinator of the ELISPOT work, involved in design of all the studies, analysis and write up of immunological aspects. |
| Kees Franken | Immunology Laboratory technician. Produced the fusion protein for the study of chapter 4. Involved in the write-up of the study of chapter 4. |
| Abdulrahman Hammond | Immunology Laboratory technician. Performed ELISPOT assays in all the studies of the thesis. |
| Dolly Jackson-Sillah | Research Clinician. Involved in recruitment and follow-up of all the study participants. |
| David Jeffries | Statistician, MRC Gambia. Led the statistical analysis of chapter 6 and advised the author on the statistical analysis of chapters 2, 3, 4, 5 and 7. Designed the database for all the studies and was involved in the analyses and write up of all the studies. |
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| Moses Lugos | Immunology Laboratory technician. Performed the majority of the ELISPOT assays of all the studies of the thesis. |
| Roger Marshall | Statistician, University of Auckland. Assisted with the analysis of the studies of chapters 3 and 5, specifically the creation of rectangular Venn diagrams. |
| Keith McAdam | Director of MRC The Gambia and head of the TB programme until March 2003. Involved in initiation and design of the TB case contact work in The Gambia. In particular, involved in design, analysis and write up of the studies of chapters 2, 3, 5, 6 and 7. |
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| Patrick Owiafe | Immunology Laboratory technician. Performed ELISPOT assays in the study of chapter 2 of the thesis. |

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# Abbreviations

| | |
|---|---|
| BCG | Bacille Calmette-Guérin |
| C | Centigrade |
| CI | Confidence Interval |
| E | Eastern |
| EC | ESAT-6/CFP-10 |
| ELISPOT | Enzyme Linked Immunospot assay |
| IFN-γ | Interferon gamma |
| kDA | kilo-Dalton |
| LAL | Limulus Amebocyte Lysate |
| L-J | Lowenstein-Jensen |
| MRC | Medical Research Council |
| n | Number |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| PHA | Phytohaemaglutinin |
| PPD | Purified Protein Derivative |
| RD | Region of Difference |
| rt-PCR | Reverse transcriptase Polymerase Chain Reaction |
| SFU | Spot Forming Units |
| SSI | Statins Serum 'Institut' |
| TB | Tuberculosis |
| TH | T Helper cell |
| TST | Tuberculin Skin Test |
| TU | Test Units |
| UK | United Kingdom |
| WHO | World Health Organisation |
| ZN | Ziehl-Neelsen |

## Articles arising from these studies

1. Hill P.C, Brookes RH, Fox A, Fielding K, Jeffries DJ, Jackson-Sillah D, Lugos M, Owiafe PK, Donkor SA, Hammond AS, Otu JK, Corrah T, Adegbola RA, McAdam KPWJ. 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 2004 ; 38 :966-73.[1]

2. Jeffries DJ, Donkor S, Brookes RH, Fox A, and Hill PC. Design and implementation of relational databases relevant to the diverse needs of a tuberculosis case contact study in The Gambia. Int J Tub Lung Dis 2004 ; 8:1095-99.[2]

3. Hill PC, Fox A, Jeffries DJ, Jackson-Sillah D, Lugos MD, Owiafe PK, Donkor SA, Hammond AS, Corrah T, Adegbola RA, McAdam KPWJ, Brookes RH. Quantitative T cell assay reflects infectious load of *Mycobacterium tuberculosis* in an endemic case contact model. Clin Infect Dis 2005; 40:273-8.[3]

4. Hill PC, Jackson-Sillah D, Fox A, Franken KL, Lugos MD, Jeffries DJ, Donkor SA, Hammond AS, Adegbola RA, Ottenhoff THM, Klein M, Brookes RH. ESAT-6/CFP-10 fusion protein and peptides for optimal detection of *Mycobacterium tuberculosis* infection by ex vivo ELISPOT in The Gambia. J Clin Microbiol 2005 ;43 :2070-74.[4]

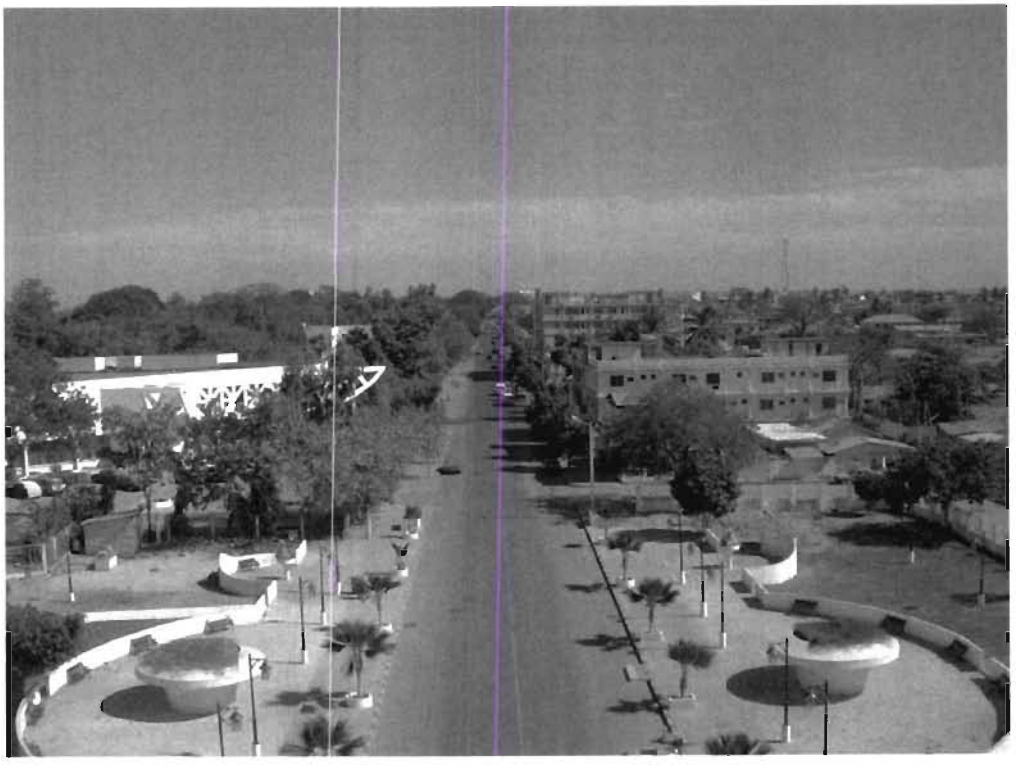
5. Jeffries DJ, Hill PC, Fox A, Lugos M, Jackson-Sillah DJ, Adegbola RA, Brookes RH. Determination of ELISPOT assay and PPD skin test cut-offs for diagnosis of *Mycobacterium tuberculosis* infection in The Gambia. Int J Tub Lung Dis (In press) .

6. Hill PC, Brookes RH, Adetifa IMO, Fox A , Jackson-Sillah D, Lugos M, Donkor S, Marshall RJ, Howie SR, Corrah T, Jeffries DJ, Adegbola RA, McAdam KP. Comparison of ELISPOT assay and tuberculin skin test in healthy children exposed to *Mycobacterium tuberculosis.* (submitted to Pediatrics).

7. Hill PC, Fox A, Jackson-Sillah D, Jeffries DJ, Lugos MD, Adegbola RA, McAdam KP, Brookes RH. Longitudinal assessment of the ELISPOT assay for *Mycobacterium tuberculosis* infection. (In preparation).

# 1

## *Mycobacterium tuberculosis* infection



**Banjul, The Gambia**

## 1.1 *Mycobacterium tuberculosis*

*M. tuberculosis* is a slim, strongly acid-alcohol-fast, rod. It grows at 37°C, but not at room temperature, with a mean generation time under enhanced conditions of 12-24 hours. On the classical Löwenstein-Jensen medium, colonies usually appear after 3-6 weeks of incubation. Enhanced speed to diagnosis and sensitivity has been obtained using rapid radiometric detection systems and liquid culture, although a combined liquid and solid media approach offers maximum sensitivity.[5,6] Mycobacteria that comprise the *Mycobacterium tuberculosis* complex include *M. tuberculosis, M. africanum, M. canetti, M. microti* and *M. bovis*. It was previously thought that human tuberculosis evolved from the bovine disease and *M. tuberculosis* evolved from *M. bovis*, which has a wider host range. However, studies exploiting the sequencing of the *M. tuberculosis* genome have found this scenario to be unfounded – rather it appears that the members of the *M. tuberculosis* complex have a common ancestor.[7] 'Endemic' ancestral strains of *M. tuberculosis*, mostly of African origin, co-exist in populations with 'epidemic modern' strains, a finding that is influencing the understanding of tuberculosis epidemics across nations and continents.[8]

'Primary' infection with *M. tuberculosis* is usually pulmonary and develops at the periphery of the mid-zone of the lung. Tubercle bacilli that reach the small bronchi or alveoli within inhaled droplets are engulfed by macrophages. The interaction between *M. tuberculosis* and the macrophage is the subject of intense study as the first major confrontation with the immune system.[9-11] While most organisms die and bacterial multiplication ceases, those bacilli that survive multiply within macrophages and are carried to the hilar lymph nodes that drain the infected site. Dissemination of some bacilli commonly occurs through lymphatic and blood vessels, whereby they may be deposited in multiple organs. Cell mediated immunity to *M. tuberculosis* is engaged 2-6 weeks after initial infection, with formation of classical histological tubercules at the sites of multiplication. The primary infected lesion, and those at sites of dissemination, fibroses and may calcify to be detectable radiologically. T cells both activate the host cell to kill the organism, and directly kill the organism themselves (cytotoxic T cells).[12] The prominent role that T cells play in the defence against *M. tuberculosis* has been demonstrated in mice[13] and is most strongly demonstrated in humans through the clear association of TB disease with HIV infection.[14,15]

A key advance in TB laboratory based research in the last decade has been the identification of important and immunogenic secreted antigens of *M. tuberculosis.* A number of selection criteria have been employed to identify immunogenic molecules of *M. tuberculosis*: recognition by sera, abundance in mycobacteria, up regulation under various stress conditions and strong T-cell recognition. Proteins released from dividing bacteria have been of particular interest. By separating culture filtrate proteins into narrow molecular mass fractions, antigen recognition has been studied in both animals and humans. The sequencing of the *M. tuberculosis* genome and the development of animal models to mimic different stages in the life cycle of *M. tuberculosis* have fast-tracked the progression from discovery to cloning and recombinant expression so these antigens can be assessed for their diagnostic potential.[16]

Secreted antigens contained in *M. tuberculosis* short-term culture filtrate are target molecules for memory immune T cells. Highly active memory effector cells accumulate rapidly after infection and recognize distinct target molecules among the secreted proteins. The first articles describing a secreted 6-kDa protein (ESAT-6) recognised in this way were published in 1995.[17,18] This antigen was shown to be present in strains belonging to the *M. tuberculosis* complex and, of the environmental mycobacteria, it was shown to be present in only *M. Kansasi, M. sugar, and M. marinum.*[18] Its gene was also shown to be lacking in BCG.[19] It became clear that ESAT-6 is a major target antigen recognized at an early stage of *M. tuberculosis* infection in particular,[20,21] part of a restricted repertoire of mycobacterial antigens available for immune system recognition at that point.[22,23] Berthet et al[24] found that ESAT-6 is co-transcribed with *lhp,* a new gene encoding a low-molecular-mass protein found in *M. tuberculosis* short-term culture filtrate. This 10 kDa protein was referred to as CFP-10. The *lhp gene* is located directly upstream of ESAT-6 in the same 'region of difference' (RD1) missing from *M. bovis* BCG and CFP-10 is a potent T-cell antigen.[25] It has been suggested that the biologically active form of ESAT-6 and CFP-10 is as a complex, which they form in a tight 1:1 manner.[26,27] There is also evidence that ESAT-6 and CFP-10 affect the anti-mycobacterial response to *M. tuberculosis*[28,29] and that they work together with other RD1 genes to form a virulence determinant important for the pathogenesis of the organism.[30] Indeed, it is postulated that the primary attenuating mechanism of BCG is loss of cytolytic activity mediated by ESAT-6, and possibly other RD1 antigens, resulting in decreased tissue invasiveness.[31-34]

The lifecycle of *M. tuberculosis* is such that the organism, in the majority of infected individuals, cycles into a dormant state rather than progressing directly to cause

disease. *In vitro* animal models and genomics have provided new insights into this phenomenon.[35-38] The DosR regulon is a set of 48 mycobacterial genes expressed when tubercle bacilli are exposed *in vitro* to low-dose nitric oxygen, hypoxia and *in vivo* in IFNγ activated macrophages.[39] It is therefore considered important in order for *M. tuberculosis* to persist in the human host. Proteins secreted as a result of the up regulation of genes from the DosR regulon are thought to be important in the maintenance of the dormancy state. They appear to be preferentially secreted at that stage in the organism's life cycle. To date, α-crystalin is the most immunogenic protein that has been found to be preferentially secreted in dormancy.[40,41]

Recently, animal models have been developed to identify antigens that are preferentially secreted in the reactivation state of *M. tuberculosis.*[42,43] Immunogenic antigens secreted preferentially at this point in the *M. tuberculosis* life cycle could potentially be developed within diagnostic tools to signal early reactivation and heightened risk of progression to disease. Indeed, there is an urgent need to link these advances in the understanding of *M. tuberculosis* antigen secretion to real human situations to identify how they can be incorporated into efforts to control TB.

## 1.2 Tuberculosis disease: epidemiology

Tuberculosis is a disease of antiquity that has undergone a global resurgence to the extent that it has been declared a global emergency by the World Health Organisation (WHO),[44,45] with specific concerns in Africa.[46] It is estimated that approximately 9 million cases of Tuberculosis emerge annually, resulting in 2-3 million deaths, accounting for twenty six percent of avoidable deaths.[47] Three regions dominate the worldwide distribution of tuberculosis, according to official notification figures (table 1.1):[48] South East Asia (36% of cases), Africa (24%) and the Western Pacific region (20%). The incidence rate of TB is highest in Africa. Indeed, while the global incidence rate of TB is growing at approximately 1% per year, this is to a great degree fuelled by a much faster increase in sub-Saharan Africa and the countries of the former Soviet Union. Other regions of the world have experienced a steady, albeit slowing, decline in TB burden. If Africa and Eastern Europe are excluded from global statistics, it is estimated that the prevalence rate of TB in 2015 would be approximately half that in 1990.[49]

**Table 1-1: Tuberculosis case notifications and rates by WHO region (2002)[48]**

| WHO region | Cases notified (all forms) | Proportion of total (%) | Rate (per 100,000 population) |
|---|---|---|---|
| Africa | 992,054 | 24 | 148 |
| Americas | 233,648 | 9 | 27 |
| E. Mediterranean | 188.458 | 6 | 37 |
| Europe | 373,497 | 5 | 43 |
| SE. Asia | 1,487,985 | 36 | 94 |
| W. Pacific | 806,112 | 20 | 47 |
| Global | 4,081,754 | 100 | 66 |

The limitations of TB notifications and difficulties in obtaining direct measures of the numbers of cases and deaths, have led WHO to attempt to estimate the size of the burden of TB globally (Table 1-2). The estimated incidence rate of TB worldwide was 141 per 100,000 population in 2001, 22 countries account for approximately 80% of all TB cases while 95% of all cases and 98% of all deaths occur in the developing world.[48] Thirteen of the 15 highest incidence countries are in sub-Saharan Africa, where HIV positivity is known to be high.

**Table 1-2: TB estimates of disease and TB deaths by WHO region, 2002. [48]**

| | Africa | Americas | E. Medit. | Europe | SE Asia | W. Pacific | Total |
|---|---|---|---|---|---|---|---|
| Population (millions) | 672 | 857 | 507 | 877 | 1591 | 1718 | 6222 |
| TB cases (all forms) | | | | | | | |
| Number ('000s) | 2354 | 370 | 622 | 472 | 2890 | 2090 | 8798 |
| Incidence | 350 | 43 | 123 | 54 | 182 | 122 | 141 |
| Deaths ('000s) | | | | | | | |
| Number ('000s) | 556 | 53 | 143 | 73 | 625 | 373 | 1823 |
| Incidence | 83.0 | 6.2 | 28.0 | 8.3 | 39.0 | 22.0 | 29.0 |

HIV is clearly the most important risk factor for progression of latent *M. tuberculosis* infection to TB disease: in 2004 it is estimated that 600,000 individuals doubly infected with HIV and TB died.[50] HIV is a major driving force in the expanding epidemic of TB in sub-Saharan Africa.[51] Of the estimated 1.4 million adults co-infected with *M. tuberculosis* and HIV in the world in 2000, 70% were in sub-Saharan Africa.[52] It is estimated that the HIV prevalence in TB patients in nearly all countries of sub-Saharan Africa is greater than 20%, and is greater than 50% in most countries of the southern cone.[51] It was estimated in 2000 that 31% of adult TB cases were attributable to HIV in the entire African region.[52] While there has been a dramatic increase in the incidence rate of TB disease in sub-Saharan Africa over the last 3 decades, there is some evidence that at least the rate of increase is slowing.[52] Because HIV infection rates are higher in women than men, in several African countries with high rates of HIV infection the majority of TB cases are now women.[48]

## 1.3 *M. tuberculosis* infection

'Latent' *M. tuberculosis* infection is thought to affect approximately one third of the world's population.[53] However, the data to support this are scanty. They come from special studies in which the *M. tuberculosis* infection status of a population was estimated by tuberculin skin testing, usually using a cross-sectional approach. Inferences were then made from the prevalence of tuberculin reactors regarding the incidence of infection.[54] The simple model assumes a constant tuberculin test conversion rate (negative to positive) and no reversion (positive to negative).[55,56] Inclusion of a reversion rate has been shown to have a significant influence on estimates of the annual risk of infection.[57] Estimates derived from the 'annual risk of infection' approach are also vulnerable to the variability of the tuberculin skin test's specificity in different settings. Furthermore, variations in the quality of intervention and in the risks related to progression to disease, limit the provision of a standard parameter to derive the incidence of secondary cases. The methodology also precludes rapid assessment of changes in transmission patterns.[58]

Much of the groundwork for the 'annual risk of infection' approach was undertaken in a 'non-BCG' setting, the United States, by Palmer et al.[59] Several important trends emerged from detailed population-based longitudinal analyses of tuberculin reaction: firstly there is an increase in the frequency of positive tuberculin reactors from birth up to approximately 60 years of age;[60] secondly, the risk of acquiring new infection does not vary markedly with age, while peaking in adolescence and early adulthood;[61] thirdly, while there is little difference in tuberculin reactivity between males and females

early in life, there is an increasing excess among males with increasing age;[60] and fourthly, by far the most important determinant of whether or not a contact of a case becomes tuberculin positive is the severity of disease in the case (bacteriologically and radiologically).[62]

Several studies have attempted to define the risk of disease after infection with *M. tuberculosis*. These have been characterised by a marked degree of geographic variation. Annual attack rates during the first year after tuberculin positivity were found to be 101/100,000 in South India,[63] 29/100,000 in Denmark,[64] 61/100,000 in the southern United States,[65] and 1520/100,000 in Alaska.[66] While case rates of tuberculosis in tuberculin reactors vary with age (being highest in children[67]) and, to a lesser extent, gender,[64] these factors cannot account for the marked geographic variation that has been observed – it remains unexplained.

Studies have demonstrated that prophylactic isoniazid reduces the chances of *M. tuberculosis* infected individuals progressing to active TB by approximately 60% (25-92%): the use of isoniazid has been evaluated in various randomised controlled trials conducted by the US Public Health service that included more than 70,000 participants.[66,68] Furthermore, the optimal period for isoniazid therapy has been concluded to be nine months.[69] Other options for prophylaxis include rifampicin[70] or rifampicin and pyrazinamide in combination,[71] although the latter may have an unacceptable side effect profile for use on a large scale.[72] While not part of the WHO plan for global control of the epidemic, the case for prophylactic treatment of *M. tuberculosis* treatment is building. In 2001 Pitman et al[73] used mathematical modelling to show that the effect of prophylactic treatment of *M. tuberculosis* infection in developed countries may have been greater than the effect of treating cases. The arguments against using prophylaxis against the development of TB disease in tropical developing countries include cost, non-compliance, the fear of using monotherapy in those who may actually have disease in the absence of x-ray facilities, and the cross-reactivity of abundant non-tuberculous mycobacteria with the PPD skin test. More specificity in the diagnosis of the *M. tuberculosis* infection phenotype might be an advantage with regard to the latter.

## 1.4 Diagnosis of *M. tuberculosis* infection

### 1.4.1 The PPD skin test (tuberculin test)

Tuberculosis screening tests (TSTs) were first introduced in 1909 by von Pirquet.[74] Originally TSTs were prepared by dissolving the residue from heated cultures of *M. tuberculosis* in a glycerin-containing solvent.[75] Seibert produced purified protein derivative (PPD) by steaming cultures of *M. tuberculosis* and purifying proteins;[76] a large 'lot' became the reference standard (PPD-S). Five test units (TU) are referenced as the bio-assayable skin test activity of 0.0001 mg of PPD-S.[77] Subsequently, master lots of PPD products were standardised by bioassay in both tuberculin-sensitised guinea pigs and skin test-positive humans. Outside of the United States, PPD RT-23 is used, having been introduced by the WHO in 1958 through the Statins Serum Institut (SSI).[78] The tuberculin reaction is a delayed hypersensitivity reaction, read at 48-72 hours. There have been several different methods used for tuberculin skin testing. Multiple puncture tests, such as the Heaf test, tine test and MONO-VACC test, have been shown to be less sensitive and specific than the Mantoux test.[79]

Repeated tuberculin skin testing does not cause a truly tuberculin-negative person, never previously infected with *M. tuberculosis* or sensitised to other mycobacteria, to become TST positive.[80] However, tuberculin reactions may decrease in size or increase in size because of: random variability (from differences in administration, reading or biological response), boosting through recall of pre-existing delayed type hypersensitivity, or new infection (conversion). [81] Biological variation is small compared with the variation from differences in administration and reading.[82] Overall, chance variation is thought to result in differences of less than 6mm of induration in 95% of subjects. Therefore, a criterion of an increase of at least 6mm of induration has been proposed to define conversion or boosting.[81]

Tuberculin boosting is thought to result from recall of waned cell-mediated immunity. Boosting is maximal if the interval between the first and second test is between 1 and 5 weeks.[83] It also tends to be higher, other things being equal, in those who have previously been vaccinated with BCG,[84] although this is not a universal finding.[85] It most commonly occurs in those with a prior tuberculin response in the mid-range of 8-13mm of induration,[86] suggesting that enhancement may be most marked in communities with a high prevalence of non-specific allergy. Furthermore, 12-13% of individuals who react to non-tuberculous mycobacteria will demonstrate the booster

phenomenon.[87] Boosting is therefore a non-specific response to any prior mycobacterial exposure.

There is evidence of an increased relative non-specific nature of a boosted tuberculin response, compared to an initial positive response. Individuals screened for inclusion into some of the large trials of BCG vaccine,[88] who were positive by initial tuberculin test or through a 2-step procedure, were often followed in parallel with those who were tuberculin test negative that were entered into the studies. The highest rates of TB disease at follow-up tended to be in those with an initial strongly positive tuberculin test. The MRC tuberculosis vaccines clinical trials committee presented such a follow-up of 56,000 adolescents in 1956.[89] The incidence rate of definite TB disease in those initially tuberculin positive was 175/100,000 per year compared to 74/100,000 per year in those only positive in a two-step tuberculin test. Similarly, in 1969, Comstock and Webster reported a 20 year follow-up of school children in Georgia, where 29/1492 (2%) of those initially tuberculin positive became definite TB cases versus 7/3768 (0.2%) of those positive through a two-step procedure.[90]

Tuberculin test conversion is defined as the development of new positivity following new infection with *M. tuberculosis,* non tuberculous mycobacteria,[91] or BCG vaccination.[81] Ferebee reported that, with repeated tuberculin tests at 1year in initially tuberculin negative individuals, the risk of TB disease associated with a reaction of greater or equal to 5mm was six times higher in a population of household contacts assumed to have tuberculin conversion, compared with a population without known exposure and assumed to have boosting.[68] The interval between initial exposure and tuberculin conversion, the 'window' period, is variable. Following BCG vaccination it has been shown to occur in over 99% of individuals by 6 weeks.[92] Following known *M. tuberculosis* exposure, conversion has been shown to occur within 3-7 weeks.[93]

It is not a straightforward matter to distinguish tuberculin test boosting from conversion. However three approaches have been described. Firstly, on clinical grounds, boosting may be identified when the increase in reaction is seen after an interval of 1 to 5 weeks in the absence of any possibility of *M. tuberculosis* exposure. In contrast, conversion may be identified after a known BCG vaccination[94,95] or following a significant known exposure, such as an outbreak of TB. Secondly, the size of the induration is important, especially if prior two-step testing has been negative. However, definitions range from an increase of at least 6mm to at least an induration of 10mm[96] up to 18mm of induration,[97] and no consensus has been reached. It does appear to be clear that as

the size of the reaction to a second tuberculin test increases, the likelihood that it is due to conversion increases.[98,99] Degree of turgidity may also be important in this regard; a more turgid reaction may be more predictive of true *M. tuberculosis* infection.[100] Thirdly, in different clinical situations and populations, the predictive value of a positive second test varies markedly as does the related risk of disease.[81] Therefore level of endemnicity is important.

There has been considerable evolution over many decades in the setting of a cut-off for positivity of the tuberculin test. Palmer and Bates[101] reported a mean diameter of induration of 18.3mm +/- 5.3mm in predominantly pulmonary tuberculosis disease. Studies in healthy Alaskan Indians, assumed to have no exposure to cross-reacting environmental mycobacteria, and known to have significant *M. tuberculosis* exposure, revealed a classical bimodal distribution of induration, with peaks at 0 and 18mm, and few reactions between 2-5mm.[102] It was concluded that Alaskan Indians should be considered to have *M. tuberculosis* infection with induration of >5mm. A much less clearly bimodal distribution of tuberculin reactions was seen in US male navy recruits with a peak at 0mm and 5mm of induration and then a gradual 'tailing off.'[103] A distribution of reactions due to environmental mycobacteria was inferred through subtraction of the imputed distribution of reactions in *M. tuberculosis* infected people. This led to a description of 3 categories of individual: negative (induration ≤5mm), intermediate (6-10mm) and positive (>10mm).

While it is generally accepted that increasing size of the tuberculin reaction is associated with increasing specificity for *M. tuberculosis*,[104] contextual factors do need to be considered: pre-test risk has been increasingly emphasized when interpreting a tuberculin skin test result. The American Thoracic Society[79] and Centers for Disease Control and Prevention[105] recommendations suggest 3 pre-test risk levels on the basis of the prevalence of infection and risk of progression to disease. These correspond to 5mm, 10mm and 15mm cut-offs, the higher the risk the lower the proposed cut-off. Rose et al,[106] through comparison of tuberculin test results in two different settings, confirmed that sensitivity and specificity do not depend on the prevalence of tuberculosis in a population. However, the predictive value of the test does relate to the prevalence of tuberculosis, hence the value of an assessment of pre-test risk. Recently, other mathematical tools have been utilised to identify appropriate tuberculin skin test cut-offs by comparing the distribution of induration reaction in notified cases with non-BCG vaccinated community controls, with adjustment for presumed latent *M. tuberculosis* infection in the controls. Berkel et al[107] used a mixture model to achieve

this in a non-endemic population in the Netherlands. In The Gambia, we have used the TB case and case contact data from the studies in this thesis and applied a multivariate discrimination algorithm to identify appropriate cut-offs for the tuberculin skin test and the ELISPOT assay.[108]

Waning, or reversion, of the tuberculin skin test response is best described after BCG vaccination and revaccination,[109] but has also been described in relation to an assumed *M. tuberculosis* infection.[110] If tuberculin tested, after 10 years or more, only 15-25% remain positive on an initial tuberculin test.[94] It appears that tuberculin reversion after BCG vaccination may be more rapid in tropical than non-tropical settings.[111] A rapid loss of tuberculin sensitivity after BCG was described in the South India/Chingleput vaccine trial,[112] and also in Malawi.[113] The loss of tuberculin reactivity is most marked in the period 6-12 months after vaccination,[114] and may be more likely to occur if the initial reaction was due to exposure to environmental mycobacteria.[87] It has also been demonstrated that reversion rates decline with increasing age.[115] This phenomenon has not been explained. Anergy associated with HIV infection, disseminated tuberculous disease or immunosuppression due to haemodialysis, transplantation or medication may also lead to a falsely negative tuberculin reaction.[116] However, standard tests utilised to detect anergy may be unhelpful in identifying those who are have a falsely negative tuberculin reaction.[117-119]

### 1.4.2 Assays employing *M. tuberculosis*-specific antigens

Building on the advances in the understanding of *M. tuberculosis* and the tools available, assays have been developed based on the expression of interferon-γ (IFN-γ) in response to *M. tuberculosis* antigens. *M. tuberculosis* infection evokes a strong T-helper 1 (Th1) type cell-mediated immune response with release of IFN-γ. *In vitro* assessment of IFN-γ production in response to mycobacterial antigens is used to detect infection with *M. tuberculosis*.[120,121] Peripheral blood mononuclear cells or, alternatively, whole blood, are stimulated with PPD or other antigens and the concentration of IFN-γ is measured,[122] or the frequency of cells secreting IFN-γ is quantified.[123]

Two immunogenic secreted proteins, ESAT-6 and CFP-10, are candidates for more specific diagnostic tests than those utilising PPD.[124] There are two commercial tests that quantify an IFN-γ response to ESAT-6 and CFP-10: QuantiFERON-TB Gold (Cellestis Ltd, Carnegie, Victoria, Australia) is a whole-blood assay that has evolved from a PPD based assay;[125] the T SPOT-TB test (Oxford Immunotech, Oxford,

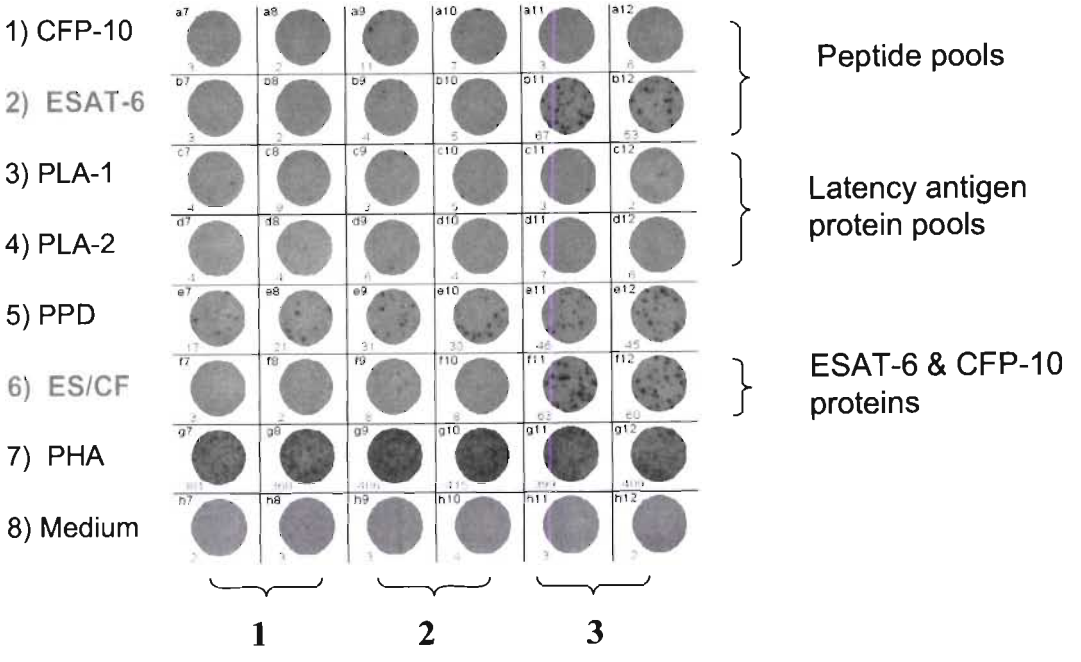
England) is an enzyme-linked immunospot (ELISPOT) assay,[126,127] with a blood cell separation step, that quantifies the frequency of cells secreting IFN-γ in response to stimulation with ESAT-6 and CFP-10.

The ELISPOT assay is an analysis of cell activation through cytokine production at the single-cell level. The assay is performed in 96-well microtiter plates (Figure 1.1). Firstly, wells are coated with high affinity monoclonal antibodies to the cytokine to be investigated. Cells are added and incubated for approximately 24 hours in the presence of antigen. During this period antigen-specific responding cells release cytokine, captured in the immediate vicinity of the cells. Cells are removed by washing and a biotinylated antibody directed to a second epitope of the cytokine is added. Streptavidin conjugated with enzyme (ALP or HRP) is added. Finally, a precipitating substrate for ALP or HRP is added and the plates are incubated until spots emerge at the site of the responding cells. The spots can be examined and counted in a dissection microscope or an image analyser system. The number of spots per 100,000 cells added to each well gives the frequency of responding cells.

Assessment of new T cell assays has been hampered by the lack of a gold standard for the diagnosis of *M. tuberculosis* infection in the absence of disease.[128] Investigators have applied two approaches to address this issue. The first is simply to assess the agreement between a T cell assay and the PPD skin test.[129] This is a helpful approach, although it is not clear what proportion of discordance found is due to the use of different antigens versus different types of response to antigenic challenge – it appears that considerable discordance occurs simply because of the different responses measured.[130] The second approach, which also may simply identify differences that are simply due to the different type of response measured, has been to stratify subjects to reflect a spectrum of likelihood of *M. tuberculosis* infection according to risk profile and assess test performance across the relevant strata.[131] A 'dose-response' effect is reflective of a causal relationship.

Table 1-3 is a summary of the studies, until the end of 2004, that formally assessed a new T cell assay for the diagnosis of *M. tuberculosis* infection. This is excluding the studies presented in this thesis. Figure 1-2 shows the association of tests for *M. tuberculosis* infection and exposure in the studies with adequate data, expressed as Odds ratios and 95% confidence intervals, including the studies of this thesis that have now been published.

**Figure 1-1. Half of a 96-well ELISPOT plate. The antigens used for a particular experiment are represented on the left hand side with supplementary details on the right hand side. Three individuals are represented, tested in duplicate. Each spot represents Interferon-γ release from a single T-cell in response to antigen.**




| | |
|---|---|
| PHA | Phytohaemaglutinin (positive control immunogen) |
| Medium | Negative control |

**Table 1-3. Evidence for the usefulness of ESAT-6 and/or CFP-10 in the diagnosis of *Mycobacterium tuberculosis* infection**

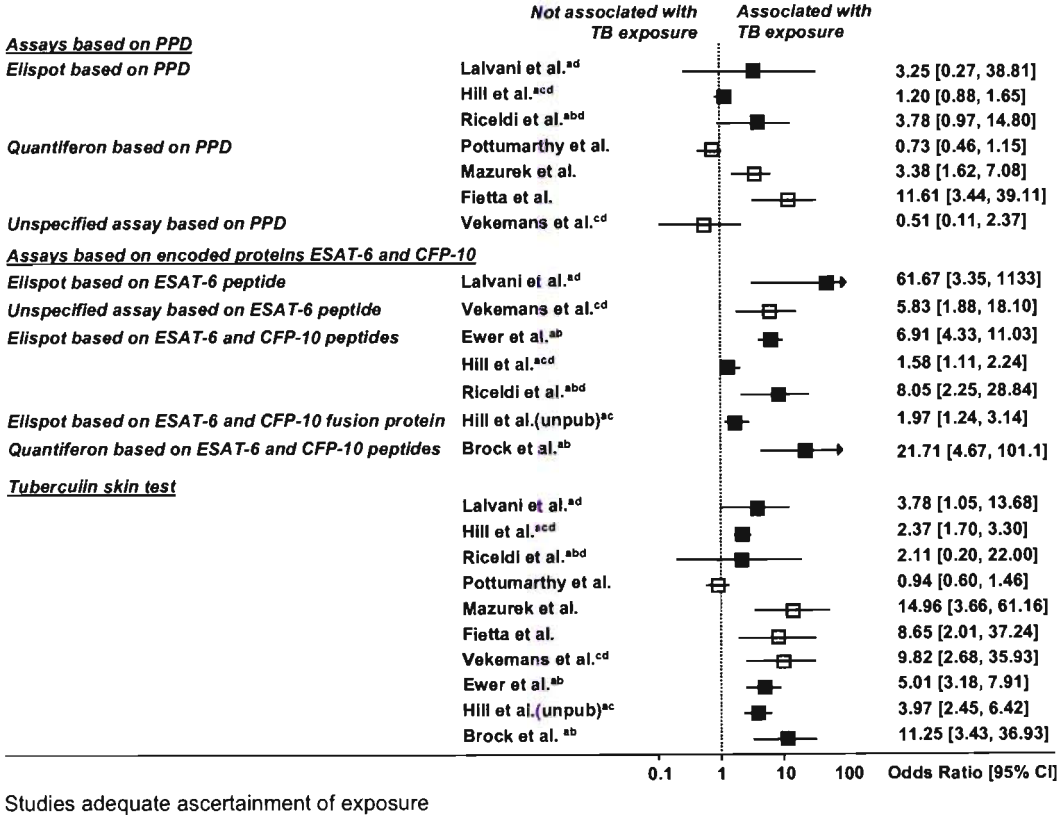
| Author & year | Setting | Participants | Exposures/ comparisons | Results | Comments |
|---|---|---|---|---|---|
| Urlichs 1998[132] | Berlin, Germany, volunteers from a research institute and TB patients from one chest hospital. | 19 healthy volunteers with previous PPD skin test (10 skin test –ve, 9 +ve). 31 culture positive TB patients at various stages of treatment. | ESAT-6 peptides and protein plus PPD, by 5 day ELISA assay. | 0/19 healthy volunteers were ESAT-6 or PPD positive. 15/17 untreated patients were ESAT-6 positive, 17/17 were PPD positive. Unclear, but appears that all treated patients were positive by both ESAT-6 and PPD. | Selection process and refusals were not documented. Unclear how recently the PPD skin test was performed, or its cut-off for positivity. |
| Van Pinxteren 2000[133] | Copenhagen, Denmark, one hospital and controls from unknown source | 24 sputum culture positive TB patients with 'minimal disease', 8 BCG vaccinated and 6 unvaccinated volunteer controls. | PPD, ESAT-6 and CFP-10 protein by 5 day ELISA assay. | 19/20 TB patients tested were PPD positive, 14 were ESAT-6/CFP-10 positive, 4 positive to only 1 antigen. 14/14 controls were PPD positive, 4//14 were ESAT-6/CFP-10 positive. | Selection process and refusals were not documented. Unclear exactly the BCG vaccination effect on controls. |
| Arend 2000[134] | Leiden, Holland, one hospital | 37 adult culture positive TB patients; 12 PPD skin test converters; 8 PPD skin test negative, BCG scar negative health workers. | 6 day ELISA for ESAT-6 and CFP-10 proteins in all subjects. | 35/37 patients were ESAT-6/CFP-10 positive. 9 and 5 of 12 PPD skin test converters were ESAT-6 or CFP-10 positive respectively. 0 health care workers were positive. | Selection process and refusals were not documented. |
| Pathan 2001[135] | London/Oxford, one hospital at each location | 27 PPD skin test positive contacts of sputum smear positive TB cases; 32 unexposed hospital workers; 25 culture positive cases; 8 'self-healed' TB cases; 11 TB cases with lymphadenitis; 17 TB cases with extra pulmonary TB. | ELISPOT in all volunteers for ESAT-6 peptides. 30 cases had ELISPOT for ESAT-6 protein. Cut-off 5 spots/well. | 40/44 TB cases were positive by ELISPOT. 23/27 TB contacts were positive. 0/32 hospital workers were positive (28 BCG vaccinated). All peptide positive cases tested were ESAT-6 protein positive. | ELISPOT spot counting by magnifying glass. Selection process and refusals not documented. |
| Arend 2001[136] | Leiden, Holland. | 44 contacts of a sputum smear positive TB case, 4 BCG vaccinated. | PPD skin test in all but 8; 6 day ELISA for ESAT-6 and CFP-10 proteins and peptides. | 17/36 contacts were PPD skin test positive (>=10mm); 9/12 PPD skin test positive contacts tested were ESAT-6/CFP-10 positive. 0/18 tested of the PPD skin test negative contacts were ESAT-6/CFP-10 positive. | Selection process and refusals were not documented. Probable 3 months between PPD skin testing and blood testing. |

| Author & year | Setting | Participants | Exposures/ comparisons | Results | Comments |
|---|---|---|---|---|---|
| Vekemans 2001[137] | Banjul, The Gambia, MRC Laboratories. | 30 sputum smear positive TB cases, 28 household contacts and 30 community controls. | PPD skin test (10mm cut-off), PPD and ESAT-6 protein by 6 day ELISA. | 11/29 controls tested were PPD skin test positive, 24/28 contacts, 27/29 cases. 27/30 controls were PPD blood positive, 23/28 contacts and 25/30 cases. For ESAT-6 9/30 controls were positive, 20/28 contacts, 13/30 cases. | Selection process well described but unclear if consecutive recruits. Unclear why TB cases had such a poor ESAT-6 response. |
| Lalvani 2001[138] | Bombay, India, one hospital and a clinic; Oxford, UK, unclear source. | 50 culture positive TB cases (India); 108 non-TB clinic attendees (India); 40 Oxford residents. | ESAT-6 peptides ESAT-6/CFP-10 peptides and PPD ELISPOT in all other subjects. Cut-off 5 spots/well. | 40/50 patients were ESAT-6 positive. 56/100 clinic attendees were ESAT-6 positive, 76 were CFP-10 positive. 80% were positive to one or the other antigen. 98 responded to PPD. 47/56 ESAT-6 peptide responders were ESAT-6 protein positive. No oxford residents responded to ESAT-6/CFP-10, 33/40 were PPD positive (33/40 were BCG vaccinated). | Selection process and refusals not documented. That 9 ESAT-6 peptide responders were ESAT-6 protein negative is unexpected. High percentage of responders is not consistent with other studies. |
| Lalvani 2001[123] | London and Oxford UK. | 47 culture positive TB cases; 47 group matched control patients (36 BCG vaccinated). 26 healthy household contacts of TB cases. 26 community controls. | ESAT-6 peptides ELISPOT and tuberculin skin testing. Some had ESAT-6 protein ELISPOT.5 spot cut-off. | 45/47 patients were ESAT-6 peptides positive; all 17 tested were ESAT-6 protein positive. 18/26 TB patients were PPD skin test positive. 4/47 control patients were ESAT-6 positive. 22/26 PPD skin test positive case contacts were ESAT-6 positive. 0/26 community controls were positive. | Selection process and refusals were not documented. |
| Lalvani 2001[139] | London, UK, one hospital. | 50 contacts of sputum smear positive TB cases. | Chest x-ray (46/48 tested were normal); Heaf test (grade 3-4 cut-off). ESAT-6 protein ELISPOT (10 spot cut-off), manual counting. Logistic regression models relating exposure intensity to test result. | ESAT-6 positivity was strongly related to exposure (OR 9; 95% CI 2.6-31.6); PPD skin test had weaker relation (1.9; 1.0-3.5); ELISPOT was not influenced by BCG status, PPD skin test was (12.1; 1.3-115.7). | Relatively small size, epidemiologically, limits the conclusions that can be made from an otherwise important study. |

| Author & year | Setting | Participants | Exposures/ comparisons | Results | Comments |
|---|---|---|---|---|---|
| Brock 2001[140] | Copenhagen, Denmark: community volunteers, one hospital. | 19 BCG vaccinated and 16 unvaccinated community controls, 13 smear positive pulmonary cases, 5 extra pulmonary cases. | PPD, ESAT-6 and CFP-10 protein whole blood assay according to Quantiferon TB commercial kit. | 0/19 non-BCG vaccinated volunteers were positive by PPD, ESAT-6 of CFP-10. Of BCG vaccinated, 2/19 responded to ESAT-6/CFP-10, 9/19 to PPD. 79% of cases responded to PPD, 72% to ESAT-6/CFP-10. | Selection process and refusals were not documented. |
| Doherty 2002[141] | Hossanna, Ethiopia, one regional hospital | 24 HIV negative household contacts of sputum positive TB cases. | PPD and ESAT-6 protein by 5 day ELISA assay. 2 years' follow-up for development of TB disease. | 7/24 contacts developed TB (clinical diagnosis: x-ray, sputum positivity in 2) at 2 years follow-up. PPD ELISA was positive in 21/24 at recruitment and ESAT-6 was positive in 9/24 at recruitment. 6/9 ESAT-6 positive became cases vs. 1/15 negative ($p<0.001$). No significant PPD difference. | Selection process and refusals/exclusions were not clearly documented. High rate of secondary cases is extraordinary and suspicious. |
| Bellete 2002[142] | Baltimore, United States, one public health service; Ethiopia, one research institute. | 202 subjects undergoing screening in Baltimore, 275 subjects undergoing screening in Ethiopia. | PPD skin test, 5mm cut-off in Ethiopia, 10mm in high risk subjects, 15mm in low risk. Quantiferon-TB commercial whole blood 2-day assay for CFP-10 and ESAT-6. | Estimated assay sensitivity was 71-85% in treated cases and specificity 75-84% in likely uninfected; skin test: 95% and 96% respectively. 1 of 11 3/12 turned assay negative from positive. None turned skin test negative. Test agreement was 79.4% in Baltimore and 35% in Ethiopia. Possible poor performance of blood test in skin test positive HIV positive subjects. | Selection process was not fully described. However exclusions were well described. Low cut-off used for skin test in Ethiopia. Cut-off for assay was determined using the data of the study polarising the subjects. |
| Ewer 2003[143] | Leicester, UK, school outbreak | 594 school students aged 11-15 years. | Heaf test (grade 3-4 cut-off), ELISPOT to ESAT-6 and CFP-10 peptides and ESAT-6 protein (5 spot cut-off). Students divided according to 4 levels of exposure to sputum smear positive TB case. | 18/20 highest exposure students were PPD skin test positive, 20/20 ELISPOT positive. 76/387 vs. 66/387 in lowest exposure category. Difference in correlation with exposure was significant ($p=0.03$). BCG vaccination was associated with PPD skin test ($p=0.002$), but not the ELISPOT. Concordant results were in 89% of children. | Reasons for refusal are not given, but demographic comparison provided. Apparently the PPD skin test was conducted several weeks before the ELISPOT (Ewer, personal communication). |

| Author & year | Setting | Participants | Exposures/ comparisons | Results | Comments |
|---|---|---|---|---|---|
| Sester 2004[144] | Saarland, Germany; one hospital | 127 haemodialysis patients; controls: 107 health care workers, 59 patients, 52 blood donors. | Whole blood PPD by CD4 Flow cytometry; Mantoux skin test (5mm cut-off); ESAT-6 and CFP-10 by flow cytometry. Comparison: difference in positivity between blood and skin test responses. | 48.6% of health care workers were PPD blood +ve, 91.7% of these were PPD skin test positive. 53.5% of haemodialysis patients were PPD blood +ve but only 51.4% of these were PPD skin positive. 50% of PPD blood +ve subjects were ESAT-6/CFP-10 positive, not different by subject category | Selection criteria and refusals were not documented. Assumption that ESAT-6/CFP-10 are reflective of latent infection. Skin test cut-off low. BCG status unclear. |
| Brock 2004[145] | Denmark, high school TB outbreak | 125 contacts of a TB case (85 not BCG vaccinated): 45 defined as high exposure, 40 as low exposure. | PPD skin test (cut-off 10mm), Whole blood 2-day assay for response to PPD, ESAT-6 and CFP-10 proteins. | 28/53 high exposure contacts were ESAT-6/CFP-10 positive, 4/72 were positive in the low exposure group. PPD ELISPOT: 24/53 and 14/72 respectively (12 of the latter 14 were of the 32 BCG vaccinated in that category). PPD skin test (only done in BCG unvaccinated): 25/45 and 4/40 respectively. Agreement between whole blood ESAT-6/CFP-10 and PPD skin test was 94% (kappa value 0.866). | Selection criteria and refusals were not documented. Health authorities originally investigated 700 contacts. Lack of PPD skin testing in BCG vaccinated subjects is a weakness of the study. |

**Figure 1-2. Association of tests for *M. tuberculosis* infection and exposure, summarised by Kunst et al.[146] Odds ratios and 95% confidence intervals were generated using RevMan 4.2 software.**




[a] Studies adequate ascertainment of exposure
[b] Studies investigating an outbreak
[c] Studies in high TB prevalence country
[d] Encoded antigen based assays more strongly associated with TB exposure than PPD based assays in direct comparison

The following general conclusions can be made from the assessment of published studies:

- T cell assays based on PPD are not as clearly associated with *M. tuberculosis* exposure as those based on ESAT-6/CFP-10.
- Estimates of test sensitivity and specificity of T cell assays vary considerably across studies.
- A number of the studies had significant weaknesses in presentation at least. For example, the selection process was commonly not described and the study subjects were often not fully accounted for in the results.

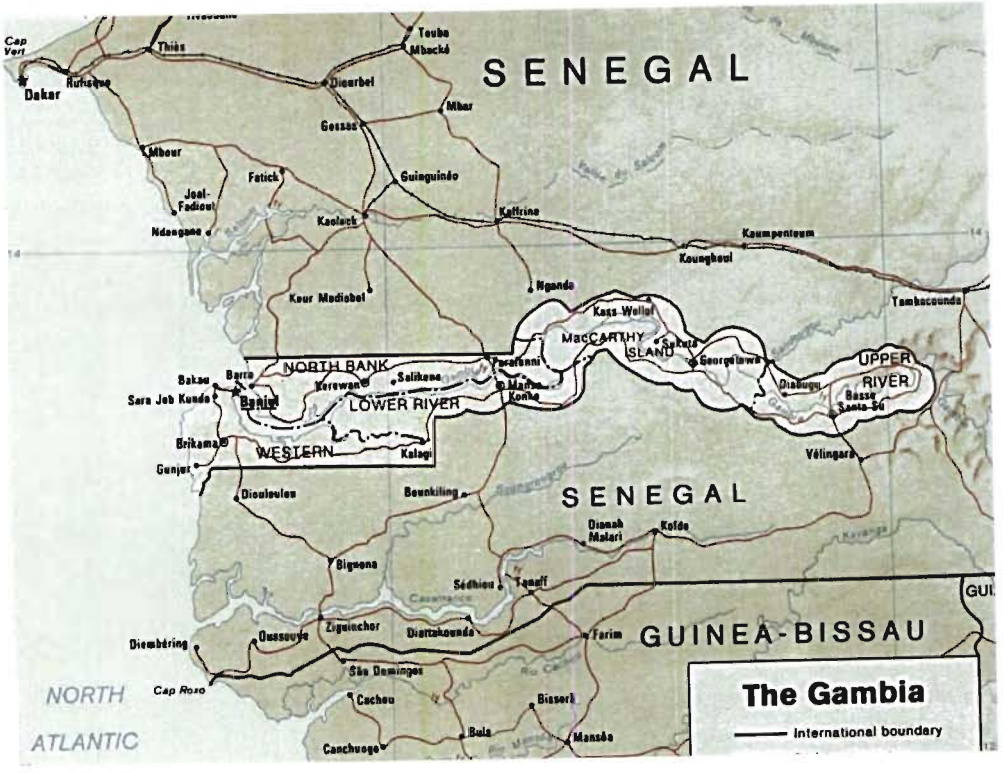
- Some studies were difficult to compare because of the use of different techniques (e.g. Use of a magnifying glass to detect immunospots versus an automated reader) or criteria for test positivity.
- Only one study provided any longitudinal assessment of a T cell assay.
- The majority of the studies were from developed countries, leaving considerable doubt over how T cell assays that use ESAT-6 and CFP-10 will perform in developing countries where environmental mycobacteria are abundant.[147]

## 1.5 Research setting

### 1.5.1 The Gambia

The Republic of The Gambia is a small country on the bulge of the West African coast bordered by Senegal on the North, East and South, and by the Atlantic Ocean on the West. It has a population of approximately 1.3 million people comprising several ethnic groups, mainly Mandinka, Fulani (Fula), Wollof and Jola. English is the official language but many other languages are spoken. Approximately ninety percent of the population are Muslim; the remaining ten percent are predominantly Christian. The country has an annual population growth rate of 4.2 percent. It is rated among one of the Least Developed Countries in the world, with a Gross National Product per capita income of US $320 per annum. Agriculture is the mainstay of the country's economy, comprising mainly groundnut and livestock farming.[148]

**Figure 1-3. Map of The Gambia**



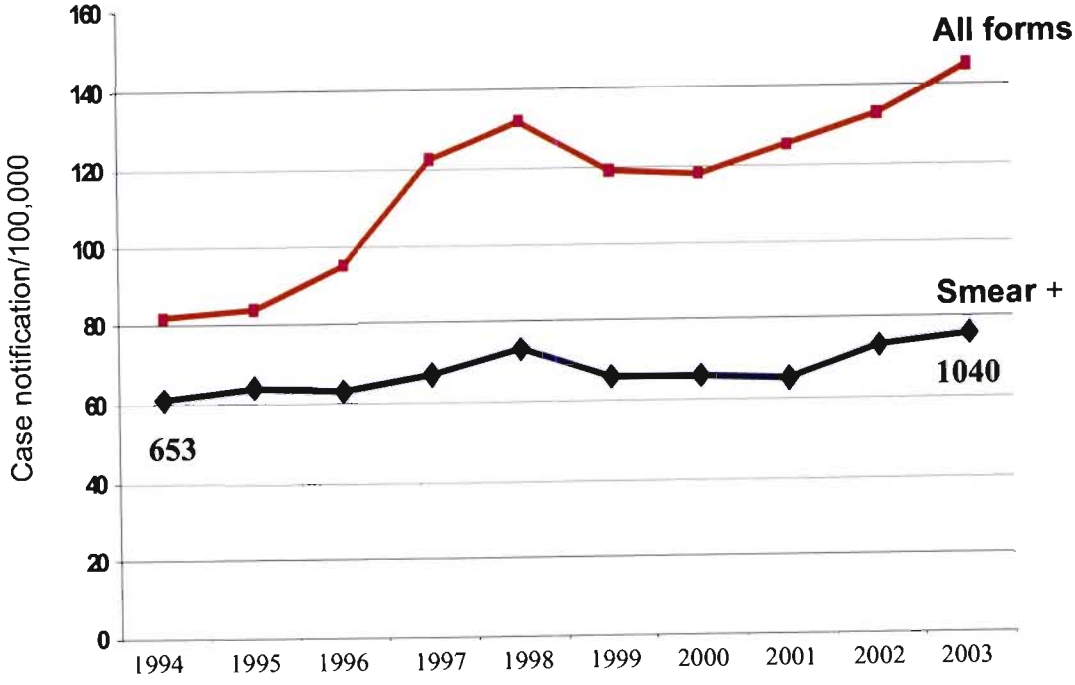

### 1.5.2 Tuberculosis in the Gambia

Tuberculosis is a significant public health problem in The Gambia – The TB case notification was 82/100,000 in 1994, rising to 140/100,000 in 2004, despite the

establishment of a programme of Directly Observed Therapy.[149] On the basis of notification figures, TB disease has the highest incidence in the Greater Banjul area, reaching 178/100,000 in 2004. HIV seroprevalence in the Gambia is relatively low for an African country, at approximately 2%.[150]

The contribution of sputum smear negative cases to the incidence rate of all-form TB disease in Gambia has been increasing over time (Figure 1-4). While a slow but steady increase in HIV seroprevalence in The Gambia may have contributed to this, other factors such as the increasing availability of chest x-rays, have been involved. The prevalence of HIV infection in sputum smear positive TB cases is 8-10%.[1,151]

**Figure 1-4: Incidence rate of all-form and smear positive TB cases by year for The Gambia, 1994-2003.[149] The numbers on the graph in bold are the actual numbers of cases.**



### 1.5.3 Research questions

The studies of this thesis seek to address the following major questions:

1. Can a reproducible framework be developed to assess new diagnostic tools for *M. tuberculosis* infection? (chapter 2).
2. Are new T cell assays that utilise 'specific' *M. tuberculosis* antigens better than the traditional PPD skin test for the diagnosis of *M. tuberculosis* infection? (chapter 2).
3. Does the extension of an *M tuberculosis* exposure gradient outside the immediate household compound of a known TB case provide useful information about the performance of new T cell assays and the PPD skin test? (chapter 3).
4. Is a fusion protein of 'specific' antigens as good as or better than their individual peptides in the diagnosis of *M. tuberculosis* infection by ELISPOT? (chapter 4).
5. Does the ELISPOT test for *M. tuberculosis* infection perform as well or better than the PPD skin test in children of different age groups? (chapter 5).
6. Noting that T cell assays and PPD skin test give a quantitative readout, converted to a qualitative yes/no readout in practice, is there added information that a quantitative analysis provides? (chapter 6).
7. Does the T cell assay response to *M. tuberculosis* change over time? Specifically, do more case contacts become positive over time and do those already positive stay positive? Further, are there risk factors for 'conversion' and 'reversion' and how do these relate to the PPD skin test over time? (chapter 7).

# 2

## Large scale evaluation of ELISPOT assay and skin test for diagnosis of *Mycobacterium tuberculosis* infection against a gradient of exposure in The Gambia

A Gambian house

## 2.1 Summary

*Background.* The purified protein derivative (PPD) skin test for *Mycobacterium tuberculosis* infection lacks specificity.

*Methods*. We assessed two more specific *M. tuberculosis* antigens (ESAT-6; CFP-10) by enzyme linked immunospot assay (ELISPOT) compared to PPD by ELISPOT and skin test in The Gambia.

*Results*. Of 735 household contacts of 130 sputum smear positive TB cases, 476 (65%) were positive by PPD ELISPOT, 300 (41%) by PPD skin test and 218 (30%) by ESAT-6/CFP-10 ELISPOT. Only 15 (2%) were ESAT-6/CFP-10 positive and PPD negative by ELISPOT. With increasing *M. tuberculosis* exposure the percentage of subjects PPD skin test positive/ESAT-6/CFP-10 ELISPOT negative increased ($p<0.001$) while the percentage of subjects PPD skin test negative/PPD ELISPOT positive decreased ($p=0.011$). Eighteen (31%) ESAT-6/CFP-10 ELISPOT positive subjects in the lowest exposure category were PPD skin test negative.

*Conclusions*. ESAT-6/CFP-10 ELISPOT probably offers increased specificity in the diagnosis of *M. tuberculosis* infection in this setting, at the cost of some sensitivity.

## 2.2 Introduction

Significant impediments to global tuberculosis (TB) control include limitations of current diagnostic tests.[152] Identification of *M. tuberculosis* infected individuals is especially difficult in tropical environments, where Bacille Calmette-Guérin (BCG) coverage is high and environmental mycobacteria are commonly encountered. The conventional Purified Protein Derivative (PPD) skin test has unknown sensitivity and specificity for *M. tuberculosis* infection and its response after BCG vaccination varies by latitude.[114] New tests are required to assist development of entry criteria for trials of new generation TB vaccines and to make preventive TB treatment feasible where resources are scarce.

Certain highly immunogenic secreted antigens of *M. tuberculosis*, such as ESAT-6 and CFP-10, offer hope for more specific diagnosis of *M. tuberculosis* infection. Their genes were deleted from *M. bovis* in the development of BCG and are found in few environmental mycobacteria.[24] Immunoassays for gamma interferon (IFN-γ) production in response to ESAT-6/CFP-10 have been tested in TB cases, their contacts and members of the general community. In non-tropical settings, they have been shown to be relatively sensitive in TB cases (>80%), approaching 100% specificity in non-endemic general communities, and have proven capable of distinguishing those who are positive on PPD skin test due to previous BCG vaccination.[137-139]

That ESAT-6 and CFP-10 are secreted by some non-tuberculous mycobacteria,[124] has led to the suggestion that the high prevalence of such organisms in the environment in tropical countries may render the assays non-specific.[147] We hypothesized that measurement of the immune responses to ESAT-6 and CFP-10 offers improved specificity over PPD for the diagnosis of *M. tuberculosis* infection in a tropical setting, without compromising sensitivity. Therefore we compared the IFN-γ responses to ESAT-6/CFP-10 by enzyme linked immunospot assay (ELISPOT) to the IFN-γ responses to PPD by ELISPOT and the delayed hypersensitivity response (DTH) to PPD by skin test, in The Gambia.

## 2.3 Methods

***Participants***. Sputum smear positive TB index cases over 15 years of age were recruited in Greater Banjul, an area housing 450,000 people, where the incidence rate of newly diagnosed sputum smear positive TB is approximately 80 per 100,000 population per year.[153] Included cases had two sputum samples positive for acid-fast bacilli by Ziehl-Neelson stain and *M. tuberculosis* on culture. They were identified at the major government health centre and the Medical Research Council Laboratories' (MRC Labs) outpatients' clinic, examined, had a chest X-ray reported by two specialist physicians, and invited, after counselling, to have an HIV test.

Household contacts were included if they were at least 6 months of age and lived the majority of the time on the same compound as the case. They were not eligible if treated for TB in the past year, or if recruited after 60 days of the case, and were excluded if diagnosed with TB within 1 month of recruitment and coughing for longer than the case. Subjects were brought to the MRC Labs, invited to give informed consent, interviewed, examined, and a blood sample taken for ELISPOT and HIV test. Fresh samples from all participants were processed onsite. To meet a logistic ceiling, a maximum of 12 contacts per day were screened, the others randomly excluded. Subject details were recorded on a standardised form.

Contacts underwent a PPD skin test (2 TU, PPD RT23, Statins Serum Institut, Copenhagen, Denmark). Induration was recorded at 48-72 hours. Regular 'spot-checks' were performed plus a second reading on 100 consecutive skin tests. Subjects with a positive skin test (mean induration diameter ≥ 10mm) were offered a chest X-ray and those with symptoms underwent a clinical assessment. Those with TB disease were referred to the National Programme for free treatment. There is no current practice of preventive treatment in The Gambia.

This study was approved by the Gambia Government/MRC joint Ethics Committee.

***Laboratory procedures.*** Sputum smears were prepared and stained with auramine-phenol[154] and confirmed by Ziehl-Neelsen (Z-N). Decontaminated specimens were inoculated into one slope each of Lowenstein-Jensen medium (L-J) containing glycerol and sodium pyruvate respectively and one vial of BACTEC 9000 MB media for isolation

of *M. tuberculosis*. All mycobacterial cultures were identified and confirmed using standard procedures.

The *ex vivo* ELISPOT assays for IFN-γ were performed as previously described.[155] For this study, synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (ABC, 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 two pools of 8 or 9 peptides (4 pools in total). Purified Protein Derivative (*M. tuberculosis,* RT49, Statins Serum Institut, Copenhagen, Denmark) was used at 10 μg/ml. The positive control was Phytohaemagglutinin (PHA; Sigma-Aldrich, UK). All antigens were tested in duplicate wells.

Assays were scored by an ELISPOT counter (AID-GmbH, Strassberg, Germany). The spot forming unit (SFU) numbers counted in each well were automatically entered into a database. Supplementary details were added by double data entry by two immunologists blinded to subject details. Positive test wells were pre-defined as containing at least ten SFUs more than, and at least twice as many as, negative control wells. For a positive ESAT-6/CFP-10 result it was necessary for one or more pools of overlapping peptides to be positive. PHA positive control wells were set to at least 150 SFUs above negative control wells. Negative control wells were required to have less than 30 SFUs.

Testing for HIV-1 or HIV-2 infection was by competitive enzyme linked immunosorbent assays (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France).

***Ascertainment of exposure.*** Various factors were assessed as surrogate markers of *M. tuberculosis* exposure. Similar to a previous Gambian study,[156] TB contacts were categorised according to where they slept: in the same bedroom as the case, a different bedroom in the same house, or in a different house. The average amount of time spent daily on the compound with the case was also assessed. The following TB case factors were assessed: duration of cough, sputum smear grade, and number of diseased quadrants on chest X-ray.

***Data management and analysis.*** All data were entered using double data entry into an ACCESS database and checked for errors. A random effects logistic regression

model, taking into account household clustering, was used to assess the relationship between risk factors and test results. Results were reported as unadjusted and adjusted odds ratios and their 95% confidence intervals (CI). The likelihood ratio test was used to test for interaction and for trend. All statistical analyses were conducted using Stata software (version 7; Stata Corp, College Station, TX).

## 2.4 Results

From May 2 2002 to April 24 2003, 143 cases and 135 households were investigated. Of 1059 eligible case contacts, 983 gave consent and 856 were selected for ELISPOT and had an adequate blood specimen. Of these, 735 (86%) contacts of 130 cases had a PPD skin test result plus ELISPOT results that met inclusion criteria (Tables 2-1 and 2-2). Skin tests from 231 subjects were read twice, giving a kappa statistic of 0.83 and 95% concordance.

Overall, 476 (65%) subjects were positive by PPD ELISPOT, 300 (41%) by PPD skin test and 218 (30%) by ESAT-6/CFP-10 ELISPOT. Only 15 (2%) subjects were ESAT-6/CFP-10 ELISPOT positive and PPD ELISPOT negative, whereas 56 (8%) were ESAT-6/CFP-10 ELISPOT positive and PPD skin test negative, and 240 (33%) were PPD ELISPOT positive and PPD skin test negative (Figure 2-1).

The univariable odds of a result being positive for each test by various possible surrogate measures of increasing exposure to *M. tuberculosis* are presented in Table 2-3. All the tests were significantly more likely to be positive in contacts with closer sleeping proximity to the case. This increase was most dramatic for PPD skin test. The odds of being PPD ELISPOT positive was higher for those subjects who reported spending over 12 hours on average per day on the compound with the case compared with the baseline of ≤ 4 hours. The duration of cough of the case and the number of diseased quadrants on X-ray were not useful measures.

The odds of test positivity were increased for the presence of a BCG scar for PPD ELISPOT and PPD skin test, but this was not statistically significant (OR=1.37; 95%CI 0.95-1.97; OR=1.1; 95%CI 0.75-1.60 respectively). The odds of test positivity decreased with the presence of a BCG scar for the ESAT-6/CFP-10 ELISPOT, also not significant (OR=0.74; 95%CI 0.5-1.1).

In the multivariable analysis, the odds of being positive by ESAT-6/CFP-10 ELISPOT for those sleeping in the same room as the case relative to those in a different house, adjusted for age, sex and ethnicity, remained significant (OR=1.85; 95% CI 1.06-3.23). After adjustment for age, sex, ethnicity and time spent on the compound with the case, the odds of being PPD ELISPOT positive for those in the same room as the case relative to those sleeping in a different house was no longer significant (OR=1.5; 95% CI 0.85-2.64). There was also an interaction found between smear grade and sleeping

proximity (p=0.04): for those sleeping in a different house the OR for smear grade 3+ was 3.05 (95% CI 1.43- 6.53), whereas for those sleeping in a different room or the same room the OR for smear grade 3+ was close to 1. For PPD skin test positivity, sleeping proximity and sputum smear grade remained independent risk factors and there was a significant interaction between age and sex. The adjusted OR for sleeping in a different room relative to a different house was 2.12 (95% CI 1.29-3.48), and for sleeping in the same room relative to a different house 4.74 (95% CI 2.67-8.46). The distributions of age and sex by proximity, together with ethnicity, are shown in Table 2-4.

The proportions of subjects positive by PPD ELISPOT, ESAT-6/CFP-10 ELISPOT, or PPD skin test across three exposure categories are shown graphically in Figure 2-2, together with a matrix showing, within each category, the percentage of subjects with each combination of the test results. The proportion of subjects positive by ESAT-6/CFP-10 ELISPOT or by PPD skin test were not as high as the proportion positive by PPD ELISPOT in any category. PPD skin test positivity diverged from ESAT-6/CFP-10 with increasing TB exposure, and from PPD ELISPOT with decreasing TB exposure. Consistent with the divergence between PPD skin test and ESAT-6/CFP-10 ELISPOT the percentage of subjects who were positive by PPD skin test and negative on ESAT-6/CFP-10 ELISPOT increased from 11% of those sleeping in a different house from the case to 32% ($p<0.0001$) of those sleeping in the same room (Figure 2-2, matrix). Consistent with the divergence of the two measures of PPD immune response, the percentage of subjects positive on PPD ELISPOT and negative on PPD skin test increased from 22% of those sleeping in the same room as the case to 41% of those sleeping in a different house (p=0.011). The percentage of subjects positive on PPD skin test and negative on PPD ELISPOT was 8.7% overall and did not change significantly across the exposure categories (p=0.17). Almost 1/3 of subjects (31%, n=58) sleeping in a different house as the case and positive by ESAT-6/CFP-10 ELISPOT were negative by PPD skin test.

| Characteristics | |
|---|---|
| **Demographic** | |
| Mean (median: range) age (years) | 32 (28:15-75) |
| Male | 93 (71%) |
| **Ethnic group** | |
| Mandinka | 42 (32%) |
| Jola | 24 (18%) |
| Wolof | 22 (17%) |
| Fula | 11 (8%) |
| Other | 31 (24%) |
| **Clinical** | |
| Mean (median; range) duration of cough (weeks) | 7 (4; 1-20) |
| Smear grade (1+ / 2+ / 3+) | 2/ 23/ 105 |
| BCG scar present (n=124)[a] | 20 (16%) |
| Mean (range) number of quadrants involved on X-ray | 2 (0-4) |
| HIV positive (n=127) | 9 (7%) |

[a] 6 subjects had uncertain scar status

**Table 2-1**: **Characteristics of 130 Sputum Smear and Culture positive Cases**

| Characteristics | |
|---|---|
| **Demographic** | |
| Mean (median: range) age (years) | 20 (16:0-100) |
| Male | 323 (44%) |
| Ethnic group | |
| Mandinka | 239 (33%) |
| Jola | 144 (20%) |
| Wolof | 140 (19%) |
| Fula | 47 (6%) |
| Other | 165 (22%) |
| Proximity to case (%) | |
| Same room | 149 (20%) |
| Different room | 340 (46%) |
| Different house | 246 (33%) |
| Mean (median: range) hours per day with case | 10 (8:1-24) |
| **Clinical** | |
| BCG scar present (n=629)[a] | 282 (45%) |
| HIV positive (n=724) | 22 (3%) |

[a] 106 subjects had uncertain BCG scar status

**Table 2-2: Characteristics of 735 Contacts of Cases**

| Marker | | PPD ELISPSOT | | ESAT-6/CFP-10 ELISPOT | | PPD skin (≥ 10 mm) | |
|---|---|---|---|---|---|---|---|
| | | % (n) positive | OR (95% CI) | % (n) positive | OR (95% CI) | % (n) positive | OR (95% CI) |
| **Sleep Proximity to case** | Different house | 62 (152) | 1 | 24 (58) | 1 | 28 (68) | 1 |
| | Different room | 63 (215) | 1.26 (0.81, 1.95) | 30 (103) | 1.45 (0.89, 2.35) | 41 (138) | 2.06 (1.28, 3.33) |
| | Same room | 72 (108) | 1.76 (1.04, 3.00) p=0.04[a] | 38 (57) | 1.96 (1.13, 3.38) p=0.02[a] | 62 (93) | 5.02 (2.87, 8.78) p<0.0001[a] |
| **Time spent in compound per day with case[b]** | ≤ 4 hours | 63 (120) | 1 | 26 (50) | 1 | 34 (65) | 1 |
| | 5-8 hours | 62 (116) | 1.00 (0.63, 1.60) | 25 (47) | 0.93 (0.54, 1.59) | 43 (80) | 1.50 (0.90, 2.50) |
| | 9-12 hours | 62 (119) | 1.05 (0.65, 1.71) | 33 (64) | 1.29 (0.76, 2.20) | 43 (82) | 1.19 (0.70, 2.01) |
| | > 12 hours | 76 (115) | 1.84 (1.07, 3.16) | 56 (37) | 1.41 (0.80, 2.47) | 44 (67) | 1.43 (0.81, 2.51) |
| **Case smear grade** | 1+/2+ | 57 (93) | 1 | 23 (37) | 1 | 25 (41) | 1 |
| | 3+ | 67 (382) | 1.66 (0.96, 2.88) | 31 (181) | 1.53 (0.80, 2.93) | 45 (258) | 2.88 (1.47, 5.65) |
| **Duration of case cough[c]** | 1-3 weeks | 65 (107) | 1 | 29 (48) | 1 | 40 (66) | 1 |
| | 4-7 weeks | 66 (171) | 0.94 (0.50, 1.76) | 31 (79) | 1.09 (0.54, 2.19) | 42 (111) | 1.24 (0.58, 2.64) |
| | 8-11 weeks | 67 (78) | 1.34 (0.62, 2.87) | 35 (41) | 1.48 (0.65, 3.37) | 44 (51) | 1.33 (0.54, 3.26) |
| | ≥ 12 weeks | 61 (117) | 0.83 (0.42, 1.63) | 25 (49) | 0.81 (0.38, 1.74) | 36 (70) | 0.93 (0.41, 2.11) |
| **No. quadrants on Case X-ray** | 0-1 | 71 (107) | 1 | 34 (51) | 1 | 42 (63) | 1 |
| | 2 | 62 (194) | 0.60 (0.32, 1.12) | 28 (84) | 0.72 (0.36, 1.44) | 40 (119) | 1.29 (0.60, 2.75) |
| | 3 | 61 (88) | 0.63 (0.30, 1.31) | 23 (34) | 0.53 (0.23, 1.22) | 43 (62) | 1.36 (0.57, 3.28) |
| | 4 | 66 (96) | 0.72 (0.34, 1.51) | 34 (49) | 0.88 (0.39, 1.98) | 38 (55) | 0.89 (0.36, 2.19) |

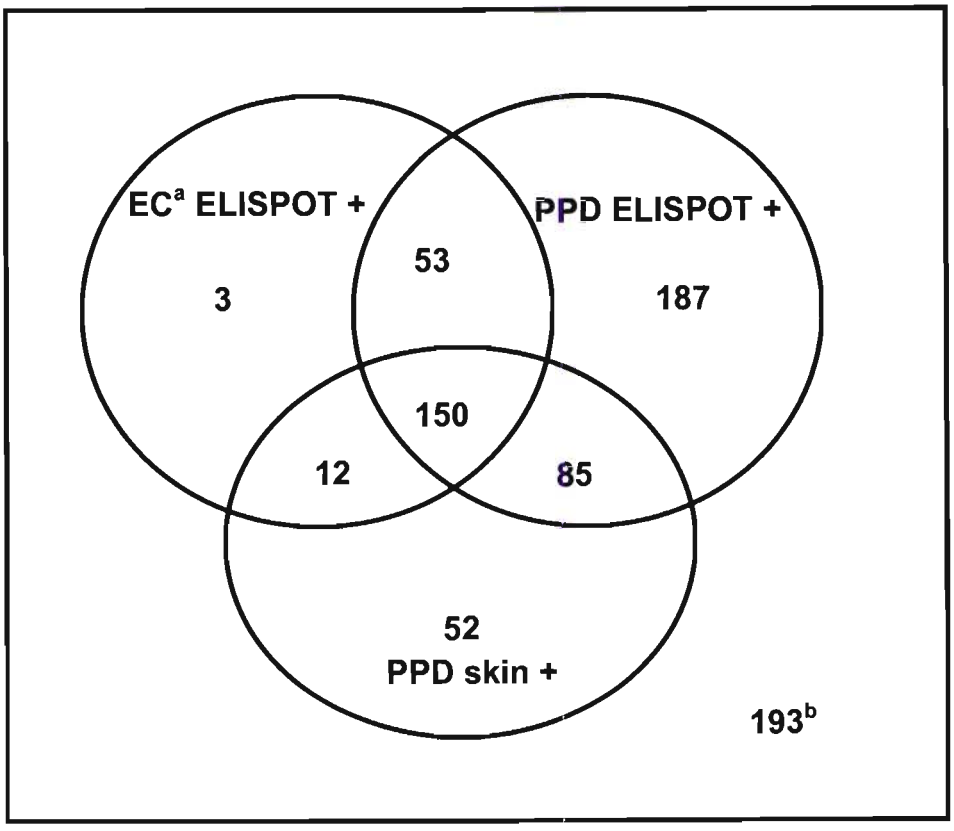
[a] p for linear trend, [b] missing for 15 contacts, [c] missing for one case who contributed two contacts to the study

**Table 2-3: Univariable Odd ratios by logistic regression (household as a random effect) for possible surrogate markers of exposure**

| Characteristic | Same room (n=149) | Different room (n=340) | Different house (n=246) |
|---|---|---|---|
| **Age** | | | |
| **Median years (range)** | 18 (0-81) | 14.5 (0-100) | 18 (0-80) |
| **% (n) 0-9 years** | 31 (46) | 33 (111) | 23 (56) |
| **% (n) 10-18 years** | 23 (34) | 31 (105) | 34 (83) |
| **% (n) 19 years +** | 46 (69) | 36 (124) | 43 (107) |
| **% (n) Male** | 50.3 (75) | 40.6 (138) | 44.7 (110) |
| **Ethnic group** | | | |
| % (n) Mandinka | 31 (46) | 31 (106) | 35 (87) |
| % (n) Jola | 17 (26) | 17 (57) | 23 (57) |
| % (n) Wolof | 22 (33) | 21 (71) | 16 (40) |
| % (n) Fula | 5(8) | 5 (16) | 9 (23) |
| % (n) Other | 24 (36) | 26 (90) | 16 (39) |

**Table 2-4: Characteristics of 735 contacts by exposure category**

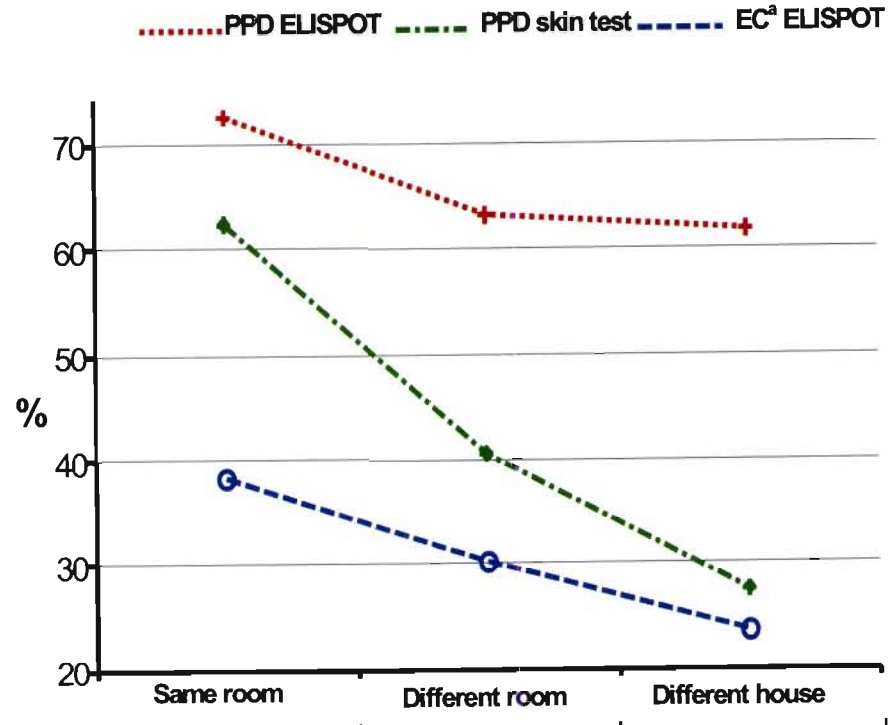
Figure 2-1: Venn diagram for the number of subjects with each combination of ESAT-6/CFP-10 ELISPOT, PPD ELISPOT and PPD skin test results (n=735).




[a] ESAT-6/CFP-10

[b] negative for all tests

**Figure 2-2: Percent positive for ELISPOT, Skin test and for combinations of results, by exposure category**




| PPD ELISPOT | EC[a] ELISPOT (n=149) | | EC[a] ELISPOT (n=340) | | EC[a] ELISPOT (n=246) | | PPD skin test |
|---|---|---|---|---|---|---|---|
| | POS | NEG | POS | NEG | POS | NEG | |
| POS | 28.2 | 22.1 | 21.2 | 10.6 | 14.6 | 6.5 | POS |
| | 8.1 | 14.1 | 7.1 | 24.4 | 6.9 | 33.7 | NEG |
| NEG | 2.0 | 10.1 | 1.5 | 7.4 | 1.6 | 4.9 | POS |
| | 0.0 | 15.4 | 0.6 | 27.4 | 0.4 | 31.7 | NEG |

[a] ESAT-6/CFP-10

## 2.5 Discussion

These results now provide a large-scale evaluation of the ELISPOT assay alongside the PPD skin test in TB contacts above 6 months of age in a tropical country. The ELISPOT assay performed predictably: with increasing recent exposure to *M. tuberculosis* and a likely background of intense mycobacterial exposure, the percentage of subjects positive by PPD was consistently high and increased slightly, while the percentage of those positive by ESAT-6/CFP-10 was less in all categories and also increased. Only 2% of subjects were ESAT-6/CFP-10 positive and PPD negative. It can be concluded that the ESAT-6/CFP-10 ELISPOT assay improved specificity over PPD ELISPOT. The PPD skin test results showed the most marked change across the *M. tuberculosis* exposure categories. With respect to the relationship between the PPD skin test and the two ELISPOT tests, two key findings from this study provide valuable insights: the percentage of subjects who were PPD skin test positive and ESAT-6/CFP-10 ELISPOT negative increased significantly with increasing *M. tuberculosis* exposure and a large percentage of subjects, especially in the lowest exposure group, were PPD ELISPOT positive and PPD skin test negative.

That the proportion of subjects positive on the PPD skin test and negative on ESAT-6/CFP-10 ELISPOT increased with increasing *M. tuberculosis* exposure (11% to 32%) supports the assertion that two *M. tuberculosis*-specific antigens may not be sufficient for the diagnosis of *M. tuberculosis* infection.[132] Comparison of the two ELISPOT tests also provides more subtle evidence for a sensitivity loss for ESAT-6/CFP-10 ELISPOT: the difference in PPD ELISPOT positivity between those sleeping in a different room and those sleeping in the same room as the case was similar to that for ESAT-6/CFP-10 ELISPOT (9.2% and 8.0% respectively), but arising from a much higher 'baseline' (the lowest exposure category). The discovery of more *M. tuberculosis* specific reagents may lead to increased sensitivity. Antigen mining from the *M. tuberculosis* genome has been utilised to identify potentially useful diagnostic antigens for *M bovis* in cattle.[157] A study of an outbreak in England suggested high sensitivity for ESAT-6/CFP-10 by ELISPOT, although the source case had been coughing for over a year and the cut-off for positivity was set at 5 spots above background.[143] Even in a study of the size reported here the number of subjects is insufficient for the sensitivity analysis required to define precisely the number of spots for a cut-off in the diagnostic antigen or control wells.

The PPD ELISPOT positive, PPD skin test negative results are similar to those noted in Malawi by Black et al, where 63% of subjects over 12 years old were positive on a 6-day assay and 37% were positive by PPD skin test at a 5mm cut-off.[158] They, along with a previous Gambian study,[156] also noted a similar age and sex pattern with the PPD skin test as our study. The discordance between the skin and blood responses may represent a specific DTH skin anergy caused by regular mucosal exposure to environmental mycobacteria. This is consistent with an experiment by Hoft et al[159] showing oral ingestion of BCG caused inhibition of the DTH skin test while inducing significant increases in mycobacteria-specific interferon-γ responses in peripheral blood mononuclear cells. That almost 1/3 of those ESAT-6/CFP-10 positive in the lowest exposure category were negative by PPD skin test suggests a significant sensitivity problem for the skin test in these subjects, and a proportion of the dramatic change in positivity for the PPD skin test across the exposure categories is likely to be due to boosting of a suppressed DTH response by recent *M. tuberculosis* exposure.[113] Follow-up to identify secondary cases will give further indication of the relative sensitivities of these tests, as one would expect those who become secondary cases to be positive at recruitment.

That nearly 35% of all subjects tested were negative on either ELISPOT test, in a TB-endemic setting with frequent environmental mycobacteria exposure, may indicate waning over time. It is likely that the *in vitro* IFN-γ ELISPOT detects recently activated lymphocytes with immediate effector function and effector-memory cells that persist for a limited time in circulation once antigen is cleared.[135,160] Antigens may well be differentially expressed by *M. tuberculosis* during the lifecycle from early to stationery phase to latency. ESAT-6 and CFP-10 are secreted early in infection, although ESAT-6 gene expression has also been shown to occur under hypoxic, latency-type conditions.[161] Other antigens, such as alpha-crystallin, have been shown to be preferentially secreted by *M. tuberculosis* in hypoxia.[162] Therefore early and latent infection may be best distinguished through measuring the immune response to different antigens. Such studies are underway in The Gambia.

The lack of a significant effect of BCG, as evidenced by a BCG scar, on either PPD test is consistent with other studies.[113,156] However, the measured odds may be an underestimate as a sixth or more of subjects given BCG at birth do not develop a visible scar,[163] and the OR for ESAT-6/CFP-10 ELISPOT of 0.74 (95% CI: 0.5-1.1) indicates that BCG vaccination may offer some protection against *M. tuberculosis* infection.

These results are of particular importance for trials of new generation vaccines and preventive drug treatment strategies.[73] Vaccine trials require a realistic approach to recruitment in endemic settings. Concern about hypersensitivity reactions (Koch phenomenon) demands avoidance of those with active *M. tuberculosis* infection in early safety trials. At the present time a combination of tests may be best: we suggest recruitment be limited to individuals negative by HIV test, PPD skin test and ESAT-6/CFP-10 ELISPOT, who have a normal chest X-ray plus no known TB contact. Later, randomised trials will be required to assess both the preventive and therapeutic effect of new vaccines. The ex *vivo* ELISPOT would offer real advantages over 6-day assays here, as it is likely to become negative as effector T-lymphocytes wane in the months after clearance of infection. The specificity gain with the ESAT-6/CFP-10 ELISPOT test is of importance with respect to strategic preventive drug treatment. To cause a significant shift in the reproductive index of the epidemic, it would not be necessary to treat all those who are infected. Furthermore, economic considerations, adverse drug reactions and logistics demand that as many as possible of those treated are truly infected.

The ELISPOT assay has advantages over the skin test, which has to be read between 48 and 72 hours and is influenced by age and sex factors. Furthermore, injection of PPD in skin tests may sensitise the individual to *M. tuberculosis* antigens affecting future diagnostic test results. Frequent ingestion of environmental mycobacteria requires diagnostic tests that employ *M. tuberculosis*-specific antigens. Routine use of the ELISPOT where the burden of TB is greatest requires further research, including studies in HIV positive subjects, and a large investment to provide laboratory scientists and adequate laboratories. While the ELISPOT is a relatively straightforward assay, technical expertise is required, especially to conduct cell separations. An alternative is whole-blood cultures and ELISA technology, which has been used to measure the IFN-$\gamma$ response to PPD, but not to more specific antigens.[164] The ELISPOT should be an important part of the armamentarium for those planning therapeutic and vaccine trials.

# 3

## Comparison of Enzyme Linked Immunospot Assay and PPD skin test for diagnosis of *Mycobacterium tuberculosis* infection in case contacts and community controls in The Gambia.

**MRC Field workers conducting interviews with study participants**

## 3.1 Summary

*Background.* In household contacts of TB cases, discordance between the PPD skin test and the ELISPOT test for *Mycobacterium tuberculosis* infection increases with decreasing exposure. We compared the PPD skin test and ELISPOT test results in community controls with their respective results in household TB case contacts in The Gambia.

*Methods*. Household contacts over 6 months of age of sputum smear positive TB cases and frequency matched community controls were recruited in The Gambia. They underwent a PPD skin test plus PPD and ESAT-6/CFP-10 ELISPOT tests. Test positivity and discordance between the tests were compared using logistic regression across an exposure gradient according to sleeping proximity to an index case.

*Results*. Six hundred and fifteen household contacts and 105 frequency matched community controls were recruited. All three tests assessed showed significant increases in positivity with increasing *M. tuberculosis* exposure; the PPD skin test had the most dramatic change. Both ELISPOT measures appeared to 'level off' in the community; in contrast the percentage positive by PPD skin test continued to trend downwards. There was a significant increase in the percentage of ELISPOT positive volunteers who were PPD skin test negative from household contacts (45/167, 26.9%) to community controls (20/26, 76.9%).

*Conclusions*. While the PPD skin test is relatively sensitive to recent *M. tuberculosis* exposure, it is heavily down-regulated in the community in our setting. Screening for safety studies of highly immunogenic new-generation TB vaccines should employ tools such as the ELISPOT in addition to the PPD skin test.

## 3.2 Introduction

The assessment of new diagnostic tests for *Mycobacterium tuberculosis* infection in tuberculosis (TB)-endemic tropical settings presents specific challenges. Three issues in particular are important. First, the abundance of environmental mycobacteria makes any test using purified protein derivative (PPD) potentially non-specific due to cross-reaction with *M. tuberculosis* antigens. Second, the PPD skin test appears to wane more quickly in tropical countries.[114] Third, specificity is very difficult to assess, as it is not possible to identify a sub-population that can be safely assumed not to be *M. tuberculosis* infected.

Recently we presented a reproducible model for assessing new diagnostic tests for *M. tuberculosis* infection in such a setting.[1] In a study of household contacts of sputum smear positive index cases in The Gambia we showed that an ELISPOT test for the *M. tuberculosis* specific antigens ESAT-6 and CFP-10 offered improved specificity over PPD by ELISPOT, at the cost of some sensitivity. A comparison of the ELISPOT results with the traditional PPD skin test showed that, while the PPD skin test was more sensitive than the ESAT-6/CFP-10 ELISPOT to recent *M. tuberculosis* exposure, an increasing percentage of ESAT-6/CFP-10 positive contacts were PPD skin test negative with decreasing known exposure. In the present study we expanded our exposure model to include community controls in order to assess the ELISPOT and PPD skin test concordance/discordance inside and outside households of a known TB case.

## 3.3 Methods

***Participants***. Sputum smear positive TB index cases over 15 years of age and their household contacts at least 6 months of age were recruited as previously described.[1] They were categorised according to where they slept as a proxy of exposure to *M. tuberculosis*: in the same bedroom as the case, a different bedroom in the same house, or in a different house on the same compound.

Frequency matched community controls were recruited as follows. The age and sex of consecutive household contacts of TB cases were obtained from a previous case contact study in The Gambia and allocated randomly (through a computer-generated list of random numbers) to consecutively recruited index case households. Index case households were asked if they were happy for a community control to be sought in the neighbourhood. Similar to a previous selection process in The Gambia[165] and other developing countries,[166] community controls were selected by choosing a random direction from the case's home (by spinning a pen in the air) and visiting the second compound on the right. The field-researchers then asked whether there was anyone living the majority of the time in that compound of the required age band (5 year age bands for those under 15 years of age, 10 year age bands for those 15 years and older) and gender. If there was more than one possible match, the control was selected randomly by the toss of a coin or drawing blindly a numbered piece of paper from a container. If there was no possible control at that compound or refusal to participate the whole process was repeated once more.

Household contacts and community controls underwent a PPD skin test (2 TU, PPD RT23, Statins Serum Institut, Copenhagen, Denmark). Induration was recorded at 48-72 hours. Subjects with a positive skin test (mean induration diameter ≥ 10mm) were offered a chest X-ray and those with symptoms underwent a clinical assessment. Those with TB disease were referred to the National Programme for free treatment. There is no current practice of preventive treatment in The Gambia.

This study was approved by the Gambia Government/MRC joint Ethics Committee.

***Laboratory procedures.*** Sputum smears were prepared, stained, cultured, identified and confirmed as previously described.[154] The *ex vivo* ELISPOT assays for IFN-γ were performed as previously described.[155] For this study, synthetic, sequential peptides

spanning the length of ESAT-6 and CFP-10 (ABC, 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 in pools of peptides. Purified Protein Derivative (*M. tuberculosis,* RT49, Statins Serum Institut, Copenhagen, Denmark) was used at 10 µg/ml. The positive control was Phytohaemaglutinin (PHA; Sigma-Aldrich, UK). All antigens were tested in duplicate wells.

Assays were scored by an ELISPOT counter (AID-GmbH, Strassberg, Germany). The spot forming unit (SFU) numbers counted in each well were automatically entered into a database. Supplementary details were added by double data entry by two immunologists blinded to subject details. Positive test wells were pre-defined as containing at least ten SFUs more than, and at least twice as many as, negative control wells. For a positive ESAT-6/CFP-10 result it was necessary for one or more pools of overlapping peptides to be positive. PHA positive control wells were set to at least 150 SFUs above negative control wells. Negative control wells were required to have less than 30 SFUs.

Testing for HIV-1 or HIV-2 infection was by competitive enzyme linked immunosorbent assays (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France).

***Data management and analysis.*** All data were entered using double data entry into a relational ACCESS database[2] and checked for errors. A random effects logistic regression model, taking into account household clustering, was used to assess the relationship between risk factors and test results. Results were reported as unadjusted and adjusted odds ratios and their 95% confidence intervals (CI). The likelihood ratio test was used to test for interaction and for trend. All statistical analyses were conducted using Stata software (version 7; Stata Corp, College Station, TX).

## 3.4 Results

From 1 July 2002 to 9 February 2004 209 index cases households were recruited and had ELISPOT and skin test results. A consenting control was identified for 119 (57%) households and 105 (88%) had a result that met the criteria for analysis. Six households were not happy for a community control to be sought, one could not be found for 47 households and for 37 households a control was found but chose not to participate. The characteristics of the community controls were similar to case contacts with respect to age, sex and HIV status, but differed by ethnic group (a larger proportion were Mandinka, a lower proportion were Wolof) and a higher proportion had a BCG scar (Table 3-1).

Table 3-2 shows the odds ratios and 95% confidence intervals for the change in ELISPOT (PPD and ESAT-6/CFP-10) and PPD skin test positivity across the exposure gradient from the community to the bedroom of a known index case. The percentage of volunteers positive increased significantly for all three tests, most markedly for the PPD skin test (OR 15.7, 95% CI 6.6-30.3 for same bedroom compared to different compound). Figure 3-2 shows the percent positive for each test across the exposure gradient for all those with both ELISPOT and skin test results. Of note both ELISPOT measures appeared to 'level off' outside the compound whereas the PPD skin test continued to trend downwards.

Changes in discordance and concordance between the three tests across the exposure categories are presented in Figure 3-2. The frequencies of a positive result for each of the three tests are illustrated by the use of scaled rectangle diagrams. These are scaled Venn diagrams, with areas of overlap proportional to frequency.[167] The concordance between the tests increased markedly from the community controls (Figure 3-2A) to those sleeping in the same room as an index case (Figure 3-2D). Maximum discordance was present in the community controls where only 10 of 64 (15.6%) PPD ELISPOT positive volunteers and 6 of 26 (23.1%) ESAT-6/CFP-10 positive volunteers were PPD skin test positive. These results were a continuation of the significant trend ($p<0.0001$) of increasing PPD skin test negativity in the presence of ELISPOT positivity with increasing distance from a known TB case, seen inside the same compound (Figure 3-2D to 2B). In contrast, 11 of 14 (78.6%) of PPD skin test positive volunteer community controls were positive by ELISPOT.

| Characteristic | Same room (n=135) | Different room (n=282) | Different house (n=198) | Different compound (n=105) | All (n=720) |
|---|---|---|---|---|---|
| **Age (years)** | | | | | |
| **Mean (median: range)** | 19.4(20:1-60) | 20.3(16:1-100) | 20.9(18:1-66) | 26.8(25:1-65) | 21.3(19:0-100) |
| **% (n) Male** | 53 (71) | 40 (112) | 49 (98) | 42 (44) | 45 (325) |
| Ethnic group | | | | | |
| % (n) Mandinka | 24 (32) | 32 (89) | 28 (55) | 64 (67) | 34 (243) |
| % (n) Jola | 22 (30) | 23 (65) | 19 (37) | 17 (18) | 21 (150) |
| % (n) Wolof | 19 (25) | 17 (47) | 21 (41) | 2 (2) | 16 (115) |
| % (n) Fula | 10 (14) | 9 (25) | 11 (22) | 8 (8) | 10 (69) |
| % (n) Other | 25 (34) | 20 (56) | 22 (43) | 10 (10) | 20 (143) |
| **BCG scar %(n)[a]** | 45 (61) | 46 (129) | 36 (70) | 59 (61) | 45 (321) |
| **HIV positive %(n/total)[b]** | 3.7 (5/134) | 2.5 (7/280) | 2.0 (4/197) | 1.9 (2/103) | 2.2 (16/714) |

[a] 95 volunteers had uncertain BCG scar status
[b] 6 volunteers did not have an HIV test result

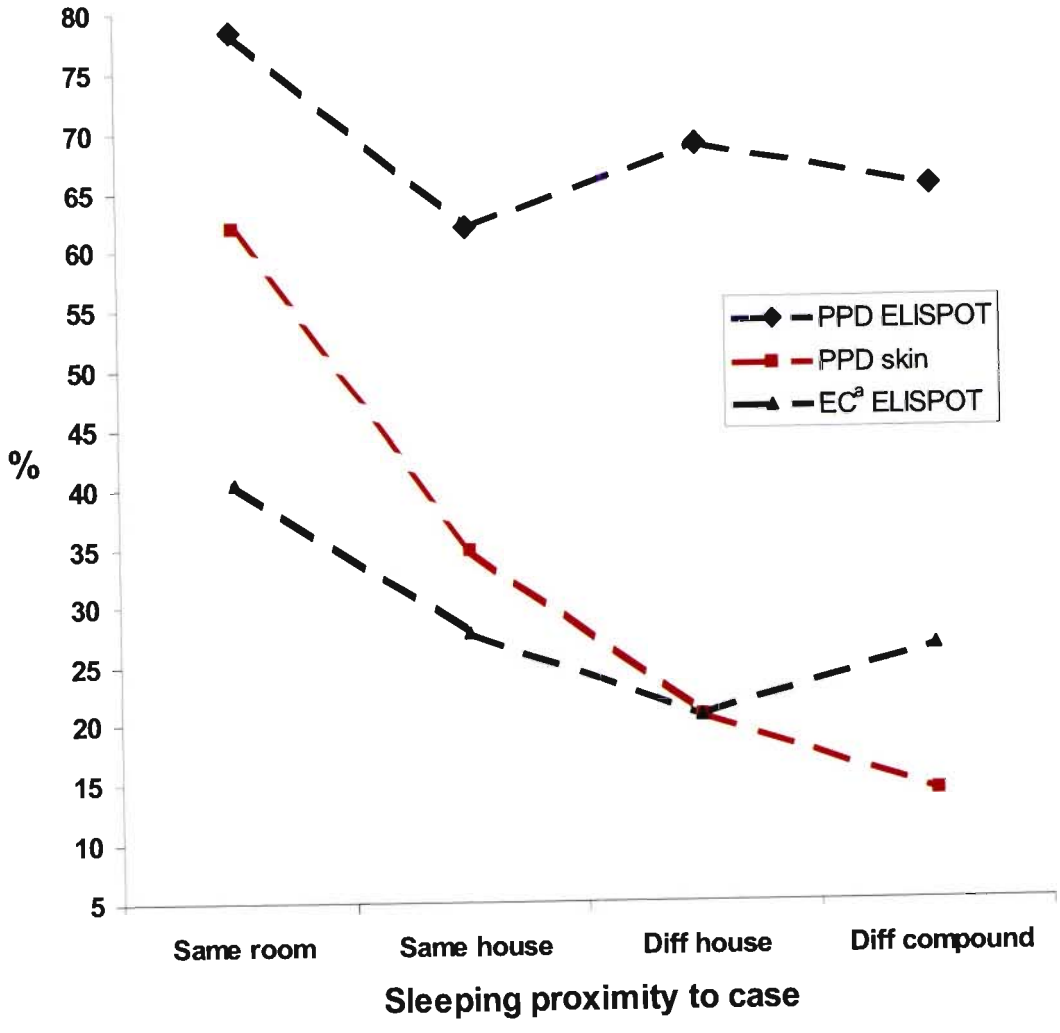
**Table 3-1**: **Characteristics of 720 contacts and community controls by exposure category**

| Antigen | Sleeping proximity | % (n) positive | OR (95% CI) | Adj. OR[a] (95% CI) | p[b] |
|---|---|---|---|---|---|
| **ESAT-6/CFP-10 (n=720)** | Different compound | 24.7 (26) | 1.0 | 1.0 | |
| | Different house | 42 (21.2) | 0.8 (0.4-1.6) | 0.7 (0.4-1.4) | |
| | Different room | 24.4 (80) | 1.3 (0.7-2.2) | 1.1 (0.6-2.0) | |
| | Same room | 40.7 (55) | 2.4 (1.3-4.5) | 2.1 (1.1-4.1) | 0.004 |
| **PPD ELISPOT (n=720)** | Different compound | 64.8 (68) | 1.0 | 1.0 | |
| | Different house | 69.7 (138) | 1.1 (0.6-2.0) | 1.3 (0.7-2.4) | |
| | Different room | 62.3 (177) | 1.0 (0.6-1.7) | 1.2 (0.7-2.0) | |
| | Same room | 78.5 (106) | 2.1 (1.1-4.0) | 2.4 (1.2-4.8) | 0.02 |
| **PPD skin test (n=691)** | Different compound | 14.3 (14) | 1.0 | 1.0 | |
| | Different house | 20.7 (40) | 1.7 (0.8-3.5) | 1.8 (0.8-3.8) | |
| | Different room | 35.0 (96) | 4.1 (2.1-8.2) | 4.6 (2.2-9.6) | |
| | Same room | 61.9 (78) | 14.2 (6.6-30.3) | 15.7 (7.0-35.3) | 0.0001 |

[a] Variables included: age, gender, ethnic group, BCG scar status
[b] p –value for linear trend

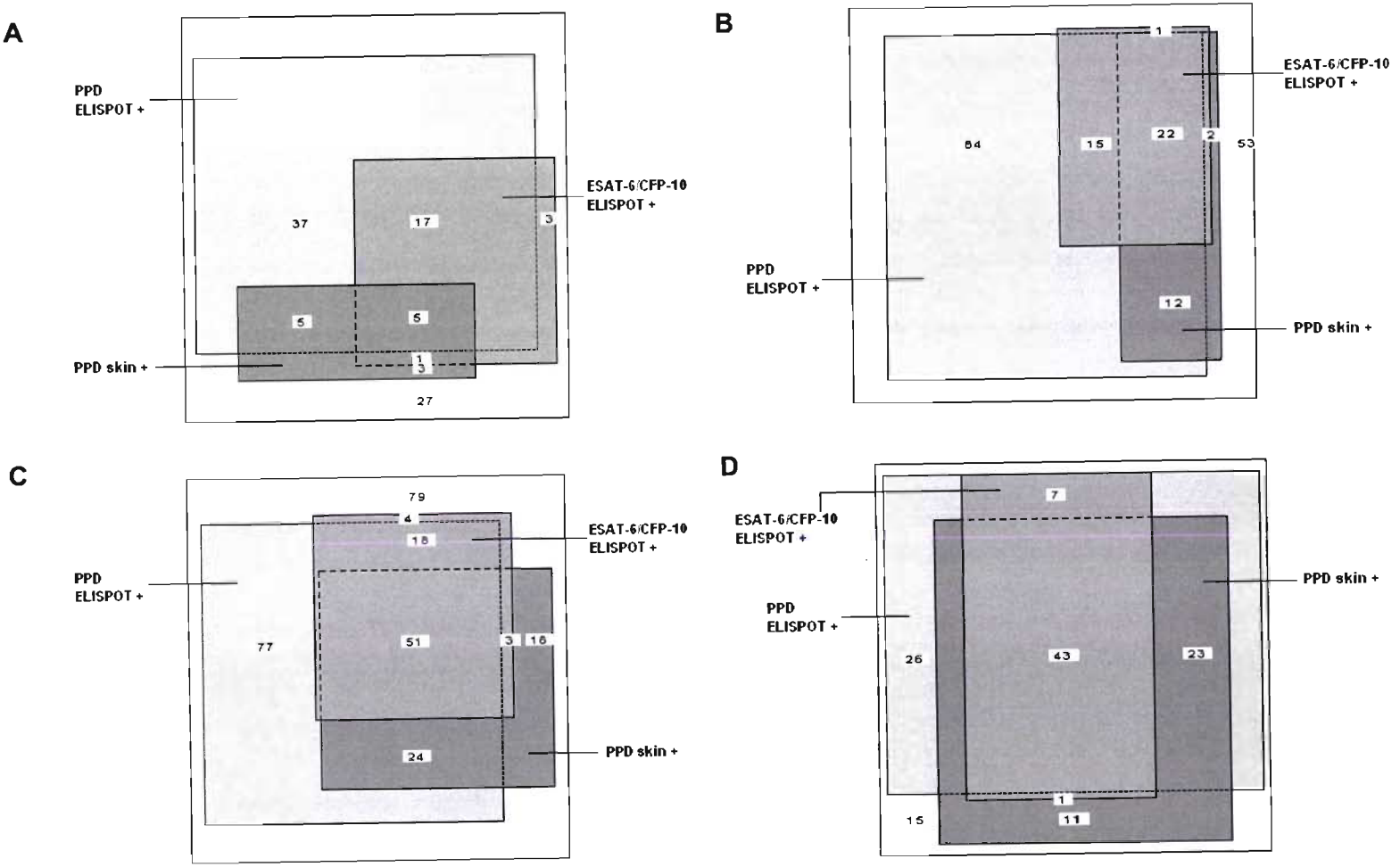
**Table 3-2: Univariable and multivariable odds ratios for the ELISPOT and PPD skin tests by logistic regression (household as a random effect) according to sleeping proximity to a case**

**Figure 3-1: Percent positive for each test by exposure category for those with a result for all tests (n=691). Age standardisation led to less than 1% change for any measure, therefore unadjusted percentages are presented.**




[a]ESAT-6/CFP-10

Figure 3-2: The number and relative proportion of subjects with each combination of ESAT-6/CFP-10 ELISPOT, PPD ELISPOT and PPD skin test results (n=691). Each diagram represents an exposure category by sleeping proximity to a case,: (A) Different compound, n=105 (B) Different house, n=198 (C) different room, n=283 and (D) same room, n=135. The size of each box represents the proportion of positive volunteers


A
PPD
ELISPOT +
ESAT-6/CFP-10
ELISPOT +
PPD skin +
37
17
3
5
5
1
3
27
B
ESAT-6/CFP-10
ELISPOT +
PPD
ELISPOT +
PPD skin +
84
1
15
22
2
53
12
C
79
4
18
ESAT-6/CFP-10
ELISPOT +
PPD
ELISPOT +
77
51
3
16
24
PPD skin +
D
ESAT-6/CFP-10
ELISPOT +
7
PPD skin +
PPD
ELISPOT +
26
43
23
1
15
11

## 3.5 Discussion

In this study we compared ELISPOT and PPD skin test results between 615 household contacts of sputum smear positive TB cases and 105 frequency matched community controls in a tropical TB-endemic setting. All three tests showed significant increases in the percentage of volunteers positive with increasing exposure to a known index case; the PPD skin test had by far the most dramatic change. Both ELISPOT measures appeared to 'level off' with a similar percentage of volunteers positive in the community as those who sleep in a separate house on the same compound as a known case. In contrast the percentage positive by PPD skin test continued to trend downwards. This divergence was accompanied by a dramatic increase in the percentage of ELISPOT positive volunteers who were PPD skin test negative between household contacts (45/167, 26.9%) and community controls (20/26, 76.9%).

The PPD ELISPOT positive, PPD skin test negative results in our study are consistent with other work in Africa[158] and similar to those in our previous study where we showed the same phenomenon in relation to the ESAT-6/CFP-10 ELISPOT. The significant sensitivity problem for the skin test seen in those with the least exposure to a TB case but within the same household is now shown to be even greater in the community where over three quarters of individuals who make an effector T-cell immune response to ESAT-6/CFP-10 do not make a positive PPD skin test response. This is consistent with the observation that there is reversion of a positive PPD skin test in tropical settings[113] and may be related to regular mucosal exposure to environmental mycobacteria causing inhibition of the DTH skin response.[159] The *ex vivo* IFN-γ ELISPOT detects recently activated lymphocytes with immediate effector function and effector-memory cells that persist for a limited time in circulation once antigen is cleared.[135,160] Therefore it is possible that the ELISPOT also undergoes reversion and this is being assessed in The Gambia.

Our study has several potential sources of bias. First, refusal by the case households and potential community controls was understandable as many households were afraid that neighbours would find out that they had TB and many individuals in the community are reluctant to be bled in The Gambia. Stigma and fear of being bled made it difficult to obtain community controls for this study. However this selection process is most accepted for our setting and we note that the characteristics and test results of the households in this study

were very similar to those of the households in our previous study,[1] making the refusals unlikely to be a source of bias. Second, the community controls were slightly older, presumably because the flexibility within age bands enabled selection of slightly older children who were easier to bleed. Age standardised ELISPOT and skin test results were not different from the crude results however. Third, there was over-representation of Mandinka and under-representation of Wollof groups in the community controls. A variety of sources (1993 census and various MRC studies) indicate that, overall, 30-50% of Gambians are Mandinka and 6-20% are Wollof. We have not found that these two ethnic groups have immune responses that are different from the rest of the Gambian population. Fourth a higher proportion of community controls had a BCG scar. While we have noted no significant effect of the BCG scar on the PPD skin test or ELISPOT results in The Gambia, it could only be expected to lead to increased positivity by PPD skin test. The basic finding of increasing discordance between the PPD skin and ELISPOT tests outside compounds with known TB cases is clear and consistent with the changes across the intra-compound exposure gradient.

These results are of particular relevance to those planning safety studies of new generation TB vaccines in tropical TB-endemic settings. In 1891 Robert Koch reported that, after establishment of infection in guinea pigs, intradermal challenge with whole organisms or culture filtrate resulted in necrosis locally and in the original tuberculous lesion.[168] Injection of larger quantities of culture filtrate subcutaneously into TB patients evoked necrosis in established TB lesions at distant sites, with disastrous results in the spine and lungs. There is a real concern that highly immunogenic vaccines may induce a so-called 'Koch phenomenon'. We are presently conducting the first safety studies in a tropical TB endemic setting of the new generation TB vaccine, MVA-85A,[169,170] which has now completed a series of Phase I studies in humans in the United Kingdom. Because of the results of the present study we have introduced both ELISPOT and PPD skin test entry criteria to avoid introducing vaccine into any volunteer making an immune response to *M. tuberculosis* antigens. Recruitment has been limited to adult men negative by HIV test, PPD skin test and ESAT-6/CFP-10 ELISPOT, who have a normal chest X-ray plus no known TB contact. We have set a PPD ELISPOT count cut-off of 100 SFU/million: volunteers may be included with counts under this cut-off.

The PPD skin test appears to be more sensitive than the ESAT-6/CFP-10 ELISPOT to recent *M. tuberculosis* exposure in The Gambia. However it appears to be subject to significant suppression/down-regulation in the community and is inadequate as a diagnostic tool for *M. tuberculosis* infection in those individuals. Longitudinal studies will be required to identify whether ELISPOT 'reversion' also occurs and to what degree. Such studies are underway in The Gambia. A combination of both tests is indicated for those conducting safety studies for new vaccines in TB-endemic tropical settings.

# 4

## Performance of ESAT-6/CFP-10 fusion protein versus peptides for diagnosis of *Mycobacterium tuberculosis* infection by ELISPOT in The Gambia

**Baobab tree**

## 4.1 Summary

*Background.* Two *Mycobacterium tuberculosis* antigens (ESAT-6; CFP-10) offer increased specificity over PPD by enzyme linked immunospot assay (ELISPOT) when their overlapping peptides are utilised.

*Methods.* We assessed whether equivalent results could be obtained for a fusion protein of the two antigens and whether a combined protein and peptide readout would offer increased sensitivity in the diagnosis of *M. tuberculosis* infection against a gradient of exposure in The Gambia.

*Results.* Of 488 household contacts of 88 sputum smear positive TB cases, 88 (18%) subjects were positive by CFP-10 peptides, 148 (30%) by ESAT-6 peptides, 161 (33%) by either or both CFF-10 and ESAT-6 peptides and 168 (34%) by the fusion protein. 188 (39%) subjects had a positive result to one or more peptide or fusion protein. The individual peptide responses, the combined peptide responses and the fusion protein all increased with increasing *M. tuberculosis* exposure.

*Conclusions.* A fusion protein of ESAT-6 and CFP-10 is as sensitive to *M. tuberculosis* exposure as the respective peptides. A combination of the addition of CFP-10 and a protein adds increased sensitivity over ESAT-6 peptides alone.

## 4.2 Introduction

There is an urgent need for reproducible and rigorous frameworks to assess new diagnostic tests for *Mycobacterium tuberculosis* infection.[152] We have recently presented a reproducible model, which utilizes an established gradient of recent *M. tuberculosis* exposure according to sleeping proximity to an index TB case. Using this model, we showed that an *ex vivo* enzyme linked immunospot (ELISPOT) assay for IFNγ with secreted antigens of *M. tuberculosis*, ESAT-6 and CFP-10, offers improved specificity over purified protein derivative (PPD) for the diagnosis of latent *M. tuberculosis* infection in The Gambia, a TB endemic tropical setting.[1] However, when the results were compared to the PPD skin test, it appeared that the specificity gain was at the cost of some sensitivity, supporting the assertion that two *M. tuberculosis*-specific antigens may not be sufficient for the diagnosis of infection.[132] Longitudinal follow-up studies to find secondary cases will shed more light on this issue.

It is not known whether it is preferable to conduct the ELISPOT assay with recombinant protein or pools of peptides, or whether increased sensitivity could be achieved through measurement of a combined peptide and protein response.[171] In addition, a fusion protein of antigens is likely to offer a far cheaper and more realistic alternative for large-scale production and field-testing. Therefore, we engineered a fusion protein of ESAT-6 and CFP-10 and formally evaluated its diagnostic value against, and in addition to, the respective peptides of the single antigens for the ability to detect *M. tuberculosis* infection in The Gambia.

## 4.3 Methods

***Participants***. TB index cases over 15 years of age were recruited from the major government health centre in Greater Banjul and the outpatient clinic at the Medical Research Council Laboratories. Included cases had two sputum smear samples positive for acid-fast bacilli and with *M. tuberculosis* isolated upon culture. Household contacts were eligible for inclusion in the study if they were at least 6 months of age and lived the majority of the time in the same compound as the case. They were not invited to participate in the study if treated for TB in the past year, or if recruited after 60 days of the case. Contacts were excluded from further study if diagnosed with TB within 1 month of recruitment and coughing for longer than the case. Written informed consent was obtained from the adults as well as from the parents or legal representatives of participating minors. Subjects were interviewed, examined, and a blood sample taken for ELISPOT and HIV test. Fresh samples from all participants were processed onsite.

The study was approved by the Gambia Government/MRC joint Ethics Committee.

***Laboratory procedures.*** Sputum smears were prepared and stained with auramine-phenol[154] and confirmed by Ziehl-Neelsen (Z-N). Decontaminated specimens were inoculated into Lowenstein-Jensen media (L-J) and BACTEC 9000 MB liquid media for isolation and identification of *M. tuberculosis*, as previously described. [1]

Testing for HIV-1 or HIV-2 infection was by competitive enzyme linked immunosorbent assays (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France).

Synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (ABC, 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 used in one pool each of 17 peptides. The positive control was phytohaemaglutinin (PHA; Sigma-Aldrich, UK). All antigens were tested in duplicate wells.

The fusion protein of ESAT-6 (Rv3875) and CFP10 (Rv3874) was engineered as follows: The individual genes were amplified by PCR from *M. tuberculosis* H37Rv genomic DNA. During the amplification steps the genes were fused with a linker encoding for asparagine-valine-alanine. The product was subsequently cloned by Gateway technology into bacterial expression vector pDEST17 (Invitrogen, San Diego, CA) containing an N-terminal hexa-histidine tag. Sequencing was performed to confirm identity of the cloned DNA fragment. The recombinant fusion protein was over-expressed in *Escherichia coli* BL21 (DE3) and purified as previously described.[172] Recombinant protein batches were analysed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining and Western-blotting with an anti-His antibody (Invitrogen) to confirm size and purity. Residual endotoxin levels were determined with a Limulus Amebocyte Lysate (LAL) assay (Cambrex) and found to be below 50 IU/mg recombinant protein. Protein batches were subsequently tested for non-specific T cell stimulation and for potential cellular toxicity in IFN-γ release assays using PBMC of *M. tuberculosis*-unexposed and BCG-unvaccinated healthy Mantoux negative donors. The fusion protein was used in a concentration of 10 µg/ml in the ELISPOT assays (see below).

The *ex vivo* ELISPOT assays for IFNγ were performed in duplicate as previously described.[155] Assays were scored by an ELISPOT counter (AID-GmbH, Strassberg, Germany). The number of spot forming units (SFU) in each well were automatically entered into a database using Matlab® software (MathWorks). Supplementary details were added by double data entry by two immunologists blinded to subject details. Positive test wells were pre-defined as containing at least ten SFUs more than, and at least twice as many as, negative control wells. For a positive ESAT-6/CFP-10 result it was necessary for one or both pools of overlapping peptides to be positive. PHA positive control wells were set to at least 150 SFC/well/$2x10^5$ above negative control wells[1]. Negative control wells were required to have less than 30 SFUs.

***Data management and statistical analysis.*** All data were entered using double data entry into an ACCESS database and checked for errors. Agreement between the qualitative test results was assessed by the Kappa statistic and the significance of the discordance was assessed by McNemar's test. For analyses by exposure, contacts were stratified according to proximity to the index case, as previously defined. The following categories were used: sleeping in the same bedroom as the index case, in a different bedroom in the same house, or in a different house on the same compound. A random

effects logistic regression model, taking into account household clustering, was used to assess the relationship between exposure and test results. All statistical analyses were conducted using Stata software (version 8; Stata Corp, College Station, TX, USA).

## 4.4 Results

From May 20 2003 to April 29 2004, 603 consenting TB case contacts were selected for *ex vivo* ELISPOT assays for IFNγ and had an adequate specimen taken. Of these, 488 (81%) contacts of 88 cases had both fusion protein and peptide ELISPOT results that met inclusion criteria (Table 4-1). Overall, 88 (18%) subjects were positive by CFP-10 peptides, 148 (30%) by ESAT-6 peptides, 161 (33%) by either or both CFF-10 and ESAT-6 peptides and 168 (34%) by the fusion protein. 188 (39%) subjects had a positive result to one or more peptide or fusion protein.

There was reasonable agreement between the combined peptide and the fusion protein qualitative results with a kappa statistic of 0.78 (95% CI: 0.69-0.87; p<0.0001), and no significant discordance (p=0.38). Twenty subjects were peptide positive and fusion protein negative, and 27 were fusion protein positive and peptide negative (Figure 4-1). Combining the tests, the proportion of subjects positive for ESAT-6 peptides or fusion protein was significantly higher than the proportion positive for ESAT-6 peptides alone (p=0.036) and the proportion of subjects positive for either the combined ESAT-6, CFP-10 peptides or fusion protein was significantly higher than the proportion positive for ESAT-6 peptides alone (p=0.007). Therefore combining the peptide and protein results suggested some additional benefit in terms of sensitivity.

The univariable odds of a result being positive for each test by exposure to *M. tuberculosis,* according to sleeping proximity, are shown in Table 4-2. All the tests were significantly more likely to be positive in contacts with closer sleeping proximity to the case. In the multivariable analysis, the odds of being positive by all the antigens and combinations with closer proximity to the case, adjusted for age, sex and ethnicity, remained significantly increased. The odds of test positivity decreased, but not significantly, with the presence of a BCG scar for the ESAT-6/CFP-10 peptide result (OR=0.74; 95%CI 0.5-1.25) and for the fusion protein (0.9; 0.6-1.4).

The median combined peptide SFU count, when positive was 48.5 SFU (81.2; 10-352.5), significantly higher than the median SFU count for the fusion protein of 38.5 SFUs (mean 70, range 10-347.5; p=0.0001). The quantitative test results are presented against each other in 4 scatter graphs in Figure 4-2. There was a strong correlation between the fusion

protein and the combined peptide SFC counts (r=0.9). The correlation between the fusion protein and ESAT-6 alone was also strong (r=0.87). However, the significantly lower positivity for CFP-10 overall lead to low correlation with the fusion protein and ESAT-6 peptide responses. These findings confirmed ESAT-6 as the immunodominant antigen in The Gambia.

| Characteristics | |
|---|---|
| **Demographic** | |
| Mean (median: range) age (years) | 20 (17:0-80) |
| Male | 242 (49.6%) |
| Ethnic group | |
| Mandinka | 144 (30%) |
| Jola | 147 (30%) |
| Wolof | 44 (9%) |
| Fula | 62 (13%) |
| Other | 91 (19%) |
| Proximity to case (%) | |
| Same room | 142 (29%) |
| Different room | 217 (45%) |
| Different house | 129 (26%) |
| **Clinical** | |
| BCG scar present | 200 (42%)[a] |
| HIV positive | 8 (2%)[b] |

[a] 71of 409 (15%) subjects assessed had uncertain BCG scar status
[b] HIV serostatus was determined in 480 subjects

**Table 4-1: Characteristics of 488 Contacts of 88 Cases**

| Antigen | Sleeping proximity | % (n) positive | OR (95% CI) | Adj. OR (95% CI) | p[a] |
|---|---|---|---|---|---|
| **ESAT-6 peptides** | Different house | 17 (22) | 1.0 | 1.0 | |
| | Different room | 32 (70) | 2.5 (1.3, 4.7) | 2.3 (1.2,4.2) | p=0.0010 |
| | Same room | 39 (56) | 3.3 (1.7, 6.4) | 3.4 (1.8,6.6) | |
| **CFP-10 peptides** | Different house | 11 (14) | 1.0 | 1.0 | |
| | Different room | 18 (38) | 1.7 (0.8, 3.7) | 1.7 (0.8,3.5) | p=0.015 |
| | Same room | 25 (36) | 3.0 (1.4, 6.6) | 3.5 (1.6,7.8) | |
| **Combined peptides** | Different house | 20 (27) | 1.0 | 1.0 | |
| | Different room | 35 (75) | 2.1(1.2, 3.8) | 2.0 (1.1,3.4) | p=0.0024 |
| | Same room | 42 (59) | 2.9 (1.5, 5.3) | 3.1 (1.7,5.7) | |
| **Fusion protein** | Different house | 24 (31) | 1.0 | 1.0 | |
| | Different room | 35 (76) | 2.0 (1.1, 3.6) | 1.8 (1.0,3.2) | p=0.0055 |
| | Same room | 43 (61) | 2.7 (1.4, 5.0) | 2.9 (1.5,5.3) | |
| **Peptides & Fusion protein combined** | Different house | 28 (36) | 1.0 | 1.0 | |
| | Different room | 39 (84) | 1.9 (1.0, 3.3) | 1.7 (1.0,3.0) | p=0.0064 |
| | Same room | 48 (68) | 2.6 (1.4, 4.8) | 2.9 (1.6,5.4) | |

[a] p –value for linear trend

**Table 4-2: Univariable and multivariable odds ratios by logistic regression (household as a random effect) according to sleeping proximity to a case**

**Figure 4-1**: **Venn diagram for the number of subjects with each combination of fusion protein and peptide results (n=488).**

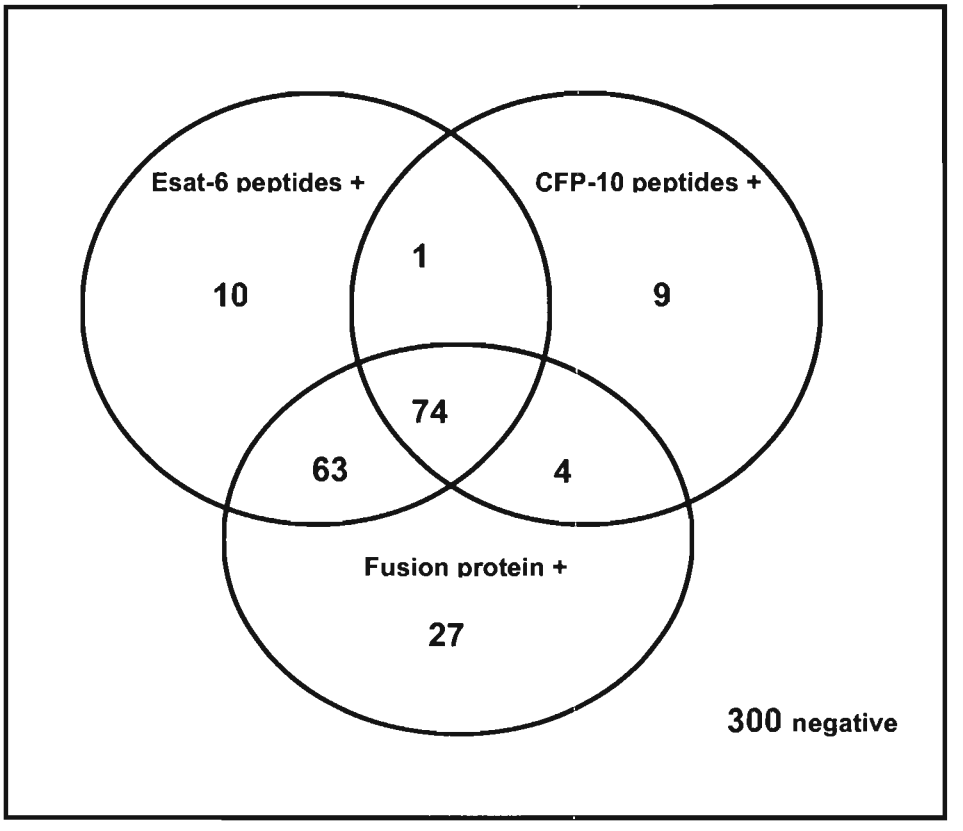
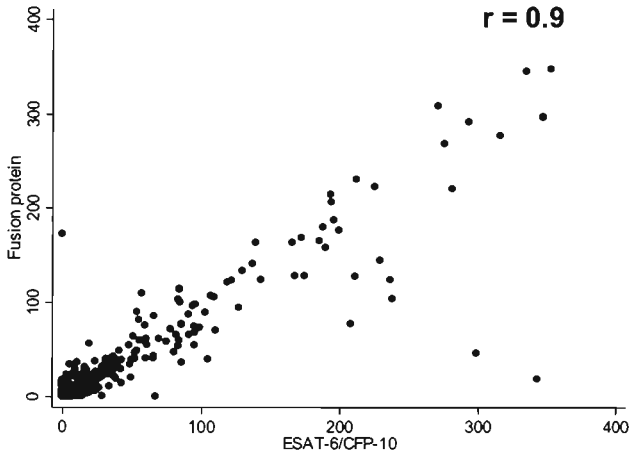
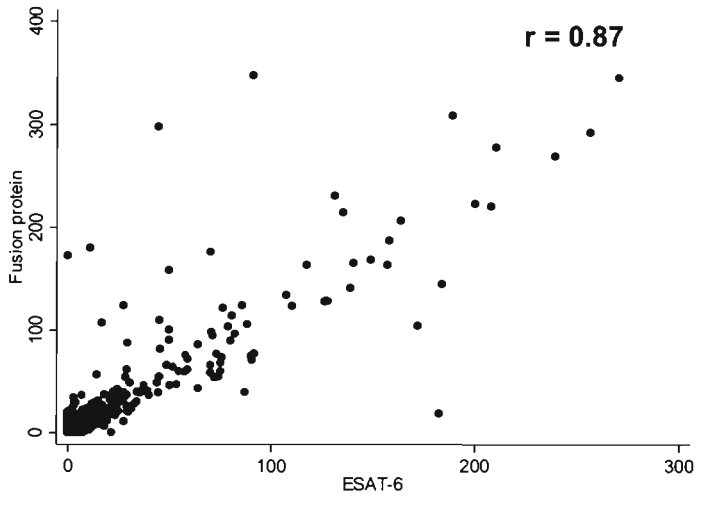
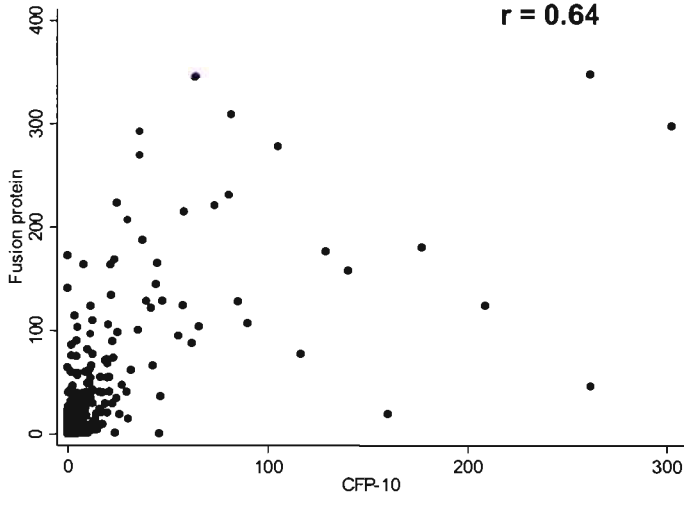
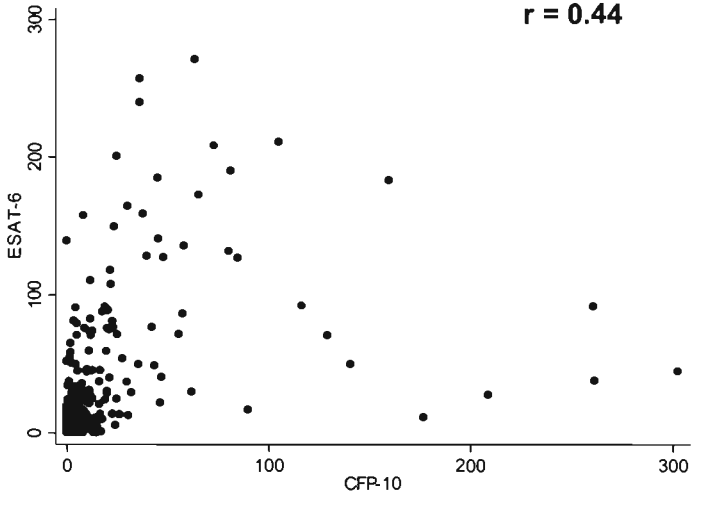


**Figure 4-2: Scatter graphs showing correlations between quantitative ELISPOT test results.**


r = 0.9
Fusion protein
ESAT-6/CFP-10
0
100
200
300
400


r = 0.87
Fusion protein
ESAT-6
0
100
200
300
400


r = 0.64
Fusion protein
CFP-10
0
100
200
300
400


r = 0.44
ESAT-6
CFP-10
0
100
200
300

## 4.5 Discussion

We report a large-scale evaluation of a *M. tuberculosis*-derived-ESAT-6/CFP-10 fusion protein against, and in addition to, its corresponding peptides by *ex vivo* ELISPOT for IFNγ in 488 TB contacts above 6 months of age in a tropical endemic setting.

As far as we are aware this is the first formal assessment of a fusion protein of ESAT-6 and CFP-10 as a reagent to monitor natural active *M. tuberculosis* infection in humans. The ESAT-6 and CFP-10 antigens, whether used as peptides or fusion protein, all induced responses with increasing positivity according to closeness of contact to a TB index case. The changes in positivity across the TB exposure gradient are similar to those reported in our recent assessment of ESAT-6 and CFP-10 peptides by ELISPOT with the PPD skin test and PPD ELISPOT.[1] We therefore reconfirm with a fusion protein, the potential of a TB case contact study design as a model of natural infection with sensitivity across a gradient of exposure. We show the model to be reproducible and we believe ready to be applied for testing new interventions, including drugs and vaccines in efficacy trials.

There was good agreement overall between the fusion protein and the peptides in both the qualitative comparisons and the quantitative correlations. In effect the fusion protein and peptide responses are mutual and each serves as a control for the other. Interestingly, our data show that the response to CFP-10 peptide antigen is significantly less than that to ESAT-6; in other studies in Zambia,[173] India,[138] Denmark[136] and the Netherlands,[134] the converse was found. Both ESAT-6 and CFP-10 antigens are similar in size, are in one operon and are expressed simultaneously in a similar ratio.[24,27] Differences in immune responses to individual ESAT-6 and CFP-10 antigens may be explained by polymorphism in HLA-type in the population and to the actual amino-acid composition of the individual proteins. An alternative explanation might relate to differences of *M. tuberculosis* strains in Africa.

By combining peptide and fusion protein data we find a small but significant sensitivity gain. Whilst peptides might be expected to stimulate mixed CD4 and CD8 T cell responses, a response to peptide in the absence of a response to the fusion protein implies a response that is exclusively mediated by CD8 T cells and representative of about 12% of the response.

Currently there is no clear explanation for an exclusive protein response in the absence of peptide, although there may be several theoretical possibilities. Since the molar concentration of peptide antigen is at least 5 fold higher than that of the protein a lack of peptide response is unlikely to be a concentration effect. Although the peptides used were 15 amino acids overlapping by 10 amino acids, there is still a small chance that peptides do not represent every conceivable sequential T cell epitope. However, it would seem unlikely that so much of the response is due to sequentially unrepresentative epitopes. There is a possibility that 'neo-epitopes' are formed across the ESAT-6/CFP-10 junction and are therefore absent in peptide pools. Yet it is difficult to see how these might occur naturally. A more likely explanation comes from recent publications showing CD8+ T cells epitopes to be spliced to form non-sequential sequences[174] within the proteosome.[175] Our data showing difference between unprocessed (peptide) and processed (protein) antigen support the possibility that spliced epitopes might also occur through a CD4 pathway. Whilst confirmation is needed the possibility remains that as much as 14% (protein positive, peptide negative) of the response to recent *M. tuberculosis* infection could be due to spliced epitopes. We are conducting longitudinal follow-up of the subjects to assess the changes in responses to peptides and proteins over time. Finally, since the readout deals only with IFN-γ, discordance in response between peptide and protein could relate to the secretion of different cytokines.

Of note, since this study was nested in a large case contact study, 464 of the 488 subjects also had a PPD skin test with a change in positivity across the exposure gradient of 20.8% to 61% (data not shown), which is very similar to the data we previously reported.[1] It is likely therefore that the gain in sensitivity in these studies is not enough to counter the sensitivity loss incurred by using only two antigens. We are exploring other ways to improve the sensitivity of the ELISPOT in our setting, including using mathematical modelling to access a lower cut-off for positivity and searching for new immunogenic, but *M. tuberculosis*-specific, diagnostic antigens.

In terms of practicality the fusion protein will be much cheaper to manufacture than bulk peptides and is naturally half the expense of individual proteins. However the use of a combination of fusion protein with peptides is advantageous. Firstly the combination gives optimal detection of latent *M. tuberculosis* infection, at least with the *ex vivo* ELISPOT assay, and secondly protein and peptide antigens act as quality control for

one another. The quality control issue becomes a lot more relevant when used in large field studies as we have done.

In conclusion, the present study has shown good agreement between the measurement of the immune response by ELISPOT assay to a fusion protein of ESAT-6 and CFP-10 and the respective overlapping peptides. Improved sensitivity was gained through the addition of CFP-10 to ESAT-6 peptides, and through the combined assessment of peptide and fusion protein responses. The fusion protein could be utilised as an alternative to pools of overlapping peptides or in addition in settings where maximal sensitivity is a priority. Further studies will be required to explore the significance, if any, of exclusive responses to either the protein or peptides, especially in relation to the time from exposure and the development of secondary TB disease.

# 5

## Comparison of ELISPOT assay and tuberculin skin test in healthy children exposed to *Mycobacterium tuberculosis*

**Training field staff for the studies**

## 5.1 Summary

*Background*. The objective of this study was to compare the ELISPOT assay with the tuberculin skin test (TST) in children for the diagnosis of *M. tuberculosis* infection in The Gambia.

*Methods*. We divided child contacts of sputum smear positive TB cases into 3 age categories (<5, 5-9 and 10-14 years) and assessed agreement between the two tests, plus their relationship to prior BCG vaccination. We categorised a child's level of *M. tuberculosis* exposure according to where s/he slept relative to a case: the same room, same house, or a different house. The relationship between exposure and test result was assessed by multiple logistic regression.

*Results*. In child contacts of 287 cases, 225/693 (32.5%) were positive by TST and 232/718 (32.3%) by ELISPOT. The overall agreement between tests was 83% (k=0.62), the discordance not significant (p=0.64). Both tests responded significantly to the *M. tuberculosis* exposure gradient in each age category. The percentage of those who were TST positive/ELISPOT negative increased (p=0.006) with increasing exposure. At the lowest exposure level, the percentage of ELISPOT positive children who were TST negative was relatively increased (p=0.008). Neither test had evidence of false positive results due to BCG.

*Conclusions*. In Gambian children, the ELISPOT is slightly less sensitive than the TST in the diagnosis of *M. tuberculosis* infection from recent exposure and neither test is confounded by prior BCG vaccination. Evidence of reduced TST sensitivity in subjects with the lowest known recent *M. tuberculosis* exposure dictates that, when maximal sensitivity is important, the two tests may be best used together.

## 5.2 Introduction

Tuberculosis (TB) in children is an important, but neglected, global health problem.[176] It was estimated in 1991 that from a reservoir of 180 million children with primary or latent *Mycobacterium tuberculosis* infection, there were 1.3 million cases of childhood tuberculosis and 450,000 deaths.[177] The overwhelming majority of these cases occur in developing countries. Children with TB tend not to be the focus of public health strategies against the disease, as over 95% have negative sputum smears and do not contribute substantially to the immediate course of the epidemic. However, many adults who develop smear positive disease acquire a primary infection during childhood. [178]

Tracing is recommended to identify *M. tuberculosis* infected childhood contacts of adult TB cases so that they can receive prophylaxis against the development of disease. However, prophylaxis is rare in developing countries through lack of resources, poor diagnostic tools and fear of giving monotherapy to children who actually have disease. The tuberculin test (TST) is the only universally used method for detecting *M. tuberculosis* infection, for which it has unknown sensitivity and specificity; the latter being compromised by cross reactivity with antigens of environmental mycobacteria and Bacille Calmette-Guérin (BCG) vaccine. It is also subject to the booster phenomenon, reader variability and false negative results in the immunocompromised.[179,180] Recently, T-cell interferon gamma (IFN-γ) responses to 2 relatively specific *M. tuberculosis* antigens (ESAT-6 and CFP-10) have been investigated for diagnosis of *M. tuberculosis* infection.[137,143,181] In children, this immunospot assay (ELISPOT) has been reported to correlate more closely with *M. tuberculosis* exposure and to be unaffected by BCG vaccination.[143] We recently reported that an ELISPOT assay offered increased specificity in the diagnosis of *M. tuberculosis* infection in The Gambia, at the cost of some sensitivity.[1] In order to more fully evaluate this assay against the TST in children we expanded our study and present a detailed comparison in TB case contacts under the age of 15 years in a TB-endemic tropical setting.

## 5.3 Methods

***Participants***. Sputum smear positive TB index cases over 15 years of age were recruited in Greater Banjul as previously described.[1] Included cases had two sputum samples positive for acid-fast bacilli by Ziehl-Neelson stain and *M. tuberculosis* on culture. They were identified at the major government health centres and the Medical Research Council Laboratories' (MRC Labs) outpatients' clinic, where, after counselling, they had an HIV test.

Household contacts were included if they were between 6 months to 15 years of age and lived the majority of the time on the same compound as a case. They were not eligible if treated for TB in the past year and were excluded if diagnosed with TB within 1 month of recruitment. Subjects were brought to the MRC Labs, invited to give informed consent, interviewed, examined, and a blood sample was taken for ELISPOT and HIV test. Fresh samples from all participants were processed onsite. On any particular day, some subjects were randomly excluded from having an ELISPOT test if laboratory capacity had been reached.

Contacts underwent a PPD skin test (2 TU, PPD RT23, Statins Serum Institut, Copenhagen, Denmark). Induration was recorded at 48-72 hours. Subjects with a positive skin test (mean induration diameter ≥ 10mm) were offered a chest X-ray and those with symptoms underwent a clinical assessment. Those with TB disease were referred to the National TB Control Programme for free treatment. There is no current practice of preventive treatment in The Gambia.

The Gambia Government/MRC joint Ethics Committee approved this study.

***Laboratory procedures.*** Sputum smears were prepared and stained with auramine-phenol and confirmed by Ziehl-Neelsen (Z-N).[154] Decontaminated specimens were inoculated into one slope each of Lowenstein-Jensen medium (L-J) containing glycerol and sodium pyruvate respectively and one vial of BACTEC 9000 MB media for isolation of *M. tuberculosis*. All mycobacterial cultures were identified and confirmed using standard procedures.

The *ex vivo* ELISPOT assays for IFN-γ were performed as previously described.[135] For this study, synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10

(ABC, 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 peptide pools were used at 5 μg/ml. The positive control was Phytohaemaglutinin (PHA; Sigma-Aldrich, UK). All antigens were tested in duplicate wells.

Assays were counted with an automated ELISPOT reader (AID-GmbH, Strassberg, Germany). The spot forming unit (SFU) numbers counted in each well were automatically entered into a database. Supplementary details were added by double data entry. Positive test wells were pre-defined as containing at least 8 SFUs (40 SFU/million cells) more than negative control wells. For a positive ESAT-6/CFP-10 result it was necessary for one or more pools of overlapping peptides to be positive. PHA wells were set to at least 150 SFUs above negative control wells. Negative control wells were required to have less than 20 SFUs.

Testing for HIV-1 or HIV-2 infection was by competitive enzyme linked immunosorbent assays (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France).

***Ascertainment of exposure.*** TB contacts were categorized according to where they slept: in the same bedroom as the case, a different bedroom in the same house, or in a different house in the same compound.

***Data management and analysis.*** All data were entered using double data entry into an ACCESS database and checked for errors. The concordance between the ELISPOT and TST was assessed by the calculation of a kappa statistic and the discordance by McNemar's test. A random effects logistic regression model, taking into account household clustering, was used to assess the relationship between sleeping proximity to an index case and test results. Age and sex were included in the analysis at the outset. Other variables assessed for possible inclusion in the model were ethnicity, BCG scar status and duration of cough of the respective index case. The likelihood ratio test was used to test for trend and for interaction between variables. All statistical analyses were conducted using Stata software (version 8; Stata Corp, College Station, TX).

## 5.4 Results

From 19th June 2002 until September 2004, 1132 children were recruited from the households of 287 TB cases; 917 were selected, and eligible, for the study. Of these, 856 had an adequate sample taken from whom 718 (83%) had an ELISPOT result that met the criteria for analysis. Just over half of the children were male and only 3 of 711 tested were HIV positive (Table 5-1).

Overall, 232 (32.3%) of the children had a positive ELISPOT result and 225 (32.5%) of 693 tested had a positive TST result. Figure 5-1 shows the numbers of contacts positive for the two tests in relation to each other (n=693) using a scaled rectangle diagram, where the numbers of individuals are represented by rectangles of a size that correlates with their relative proportion. Sixty (26.7%) of the TST positive children were ELISPOT negative and 55 (25%) who had a positive ELISPOT result were TST negative. The overall agreement between the two tests was 83% (kappa statistic=0.62) and the discordance was not statistically significant (p=0.64).

The univariable odds of a positive test result across the *M. tuberculosis* exposure categories (represented as sleeping proximity to a case) for each of three age groups are shown in Table 5-2. Both tests were significantly likely to be more positive with increasing exposure to the index case in all age groups. This was most marked in the under five-age group and for the TST. In the multivariable analysis, adjusting for age, sex and ethnicity, the changes in positivity across the exposure gradient remained significant for both the ELISPOT and TST.

A BCG scar was clearly visible in 330 (46.0%) of the children (Table 5-1). The univariate odds of a positive test for those BCG scar positive, compared to those with no evidence of a scar, was OR=0.8 [95% CI:0.5-1.2; p=0.34] for ELISPOT and OR=0.8 [0.5-1.2; p=0.27] for TST. When adjusted for age, sex and ethnicity in a multivariable analysis, the effect of the presence of a BCG scar on the results for both tests remained non-significant. A separate analysis of under five year old children (most recently exposed to BCG vaccine) also showed no significant relationship between scar status and test results (OR, 2.5 [0.6-11.0; p=0.21] for ELISPOT and OR, 1.7 [0.4-6.7; p=0.45] for TST).

The odds of test positivity were not related to sputum smear grade in the index case (OR, 0.8 [0.3-1.4] for ELISPOT and 1.1 [0.5-1.6] for TST). The duration of the cough in

the index case also did not affect the odds of a positive test for TST or the ELISPOT (OR, 1 [0.9-1.1] for both tests).

The exposure gradient by sleeping proximity enabled a stratified analysis of the level of concordance between the two tests. In all age groups, there was a divergence between TST and ELISPOT results with increasing exposure to the index case (Figure 5-2). Consistent with this divergence, the number of contacts who had positive TST and negative ELISPOT results increased from 3.3% of those sleeping in a different house to 12.3% of those sleeping in the same room (p=0.006). This compared to 7.7% and 6.1% respectively across the exposure gradient for the subgroup that were ELISPOT positive and TST negative (P= 0.7). Analysis of TST results in the subgroup of children that were ELISPOT positive showed that there was a significant change in the number of ELISPOT positive children that were TST negative across the exposure gradient: 40% of ELISPOT positive children sleeping in a different house as a case were TST negative, whereas only 13.7% of ELISPOT positive children in the highest exposure gradient were TST negative (p=0.008).

| Characteristic | Value |
|---|---|
| Demographic characteristics | |
| Age, years | |
| Mean | 7.5 |
| Median | 7 (0.5-14) |
| 0.5-4 | 195 (27.2) |
| 5-9 | 275 (38.3) |
| 10-14 | 248 (34.5) |
| Sex | |
| Male | 379 (52.8) |
| Female | 339 (47.2) |
| Ethnic group | |
| Mandinka | 246 (34.3) |
| Jola | 162 (22.6) |
| Wollof | 151 (21.0) |
| Fula | 57 (7.9) |
| Others | 102 (14.2) |
| Clinical findings | |
| BCG scar | |
| Absent | 313 (43.6) |
| Present | 330 (46.0) |
| Not determined | 75 (10.5) |
| HIV results* | |
| Positive | 3 (0.42) |
| Sleeping proximity to TB case | |
| Separate house | 183 (25.5) |
| Separate room | 372 (51.8) |
| Same room | 163 (22.7) |

**Table 5-1: Characteristics of 718 Childhood Contacts of Tuberculosis patients in the Gambia**

| Age group, years | ESAT-6/CFP-10 ELISPOT (n=718) | | | TST[a] (n=693) | | |
|---|---|---|---|---|---|---|
| | Positive results, No. (%) of contacts | Unadjusted OR (95% CI) | Adjusted OR (95% CI) | positive results, No. (%) of contacts | Unadjusted OR (95% CI | Adjust OR (95% CI) |
| **Age group 0.5-4** | | | | | | |
| Sleep proximity | | | | | | |
| Different house | 4 (10.3) | 1 | 1 | 4(10.0) | 1 | 1 |
| Different room | 27 (30.0) | 5.9(1.2-28.3) | 5.4(1.2-24.4) | 27(31.4) | 7.4(1.3-42.9) | 8.7(1.3-58.6) |
| Same room | 30 (45.5) | 12.2(2.4-62.0) | 11.6(2.3-57.9)[c] | 35(55.6) | 26.7(3.9-184.6) | 38.3(4.3-341.8)[d] |
| **Age group 5-9** | | | | | | |
| Sleep proximity | | | | | | |
| Different house | 14 (21.2) | 1 | 1 | 11(16.9) | 1 | 1 |
| Different room | 48 (32.4) | 2.0(0.8-4.6) | 1.9(0.8-4.4) | 43(29.7) | 3.3(0.9-12.6) | 3.2(0.8-12.4) |
| Same room | 27 (44.3) | 3.5(1.3-9.5) | 3.5(1.3-9.4)[b] | 31 (52.5) | 16.1(3.1-82.2) | 15.9(3.1-81.2)[d] |
| **Age group 10-14** | | | | | | |
| Sleep proximity | | | | | | |
| Different house | 18 (23.1) | 1 | 1 | 12(16.2) | 1 | 1 |
| Different room | 46 (34.3) | 2.3(0.8-6.3) | 2.3(0.8-6.4) | 45(60.8) | 3.8(1.5-10.0) | 3.8(1.5-9.6) |
| Same room | 18 (50.0) | 5.9(1.4-24.3) | 6.5(1.5-27.7)[b] | 17(23.0) | 7.4(2.0-26.8) | 8.0(2.3-28.5)[d] |
| **Overall** | | | | | | |
| Sleep proximity | | | | | | |
| Different house | 36(19.7) | 1 | 1 | 27(15.0) | 1 | 1 |
| Different room | 121(32.5) | 2.1(1.1-3.8) | 2.1(1.1-3.8) | 115(32.4) | 3.6(1.8-7.0) | 3.4(1.8-6.6) |
| Same room | 75(46.0) | 3.9(2.0-7.7) | 4.6(2.3-9.3)[c] | 83(52.5) | 9.2(4.3-19.6) | 10.2(4.7-21.8)[d] |

[a]Positive result defined as a mean induration diameter of ≥ 10 mm
p values for linear trend, [b]p< 0.05, [c] p< 0.01, [d] p< 0.001

**Table 5-2: Univariable and multivariable odds ratios determined by logistic regression (household as random effect), for sleeping proximity as a surrogate marker of exposure to *M. tuberculosis* by age group.**

**Figure 5-1: Scaled rectangle diagram of the number of children (rectangle size correlates with relative proportion) with each combination of ELISPOT and TST results (n=693).**

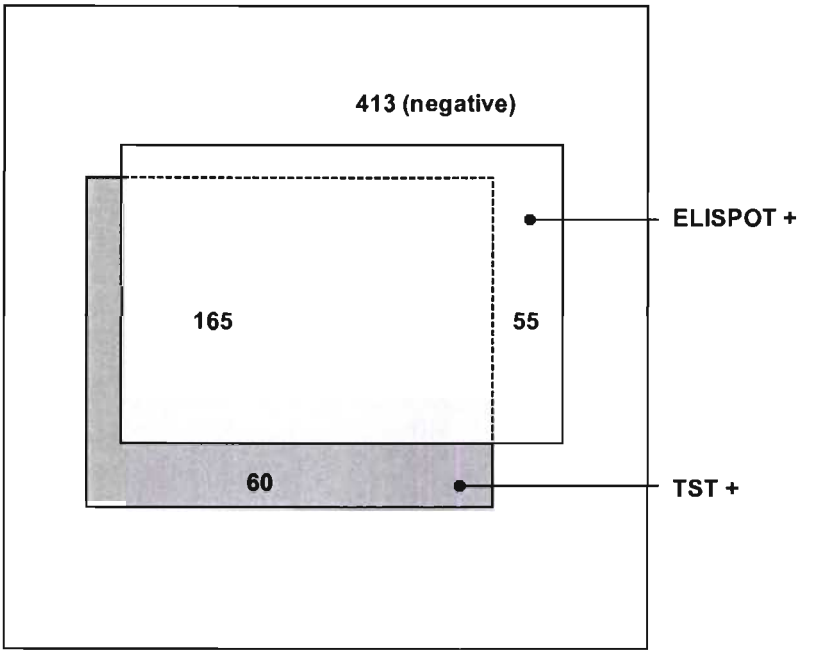
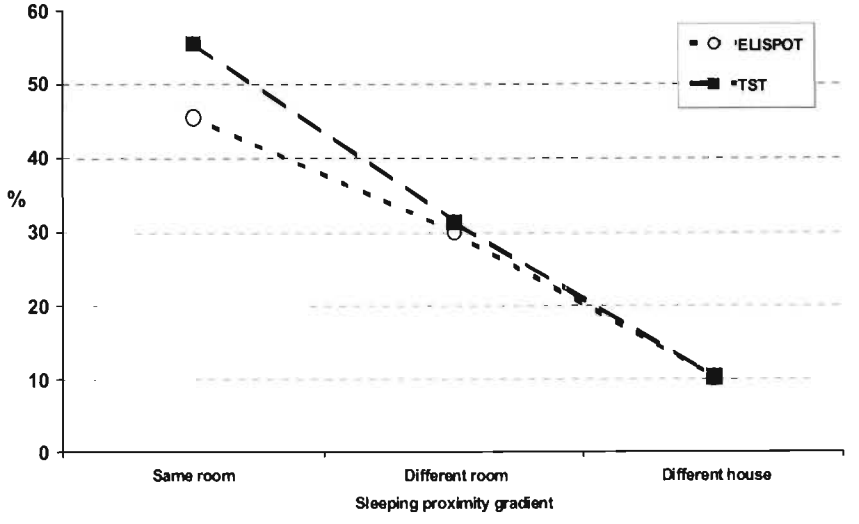
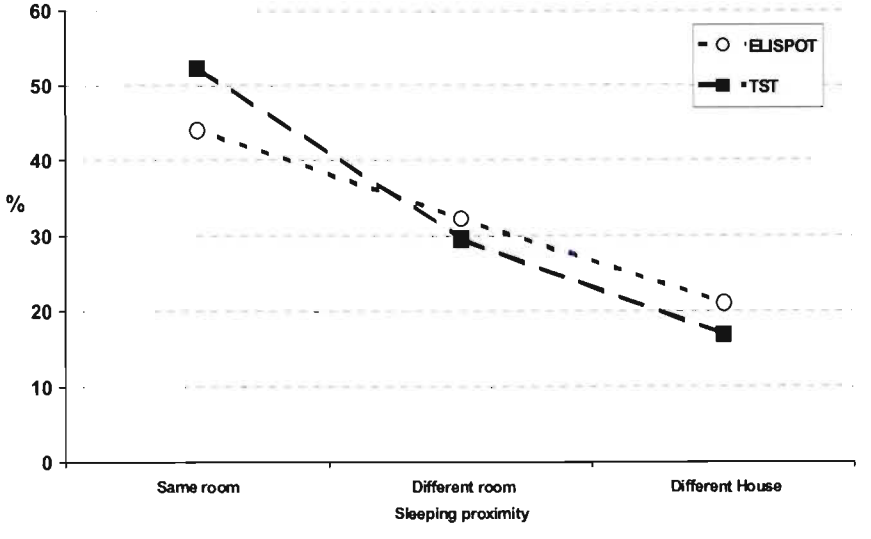


**Figure 5-2: Percentage of children positive for ELISPOT and TST by *M. tuberculosis* exposure (according to sleeping proximity to a TB case), by age group.**

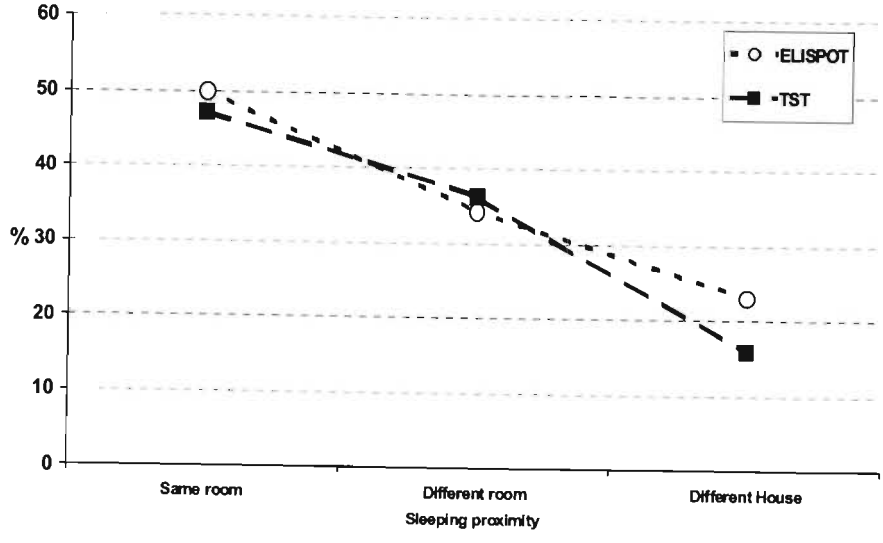
A. Age group 0.5-4 years




**B. Age group 5-9 years**



C. Age group 10-14 years



## 5.5 Discussion

As far as we are aware, this study provides the first large-scale comparison of the ELISPOT with the TST for the diagnosis of *M. tuberculosis* infection in childhood contacts of TB cases in a TB-endemic tropical setting. The tests were relatively concordant with each other with no statistically significant discordance overall. In all three-age categories assessed, both tests responded appropriately to a gradient of recent exposure to *M. tuberculosis* infection, the TST most dramatically. Neither test showed evidence of false positive results in the presence of a BCG scar, even in under 5 year old children. An increase in the proportion of children who were TST positive/ELISPOT negative with increasing exposure exposed a sensitivity problem for the ELISPOT for the detection of *M. tuberculosis* infection from recent exposure. In contrast, a large proportion (40%) of ELISPOT positive children in the lowest exposure group were TST negative, implying a possible sensitivity problem for the TST in those most similar to the general community.

Our study demonstrates the usefulness of an exposure gradient in exposing subtleties in the performance of diagnostic tests for *M. tuberculosis* infection in the absence of a gold standard. While only 8% of contacts were ELISPOT positive and TST negative overall, the finding that the proportion of children with this result increased with increasing *M. tuberculosis* exposure (3.3%-12.3%) is consistent with our previous finding in all age groups, and the assertion that 2 antigens may not offer adequate sensitivity.[132] The ELISPOT test is not as sensitive as the TST for the diagnosis of *M. tuberculosis* infection from known recent *M. tuberculosis* exposure.

Conversely, for those in the lowest exposure group, we found evidence of a sensitivity problem for the TST. The reasons for the increasing discordance between the TST and ELISPOT with decreasing known *M. tuberculosis* exposure are likely to be complex, requiring a detailed understanding of the mechanisms behind each type of immune response and repeated comparisons over time (i.e. longitudinal as well as cross-sectional data). Only approximately 32% of all contacts were positive for either TST or ELISPOT in our setting with endemic TB and intense exposure to environmental mycobacteria, suggesting that both these tests wane over time. The chance that the kinetics of waning for the two tests are identical is very small. The ex vivo ELISPOT detects recently activated lymphocytes with immediate effector function and effector memory cells that are present transiently.[135] While ESAT-6 and CFP-10 are secreted mainly early in infection,[161] it is likely that *M. tuberculosis* predominantly secretes other

antigens at different times. There is a need to identify the full repertoire of secreted antigens during the organism's life cycle. Other antigens such as the EspA[182] may in combination or alone provide improved sensitivity and specificity in the diagnosis of *M. tuberculosis* infection. However, in dormancy there may be periods of time where no antigens at all are secreted. It is likely that both the ELISPOT and TST convert and revert at different rates,[113] explaining some of our findings. A longitudinal comparison of the two tests is underway in The Gambia.

The absence of any significant effect of BCG vaccine on the TST is consistent with our previous results and those from other tropical settings,[183-185] but inconsistent with a recent meta-analysis which reported increased likelihood of a positive TST in BCG vaccinated persons, in particular within 15 years post vaccination.[186] The absence of a significant effect of BCG scar status on the ELISPOT with age was not unexpected. A weakness of our study and most others (including the majority of those in the above mentioned meta analysis) is that they rely on the presence or absence of a BCG scar because of the difficulties in obtaining accurate immunization histories and/or records. Considering that 6-17% of BCG vaccinated children may not develop scars following vaccination the calculation of any effect of BCG on test results may be an underestimate.[163,187] Furthermore, it is possible that BCG protects against the development of new infection after exposure, which might also lead to an underestimate of the presence of false positive results. Therefore, a small benefit of ELISPOT over the TST with respect to the confounding effect of BCG vaccination in our setting cannot be excluded. An even larger study would be required to resolve this issue.

A blood test for *M. tuberculosis* has some theoretical advantages, even in developing countries. For example, it is a one off test with a result within 24 hours and it does not require two patient visits. Furthermore, it does not involve the injection of antigens that may affect the results of future tests. However, the capital outlay needed to set up an appropriately equipped laboratory and employ adequately skilled staff, is a significant impediment to the routine use of the ELISPOT test in developing countries. The use of whole blood assays may be a partial solution to this problem. However this study has shown that, irrespective of these issues, a T cell assay employing *M. tuberculosis* specific antigens is not as sensitive for the diagnosis of *M. tuberculosis* from recent exposure as the TST in The Gambia and offers no added benefit with respect to the confounding effect of BCG vaccination. However, for those (e.g. vaccine trialists) wishing to identify individuals in the general community with any evidence of *M.*

*tuberculosis* infection in similar settings, a T cell assay as part of the screening procedures in addition to a TST is advisable at the present time.

# 6

## Quantitative T cell assay reflects infectious load of *Mycobacterium tuberculosis* in an endemic case contact model

The 'quad', MRC Gambia (dry season)

## 6.1 Summary

*Background.* Currently, reliable efficacy markers for assessment of new interventions against tuberculosis are limited to disease and death. More precise measurement of the human immune response to infecting *Mycobacterium tuberculosis* may be important. A qualitative ELISPOT result for ESAT-6/CFP-10 offers improved specificity over the PPD-skin test reaction in the detection of *M. tuberculosis* infection. We evaluated the quantitative ELISPOT and PPD-skin test responses to recent *M. tuberculosis* exposure.

*Methods.* We studied quantitative PPD-skin test and PPD-ELISPOT results in 1052 healthy household contacts sputum smear and culture positive TB index cases in The Gambia, according to an *ex vivo* IFNγ ELISPOT response to *M. tuberculosis* specific antigens (ESAT-6/CFP-10). We then studied the quantitative PPD-skin test and PPD-ELISPOT results in ESAT-6/CFP-10 positive case contacts against a natural exposure gradient according to sleeping proximity to a TB case.

*Results.* Both PPD-skin and PPD-ELISPOT measures were significantly increased in ESAT-6/CFP-10 positive subjects ($p<0.0001$). However, when quantitative PPD-skin test and PPD-ELISPOT results were compared in ESAT-6/CFP-10 positive subjects, only the ELISPOT count was sensitive to the exposure gradient, increasing significantly according to exposure ($p=0.009$).

*Conclusions.* The quantitative ELISPOT response to PPD in specific-antigen-positive TB case contacts reflects the infectious load of *M. tuberculosis* from recent exposure. This property offers new possibilities for the assessment of the efficacy of new interventions and should be considered when relating the early immune response to progression to disease.

## 6.2 Introduction

A significant barrier to the assessment of vaccines and other interventions against pathogens that undergo a latency period is the protracted time, often decades, before the onset of progression to disease. One alternative is to use a biomarker that reflects infection status. For *Mycobacterium tuberculosis* the diagnosis of infection has been reliant on the conventional purified protein derivative (PPD) skin test, which has unknown sensitivity and specificity and is subject to variation by age, sex and latitude.[114] It is also subject to inhibition by orally ingested mycobacteria,[159] which may explain the early reversion from 'positivity' that occurs in TB-endemic tropical settings.[113] These properties make the PPD-skin test problematic for setting entry criteria and infection endpoints for studies of new interventions.

We and others have shown that a positive *ex vivo* T cell response to the 'early-secreted' antigens of *M. tuberculosis* (ESAT-6 and CFP-10) by ELISPOT identifies individuals likely to be recently infected with *M. tuberculosis* from recent exposure.[138,143,181] The use of ESAT-6 and CFP-10 provides improved specificity over PPD in the diagnosis of *M. tuberculosis* infection. Since the ELISPOT assay and PPD-skin test are sensitive measures of cell-mediated immunity, the magnitude of the response may provide important information with respect to the relationship between infecting *M. tuberculosis* and the host. Therefore, in the present study, we evaluated the quantitative ELISPOT and PPD-skin test responses to *M. tuberculosis* according to *M. tuberculosis* exposure in healthy household contacts of TB cases in The Gambia, an endemic tropical setting.

## 6.3 Methods

***Participants***. Sputum smear positive TB index cases over 15 years of age were recruited in Greater Banjul, an area housing 450,000 people, where the incidence rate of newly diagnosed sputum smear positive TB is approximately 80 per 100,000 population per year.[153] Included cases had two sputum samples positive for acid-fast bacilli by Ziehl-Neelsen stain and *M. tuberculosis* on culture. Their household contacts were included if they were at least 6 months of age and lived the majority of the time on the same compound as the case. They were not eligible if treated for TB in the past year and were excluded if diagnosed with TB within 1 month of recruitment. Subjects were invited to give informed consent, interviewed, examined, and a blood sample taken for ELISPOT and HIV test. Fresh samples from all participants were processed onsite. A maximum of 12 contacts per day were screened, the others randomly excluded.

Contacts underwent a PPD-skin test (2 TU, PPD RT23, Statens Serum Institut, Copenhagen, Denmark). Induration was recorded at 48-72 hours. Subjects with a positive skin test (mean induration diameter ≥ 10mm) were offered a chest X-ray and those with symptoms underwent a clinical assessment. Those with TB disease were referred to the National Programme for free treatment. There is no current practice of preventive treatment in The Gambia.

This study was approved by the Gambia Government/MRC joint Ethics Committee.

***Laboratory procedures.*** Sputum smears were prepared and stained with auramine-phenol[154] and confirmed by Ziehl-Neelsen (Z-N). Decontaminated specimens were inoculated into one slope each of Lowenstein-Jensen medium (L-J) containing glycerol and sodium pyruvate respectively and one vial of BACTEC 9000 MB media for isolation of *M. tuberculosis*. All mycobacterial cultures were identified and confirmed using standard procedures.

The *ex vivo* ELISPOT assays for IFN-γ were performed as previously described.[155] For this study, synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (ABC, 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. Purified Protein Derivative (*M. tuberculosis,* RT49, Statins Serum Institut, Copenhagen, Denmark) was used at 10 µg/ml. The positive control was Phytohaemaglutinin (PHA; Sigma-Aldrich, UK). All antigens were tested in duplicate wells.

Assays were scored by an ELISPOT counter (AID-GmbH, Strassberg, Germany). Positive test wells were pre-defined as containing at least ten SFUs more than, and at least twice as many as, negative control wells. For a positive ESAT-6/CFP-10 result it was necessary for one or more pools of overlapping peptides to be positive. PHA positive control wells were set to at least 150 SFUs above negative control wells. Negative control wells were required to have less than 30 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 assays (Wellcome Laboratories, Kent, UK) and Western blot (Diagnostics Pasteur, Marnes-la-Coquette, France).

***Ascertainment of exposure.*** As per previous study designs in The Gambia,[165] TB contacts were categorised according to where they slept: in the same bedroom as the case, a different bedroom in the same house, or in a different house.

***Data management and analysis.*** The number of SFUs in each well were automatically entered into a relational ACCESS database[2] using Matlab® software (MathWorks). Supplementary details were added by double data entry by two immunologists blinded to subject details and checked for errors. All other data were entered using double data entry, and checked for errors. A linear mixed model, with household clustering as a random effect, was fitted to the square root transformed quantitative measures. All other variables were fitted as fixed effects and the significance across proximity was quantified, allowing for the effects of possible confounding factors. All statistical analyses were conducted using Stata software (version 8; Stata Corp, College Station, TX).

## 6.4 Results

From May 2 2002 to November 30 2003, 1,382 healthy contacts of 188 sputum smear positive cases were recruited, 1187 were selected for ELISPOT for IFNγ and 1052 (89%) of these had results that met inclusion criteria. 1018 (97%) of these had PPD-skin test results. The median age of the cases was 32 years (range 15-75), 130 (70%) were male, and 12 (7%) of 184 tested were HIV positive. The median age of the case contacts was 17 years (6 months to 100 years) and 473 (45%) were males (Table 6-1). Overall, 313(30%) subjects were ESAT-6/CFP-10 ELISPOT positive.

Quantitative PPD-skin test and PPD-ELISPOT results according to evidence of recent *M. tuberculosis* infection (Figure 6-1) revealed that subjects who tested positive to ESAT-6/CFP-10 tended to have a higher PPD-ELISPOT count and a larger skin test reaction than those who were negative ($p<0.0001$ for both analyses). These data show that recent *M. tuberculosis* exposure associates with an increased immune response to PPD as measured by ELISPOT count or skin test reaction.

Exposure to *M. tuberculosis* in case contacts was classified by sleeping proximity to a case. Of the 313 ESAT-6/CFP-10 ELISPOT positive contacts, 97 (31%) slept in the same room as a case, 144 (46%) in a different room in the same house, and 72 (23%) in a different house on the same compound. When quantitative PPD-ELISPOT and PPD-skin test values were analysed according to exposure in ESAT-6/CFP-10 positive contacts (Figure 6-2), PPD-ELISPOT counts increased significantly according to level of exposure to *M. tuberculosis* (Figure 6-2A, adjusted $p=0.009$, allowing for the effects of ethnicity, gender, age, and household clustering), but no such relationship across the exposure gradient was found for the PPD-skin test results (Figure 6-2B).

There were no significant interactions found between variables. With respect to the quantitative responses to individual *M. tuberculosis* antigens in those that made a positive response, a similar trend was observed for ESAT-6 ($n=252$, $p=0.08$; data not shown), but there were too few CFP-10 positive subjects for such an analysis ($n=124$).

| Characteristics | |
|---|---|
| **Demographic** | |
| Mean (median: range) age (years) | 21 (17:0-100) |
| Male | 473 (45%) |
| Ethnic group | |
| Mandinka | 294 (28%) |
| Jola | 269 (26%) |
| Wolof | 187 (18%) |
| Fula | 72 (7%) |
| Other | 230 (22%) |
| Proximity to case (%) | |
| Same room | 232 (22%) |
| Different room | 494 (47%) |
| Different house | 326 (31%) |
| **Clinical** | |
| BCG scar present (n=883)* | 419 (47%) |
| HIV positive (n=1047) | 25 (2.4%) |

*169 subjects had uncertain BCG scar status.

**Table 6-1**: **Characteristics of 1052 TB case contacts**

**Figure 6-1: ELISPOT counts and PPD-skin test diameter by response to *M. tuberculosis*-specific peptides. (A) ELISPOT counts (Spot forming units/well/2x10$^{5)}$) in response to PPD in those subjects who have negative responses to ESAT-6/CFP-10 peptides (n=739). (B) PPD-skin test diameter in subjects in category A (n=717). (C) ELISPOT counts in those positive to ESAT-6/CFP-10 peptides (n=313). (D) PPD-skin test diameter in subjects in category C (n=301). The quantitative ELISPOT results were cut-off at 250 spots/well above background, so the results of 10 subjects with counts above 300 are not presented (9/10 were ESAT-6/CFP-10 positive).**

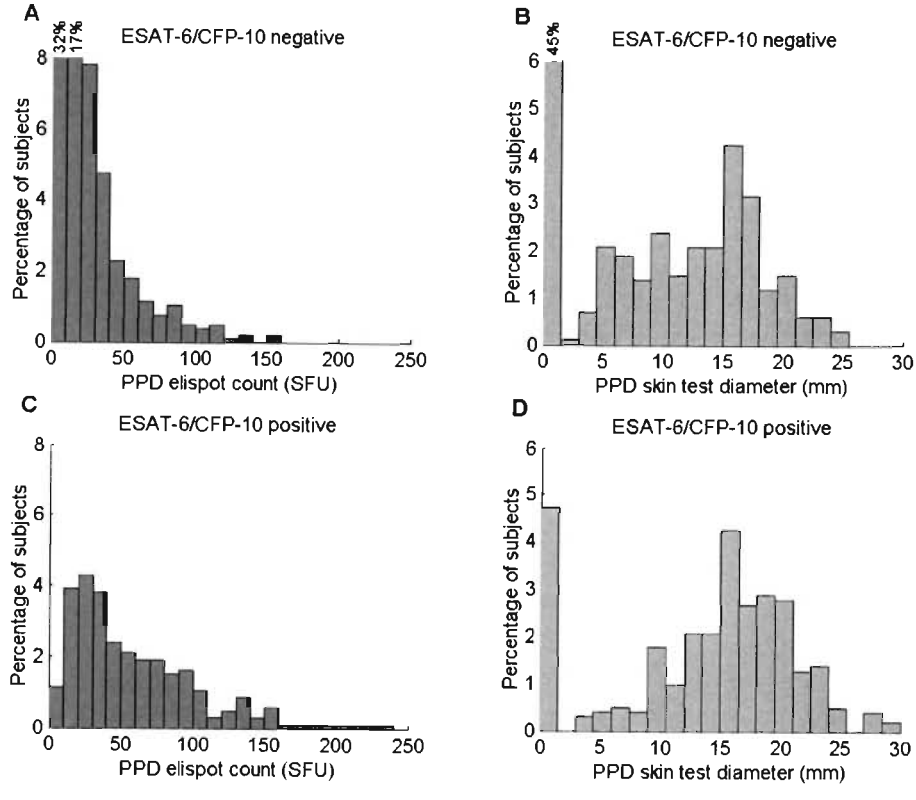
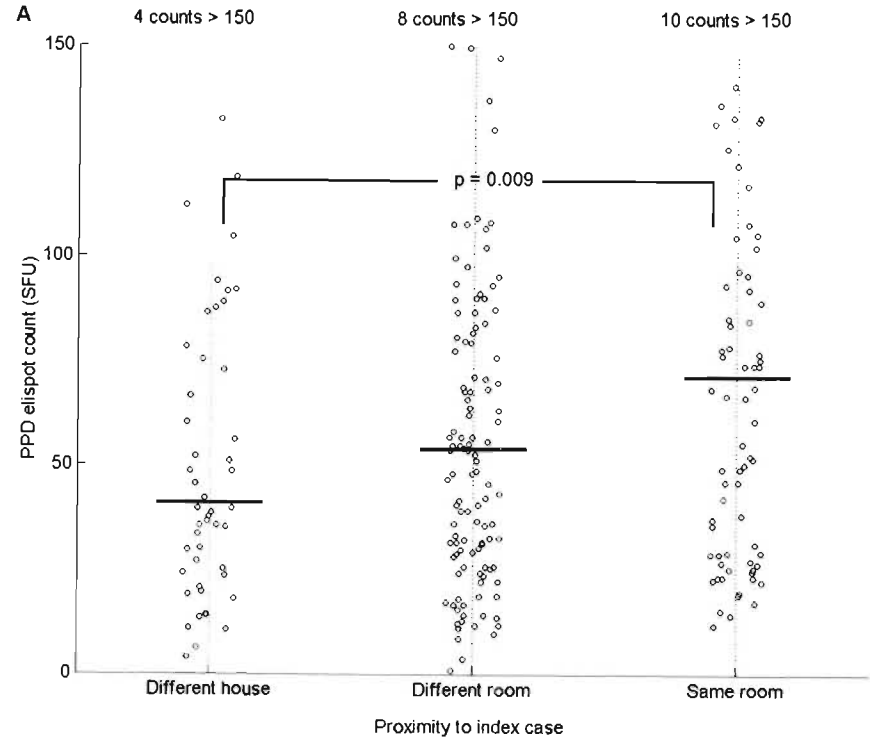
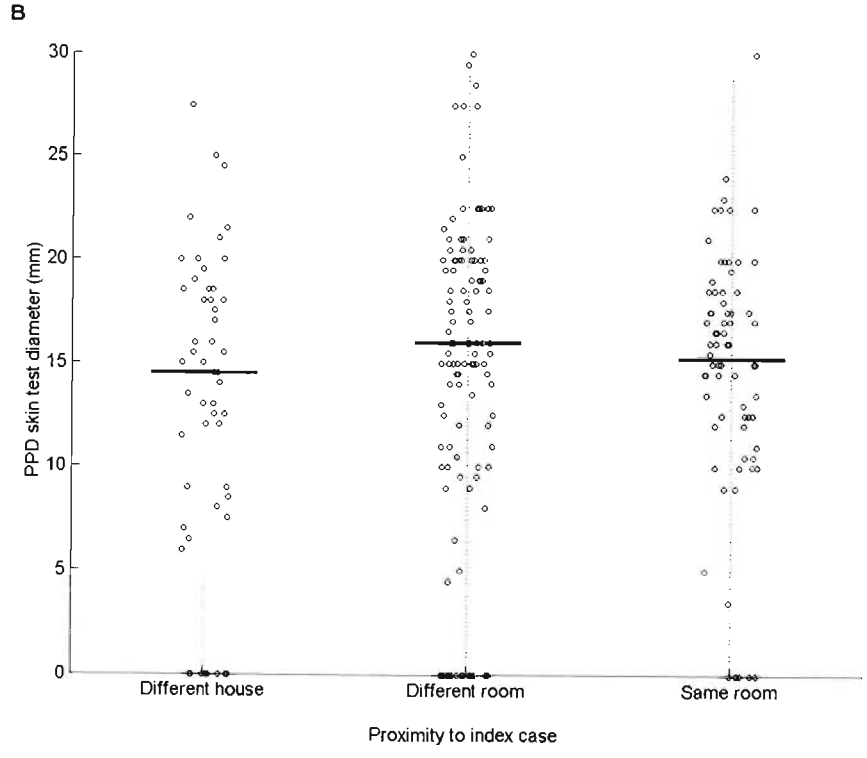


**Figure 6-2**: **Quantitative PPD-ELISPOT (A, n=313) and PPD-skin test (B, n=301) results by *M. tuberculosis* exposure category in ESAT-6/CFP-10 ELISPOT positive subjects. The horizontal lines represent the median values.**


A
4 counts > 150
8 counts > 150
10 counts > 150
150
100
50
0
p = 0.009
PPD elispot count (SFU)
Different house
Different room
Same room
Proximity to index case


B
30
25
20
15
10
5
0
PPD skin test diameter (mm)
Different house
Different room
Same room
Proximity to index case

## 6.5 Discussion

The results presented here provide an analysis of the quantitative immune response to natural *M. tuberculosis* exposure in an endemic setting in over a thousand healthy, largely HIV-negative (97.6%), household contacts of sputum smear positive TB cases. The quantitative PPD results for those subjects who were ESAT-6/CFP-10 positive were significantly higher than for those who were ESAT-6/PPD-10 negative. This was true whether the response to PPD was measured by ELISPOT or by skin test. When the quantitative PPD results of ESAT-6/CFP-10 positive subjects were compared across a natural exposure gradient, the ELISPOT assay revealed a positive dose-immune response relationship whereas the skin test showed no such relationship. To our knowledge, this is the first time that a dose-immune response has been demonstrated in a natural model for any infecting organism and these results provide evidence for the utility of the ELISPOT assay beyond a specificity gain for the diagnosis of *M. tuberculosis* infection.

The concept of utilising sleeping proximity as a measure of exposure dates back at least to a classic report by Glover in 1920 with respect to meningococcal carrier rates.[188] The model is particularly appropriate for airborne infections such as tuberculosis where cases cough mostly at night. The sleeping proximity gradient has been found to be both reproducible and reliable in The Gambia.[1,156] Since the model is natural there will be a number of environmental variables.[189] We overcome such background 'noise' by analysis of large numbers of case contacts.

To give close quantitation of the response to the whole organism without losing specificity, we conducted the ELISPOT analyses using an 'inverse' combination that combines qualitative, antigen-specific, responses with those that are quantitative and non-specific. The first combination identifies individuals likely to be infected with *M. tuberculosis* (showing a positive response to ESAT-6/CFP-10 peptides), while the second enumerates these by a response to PPD. The evaluation of PPD responses also enabled a direct comparison between the immune responses of the skin and blood compartments using the same antigenic challenge.

The *ex vivo* ELISPOT is reported to be specific for recently activated lymphocytes with an effector/memory phenotype that wanes as antigen is cleared.[160] The specific transient nature of *ex vivo* responsive cells may be a key to the sensitivity to recent infectious load that is lacking in the skin test reaction. Such a function, beyond a

qualitative (yes/no) test for infection, has several important possible applications. For example, a relative difference in ELISPOT frequency overall between intervention and placebo recipients could provide an early indication of protection in vaccine efficacy trials, either against new infection in those with known TB exposure after prophylactic vaccination, or against existing infection (therapeutic intervention).

Furthermore, we hypothesize that the quantitative ELISPOT frequency will decrease rapidly and reliably with successful intervention against *M. tuberculosis* infection, and have commenced a randomised trial to assess ELISPOT reversion with isoniazid treatment in 300 TB case contacts in The Gambia. A small study in cases has shown promising results in this regard.[190] A surrogate marker of efficacy would assist greatly in the assessment of new interventions.

The complexities of the organism-host interaction include organism factors such as differences in virulence between organism strains[191] plus, as shown in this study, differences in the infectious load received. It has been suggested that the strength of the immune response to recent infection could be a predictor of progression to disease over time.[141] Our results dictate that the infectious load must now be taken into account in longitudinal studies that address this question. For example, in a case contact study, a measure of the amount of exposure to an index case should be obtained when assessing the quantitative immune response to recent *M. tuberculosis* infection in relation to the later development of secondary disease.

As the immunological features of protection from tuberculosis are delineated, it is important that the best tools available are utilised. Improved precision in the quantitative measurement of the immune response to recent infection is possible through a functional T-cell assay that measures a transient component of the immune response. This provides new possibilities for the assessment of the efficacy of new interventions against TB and new considerations for those evaluating the initial immune response to *M. tuberculosis* in relation to the progression to disease. These results are also of relevance to studies of interventions against other major infectious diseases.

# 7

## Longitudinal assessment of the ELISPOT assay for the diagnosis of *Mycobacterium tuberculosis* infection

**MRC Labs**

## 7.1 Summary

*Background.* Cross-sectional evaluations the ELISPOT assay for *M. tuberculosis* infection have shown its promise in the diagnosis of infection after recent exposure. It is not known whether ELISPOT reversion and/or conversion occur over time.

*Methods.* We conducted tuberculin skin and ELISPOT tests in consecutively recruited TB case contacts: a blood sample for ELISPOT after 3 months and 18 months; those that were PPD skin test negative at recruitment had a repeat PPD skin test and up to 100 consecutively recruited contacts had a repeat PPD skin test at 18 months. We compared conversion and reversion of both tests and assessed risk factors for these phenomena by multiple logistic regression.

*Results.* At both recruitment and at 18 months the agreement between the PPD skin test and the ELISPOT was approximately 70%. ELISPOT conversion occurred at 3 months in 29% of contacts; conversion occurred at 18 months in 33% of those negative at 3 months. ELISPOT reversion occurred at 3 months in 39%; reversion occurred at 18 months in 29% of those who were positive at 3 months. A positive PPD skin test at recruitment was associated with ELISPOT conversion at 3 months (OR=2.6; 1.2-5.7; p=0.014); a positive ELISPOT at recruitment was associated with conversion from 3 months to 18 months (OR 5.0; 1.3-19.6; p=0.022). A positive PPD skin test at recruitment was associated with decreased ELISPOT reversion at 3 months (OR 0.2; 0.1-0.8; p=0.016); a positive recruitment ELISPOT was associated with decreased ELISPOT reversion between 3 and 18 months (OR 0.13; 0.03-0.68; p=0.015). Only 10% of PPD skin test positive individuals at recruitment reverted to negative at 18 months; 34% of those initially negative became positive at 3 months and 46% of those initially negative became positive at 18 months. Sleeping in the same room as a case was associated with PPD skin test conversion at 3 months.

Conclusions. The ELISPOT test may play an important role in the diagnosis of early *M. tuberculosis* infection after a known exposure and a repeated test may be required to capture as many infected individuals as possible. A high rate of ELISPOT reversion compared to the PPD skin test suggests the assay may be unreliable in the diagnosis of latent infection in a state of dormancy.

## 7.2 Introduction

*Mycobacterium tuberculosis* undergoes a complex natural lifecycle involving early 'active' infection, dormancy and reactivation. Early infection and reactivation may both progress to disease. A more accurate gauge of where *M. tuberculosis* is located in this cycle of infection and the factors that influence transition between each stage are crucial issues in tuberculosis (TB) research. New diagnostic tools plus detailed immunological and molecular studies are needed.

Key cross-sectional evaluations of a new T cell based *ex vivo* Enzyme-Linked Immunospot Assay for interferon gamma (ELISPOT), specific for early secreted antigens of *M. tuberculosis*, have shown its considerable promise in the diagnosis of infection after recent exposure.[1,143] However, it is not clear whether positive or negative ELISPOT responses in TB case contacts are maintained over time.[185] ELISPOT conversion, after an initially negative response, might be expected where there is ongoing exposure to a case in the early stages of treatment. Reversion of a previously positive response would be anticipated as *M. tuberculosis* makes a transition into dormancy, since the ELISPOT assay is thought to be specific for effector-memory T cells that disappear from the circulation without the ongoing presence of antigen.[160] Within the context of ongoing prospective studies of TB case contacts in The Gambia,[1] we hypothesized that conversion and reversion of the ELISPOT test would occur and present test results at recruitment and after 3 and 18 months, with comparison to the PPD skin test at recruitment and 18 months.

## 7.3 Methods

***Participants.*** Recruitment of sputum smear positive index cases and inclusion or exclusion of their household contacts for the Gambian case contact studies has been described previously. Household contacts of newly diagnosed TB cases were eligible for inclusion in this study if they were at least 15 years of age and had recruitment ELISPOT result but were not diagnosed with TB disease. Those with TB disease were referred to the National Programme for free treatment. There is no current practice of preventive treatment in The Gambia. Contacts were interviewed, examined, and a blood sample taken for ELISPOT and HIV test at recruitment. They also underwent a tuberculin skin test (2 TU, PPD RT23, Statins Serum Institut, Copenhagen, Denmark). The contacts were asked to provide a blood sample for ELISPOT after 3 months and 18 months. Those who were PPD skin test negative at recruitment were asked to have a repeat PPD skin test and up to 100 consecutively recruited contacts were asked to have a repeat PPD skin test at 18 months (A decision was made to seek approval for a repeat PPD skin test at 18 months after a preliminary analysis of 18 month ELISPOT results). All participants underwent clinical follow-up at 3 months, 6 months, 12 months and 18 months. Those with symptoms consistent with TB underwent a clinical examination and a chest x-ray if indicated and those able to produce sputum underwent sputum analysis. Those diagnosed with TB disease were referred to the National Programme for free treatment and excluded from the analysis.

***Laboratory procedures.*** Sputum smears were prepared, stained and cultured, plus HIV tests performed as previously described. ELISPOT assays for IFNγ were performed in duplicate as previously described.[123] Synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (ABC, Imperial College, London, UK) were used. Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. The ESAT-6 CFP-10 peptide pools were used at a final concentration of 2.5μg/ml for each peptide. The positive control was Phytohaemaglutinin (PHA; Sigma-Aldrich, UK). The *ex vivo* ELISPOT assays were enumerated using an ELISPOT reader (AID-GmbH, Strassberg, Germany). Positive test wells were pre-defined as containing at least eight spot forming units (SFU) more than negative control wells. For a positive result it was necessary for at least one of the pools of overlapping peptides to be positive. PHA wells were set to at least 150 SFU/well/$2x10^5$ above negative control wells. Negative control wells were required to have less than 20 SFU/well/$2x10^5$.

***Reliability of the ELISPOT assay.*** We assessed the reliability of the ELISPOT assay in two ways. Firstly, as we tested each antigen in duplicate for each subject, we compared the between and the within subject variability by fitting a random effects model for subject. We used ELISPOT results from consecutively recruited TB case contacts: 1223 duplicate results for ESAT-6 peptides and 1182 duplicate results for CFP-10 peptides. For CFP-10 the between subject standard deviation was 29.4 spots and the within subject standard deviation was 3.6 spots. For ESAT-6, the between subject standard deviation was 38.9 spots and the within subject standard deviation was 4.2 spots. Therefore the within variability was very small compared to the between variability. Secondly, we recruited 22 adult volunteers from the general community and conducted an ELISPOT test followed by a repeat test after 1 week. Eleven volunteers had a negative test initially: none of these had a positive test after 1 week. Eleven volunteers had a positive test initially: only one of these had a negative test after one week.

***Data management and analysis.*** The number of SFU in each well were automatically entered into a database using Matlab® software (MathWorks). Supplementary details were added by double data entry. All other data were entered using double data entry into an ACCESS database and verified.[2] Random effects logistic regression models, taking into account household clustering, were used to assess the relationship between conversion and reversion of test results to possible risk factors. Age was included in the models a priori. Statistical analyses were conducted using Stata software (version 8; Stata Corp, College Station, TX, USA).

## 7.4 Results

Between April 1 2003 and October 10 2004, 731 healthy household contacts, over the age of 15 years, of 175 TB cases were recruited. Of these, 608 were selected for ELISPOT and had an adequate sample taken: 566 (93%) had a result that met the inclusion criteria for analysis. At 3 months follow-up, 390 contacts were located, agreed to have a repeat ELISPOT test and had an adequate sample taken. Of these, 348 (89%) from 126 households had an ELISPOT result that met the criteria for analysis. Two hundred and fifty nine consecutively recruited contacts with ELISPOT results at 3 months follow-up were visited at 18 months: 188 were found and agreed to have an ELISPOT test and 153 had an adequate specimen. Of these 136 (89%) from 62 households had an ELISPOT result that met criteria for analysis. One hundred and eighty six contacts who were PPD skin test negative at recruitment had a repeat PPD skin test at 3 months. Seventy-three consecutively visited contacts found at 18 months were asked and agreed to have a repeat PPD skin test. The age, sex, ethnicity and HIV status were similar for those contacts with results at recruitment, 3 months and/or 18 months (Table 7-1).

Of the 210 ELISPOT negative contacts at recruitment that had a repeat ELISPOT test at 3 months, 61 (29%) became positive. Of the 138 contacts who had a positive ELISPOT at recruitment and a repeat ELISPOT, 54 (39%) reverted to negative at 3 months. Figure 7-1A shows, for those who had results at recruitment, 3 months and 18 months, the number of contacts who were ELISPOT positive at each time point and the number of those who were persistently negative. Of the 81 contacts who had a negative ELISPOT at 3 months and had a repeat ELISPOT at 18 months; 26 (33%) became positive. Of the 55 contacts that were ELISPOT positive at 3 months and had a repeat test, 16 (29%) became negative.

Table 7-2 shows the analysis of risk factors for conversion of a negative ELISPOT test after 3 and 18 months. In the univariable analysis, sleeping in the same room as a case ($p=0.02$) and a positive skin test at recruitment ($p=0.002$) were found to be risk factors for ELISPOT conversion. In a multivariable model with household as a random effect, including age, sleeping proximity to a case, ethnicity and PPD skin test at recruitment, only a positive PPD skin test at recruitment remained a significant risk factor (OR=2.6; 95% CI 1.2-5.7; $p=0.014$).

In the univariable analysis, a positive ELISPOT at recruitment was a strong predictor of conversion from negative at 3 months to positive at 18 months (p=0.006). In a multivariable model with household as a random effect, including age and recruitment PPD skin test, a positive ELISPOT at recruitment remained a strong predictor of ELISPOT conversion at 18 months (OR=5.0; 1.3-19.6; p=0.022).

Table 7-3 shows the analysis of risk factors for reversion of a positive ELISPOT test after 3 and 18 months. In the univariable analysis those who slept in the same room as a case (p=0.039) were more likely to have ELISPOT reversion at 3 months than those sleeping in a different house, while males (p=0.029) and those with a positive PPD skin test at recruitment (p=0.011) were less likely to revert. In a multivariable model with household as a random effect, including age, sex, sleeping proximity to a case plus recruitment ELISPOT and PPD skin test result, those who slept in the same room as a case were no longer found to be more likely, and males were no longer less likely, to undergo ELISPOT reversion. However PPD skin test remained associated with a decreased likelihood of reversion (OR 0.2; 0.1-0.8; p=0.016).

In the univariable analysis a positive recruitment ELISPOT was associated with a decreased likelihood of reversion between 3 and 18 months (p=0.019). In a multivariable model with household as a random effect, including age, sex, recruitment PPD skin test and ELISPOT results, a positive ELISPOT at recruitment remained associated with a decreased likelihood of ELISPOT reversion between 3 and 18 months (OR 0.13; 0.03-0.68; p=0.015).

Figure 7-2 shows the quantitative ELISPOT frequencies at recruitment and at 3 months for those initially test positive (Fig 2A) and those initially test negative (Fig 2B). Few test conversions or reversions could be explained by small changes in the counts around the cut-off for test positivity. There was also, for all those initially test positive, a significant reduction in spot counts overall: the median count changed from 29.8 SFU/well/$2x10^5$ at recruitment to 15.5 SFU/well/$2x10^5$ at 3 months (p=0.0001, Wilcoxon signed rank test).

Sixty four (34%) of 186 contacts negative at recruitment became PPD skin test positive at 3 months. In the univariable analysis only sleeping in the same room as a case was significantly associated with PPD skin test conversion at 3 months, remaining significant in a multivariable model with household as a random effect, adjusting for age and sex (OR 5.0; 1.6-16.4; p=0.006). Of thirteen PPD skin test converters at 3

months, 5 (38%) became negative at 18 months. Seventy-one contacts had a PPD skin test at both recruitment and at 18 months. Only 3/30 (10%, Figure 7-1B) of those initially positive had a negative PPD skin test. In contrast, 19 of 41 (46%) of those negative at recruitment became positive. Neither age, sex, recruitment ELISPOT result, sleeping proximity to a case or ethnic group showed any significant relationship, by random effects logistic regression, with PPD skin test conversion between recruitment and 18 months. At recruitment the agreement between the ELISPOT and PPD skin test was 72% (kappa statistic 0.44) but the discordance was highly significant (p=0.0001). At 18 months the agreement between the two tests was 70% (kappa statistic 0.38) and the discordance significant at the p=0.1 level (p=0.09). Of 19 contacts who were PPD skin test negative at recruitment but positive at 18 months, 13 (68%) were ELISPOT positive at 18 months (6 had been ELISPOT positive at recruitment). Of the 14 contacts who were ELISPOT negative at recruitment but positive at 18 months, 10 (71%) were PPD skin test positive at 18 months (3 had been PPD skin test positive at recruitment).

| Demographic | Recruitment (n=566) | 3 months (n=348) | 18 months (n=136) |
|---|---|---|---|
| Mean (median: range) age (years) | 30 (25:15-80) | 30.5 (25:15-80) | 30 (25:15-80) |
| Male (%) | 277 (48.9%) | 158 (45.4%) | 61 (44.5%) |
| Ethnic group | | | |
| Mandinka | 174 (30.7%) | 106 (30.5) | 31 (22.8%) |
| Jola | 186 (32.9%) | 112 (32.2) | 38 (27.9.4%) |
| Wolof | 61 (10.8%) | 35 (10.1) | 19 (14.0%) |
| Fula | 50 (8.8%) | 40 (11.5) | 19 (14.0%) |
| Other | 95 (16.8%) | 55 (15.8) | 29 (21.3%) |
| Proximity to case (%) | | | |
| Same room | 143 (25.3%) | 83 (23.9) | 28 (20.6%) |
| Different room | 236 (41.7%) | 145 (41.7) | 62 (45.6%) |
| Different house | 187 (33.0%) | 120 (34.5) | 46 (33.8%) |
| **Clinical** | | | |
| BCG scar present | 221 (39.1%)[a] | 145 (41.7)[c] | 51 (37.5%)[d] |
| HIV positive | 18 (3.2%)[b] | 11 (3.2) | 1 (0.7%) |

[a] 86 (15.2%) had uncertain scar status, [b] n=565 tested, [c] 55 (15.8%) had uncertain scar status, [d] 29 (21.3%) had uncertain scar status.

**Table 7-1: Characteristics of TB case contacts with ELISPOT results at recruitment, 3 months and 18 months.**

| Risk factor | Category | 3 months conversion (n=210) | | 18 months conversion (n=81) | |
|---|---|---|---|---|---|
| | | % (n) | OR (95% CI) | % (n) | OR (95% CI) |
| Sleep proximity | Different house | 19.5 (15) | 1.0 | 35.7 (5) | 1.0 |
| | Different room | 31.8 (28) | 1.9 (0.9-4.1) | 33.3 (13) | 0.8 (0.2-4.0) |
| | Same room | 40 (18) | 2.7 (1.1 –6.4)[a] | 32.1 (9) | 1.2 (0.2-8.4) |
| Age (years) | 15-20 | 21.6 (16) | 1.0 | 23.1 (6) | 1.0 |
| | 21-29 | 32.8 (20) | 2.0 (0.8-4.7) | 43.3 (12) | 3.0 (0.6-13.9) |
| | 30-45 | 32.6 (14) | 1.7 (0.6-4.2) | 33.3 (4) | 2.5 (0.3-21.3) |
| | >45 | 34.4 (11) | 2.3 (0.8-6.7) | 30.8 (4) | 1.4 (0.2-10.2) |
| Gender | Female | 36.2 (17) | 1.0 | 28.3 (13) | 1.0 |
| | Male | 33.3 (15) | 1.3 (0.7-2.4) | 40 (14) | 2.2 (0.5-9.2) |
| Ethnicity | Mandinka | 24 (18) | 1.0 | 12.5 (3) | 1.0 |
| | Jola | 30.8 (20) | 1.5 (0.6-3.3) | 60.0 (15) | 16.2(1.8-147.1)[e] |
| | Wolof | 20 (4) | 0.8 (0.2-3.1) | 27.3 (3) | 3.7 (0.3-42.3) |
| | Fula | 25 (4) | 1.1 (0.3-4.2) | 12.5 (1) | 1.2 (0.1-21.0) |
| | Other | 44.1 (15) | 2,6 (1.0-6.7)[b] | 38.5 (5) | 5.6 (0.7-48.2) |
| BCG scar | Absent/uncertain | 33.9 (39) | 1.0 | 35.6 (16) | 1.0 |
| | Present | 23.2 (22) | 0.6 (0.3-1.1) | 30.1 (11) | 1.0 (0.4-4.0) |
| Recruit PPD skin test[c] | <10mm induration | 22.9 (33) | 1.0 | 25.5 (14) | 1.0 |
| | >=10mm induration | 46.4 (26) | 3.0 (1.5-6.4)[d] | 54.2 (13) | 4.2 (1.0-17.1)[f] |
| Recruit ELISPOT | Negative | NR | NR | 20.8 (11) | 1.0 |
| | Positive | | | 57.1 (16) | 6.3 (1.7-23.8)[g] |

[a] p=0.024, [b] p=0.051, [c] 200/210 3 month volunteers ELISPOT negative at recruitment had a PPD skin test; all 18 month volunteers had a recruitment PPD skin test, [d] p=0.013, [e] p=0.003, [f] p=0.043, [g] p=0.006

**Table 7-2: Risk factors for ELISPOT conversion at 3 months and at 18 months: univariable odds ratios by logistic regression (household as a random effect) according to change to positive for those negative at the immediately previous time point.**

| Risk factor | Category | 3 months reversion (n=138) | | 18 months reversion (n=55) | |
|---|---|---|---|---|---|
| | | % (n) | OR (95% CI) | % (n) | OR (95% CI) |
| Sleep proximity | Different house | 44.2 (19) | 1.0 | 38.9 (7) | 1.0 |
| | Different room | 47.4 (27) | 1.2 (0.4-3.5) | 26.1 (6) | 0.5 (0.1-2.4) |
| | Same room | 21.1 (8) | 0.3 (0.1-0.9)[b] | 21.4 (3) | 0.4 (0.1-2.3) |
| Age (years) | 15-20 | 41.3 (12) | 1.0 | 46.2 (6) | 1.0 |
| | 21-29 | 42.0 (21) | 0.9 (0.3-2.9) | 23.8 (5) | 0.4 (0.1-1.7) |
| | 30-45 | 28.2 (11) | 0.4 (0.1-1.6) | 23.1 (3) | 0.4 (0.1-2.1) |
| | >45 | 50.0 (10) | 1.4 (0.4-5.8) | 25.0 (2) | 0.4 (0.1-2.9) |
| Gender | Female | 48.0 (36) | 1.0 | 31.0 (9) | 1.0 (0.4-2.6) |
| | Male | 28.6 (18) | 0.4 (0.2-0.9)[c] | 26.9 (7) | 0.7 (0.2-3.0) |
| Ethnicity | Mandinka | 51.6 (16) | 1.0 | 28.6 (2) | 1.0 |
| | Jola | 48.9 (23) | 0.9 (0.3-2.5) | 7.7 (1) | 0.2 (0.02-2.9) |
| | Wolof | 26.7 (4) | 0.3 (0.1-1.4 | 12.5 (1) | 0.4 (0.02-5.1) |
| | Fula | 25.0 (6) | 0.3 (0.1-1.1) | 45.5 (5) | 2.1 (0.3-15.8) |
| | Other | 23.8 (5) | 0.3 (0.1-1.1) | 43.8 (7) | 1.9 (0.3-13.2) |
| BCG scar | Absent/uncertain | 38.6 (34) | 1.0 | 32.5 (13) | 1.0 |
| | Present | 40.0 (20) | 1.1 (0.5-2.7) | 20.0 (3) | 1.0 (0.3-4.0) |
| Recruit PPD skin test[a] | <10mm induration | 60.0 (21) | 1.0 | 47.1 (8) | 1.0 |
| | >=10mm induration | 32.2 (29) | 0.3 (0.1-0.7)[d] | 19.4 (7) | 0.3 (0.1-1.1)[e] |
| Recruit ELISPOT | Negative | NR | NR | 52.2 (12) | 1.0 |
| | Positive | | | 12.5 (4) | 0.1 (0.01-0.7)[f] |

[a]125/138 of those with ELISPOT results at 3 months and 53/55 with results at 18 months had a recruitment PPD skin test, [b]p=0.039, [c]p=0.029, [d]p=0.011, [e]p=0.059, [f]p=0.019.

**Table 7-3: Risk factors for ELISPOT reversion at 3 months and at 18 months: Univariable odds ratios by logistic regression (household as a random effect) according to change to negative for those positive at the immediately previous time point.**

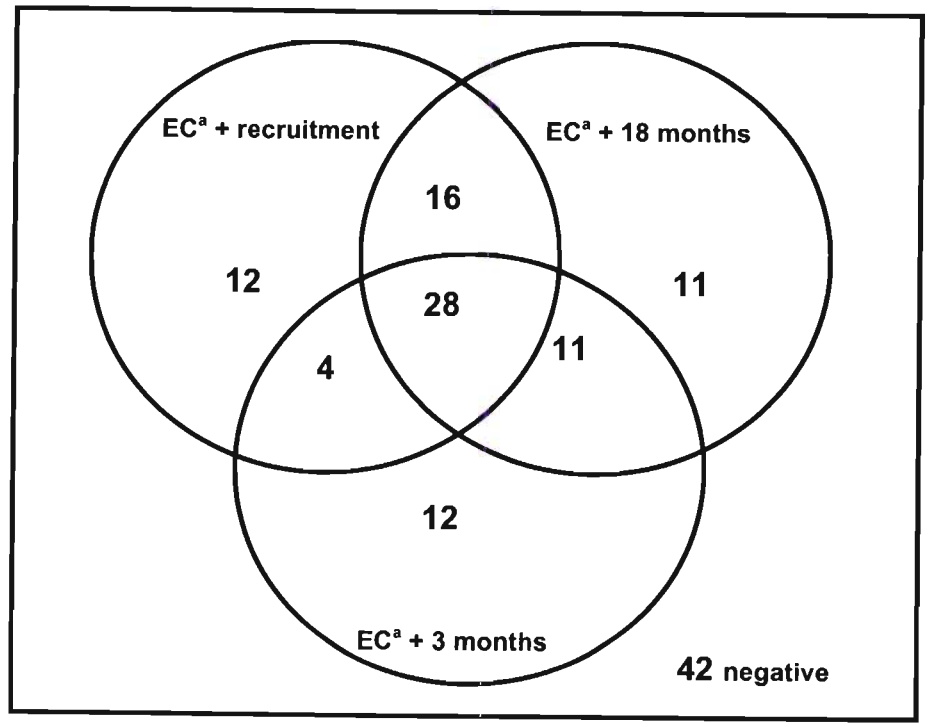
| Risk factor | Category | 3 months conversion (n=186) | |
|---|---|---|---|
| | | % (n) | OR (95% CI) |
| Sleep proximity | Different house | 27.6 (23) | 1.0 |
| | Different room | 33.3 (26) | 1.5 (0.7-3.4) |
| | Same room | 60 (15) | 4.6 (1.5 –13.8)[a] |
| Age (years) | 15-20 | 29.5 (18) | 1.0 |
| | 21-29 | 44.6 (25) | 2.1 (0.8-5.0) |
| | 30-45 | 27.8 (10) | 0.8 (0.3-2.4) |
| | >45 | 33.3 (11) | 1.2 (0.4-3.5) |
| Gender | Female | 32.1 (35) | 1.0 |
| | Male | 37.6 (29) | 1.3 (0.7-2.7) |
| Ethnicity | Mandinka | 28.2 (20) | 1.0 |
| | Jola | 41.0 (25) | 1.9 (0.8-5.0) |
| | Wolof | 31.3 (5) | 1.1 (0.3-4.9) |
| | Fula | 26.7 (4) | 1.0 (0.3-4.5) |
| | Other | 43.5 (10) | 2.2 (0.7-7.3) |
| BCG scar | Absent/uncertain | 33.9 (38) | 1.0 |
| | Present | 35.1 (26) | 1.1 (0.5-2.2) |
| Recruit ELISPOT | Negative | 33.6 (51) | 1.0 |
| | Positive | 38.2 (13) | 1.5 (0.6-3.8) |

[a] p=0.007.

**Table 7-4: Risk factors for PPD skin test conversion at 3 months.**

**Figure 7-1: Venn diagrams showing the number of household contacts positive and negative according to time tested. (A) ESAT-6/CFP-10 ELISPOT at recruitment, 3 months and 18 months follow-up (n=136); (B) PPD skin test at recruitment and 18 months follow-up (n=71).**

A




[a] ESAT-6/CFP-10

B

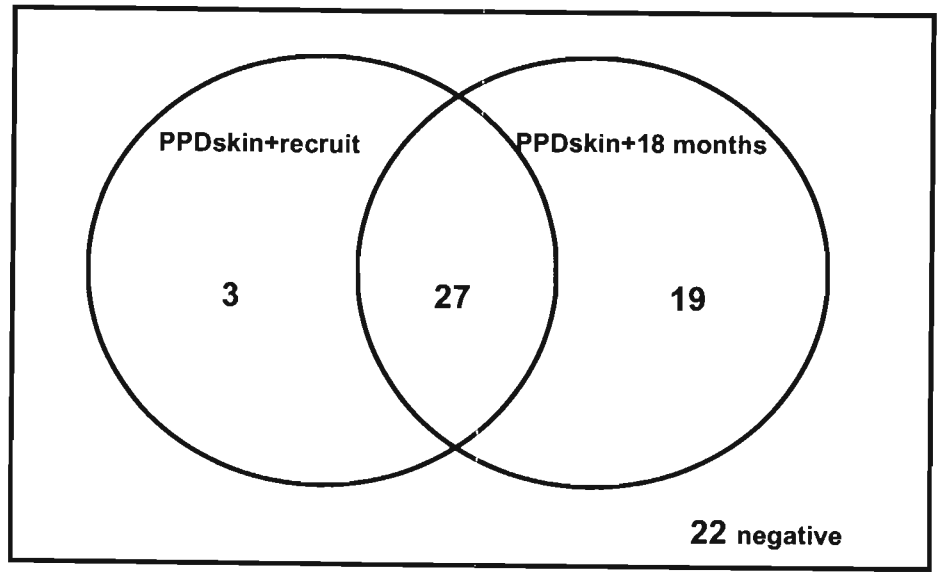
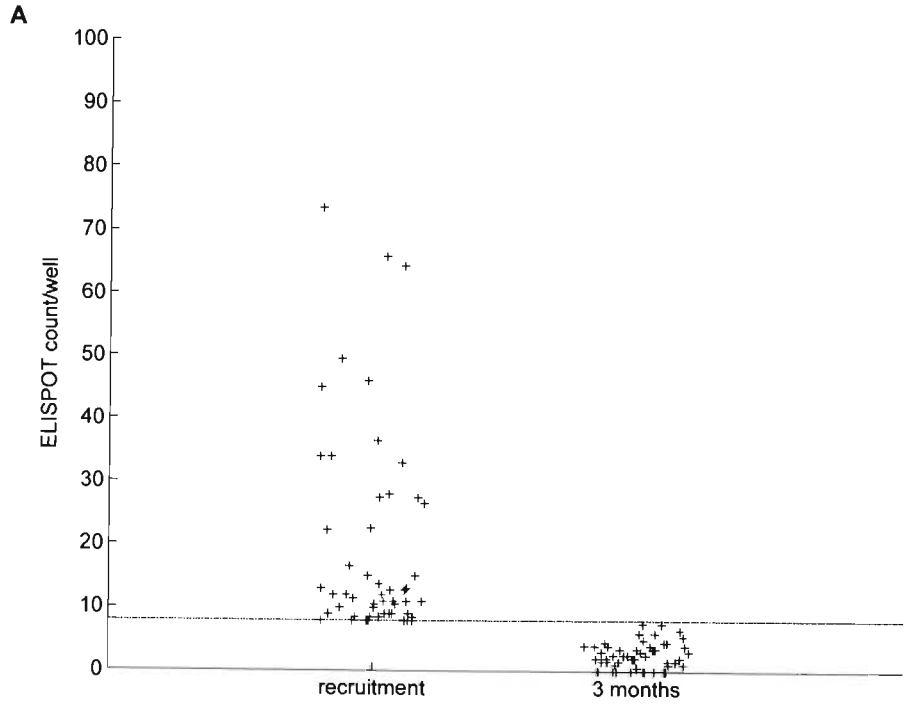


Figure 7-2: ELISPOT counts (spots/well above negative control) at recruitment and 3 months in household contacts TB cases. (A) Counts in those that underwent ELSIPOT reversion; (B) counts in those that underwent ELISPOT conversion.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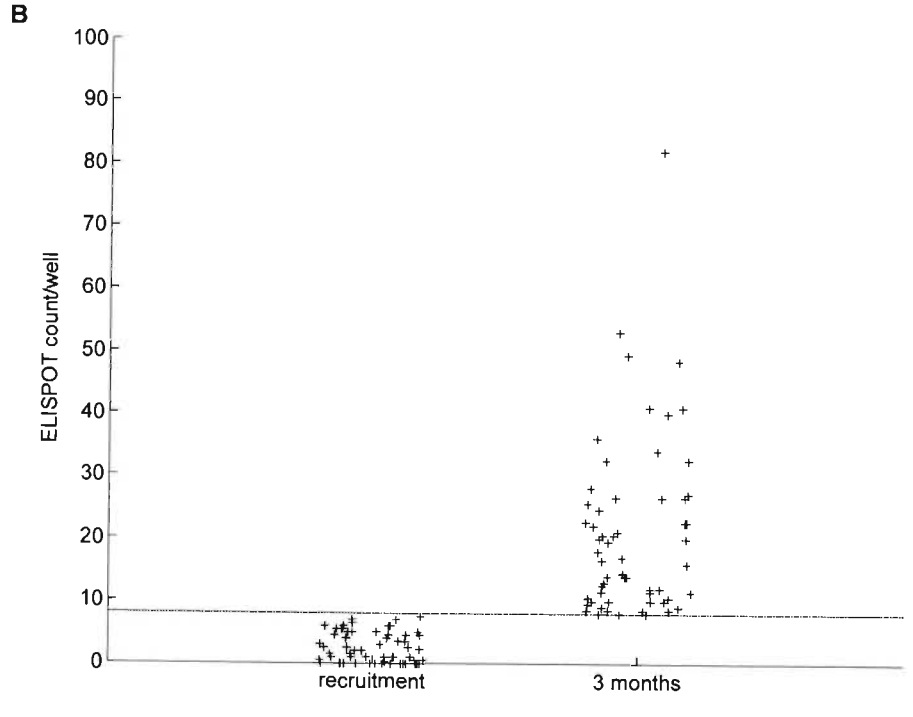
ELISPOT count/well
recruitment
3 months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ELISPOT count/well
recruitment
3 months

## 7.5 Discussion

In this study we found that both ELISPOT conversion and reversion occur. ELISPOT conversion occurred at 3 months in 29% of those negative at recruitment and at 18 months in 33% of those negative at 3 months. ELISPOT reversion occurred at 3 months in 39% of those initially positive and at 18 months in 29% of those who were positive at 3 months. A positive PPD skin test at recruitment was a strong risk factor for ELISPOT conversion at 3 months (OR=2.6; 1.2-5.7; p=0.014) while a positive ELISPOT at recruitment was a strong risk factor for conversion from 3 months to 18 months (OR 5.0; 1.3-19.6; p=0.022). In contrast a positive PPD skin test at recruitment was associated with decreased ELISPOT reversion at 3 months (OR 0.2; 0.1-0.8; p=0.016), while a positive recruitment ELISPOT was associated with decreased ELISPOT reversion between 3 and 18 months (OR 0.13; 0.03-0.68; p=0.015). Only 10% of PPD skin test positive individuals at recruitment reverted to negative at 18 months, while 46% of those initially negative became positive at 18 months. The only risk factor identified for PPD skin test conversion at 3 months was sleeping in the same room as a TB case. No risk factors for PPD skin test conversion after 18 months were identified, although the numbers for this analysis were small. At both recruitment and at 18 months the agreement between the PPD skin test and the ELISPOT was approximately 70%. These data provide new insights into the properties and possible niche for the ELISPOT test with respect to *M. tuberculosis* infection.

Changes in the ELISPOT frequency over time have been reported in TB cases in a small uncontrolled study that demonstrated a decrease in the SFU during and after TB treatment.[190] These data are consistent with the idea that ELISPOT responses are transient and require continued exposure to antigen to maintain high frequencies. To our knowledge, no longitudinal results in TB case contacts screened by ELISPOT have been reported. Conversion and reversion of the traditional PPD skin test following BCG vaccination have been reported in a TB-endemic tropical setting,[113] although PPD skin test reversion would not be expected to occur after only 3 months.[142] Here we show that both conversion and reversion of the ELISPOT occur after a relatively brief 3-month period of follow-up after known *M. tuberculosis* exposure. That a positive ELISPOT at recruitment was a strong predictor of ELISPOT conversion from 3 months to 18 months, is consistent with the concept that the test switches off quickly upon the cessation of antigen secretion and switches on upon the recommencement of secretion in those who do not clear their infection. In these individuals the mycobacterium enters dormant state whereby it may not reliably secrete ESAT-6 and CFP-10 and

preferentially secretes other antigens.[37] Therefore, the ELISPOT should be regarded as potentially unreliable in the diagnosis of latent infection, especially in situations where there is no clearly defined recent case contact. The significance of the switch on of the ELISPOT response cannot be determined from this study. It would be of great interest if the ELISPOT were found to be sensitive to *M. tuberculosis* reactivation.

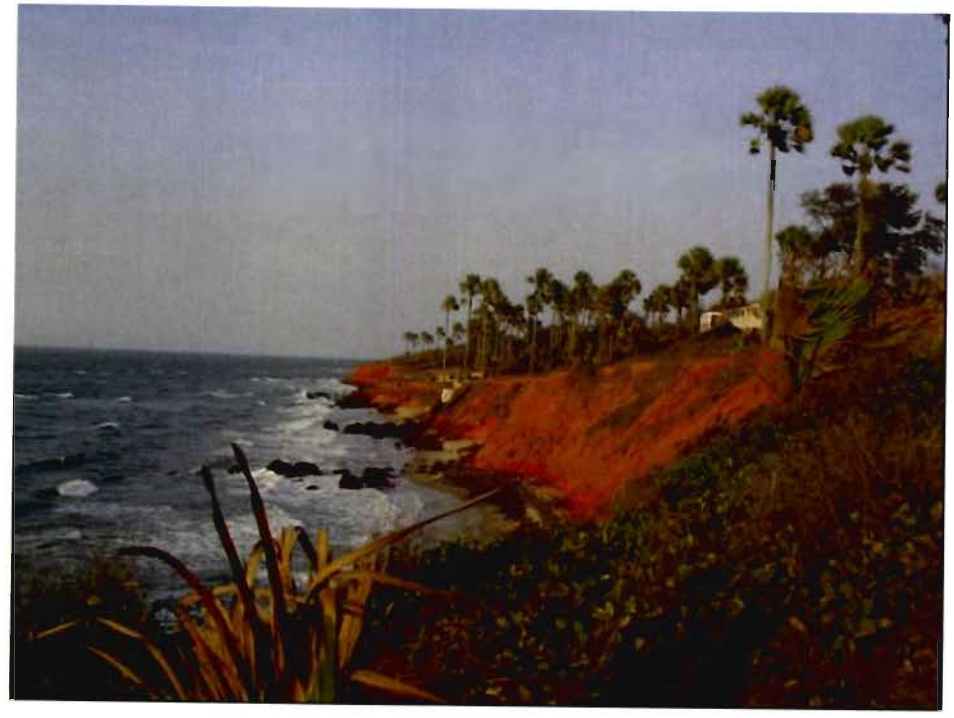
With respect to those who convert to positive either by ELISPOT or PPD skin test, it is likely that they become exposed to *M. tuberculosis* after their respective index case begins anti-tuberculous treatment. This is possible as several weeks of treatment are required before a case becomes sputum negative. It is also possible, in an endemic setting, for individuals to be exposed to an unrecognised case. The PPD skin test, which is less likely to revert over 18 months of follow-up, provides perhaps the best insight into this-34% conversion occurred at 3 months (probably largely related to ongoing exposure to the respective case), rising more slowly to 44% at 18 months (the additional percentage probably reflecting exposure other than to the known case). It is of note that 6 of 13 PPD skin test converters with 18 month results reverted to a negative PPD skin test at 18 months, compared with only 3 of 30 initially positive at recruitment. This is consistent with the premise that their conversion may have arisen from boosting of a non-specific mycobacterial response, which may be more likely to revert to negative early.[87,89,90] It is not clear why a positive PPD skin test at recruitment is predictive of ELISPOT conversion at 3 months. It appears unlikely that this is related to a difference in the incubation period of the two tests. It is possible that the PPD skin test causes boosting of an ELISPOT response-although this has not been demonstrated. Studies are underway in The Gambia to explore this possible phenomenon. Overall, it appears likely in our setting, that repeated ELISPOT and PPD skin testing, at least in the first 3-6 months after a known exposure, may offer a significant gain in sensitivity in the identification of those who become infected with *M. tuberculosis* from their exposure to a case. This should be confirmed by longitudinal studies in low TB prevalence settings.

In The Gambia we have used mathematical modelling of the results from over 1500 individuals to identify a correct cut-off for the ELISPOT test.[108] Through this we have identified a cut-off for ELISPOT positivity of 8 spots/well above the negative control well, as opposed to 5 spots/well in some other studies.[143] However, as an example, using a cut-off of 5 spots/well, we still find 21% conversion (versus 26%) and 40% reversion at 3 months (versus 39%; data not shown). Therefore significant ELISPOT conversion and reversion occur irrespective of the cut-off for positivity.

The ELISPOT test may play an important role in the diagnosis of early *M. tuberculosis* infection after a known exposure and a repeated test may be required to capture as many infected individuals as possible. A high rate of ELISPOT reversion suggests the assay may be unreliable in the diagnosis of latent infection in a state of dormancy. While some reversion could reflect clearance of the organism, we suggest it is probably due to an inability of the ELISPOT to continue to diagnose *M. tuberculosis* infection as the organism cycles into dormancy, where ESAT-6 and CFP-10 antigens may not be secreted continuously. That a positive PPD skin test at recruitment was associated with a decreased likelihood of ELISPOT reversion at 3 months, suggests that the two tests may ultimately be best used together, perhaps to identify those most probably infected. Repeatedly positive ELISPOT tests may also assist in identifying important subgroups of TB case contacts. Furthermore, we have recently reported that 'transience' of *ex vivo* responsive cells may actually give the ELISPOT assay a unique role as a precise dynamic monitor of the infecting *M. tuberculosis* load.[3] Indeed, further longitudinal studies may show it to serve an important function as early indicator of reactivation. It is clear that further studies are now required to define precisely the niche of the ELISPOT test with respect to *M. tuberculosis* infection. In particular long term follow-up of large numbers of case contacts to identify secondary cases.

# 8

# Summary and conclusions



'MRC' cliffs

The key findings and their importance can be summarised as answers to the research questions that were posed in chapter 1:

1. Can a reproducible framework be developed to assess new diagnostic tools for *M. tuberculosis* infection? (chapter 2).

A reproducible framework for the assessment of new diagnostic tools for *M. tuberculosis* infection has been developed. It comprises the following framework:

- Consecutive recruitment of TB cases and their household contacts
- Purposeful selection, within 3 categories of sleeping proximity to an index case, of contacts within the limitations of the capacity of the laboratory required for processing of the samples.
- Comparison of tests in TB case contacts using tests of concordance and discordance plus response to an 'exposure gradient' assessed by logistic regression (qualitative results) and linear regression (quantitative results).
- Longitudinal assessment with repeat testing over 18 months.
- Further comparison could be made in large numbers of case contacts with respect to development of secondary disease. To confirm that they received their infection from their respective index case, molecular fingerprinting could be performed.

2. Are new T cell assays that utilise 'specific' *M. tuberculosis* antigens better than the traditional PPD skin test for the diagnosis of *M. tuberculosis* infection?

Cross-sectional studies in The Gambia suggest that, by ELISPOT, 'specific antigens' are more specific than PPD in the diagnosis of *M. tuberculosis* infection after known exposure. However, comparison with the PPD skin test, suggests that this is at the cost of some sensitivity. Further complexities were also identified: the PPD skin test appeared to be less sensitive to *M. tuberculosis* infection in those in the lowest exposure category and the two tests were most discordant from each other in these subjects. This was thought to be related to different properties of the skin response and the blood response to antigen, requiring further study.

3. Does the extension of an *M tuberculosis* exposure gradient outside the immediate household compound of a known TB case provide useful information about the performance of new T cell assays and the PPD skin test?

The recruitment of 'community controls' revealed a further increase in the discordance between the ELISPOT and PPD skin test in The Gambia. The increasing discordance may be interpreted as a natural progression with 'distance' away from a known TB case contact. In that sense it strengthened the findings of the previous study. However, since this finding was a natural progression of the findings within case contact households, the extra work of identifying community controls for the assessment of new tests for *M. tuberculosis* infection may be difficult to justify. The results of this study have implications for those using the PPD skin test to identify individuals with no evidence of *M. tuberculosis* infection for the first safety studies of new vaccines in TB-endemic settings. Such studies are underway in The Gambia-we have used the ELISPOT and PPD skin test together to recruit individuals for the first safety studies. The screening ELISPOT results also provide an important baseline for the immunogenicity readouts.

4. Is a fusion protein of 'specific' antigens as good as or better than their individual peptides, in the diagnosis of *M. tuberculosis* infection by ELISPOT?

This study showed that the fusion protein performed similarly to the respective peptides in the diagnosis of *M. tuberculosis* infection after recent exposure. Detailed analysis revealed that a small proportion of individuals were positive by each test and not the other. Some scientific explanations for exclusive responses to protein versus peptides and vice versa were considered. In this light, there are two good reasons for considering using both tests: firstly, the small sensitivity gain may be real and of value in particular settings; secondly, the two tests provide a back-up for each other as a safeguard against test failure.

5. Does the ELISPOT test for *M. tuberculosis* infection perform as well or better than the PPD skin test in children of different age groups?

We showed that, in children, the ELISPOT assay employing *M. tuberculosis* specific antigens showed good concordance and no significant discordance with the PPD skin test. However, analysis across an exposure gradient suggested that the ELISPOT, as shown previously in all age groups, is not as sensitive for the

diagnosis of *M. tuberculosis* infection from recent exposure as the TST in The Gambia and, importantly, offers no added benefit with respect to the confounding effect of BCG vaccination-even in under 5 year olds. Also consistent with the findings in all age groups, there appeared to be a sensitivity problem for the TST in those in the lowest 'recent' exposure category.

6. Noting that T cell assays and PPD skin test give a quantitative readout, converted to qualitative yes/no readout in practice, is there added information that a quantitative analysis can provide? (chapter 6).

In this study of over 1000 case contacts we showed the benefit of an 'inverse combination,' using the qualitative response to specific *M. tuberculosis* antigens to identify those most likely to be 'processing' an infection, and the quantitative response to PPD (representing whole organism). We found that the precise quantitation of the ELISPOT identified a dose-response relationship between the human host response and received organism load, as reflected by sleeping proximity to a TB case. This finding highlights the dynamic nature of the 'T cell effector' response that the ELISPOT detects and how the assay is able to identify insights into what is happening between the host and invading organism at a specific point in time. Interventions that affect the host-pathogen interaction may be able to be assessed by repeated ELISPOT tests over time, opening new possibilities for the assessment of new vaccines and medications against *M. tuberculosis* infection.

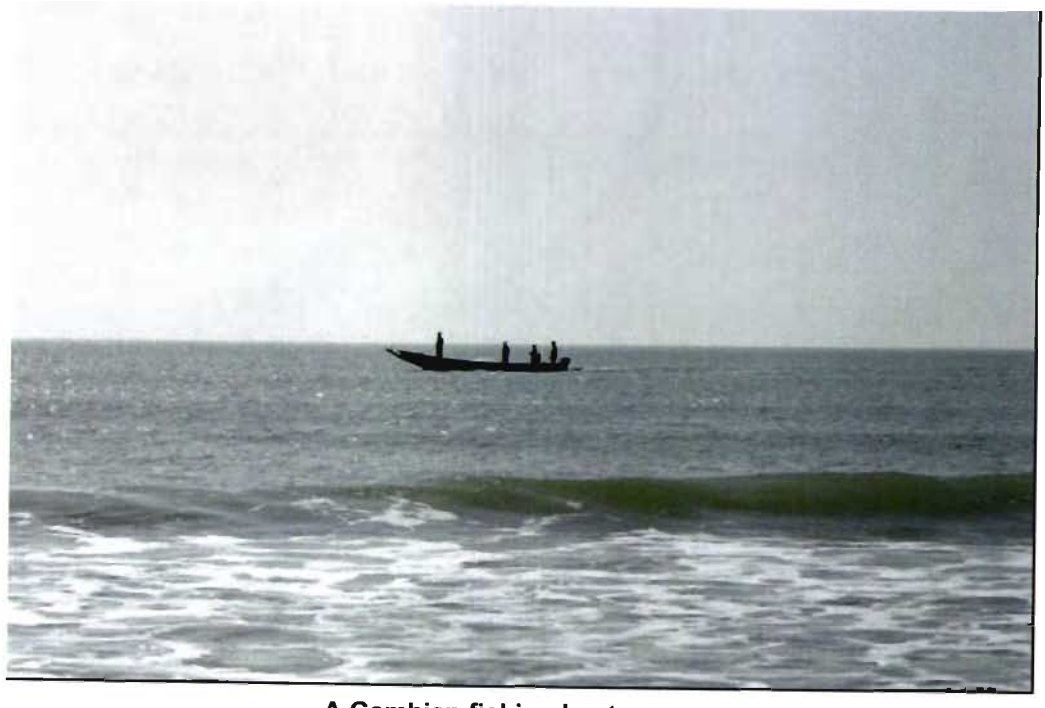
7. Does the T cell assay response to *M. tuberculosis* change over time? Specifically, do more case contacts become positive over time and do those already positive stay positive? Further, are there risk factors for 'conversion' and 'reversion' and how do these relate to the PPD skin test over time?

In this study we repeated ELISPOT tests in 348 case contacts at 3 months and 136 after 18 months; we repeated the PPD skin test in 186 PPD skin test negative contacts at 3 months and 73 consecutively recruited contacts at 18 months. At both recruitment and at 18 months the agreement between the PPD skin test and the ELISPOT was approximately 70%. ELISPOT conversion occurred at 3 months in 29% of those initially negative, and at 18 months in 33% of those negative at 3 months. ELISPOT reversion occurred at 3 months in 39% of those initially positive, and at 18 months in 29% of those who were positive at 3 months. A positive PPD

skin test at recruitment was a strong risk factor for ELISPOT conversion at 3 months, while a positive ELISPOT at recruitment was a strong risk factor for conversion from 3 months to 18 months. In contrast, a positive PPD skin test at recruitment was associated with decreased ELISPOT reversion at 3 months, while a positive recruitment ELISPOT was associated with decreased ELISPOT reversion between 3 and 18 months. Only 10% of PPD skin test positive individuals at recruitment reverted to negative at 18 months, while 46% of those initially negative became positive at 18 months. The only risk factor identified for PPD skin test conversion at 3 months was sleeping in the same room as a TB case. A high rate of ELISPOT reversion suggests the assay may be unreliable in the diagnosis of latent infection in a state of dormancy, where ESAT-6 and CFP-10 antigens may not be secreted continuously. That a positive PPD skin test at recruitment was associated with a decreased likelihood of ELISPOT reversion at 3 months, suggests that the two tests may ultimately be best used together, perhaps to identify those most probably infected. This study has laid the foundation for larger longitudinal studies to identify secondary TB cases in sufficient number to relate progression to disease to recruitment and follow-up test results.

# 9

# Future studies



A Gambian fishing boat

The following studies are ongoing or planned at the MRC laboratories in The Gambia:

1. *Determination of ELISPOT assay and PPD skin test cut-offs* for diagnosis of *Mycobacterium tuberculosis* infection in The Gambia using mathematical tools. This study has utilised the large ongoing case and contact recruitment in The Gambia to attempt to identify the right cut-off for ELISPOT positivity, noting that some of the sensitivity issue identified in this thesis could be simply related to the cut-off we used.

2. *Alternative assays.* We are using our reproducible model to assess the Quantiferon-TB assay for the diagnosis of *M. tuberculosis* infection in comparison to the ELISPOT and PPD skin test.

3. *New antigens.* We are working in collaboration with Dr Michel Klein and Professor Tom Ottenhoff in the Netherlands to assess, using our reproducible model, new immunogenic antigens for their ability to be used in the diagnosis of *M. tuberculosis* infection. This includes antigens from the 'dormancy regulon' of *M. tuberculosis*, with the aim of identifying dormant infection may be more reliably. Our record in our case contact work has led to a leadership role in a Gates Grand Challenge grant that is providing 1 million pounds over 5 years to our group in The Gambia for this work. We will be coordinating the set-up of TB case contact studies in multiple African sites.

4. *Identification of strain differences* on the results of diagnostic tests for *M. tuberculosis* infection. We have recently identified by spoligotyping that more than 1/3 of our isolates are *M. africanum.*[192] TB cases that are due to *M. africanum* and their household contacts are less likely than those due to *M. tuberculosis* to respond to ESAT-6 in an ELISPOT assay. Further strain work is being done and linked to ELISPOT and gene expression data. We have successfully submitted an application Sanger Centre in Cambridge to sequence the genome of *M. africanum* to build on the recent advances in understanding of the genetic differences between *M. africanum* and *M. tuberculosis,* in particular with respect to gene deletions.[193]

5. *Gene expression studies.* By collecting a small volume of blood into RNA fixative (PAX tubes), we have been able to create a bio bank for reverse transcription PCR (rt-PCR) studies in TB cases and case contacts. We are linking the clinical and immunological phenotype data with the rt-PCR analyses

to provide insights into the human host response to infecting *M. tuberculosis.* The first study with respect to this has been published with respect to IL-4 and IL-4δ2 cytokine gene expression.[194] In a much larger study of FOXP3 (a marker of T cell regulation) gene expression we have shown a significant reduction in FOXp3 expression in individuals with evidence of *M. tuberculosis* infection from recent exposure to a TB case.[195] The resulting article has incorporated data from chapter 3 of this thesis.

6. *Follow-up of large numbers of case contacts* over several years for the development of secondary cases will be important to determine more precisely the sensitivity and predictive value of the PPD skin test and the ELISPOT assay in our setting. We will then conduct a series of nested case control studies comparing 'progressors' to randomly selected 'non-progressors' to identify factors that are associated with progression to disease.

7. *A randomised double blind controlled trial* of isoniazid versus placebo for the reversion of a positive ELISPOT response in TB case contacts. This study, funded by the Medical Research Council and commenced in late 2004, is exploring whether isoniazid can induce ELISPOT reversion at a greater rate than naturally occurs in TB case contacts with ELISPOT and PPD skin test positive TB case contacts. If this is true, then ELISPOT reversion could be an efficacy marker for the assessment of new interventions against *M. tuberculosis* infection in the prevention of disease, a property the PPD skin test does not have.[196] It may be that ELISPOT reversion follows after an early increase in ELISPOT count, although the evidence for an early increase is based on one study in TB cases, by Nicol et al.[197]

8. *Host genetics.* We are exploring the role of host genetics in the immune response to *M. tuberculosis* infection and disease. The study of the host genetics of disease is a continuation and expansion of work that began before the present case contact study,[198,199] in collaboration with Professor Adrian Hill's group,[200-203] and the Sanger Centre in Cambridge. We are exploring possible collaborators for the study into the host genetics of infection.

Other, similar, studies should be conducted at different geographical sites of different latitudes and with different levels of endemnicity.

# 10 Appendices-Forms used for research

Note:

1. These forms have been reformatted for inclusion in this thesis

2. The data collected served the needs of the studies in this thesis, but also a number of other interlocking projects.

**MRC Laboratories**

**Programme**

**PO Box 273**
**Fajara, Banjul**
**The Gambia**

**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC Study TB Patient information sheet SCC No 892.**

Tuberculosis is a serious disease in The Gambia. It can be treated but without proper treatment can cause serious illness and death. People that are sick from TB may have a cough, chest pain, fever, weight loss and other problems. When you are sick from TB you can infect healthy people by coughing, sneezing, breathing on them and spitting. Because the drugs to treat TB must be taken 3 times a week for 6 months it would be much better if we could stop people getting the disease. The MRC with The Gambian TB Control Programme want to work with Gambians to look for reasons why some people, but not others develop TB disease when they come into contact with someone sick with TB. These factors could be things about the way you live and things that are in your blood that control your body's response to infection. We would like to find 300 TB patients, and members of their household and of a neighbour's household with no TB that would like to take part in the study. The study will take 2 years and we would visit your house after 3 months and then every 6 months in this time.

If you accept to be in this study an MRC field worker will ask you questions about your illness and the way you live. You will also be asked to visit the MRC to see a doctor, and as a standard part of care you will have a chest X-ray, which has virtually no risk. You will be asked to provide sputum samples, a saliva sample, a few strands of the hair from your head, and have a skin test to measure your body's reaction to the germ that causes TB. The skin test is done by injecting a tiny amount of liquid under the skin on your arm. This may cause a small lump with local discomfort within 2 days but will rarely last longer. We would also like to take 2 to 6 teaspoons (10-30 ml) of blood now and 2 teaspoons (10ml) of blood about 6 months after you have finished treatment for TB. Samples will be used to obtain and store the substance that contains information about your genes (DNA). By analysing this substance we can start to understand why you differ in many ways from other people. We may at some future time want to analyse this substance to identify genes associated with developing TB. Information from this analysis will not be linked to your personal information and your genetic information will be kept confidential. As part of this study, the bacteria that are causing your TB will be stored for future tests. Researchers will take cultures grown from your samples to Stanford University in the United States for analysis and further research. We would like permission to perform a HIV test on your blood sample as this is important for understanding the other tests. HIV is the virus that causes AIDS. The HIV results will be given to you if you want but will be kept confidential. Counselling will be provided if you test positive.

Fieldworkers will arrange to visit your household to give you and other members information about TB and ask if they would like to be part of the study to identify those at risk of developing TB. People that agree will have a skin test done and give a blood sample. A field worker will visit your household after 3 and 6 months and then every 6 months to check whether anyone has signs of TB and will arrange for them to see an MRC doctor and get treatment if needed. If you become sick during the time of the study you can see medical staff at MRC for free.

You should only join the study if you want to. You can ask as many questions as you like. You can leave the study at any time without giving any reason and this will not affect your medical care. You may withdraw your samples with identifiable links from the study at any time. If you have any questions or concerns, please contact Dr Dolly Sillah at MRC Laboratories, PO Box 273, Fajara, phone number 495 442. There will be no cost or payment to you for participation in this study. Any Medical information you have provided to MRC will remain confidential. Any data that may be published will not reveal your identity. Patient information may be provided to federal and regulatory agencies, as required.

Thank you very much for your co-operation.

**MRC Laboratories**
**Programme**
**PO Box 273**
**Fajara, Banjul**
**The Gambia**

**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC Study TB Household Contact information sheet** **SCC No 892**

Tuberculosis is a serious disease in The Gambia. It can be treated but without proper treatment can cause serious illness and death. People that are sick from TB may have a cough, chest pain, fever, weight loss and other problems. When you are sick from TB you can infect healthy people by coughing, sneezing, breathing on them and spitting. Because the drugs to treat TB **must** be taken 3 times a week for 6 months it would be much better if we could stop people getting the disease.

The MRC with The Gambian TB Control Programme want to work with Gambians to look for reasons why some people, but not others develop TB disease when they come into contact with someone sick with TB. These factors could be things about the way you live and things that are in your blood that control your body's response to infection. We would like to find 300 TB patients, and members of their household and of a neighbour's household with no TB that would like to take part in the study. The study will take 2 years.

If you accept to be in this study an MRC field worker will ask you questions about any past illness and the way you live. You will also be asked to have a skin test to measure your body's reaction to the germ that causes TB. The skin test is done by injecting a tiny amount of liquid under the skin on your arm. This may cause a small lump with local discomfort within 2 days but will rarely last longer. We may ask to repeat the skin tests 3 months later. We would like to meet you at home after 3 months and 6 months and then every 6 months to check whether you have signs of TB and will arrange for you to be able to see an MRC doctor and, if needed, to get treatment for TB at your local health center. We would also like to take some saliva, blood and a few strands of hair from your head. We would like 2-6 teaspoons (10-30 ml) of blood today and 2 teaspoons in 3 months. Children aged less than 5 years will only be asked for 1 teaspoon of blood, those aged 5 to10 years for 1-2 teaspoons, and those aged 10 to 14 years for 2-3 teaspoons of blood. Samples will be used to obtain and store the substance that contains information about your genes (DNA). By analysing this substance we can start to understand why you differ in many ways from other people. We may at some future time want to analyse this substance to identify genes associated with developing TB. Information from this analysis will not be linked to your personal information and your genetic information will be kept confidential. We may perform a HIV test on your blood sample as this is important for understanding the other tests. HIV is the virus that causes AIDS. The HIV results will be given to you if you want but will be kept confidential. Counselling will be provided if you test positive.

If you become sick during the time of the study you can see medical staff at MRC for free.

You should only join the study if you want to. You can ask as many questions as you like. You can leave the study at any time without giving any reason and this will not affect your medical care. You may withdraw your samples with identifiable links from the study at any time. If you have any questions or concerns, please contact Dr Dolly Sillah at MRC Laboratories, PO Box 273, Fajara, phone number 495 442. There will be no cost or payment to you for participation in this study. Your Medical information at MRC will remain confidential. Any data that may be published will not reveal your identity.

Thank you very much for your co-operation.

**MRC Laboratories**
**Programme**
**PO Box 273**
**Fajara, Banjul**
**The Gambia**

**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC Study External Control information sheet Version 1. December 2001. EC No... SCC No. 892**

Tuberculosis is a serious disease in The Gambia. It can be treated but without proper treatment can cause serious illness and death. People that are sick from TB may have a cough, chest pain, fever, weight loss and other problems. When you are sick from TB you can infect healthy people by coughing, sneezing, breathing on them and spitting. Because the drugs to treat TB **must** be taken 3 times a week for 6 months it would be much better if we could stop people getting the disease.

The MRC with The Gambian TB Control Programme want to work with Gambians to look for reasons why some people, but not others develop TB disease when they come into contact with someone sick with TB. These factors could be things about the way you live and things that are in your blood that control your body's response to infection. We would like to find 300 TB patients, and members of their household and of a neighbour's household with no TB that would like to take part in the study.

If you accept to be in this study an MRC field worker will ask you questions about any past illness and the way you live. You will also be asked to have a skin test to measure your body's reaction to the germ that causes TB. The skin test is done by injecting a tiny amount of liquid under the skin on your arm. This may cause a small lump with local discomfort within 2 days but will rarely last longer. We may ask to repeat the skin tests 3 months later. We would also like to take some saliva and 2-6 teaspoons (10-30 ml) of blood, plus a few strands of hair from your head. Children aged less than 5 years will only be asked for 1 teaspoon of blood, those aged 5 to10 years for 1-2 teaspoons, and those aged 10 to 14 years for 2-3 teaspoons of blood. Samples will be used to obtain and store the substance that contains information about your genes (DNA). By analysing this substance we can start to understand why you differ in many ways from other people. We may at some future time want to analyse this substance to identify genes associated with developing TB. Information from this analysis will not be linked to your personal information and your genetic information will be kept confidential. We may perform a HIV test on your blood sample as this is important for understanding the other tests. HIV is the virus that causes AIDS. The HIV results will be given to you if you want but results will be kept confidential. Counselling will be provided if you test positive.

If you become sick during the time of the study you can see medical staff at MRC.

You should only join the study if you want to. You can ask as many questions as you like. You can leave the study at any time without giving any reason and this will not affect your medical care. You may withdraw your samples with identifiable links from the study at any time. If you have any questions or concerns, please contact Dr Dolly Sillah at MRC Laboratories, PO Box 273, Fajara, phone number 495 442.There will be no cost or payment to you for participation in this study. Your medical information at MRC will remain confidential. Any data that may be published will not reveal your identity.

Thank you very much for your co-operation.

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**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC Study TB Patient's Consent Form (Form 1) Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study
I understand that I will:
Be interviewed by an MRC field worker
visit MRC for examination by a doctor
given a chest X-ray at MRC
given a skin test
give sputum and saliva samples
Provide a sample of hair from my head.
Give 2-6 teaspoons (10-30 ml) of blood now and 2 teaspoons 12 months after finishing treatment.
have an HIV test on my blood sample
I understand that if I have any health problem during the 2 years of the study, I can go to MRC to see a doctor there. My medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse to take part in the study, and if I choose to join, I am free to leave it at any time and without giving any reason, and that this will not affect my medical care

I would like to know the results of my HIV test: YES/NO *(circle one)*

Signature or thumb-print of control: _____

I have read the above to: _____
(PRINT NAME OF VOLUNTEER)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

**MRC Laboratories**
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**Banjul**
**The Gambia**

**TBCC Contacts Consent Form (Form2) Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that I will:
Be interviewed by an MRC field worker
have a skin test
give 2-6 teaspoons of blood now and 2 teaspoons in 3 months
give a saliva sample
Give a sample of hair from my head.
Be visited at home after 3, 6, 12, 18 and 24 months by an MRC field worker who will assess whether I have signs of illness from TB, and if so will refer me to see a doctor at MRC
I understand that if I have any health problem during the 2 years of the study, I can go to MRC to see a doctor there. My medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse to take part in the study, and if I choose to join, I am free to leave it at any time and without giving any reason, and that this will not affect my medical care

I would like to know my HIV status: YES / NO *(circle one)*

Signature or thumb-print of contact: _____

I have read the above to: _____
(PRINT NAME OF VOLUNTEER)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

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**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC External Controls Consent Form (Form3) Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that I will:
Be interviewed by an MRC field worker
have a skin test
give 2-6 teaspoons of blood
give a saliva sample
give a sample of hair from my head.
I understand that if I have any health problem during the 2 years of the study, I can go to MRC to see a doctor there. My medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse to take part in the study, and if I choose to join, I am free to leave it at any time and without giving any reason, and that this will not affect my medical care

I would like to know my HIV status: YES / NO *(circle one)*

Signature or thumb-print of control: _____

I have read the above to: _____
(PRINT NAME OF VOLUNTEER)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

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**The Gambia**

**TBCC External controls consent of Parent/Guardian for Participation by Minors Aged 1-17 (Form4)**
**Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that the child under my care will:
Be interviewed by an MRC field worker
have a skin test
give a blood sample of 1 teaspoon if aged less than 5 years, 1-2 teaspoons if aged 5-10 years and 2-3 teaspoons if aged 10-15 years
give a saliva sample
have a sample of hair removed from his or her head.

I understand that if he/she has any health problem during the 2 years of the study he/she can be seen by medical staff at MRC. His/her medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse, and if I allow the child under my care to take part, I am free to change my decision at any time without giving any reason, and that this will not affect his/her medical care

I would like to know my child's HIV status: YES / NO *(circle one)*

Signature or thumb-print of parent/guardian: _____

I have read the above to: _____ the parent/guardian of _____
(PRINT NAME OF PARENT/GUARDIAN) (PRINT NAME OF CHILD)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

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**The Gambia**

**TBCC External controls assent by Minors Aged 7-17**
**Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that I will:
Be interviewed by an MRC field worker
have a skin test
blood sample of 1-2 teaspoons if aged 7-10 years and 2-3 teaspoons if aged 10-15 years
give a saliva sample
have a sample of hair removed from my head.

I understand that if I have any health problem during the 2 years of the study I can be seen by medical staff at MRC. My medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse, and if I take part, I am free to change my decision at any time without giving any reason, and that this will not affect my medical care

I would like to know my HIV status: YES / NO *(circle one)*

I understand what is involved in this study and assent to participate in this study.

Signature or thumb-print of minor aged 7 to 17: _____

I have read the above to: _____
(PRINT NAME OF CHILD)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

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**National TB/Leprosy Control**

**Banjul**
**The Gambia**

**TBCC Consent of Parent/Guardian for Participation by Minors Aged 1-17 (Form5)**
**Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that the child under my care will:
Be interviewed by an MRC field worker
Have a skin test
give a blood sample of 1 teaspoon if aged less than 5 years, 1-2 teaspoons if aged 5-10 years and 2-3 teaspoons if aged 10-15 years at recruitment and after 3 months
give a saliva sample
have a sample of hair removed from his or her head.
be visited at home after 3, 6, 12, 18 and 24 months by an MRC field worker who will assess whether he/she has signs of illness from TB, and if so he/she will be referred to see a doctor at MRC

I understand that if he/she has any health problem during the 2 years of the study he/she can be seen by medical staff at MRC. His/her medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse, and if I allow the child under my care to take part, I am free to change my decision at any time without giving any reason, and that this will not affect his/her medical care

I would like to know my child's HIV status: YES / NO *(circle one)*

Signature or thumb-print of parent/guardian: _____

I have read the above to: _____ the parent/guardian of _____
(PRINT NAME OF PARENT/GUARDIAN) (PRINT NAME OF CHILD)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

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**The Gambia**
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**Banjul**
**The Gambia**

**TBCC assent by Minors Aged 7-17**
**Version 1. December 2001. EC No... SCC No 892.**

I have read the information sheet or it has been translated for me and I have had the chance to ask questions about the study.
I understand that I will:
Be interviewed by an MRC field worker
have a skin test
give a blood sample of 1-2 teaspoons if aged 7-10 years and 2-3 teaspoons if aged 10-15 years
give a saliva sample
have a sample of hair removed from my head.

I understand that if I have any health problem during the 2 years of the study I can be seen by medical staff at MRC. My medical information will remain confidential and will be used only for the purpose of the study.
I understand that I am free to refuse, and if I take part, I am free to change my decision at any time without giving any reason, and that this will not affect my medical care

I would like to know my HIV status: YES / NO *(circle one)*

I understand what is involved in this study and assent to participate in this study.

Signature or thumb-print of minor aged 7 to 17: _____

I have read the above to: _____
(PRINT NAME OF CHILD)

in a language which he/she understands. I believe that he/she gives his/her consent to take part in the study.

Signature of the field worker: _____

Name of the field worker: _____

Date: _____

**TBCC**
**INDEX CASE Clinical Form**

Date: |__|__| / |__|__| / |__|__|__|__| MRC N°: |__|__| /|__|__|__|__|

Patients name: first |__|__|__|__|__|__|__|__|__|__|__|__| Household number |__|__|__|

last |__|__|__|__|__|__|__|__|__|__|__|__| Interviewer |__|__|
*(initials)*

Referred from: _____

| Precise home address | Address of contact person |
|---|---|
| | |

Samples sent/tests: Sputum for culture |__| Hair |__| Blood |__| skin test |__|
*(1=yes; 2=no, 3=done before seen at clinic)*

**A. Demographics**

Age: |__|__| *(years)*
Sex: |__| *(1=male,2=female)*

Marital status |__| *(1=married, 2=divorced, 3=single, 4=widowed,)*
Number of wives/co-wives |__|
Do they live at the above address? |__| *(1=Yes, 2=No)*
If yes, since when |__| *(1=since birth, 2=since childhood, 3= for more than 5 years, 4=for more than 1 year, 5=for more than 3 months, 6=for less than 3 months)*

Ethnic group |__| *(1=Mandinka, 2=Wolof, 3=Fula, 4= Serahula, 5=Jola, 6=Aku, 7=Serere, 8= Manjago, 9=other, specify)*
What is their job? |__| _____ *(state and check code list for code)*

Kindly state the average monthly household income in dalasis _____

Did/do they go to school? |__| *(1=yes 2=no)*
If yes, i. what type? |__| *(1=secular, 2=arabic, 3=others, specify* _____ )
For how many years were they educated? |__|

**B. Previous history**

1. Has the patient ever been treated for tuberculosis ? |__| *(1=yes; 2=no; 3=don't know)*
If yes, where ? _____
When (year of treatment) |__|__|__|__|
completed treatment ? |__| *(1=yes; 2=no; 3=don't know)*
2. Has the person ever lived in the same house as someone with tuberculosis? |__|
*(1=yes; 2=no; 3=don't know)*
*if yes, specify (who, when)* _____ |__|__|__|__|

**C. Personal Risk Factors**

1. If patient is a woman, is she pregnant? |__| *(1=yes, 2=no, 3=unknown)*
If pregnant, how many months? |__|__|

2. Does the patient drink alcohol |__| *(1=yes, 2=no, 3=no response)*

If yes:
How long have they been drinking? |__| (1= <6 months, 2= 7-24 months 3= >24 months)
How frequent do they drink? |__| (1= Occasionally, 2=1-2 times/ week, 3=3 – 5 times /week, 4.=> 5 times/week
Do they drink strong alcohol more than half the time? |__| *(1=yes, 2=no)*

3. Have they been smoking in the past six months?|__| *(1=yes;2= no; 9= no response)*
If yes,
How often does he/she smoke? |__| *(1=not every day; 2=1-5 times/day; 3=6-10/day; 4=10+/d)*
How many years has he/she smoked? |__| *(1=less than one year; 2=1-5 yrs; 3=6-10 yrs; 4=10+ yrs)*

4. Have they ever experienced wheezing in the chest? |__| *(1=yes, 2=no)*
If yes, how many attacks have they Experienced over the last 3 months? |__|__|
*(state number of attacks - 99 for don't know)*

5.Has a doctor ever told them that they have asthma? |__| *(1=yes, 2=no)*

If yes, are they on treatment? |__| *(1=yes, 2=no, 3=unknown)*

6. Is the person taking any medication? |__| *(1=yes; 2=no; 3=don't know)*
*if yes, specify:* _____

7.Has the person ever been told by a doctor that he/she has diabetes? |__| (Yes=1 no=2 don't know=3)
If yes, duration of treatment : |__|__| (months)

**D. Behaviour of patient**

1. What time of the day do they cough most? |__| *(1= Almost all the time, 2= at dawn, 3= mornings, 4= afternoon, 5= evenings)*
2. Where do they spend most of their day when at home? |__| *(1=indoors, 2=outdoors)*

3. Do they cover their mouth when coughing? |__| *(1=Yes 2= no, 3=sometimes, 4=cannot remember)*

4. Do they produce sputum when they cough? |__| *(1= yes, 2=no)*

If yes, what do they do with the sputum they produce? |__|
*(1= Swallowed, 2 =Spits out openly, 3=Spits into covered container, 4=other, specify_____)*

**E. Clinical symptoms:** *for each of the following, please state : 1=yes; 2=no; 3=don't know*

| | | | |
|---|---|---|---|
| cough \|__\| | how long ? ___ *(weeks until diagnosis made)* | | |
| productive | \|__\| | chest pain | \|__\| |
| haemoptysis | \|__\| | side pain | \|__\| |
| fever/chills | \|__\| | dyspnoea | \|__\| |
| night sweats | \|__\| | loss of appetite | \|__\| |
| loss of weight | \|__\| | | |
| other symptoms *(specify)* \|__\|_____ | | | |

**F. Clinical signs:** *for each of the following, please state : 1=yes; 2=no; 3=don't know*

| | | | |
|---|---|---|---|
| pallor | \|__\| | splenomegaly | \|__\| |
| wasting: | \|__\| | hepatomegaly | \|__\| |
| clubbing: | \|__\| | oedema | \|__\| |

lymphadenopathy: |__| which sites ? _____

BCG scar: |__| size: |__|__| / |__|__| mm
Other clinical signs: |__| *(specify)*:_____

**G. Chest examination:** *for each of the following, please state: 1=yes; 2=no and tick the site : upper zone:UZ; mid zone: MZ; lower zone:LZ*

| | | Right (*tick*) | | | Left (*tick*) | | |
|---|---|---|---|---|---|---|---|
| | | UZ | MZ | LZ | UZ | MZ | LZ |
| dullness | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| crepitations | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| bronchial breathing | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| rhonchi | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| wheeze | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| no breath sounds | |__| | ___ | ___ | ___ | ___ | ___ | ___ |
| other _____ | | ___ | ___ | ___ | ___ | ___ | ___ |

**H. Anthropometry**

| | |
|---|---|
| Weight | |__|__|.|__| kg |
| Height | |__|__|__|.|__| cm |
| BMI | |__|__|.|__| kg/$m^2$ |
| Bio-electrical impedance | |__|__|__|__| ohms |
| Skin-fold thickness | |__|__|.|__| mm |

**I. Chest X-ray**

Done : |__| *(1=yes; 2=no)* date: |__|__| / |__|__| / |__|__|__|__|

Quality |__| *(1=average quality, 2=satisfactory, 3=poor quality*

| | R | L |
|---|---|---|
| 1. Predominant infiltrates : Upper | ___ | ___ |
| Middle | ___ | ___ |
| Lower | ___ | ___ |

*(small nodules=1, patchy =2, consolidation =3, cavitation =4, bullae=5, obscured =6, none=7)*

| 2. Cavitation: | R | Diameter*(cm)* | L | Diameter*(cm)* |
|---|---|---|---|---|
| Upper | ___ | ___ | ___ | ___ |
| Middle | ___ | ___ | ___ | ___ |
| Lower | ___ | ___ | ___ | ___ |

*(Enter 1(yes) or 2(no) in each zone plus corresponding diameter)*

3. Total number of zones involved with parenchymal disease out of a total number of evaluable zones:

____ zones out of ____evaluable zones.

| 4. Pleural disease: | R | L |
|---|---|---|
| | ___ | ___ |

*( effusion to the fifth rib=1, effusion to the 3rd rib =2, blunted angle =3, thickening =4, absent=5)*

5.Lymphadenopathy

Hilar |__|

Mediastinal |__|

*( 1=present, 2= doubtful, absent=3)*

6. Other
Pericardial effusion |__|
Miliary TB |__|
*( 1=present, 2=doubtful, absent=3)*
Other diagnoses _____

**J. Tests results:**

1. Sputum smear: date: |__|__| / |__|__| / |__|__|__|__|

smear 1: |__| *(1=positive +, 2=positive ++ , 3=positive +++, 4=scanty, 5=negative, 6=unsatisfactory sample, 9=not known)*
smear 2: |__|
smear 3: |__|

2. Skin test: date: |__|__| / |__|__| / |__|__|__|__|

tuberculin: |__|__| / |__|__| mm *(size along the 2 diameters)*

**K. Treatment:**

Smear Positive TB :
Is the patient referred for anti-TB treatment? |__| *(1=yes; 2=no)*
- if yes, place of treatment: _____
date treatment starts: |__|__| / |__|__| / |__|__|__|__|

**TBCC**

**Contact Recruitment Questionnaire**

**Background information**

Date |__|__| |__|__| |__|__|__|__|
*day* *month* *year*

Interviewer |__|__| *(initials)*

Identification number |__|__|/|__|__|__|__|

Household number |__|__|__|

Samples sent/tests: Saliva |__| Hair |__| Blood |__| skin test |__| *(1=yes; 2=no)*

**A. Details of Subject**

1.First Name |__|__|__|__|__|__|__|__|__|__|__|__|

2.Last Name |__|__|__|__|__|__|__|__|__|__|__|__|

3.Age in years |__|__| Age in months for children under 2 |__|__|

4.Relation to the case |__| _____ *state and check code list for code*

5.Sex |__| *(1=male, 2=female)*

6.Marital status |__| *(1=married, 2=divorced, 3=single, 4=widowed,)*

7.Number of wives/co-wives |__|

8.Ethnic group |__|*(1=Mandinka,2=Wolof,3=Fula,4=*

*Serahula,5=Jola,6=Aku,7=Serere,8= Manjago, 9=other, specify)*

9.Occupation |__| _____*state and check code list for code*

10.Do/did they go to school? |__| *(1=Yes, 2=No)*

If yes, what type |__| *(1=secular, 2=arabic, 3=other) If other, specify* _____

if yes, number of years of schooling |__|__|

11.Do they live at the above address? |__| *(1=Yes, 2=No)*

if yes, since when? |__| *(1=since birth, 2=since childhood, 3= for more than 5 years, 4=for more than 1 year,5=for more than 3 months, 6=for less than 3 months)*

**B. Personal History**

1.Vaccinated with BCG? |__| *(1=yes health card visualised, 2=yes no health card, 3=no, 4=unknown)*

2.BCG scar present? |__| *(1=yes, 2=no, 3= unknown)*
- if yes, state size |__|__|/|__|__| mm

3.Has the contact ever been treated for tuberculosis before? |__| *(1=yes, 2=no, 3= unknown, 9=no response)*
- if yes, where? _____
- if yes, when? |__|__|__|__| *(state year of treatment)*
- Completed treatment? |__| *(1=yes, 2=no, 3= unknown, 9=no response*
if no, why? _____

Total duration of treatment |__|__| *(state number of months of treatment)*

**C.Personal Risk Factors**

1.Has the contact been a smoker in the past six months? |__| *(1=yes;2= no; 9= no response)*
- if yes, how often does he/she smoke? |__| *(1=not every day; 2=1-5 times/day; 3=6-10/day; 4=10+/d)*
- if yes, how many years has he/she smoked? |__| *(1=less than one year; 2=1-5 years; 3=6-10 years; 4=10+ years)*

2.Does the contact drink alcohol? |__| *(1=yes, 2=no, 3=response)*
- If yes how long have they been drinking? |__| *(1=<6months, 2=7-24months, 3=>24months)*
- how frequent do they drink? |__| *(1=0ccassionally, 2=1-2times/week, 3=3-5times/week, 4= >5times/week)*

Do they drink strong alcohol more than half the time? |__| *(1=yes, 2=no)*

3.Has the contact ever been told by a doctor that he/she has diabetes? |__| *(1=yes, 2=no)*
- if yes, since when |__|__|__|__| *(state year of diagnosis)*
- what is their regular treatment ? –
_____

4.If a woman, does she think she could be pregnant? |__| *(1=yes, 2=no)*

5.If a child how long were they breast fed for? |__|__| *(state number of months - 99 for don't know)*

6.Have they ever experienced wheezing in the chest ? |__| *(1=yes, 2=no)*
- if yes, how many attacks have they experienced over the last 3 months ? |__|__| *(state number of attacks - 99 for don't know)*

7.Has a doctor ever told them that they have asthma? |__| *(1=yes, 2=no)*

- if yes, are they on treatment ? |__| *(1=yes, 2=no, 3=unknown)*
- what is your regular treatment?
_____

8.Have they ever had worms? |__| *(1=yes, 2=no, 3=unknown)*

- if yes, how many times over the last year? |__|__| *(state number of times - 99 for don't know)*

- do they take any drugs against worms? |__| *(1=yes, 2=no, 3=unknown)*
- if yes, when did they last take drugs ? |__| *(1=less than 1 week ago; 2=less than 1 month ago; 3=less than one year; 4= more than 1 year ago)*

**D. Level of exposure**

1.Proximity with the index case (contacts only) |__|

*(4 = sleeps in same bed; 3 = sleeps in same room but not bed; 2 = sleeps in same house but not room, 1=sleeps in separate house[house of head of household])*

2.How many hours a day on average does the contact spend on the compound at the same time as the index case? |__|__| *(state number of hours-99 for don't know)*

3.Suspect TB case? |__| *(1=yes, 2=no, 3=unknown)*

**E. Nutritional Assessment**

1.Weight |__|__|.|__|kg
2.Height |__|__|__| .|__|cm
3.BMI |__|__|.|__|kg/$m^2$
4.MUAC (children < 5 years) |__|__|.|__| cm
5.Bio-electrical impedance |__|__|__|__| ohms
6.Skin-fold thickness |__|__|.|__| mm

**F. Skin test:** date: |__|__|/|__|__|/ |__|__|__|__|

tuberculin: |__|__| / |__|__| mm *(size along the 2 diameters)*

## TBCC Household Contact Follow Up Form

Index case ID num |__||__| /|__|__||__|__|  Name : |__|__|__|__|__|__|__|__|__|__|__| |__|__|__|__|__|__|__|__|__|__|__|

Date : |__|__|/|__|__|/|__|__|__|__|  Field Worker's initials |__|__|

| No | FULL NAME (State clearly and fill in before visit) | MRC ID number | TB suspect at previous visit (Y/N) | Referred to ± attended MRC TB clinic * | Permanently absent? why? ** | Have they been diagnosed with TB? (Y/N) | Have they been taking treatment for TB? *** | Suspect TB case (Y/N/?) If yes record why**** | Height (cm) | Skin fold | Hair sample taken? (Y/N) | Weight (kg) + MUAC (children <5) | Bioelect Impede | Blood taken? (Y/N) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Head | | | | | | | | | | | | | | |
| 02 | | | | | | | | | | | | | | |
| 03 | | | | | | | | | | | | | | |
| 04 | | | | | | | | | | | | | | |
| 05 | | | | | | | | | | | | | | |
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| 07 | | | | | | | | | | | | | | |
| 08 | | | | | | | | | | | | | | |
| 09 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |

* Codes for referral and attendance **1= not referred, 2 = referred but did not attend, 3 = referred and attended (please take care to follow up those that did not attend previous)**

** **Codes for permanent absence:** 1 = not absent, 2 = moved away permanently; 3 = away most of the time; 4 = in hospital (state suspected reason), 5 = died (state suspected reason), 5 = don't know

*** Codes for TB treatment **1 = not taking treatment, 2 = taking standard treatment at the health centre, 3 =taking other treatment**

**** Codes for suspecting TB **(more than 1 number can be entered) 1 = persistent cough, 2 = blood in sputum more than once, 3 = chest pain, 4 = becoming weak or losing weight, 5 = treated for other illness with no improvement**

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