UNIVERSITY OF BERGEN

Assessment of the utility of repeat tuberculin testing: a prospective study of adolescents in a high tuberculosis prevalence setting in South India

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I LIST OF ABBREVIATIONS

ARTI Annual risk of tuberculosis infection

BCG Bacille Calmette Guerin

HCW Health care worker

HIV Human immunodeficiency virus

LTBI Latent tuberculosis infection

M.tb Mycobacterium tuberculosis

NTM Non-tuberculous mycobacteria

OT Old tuberculin

PPD Purified Protein Derivative

PPD-S Purified Protein Derivative Seibert

QFT QUANTIFERON TB Gold In Tube assay

TB Tuberculosis

TRO Tuberculosis Research Office

TST Tuberculin Skin Test

TU Tuberculin Units

WHO World Health Organization

II SUMMARY

Background –Under-nutrition is associated with sub-optimal or false –negative tuberculin responses, while BCG vaccination may produce false-positive reactions. The two-step tuberculin test may be of value in populations where under -nutrition is highly prevalent in young children as in India, to help identify individuals who could potentially boost their immune response to the

second test, thus preventing them from undergoing further serial testing and preventing their misclassification as converters. We studied the factors that were associated with a sub-optimal response to the initial tuberculin skin test (TST) as well as an enhanced response following two-step testing in a cohort of adolescents.

Materials and Methods - Adolescent subjects aged 11-18 years who attended high-schools and junior colleges in Palamaner Taluk, South India were recruited. Baseline demographic, clinical and anthropometric data were collected. A tuberculin test using 2 TU of RT23 was administered. Those who developed a sub-optimal response (< 5mm) to this test were subjected to a second test 1-4 weeks following the initial test. Chi-square test and multiple logistic regression were used to test the association between the various categorical variables-demographic, socio-economic and clinical with sub-optimal and enhanced responses following two-step testing.

Results- A total of 6643 participants were recruited, of whom 6608 underwent initial TST screening. Nearly 30% were under-nourished, and only about 62.3% had a BCG scar. 1257 (19%) developed a sub-optimal response to the initial TST (< 5mm). Younger age {AOR 1.96 (1.31-2.93)}, under-nutrition {AOR 1.22 (1.06-1.39)**}**, presence of BCG scar {AOR 0.74(0.65-0.85)**}** and higher socio-economic class {AOR 0.77(0.66-0.89**)}** were associated with a sub-optimal response. With repeat testing within 1-4 weeks of the first test (N=1098), an enhanced response (≥ 10mm, with an increment of 6mm or more over initial test) was seen in 47(4.3%) of the participants. With the sole criterion of ≥ 6mm used to define an enhanced response, 145 (13.2%) developed such a response. A history of exposure was linked to an enhanced response at this cut-off {AOR 5.86 (1.15-29.76)}. A history of exposure was associated with development of an enhanced response; both at initial as well as at repeat testing {unadjusted OR 2.56 (1.15-5.54)}.

Conclusion- The two-step tuberculin test may be useful in undernourished populations, to identify potential boosters and prevent their misclassification as converters during further tuberculin testing.

III BACKGROUND

1.1 The burden of tuberculosis

Tuberculosis (TB) as an infectious disease has accounted for millions of deaths for centuries now- the World Health Organization (WHO) estimates that every year there are at least nine million new infectious cases of TB all over the world, and nearly two million affected people succumb to it. Africa and Asia account for nearly 30% and 55% of these cases respectively, with India alone representing around 20% (1) .

The scourge of TB has now increased due to the rise in the incidence of HIV (*Human immunodeficiency virus*). HIV positive individuals are nearly 37 times more prone to develop TB (1).

1.11 History of TB

Evidence of TB in skeletal remains in the Neolithic period (5000 BC) has been well documented (2). Egyptian mummies also harbor evidence of spinal TB as early as 2400 BC. Ancient Indian literature dating to1500 BC attributes TB or consumption to excessive fatigue, worries, and hunger, pregnancy and chest wounds (2) .

Hippocrates (470-360 BC) had, in his doctrines on medicine made a mention of the occurrence of TB of the spine and its complications (3). Galen, known for his humoral theory in medicine and other anatomical discoveries, had described patients suspected to be suffering from TB as coughing up blood, yellow sputum or bits of putrefied tissue (4).

1.2 Identifying tuberculosis infection

Following exposure to tubercle bacilli, the individual is said to be infected. Three consequences following exposure may now occur- (a) the bacilli are eliminated by the innate immune system comprising natural killer (NK) cells and other immune cells (b) the adaptive immune system comprising predominantly $CD4^+$ and macrophages contain the bacilli, which may thus remain dormant and establish a state of latency; or (c) both mechanisms fail, and the individual progresses to disease (5) .

Establishing that a person has been infected with M.tb is a challenge- the state of latency; wherein the individual does not show symptoms and /or signs of active disease, yet harbors the bacilli in the body. Such a latently infected person runs the risk of reactivating to active disease; especially over the following two years.

1.21 The tuberculin skin test

The tuberculin skin test (TST) is the oldest tool to detect TB infection. Introduced over a century ago by Sir Robert Koch, it has undergone several modifications over the years; the improvisation by Charles Mantoux being the most widely accepted, which is also the commonest method of administration today.

i. Immunological basis for the TST

This test is a classic example of a delayed hypersensitivity type of immunological reaction. Delayed hypersensitivity reactions are inflammatory reactions initiated by mononuclear leukocytes. The term ‘delayed’ is used to differentiate a secondary cellular response, which appears 48-72 hours after antigen exposure, from an immediate hypersensitivity response, which generally appears within 12 minutes of an antigen challenge (6). The delayed type of reaction is mediated by T cells and monocytes/macrophages rather than by antibodies. They are also termed type IV hypersensitivity reactions. An individual generally becomes tuberculin- positive approximately 6 weeks (average duration 3-8 weeks) after being infected with the tubercle bacilli which is the time needed for the development of delayed hypersensitivity (7).

ii. Administration of the tuberculin skin test

The test is administered using the Mantoux technique, which involves injecting 0.1 ml of purified protein derivative (PPD) , a mixture of several antigens extracted from cultures of tubercle bacilli, intradermally into the inner surface of the forearm- left side usually, such that a wheal of about 6-10 mm is raised above the skin (8).

iii. Reading of the test

The test is read 48-72 hours after administration, measured in millimeters of induration (palpable swelling; not the erythema or redness) across the long axis of the forearm (8). Varying perspectives exist as regards to the time of reading of the test. This is discussed later in the review.

1.22 The interferon gamma release assays

The interferon gamma release assays (IGRA's) are novel immunological blood tests that use antigens present almost exclusively in M.tb (ESAT-6, CFP-10 and TB 7.7) to stimulate the release of the cytokine interferon gamma (INF-γ) from sensitized immune cells (9, 10). The Quantiferon TB Gold (QFT), marketed by Cellestis, Carnegie, Australia), measures the amount of INF-γ by ELISA (enzyme-linked immunosorbant assay), and is expressed in terms of IU of INF/mm^3. The TSPOT.TB, marketed by Oxford Immunotec, Abingdon, UK , uses an enzyme linked immunospot (ELISPOT) technique, and results are expressed as the number of cells (spots) in the peripheral blood mononuclear cell fraction that are producing INF-γ. Results for both tests are available with 12-18 hours of blood sample collection , and are reported as positive, negative or indeterminate/borderline (11) .

There are varying views about the utility of the IGRA's and their superiority over the TST. Evaluation needs to be done in terms of whether the IGRA's are more sensitive than the TST in identifying latent TB infection or in immune-suppressed states; whether they are more specific than the TST in BCG vaccinated or non-tuberculous mycobacteria (NTM) sensitized individuals, or if they are useful is serial testing of high risk groups namely health-care workers (HCW's).

Studies have shown that the IGRA's have better or at least similar sensitivity but a higher specificity compared to the TST (12). In a study by Casas I *et al* among health –care workers in Spain to evaluate the performance of the IGRA's to detect recent infection with M.tb it was shown that the two IGRA's- QFT and TSPOT. TB showed very good concordance with respect to each other, and also when compared with a TST in the same population (10) . None of the three tests were affected by prior BCG vaccination, but increasing age was linked to TST and TSPOT.TB positivity, while degree of occupational exposure corroborated with assay positivity

only (10) . Data have shown that the QFT may be more sensitive in detecting subjects with history of exposure to T(10, 12-14).The TST results in a greater proportion of positive results, probably due to cross-reaction with BCG (13, 15). Two studies in India and Indonesia (16, 17) have shown that indeed BCG does not affect TST response. While most of the studies have been carried out within populations with low TB prevalence; and BCG may be given at repeated intervals as in many European nations (which may render TST still susceptible to cross-reactions with BCG); the situation in India is quite different- vaccination is generally conducted within the first week of birth; and no repeat/booster doses are administered. This may explain the lack of BCG effect on TST response. The TST positivity may also be linked to receipt of a previous TST (13). While increasing age has been linked to both TST (12, 16) as well as QFT positivity (16, 18), the study in Poland showed an inverse relation with age and TST positivity (18).

i. Immune tests in the HIV infected

A systematic review and meta-analysis of 37 studies to evaluate the role of IGRA's (QFT and T.SPOT) among 5736 HIV infected individuals showed that the pooled sensitivity of T.SPOT was higher than for QFT (72 % vs. 61%) from 8 studies (19). Five other studies comparing TST with IGRA's showed either higher, similar or lower sensitivity of the IGRA's compared to the TST. But these were for low/middle income countries (as per World Bank classification), and results for high income countries showed higher sensitivity for the IGRA's (19). When concordance between TST and IGRA's was measured from 15 studies, there was either moderate or poor agreement for both low/middle income and high income countries; but was comparatively higher for the high income countries (19). Data from 21 studies comparing $CD4^+$ counts of <200 and ≥ 200 showed that the pooled proportion of positive results was significantly lower for all 3 tests for $CD4^+$ counts of <200 vs. ≥ 200 ; both in low/middle income and high income countries (19).

ii. The immune tests in health-care workers

In a systematic review of 50 studies evaluating IGRA's for screening of TB among HCW, it was shown that TST positivity in high incidence settings (5 of these studies) was higher among those countries reporting greater BCG vaccination (India , 71%) vs. Vietnam (37.3%) , however, was

significant only in the latter (20). While the 25 studies in low/middle incidence settings showed a significant difference between IGRA's and TST positivity – which was higher, regardless of the BCG vaccination status of the tested individuals. Twenty-two studies in low/middle incidence settings evaluated occupational risk and IGRA's ,14 of which showed risk linked to occupational risk factors, including higher risk for clinical staff working in a high-risk ward, TB clinic or geriatric care and increased duration of healthcare employment (20). Of the studies that evaluated all three tests, it was shown that they all correlated well with respect to occupational risk factors, but none were consistently linked to any of the indicators of occupational exposure (20).

iii. Serial testing of Health care workers (HCW's)

Of the two studies that had data on serial testing in high incidence settings, it was shown that although the rates of IGRA conversions were higher (11- 21%) compared to the TST (4%); neither study showed which test was better linked to exposure and conversion, while the values also varied with different cut-offs used for the two tests (20). Moreover, QFT reversions were reported in India ranging from 7% - 40% over a 6- 18 month period. Data from low incidence settings also showed that the IGRA results varied, with conversions/reversions subject to different cut-offs used for testing (20) . Also, these rates were higher compared to that of the TST. However, there are limited data on exposure and the superiority of IGRA's over the TST in identifying new or recent TB infection (20) .

Data from 4 studies with serial IGRA tests from India, South Africa and USA showed both conversions and reversions of the IGRA's ranging from 16% in India to 80% in South Africa. These were more common when test results were close to cut-off values, even in low incidence settings (21).

iv. Can TST boost a subsequent IGRA response?

With respect to booting effect of TST on IGRA's, 13 studies were evaluated, of which 7 showed that boosting of IGRA's did occur, when repeated between 7-28 days following a TST, this was most prominent after 3 days of the TST, and seemed to wane after 3 months (21). 5 studies showed no boosting effect of TST and these had IGRA's conducted either less than 7 days or greater than 3 months of a TST. It follows that the optimum time to conduct an IGRA after TST would be within 3 days of performing the TST (21).

1.23 Which tests are better- the TST or the IGRA's?

The QFT may have distinct advantages over the TST; it is not affected by prior BCG vaccination or most of the non-tuberculous mycobacteria (NTM) which makes it more specific that the TST (10). Also, it entails a single patient visit, and the chances of allergic reactions are minimal as no substance is administered. However, it also requires a good laboratory facility and highly skilled laboratory personnel to conduct the test. It is undoubtedly more expensive compared to the TST (9).The TST on the other hand, while displaying similar sensitivity to the QFT, is less specific since it is affected by prior BCG and/or NTM sensitization (9, 10). It is also subject to inter/intra-observer variations, while the QFT may be a more objective test. However, the TST is more cost-effective, especially in resource-limited settings. In a meta-analysis of 58 cross-sectional studies to determine the sensitivity, specificity and reproducibility of the IGRA's in identifying latent TB infection (LTBI); it was shown that the ELISPOT had the highest sensitivity, followed by the QFT; however both displayed much higher sensitivity compared to the TST (9). The discordance between the TST and IGRA's was most evident in BCG vaccinated populations, as the IGRA's were more specific, their being unaffected by BCG (9).

Studies have shown that the TST may too remain unaffected by BCG when the individuals are vaccinated during infancy (compared to vaccination at an older age, or receiving multiple vaccinations) (10). In settings such as India where BCG is only given during infancy (usually within the first week of birth); the BCG might have no discernable effect after about ten years; and subsequently on TST, as the effect of BCG immunity is known to wane with time. Hence it may be continue to be used, as it is less costly, and may be more acceptable as it does not involve blood sample collection.

From the information above, it may be garnered now that these immunological tests, either the TST or the IGRA's behave differently in high and low/intermediate incidence settings. The IGRA's are indeed more specific in BCG vaccinated populations, but this effect may not be as prominent in adolescents and adults, as the BCG immunity wanes as well. Despite the IGRA's having operational advantages over the TST, in resource limited, high burden settings, the TST might still be the more feasible alternative, and thus continue to be used for detecting latent TB infection.

Before concluding, it may be worth mentioning that neither the TST nor the IGRA's and other IGRA's distinguish between active disease and latent infection. In other words, no gold standard exists yet to identify latent TB infection (LTBI). The IGRA's may identify more recent infection, while the TST identifies remote as well as recent infection. At this juncture, the IGRA's may gain the upper hand as a more valuable alternative; as recently infected individuals (within the first 2 years) are at highest risk of progression to active disease. However, established operational practice and cost-effectiveness may continue to make the TST valuable in resource-limited settings, including India.

1.3 History of the TST

Having discovered the tubercle bacillus (*Mycobacterium tuberculosis*) in 1882, Robert Koch was next engrossed in trying to find a cure for the illness. He tried to grow the bacilli on a special nutrient medium containing glycerinated beef broth. He discerned a growth of a colony of organisms. He boiled, filtered and reduced the extract to a concentrate of one-tenth of its volume and called it 'tuberculinum'(22) . He announced at the Berlin conference in the year 1890 that he had discovered a cure for the illness, but when this extract was administered to individuals, many of them became violently ill, some even died; but all of them had developed an inflammation at the site of injection (22). Unfortunately, Koch had failed to discover a cure for TB, but had laid the foundation for the use of the tuberculin skin test as a valuable tool to detect TB infection (22).

Following the development of Koch's old tuberculin (OT), D'Arcy Hart showed in 1932 that a 1:10 dilution of OT was the maximum concentration that was needed for screening persons for mycobacterial infection, and that reactions to much higher doses may not have been due to *Mycobacterium tuberculosis* (M.tb) (23).

Seibert FB produced a more purified form of OT, by steaming cultures of M.tb in an Arnold sterilizer and purifying the proteins by repeated precipitation with neutral ammonium sulfate. This was the purified protein derivative (PPD), and became the PPD-S that was adopted as the international standard by the WHO in 1952 (23). By convention, 5 tuberculin units are defined as the bio-assayable skin test activity contained in 0.0001 mg of PPD-S. Initially, two strengths-

1TU (first strength) and 250 TU (second strength) were used. Furcolow *et al* showed that an intermediate strength of 5 TU was sufficient to elicit a response in nearly 99.6% of patients with active tuberculosis (23). In 1958, the WHO introduced another purified protein derivative, the RT 23 manufactured by the Staten Serum Institute, Denmark. The standard testing dose that is now used is 2 TU (23). However, 1 TU is the standard dose utilized in India for tuberculin testing- Chadha VK conducted a study among 5-9 year old BCG-unvaccinated children during 1998 to determine the differences in response between 1 and 2 TU and demonstrated that there was no difference between the two doses (24). Hence, I TU is commonly used in India.

1.31 Evolution of the test

Based on the findings of Robert Koch, the French physician Charles Mantoux developed the tuberculin skin test twenty years later in 1907, expanding on the idea of von Pirquet that a second injection of small pox in already immunized individuals caused a quicker and more severe response in the individual.

Five different techniques of administration have been used for the TST; however, the Mantoux technique is most popular and is widely used even today (22) :-

- Scarification test (von Pirquet, Trumbusti) - two drops of OT and diluent (control) each were placed side by side on the forearm, and three superficial scratches were made side by side with a lancet. The reaction would be indicated by a swollen, linear scar along the scratch mark.

- Moro patch test- two patches, one each of OT and control were placed either on the arm or the back with an adhesive, and test results were read 2-5 days afterward.

- Multiple puncture tests (Heaf, Sterneedle, Tine) - all tests essentially punctured the skin with sharp needles from a gun, after a drop of OT was placed at the test site by a platinum loop. Sterilization methods of the needles so used were more advanced in the tests that were introduced later in sequence.

- Mantoux intradermal test- a standard dose of tuberculin is injected intradermally into the forearm, and test results read 2-4 days afterward.

- Dermo-spray method of A. Krantz (currently under investigation) - a modification of the Mantoux technique, it uses a device to eject exactly 0.1 ml (the standard test dose) of tuberculin into the test site, that pierces the skin forcibly enough to raise a white papule.

The ***Mantoux test*** is now the standard method to determine if an individual is infected with M.tb. However, this test cannot distinguish between latent TB infection (LTBI) and active disease (25).

1.32 Classification of tuberculin reactions

Historically, reactions were classified as being either positive or negative, and one such categorization was given by Aronson in 1934, as described below (22) :-

Negative No edema at test site, even if slight redness is present
Doubtful Slight redness and a trace of edema measuring 5 mm or less
1+ Some redness and definite edema of more than 5 mm and not exceeding 10 mm
2+ Area of redness and edema varying from 10 to 20 mm in diameter
3+ Marked redness and edema and an area of necrosis exceeding 20 mm in diameter
4+ Marked redness, edema and area of necrosis

This was followed by a method of grading the reactions, as the tuberculin reactions seemed to vary not only in size but also consistency. The gradation was recorded as follows (22) :-

Type I A -reaction which is dense, hard and elevated with sharp borders, and which is surrounded by edema (swelling) and which sometimes is bullous or is dotted with vesicles.
Type II A- reaction which is also dense, hard and elevated with sharp borders but the hardness is of a lower degree than above type, which may or may not be surrounded by edema. The frequency of bullae or dotted vesicles is less than the above type.

Type III A- reaction of mild density but the borderline is distinctly palpable though not easily distinguishable to the eye.
Type IV- Soft, barely perceptible reactions which can be easily missed unless the reader is careful.
This practice was followed by measurement of induration only; and longitudinal and/or transverse measurement (whichever was larger) was taken as the final measurement (22). The current practice is to measure induration by marking the margins with a ball-pen after palpation across the longitudinal axis of the forearm, and record the same in millimeters.

1.4 Specific and non-specific response to tuberculin

Tuberculin surveys were conducted by the WHO in various regions of the world in order to determine the pattern of response to tuberculin. It was established that there were two types of responses- a high-grade or *specific* type; which was brought out by relatively low doses of tuberculin, namely 5 TU. It was commonly seen in almost all persons with active TB disease, as well as those with positive signs of TB (lesions or cavitations) on chest X-ray. It was also seen in those who had a history of contact with an infectious case of TB (26). The other type, a low-grade response producing small reactions to a lower dose of tuberculin (5TU) , and larger reactions to higher doses (100TU or 250TU) of tuberculin was designated as being *non-specific* to tuberculosis infection (26).

The WHO Tuberculosis Research Office (TRO) had also conducted a series of studies in India during 1930 and afterwards, to document the kinds of responses that developed following administration of human as well as avian tuberculins (27). It was established that when both these tuberculins were used in equal concentration by weight (as the avian tuberculin had no standard dosage, it was concentrated in equivalent doses in milligrams to the human tuberculin - 0.0002, 0.0002 and 0.002 milligrams to 1, 10 and 100 TU respectively); the human tuberculin was concentrated as per standard procedure at the Serum Institute, Copenhagen. The human tuberculin seemed to elicit a greater number of positive reactions (defined by reactions $\geq$ 6mm) at smaller doses (1TU) than the avian type, in persons who had had a tuberculous infection. However, since the two preparations were not identical, either qualitatively or quantitatively, it

was inconclusive as to which one was better. The reasons for the non-specific response had not been established as yet (27). WHO's TRO also carried out tuberculin studies using 5 TU and 100 TU to document variations in responses across geographic regions. It transpired that both the *specific* and *non-specific* responses were more common in the tropics (26).

Based on the surveys carried out by the WHO in India, Edwards L *et al* conducted a TST survey among school children in different regions in India, using 5 TU and 100 TU of PPD- Danish State Serum Institute RT XIX-XX-XXI and established that there were mainly two kinds of responses to tuberculin- a *specific* type appearing as a strong response to low doses of tuberculin; and a *non-specific* response producing weak responses to a low dose and strong response to larger doses of tuberculin (28). Further studies carried out by Narain R *et al* in India to determine prevalence of this non-specific response showed that such prevalence was higher in the lower lying plains than high altitude areas. This was true for all age groups- by the age of 15-19 years, nearly 80% of the population in high prevalence areas showed such non-specific response. In areas of low prevalence, about 40% of individuals of similar age group showed such response (29).

Thus, it may be concluded from the above statements that there is a *specific* type of response to tuberculin which is fairly uniform in degree of response in those who are infected with M.tb, and this is similar across different regions of the world, varying according to the prevalence of TB. The *non-specific* type on the other hand seems to vary in prevalence as well as degree of response in different parts of the world. This could be due to varying background rates of non-tuberculous mycobacterial infection, as also BCG vaccination.

1.5 Tuberculin surveys as a means of determining the annual risk of tuberculosis infection (ARTI)

Tuberculin surveys provide information on the prevalence of TB infection in a population, which in turn are used to calculate the annual risk of tuberculosis infection (ARTI) (30). It is defined as the proportion of the population under study which is primarily infected or re-infected in the course of one year. These are conducted mostly among school children and provide data on the

prevalence of infection in a specific age group and gender, enabling the calculation of the annual risk of TB infection (ARTI) (30). The ARTI, in turn, is used to estimate the incidence of smear positive TB. The estimates of the annual risk of infection obtained from children demonstrate a more recent disease situation and its trends (31). Older age groups might have a higher prevalence of HIV and non-tuberculous mycobacterial infection, which may affect the interpretation of TST surveys (31). Chadha VK *et al* conducted a survey among school children aged 1-9 years in India during the years 2000-03, in four defined zones in order to calculate ARTI. They showed that the ARTI for the entire country was 1.5%, with rural and urban areas having approximately 1.3% and 2.2% respectively (32).

1.6 Factors affecting tuberculin reactivity

The interpretation of the TST response is dependent on several factors. The results have to be dealt with caution especially when close to cut-off values, while corroborating with other findings of TB infection/disease such as a history of TB exposure, chest roentgenogram, sputum microscopy and culture, and /or the newer immunological blood tests.

1.61 Time of reading of the test

The size of the TST reaction may vary with the time of reading. Singh D *et al* showed that the TST readings were at least 1.7 times larger when read at 72 hours than at 48 hours, using a cut-off of 15mm for a positive reaction with a 10 TU dose of tuberculin (33). Another study by Gopi PG *et al* in the Tiruvallur district in South India carried out in children aged 1-9 years using 1 TU showed that there was no significant difference between reading the test at 48 or 72 hours (34). Serane V *et al* in their study conducted in children aged 5-9 years showed that the readings did not vary significantly when read at 24 or 72 hours, when the cut-off for a positive reaction was 10 mm or greater (35). The time interval adopted for reading the test after administration is generally between 48- 72 hours, as this is the time required for development of delayed hypersensitivity following antigenic stimulation.

1.62 Technique of reading the test

The technique of reading the test is another potential source of error- the ball-pen technique and palpation method are commonly used. Pouchot J *et al* showed that the ball-pen technique was more reliable than the palpation method (36). Kendig EL *et al* conducted an observational study wherein a known tuberculin converter (TST response 15mm) was evaluated by 107 health care professionals (only two of them had used the ball-pen technique) and showed that that there was a tendency towards under-reading the TST, as 33% of them had recorded the measurement as < 10mm (37). Ciftci E *et al*, in a study comparing the manual method of reading at 24, 48 and 72 hours, with ultrasonographic measurements of the same conducted within half an hour of the manual reading demonstrated that there was a significant under-reading of the TST reactions by the manual technique (38). Ortakoylu G *et al* conducted a study in individuals aged 16-18 years in Iran using 5 TU to determine the reliability of the palpation technique, using measurements conducted by three different readers and found that the measurements did not vary significantly. Also, they compared this with the ball-pen techniques of reading and showed that there were no significant differences between the two techniques either (39).

Other operational issues such as dosage of tuberculin administered the type of tuberculin used, depth of injection (intradermal or subcutaneous), method of storage of tuberculin, inter/intra-observer variations; along with inherent biological variation in the individual may affect the tuberculin response. However, it has been shown that when repeated TST's are administered, chance variation of reading of a reaction is less than 6mm in 95% of the subjects, taking into account biological as well as procedural variations (40). Hence, any increase of TST response over 6 mm may be considered as being true biological phenomena (either boosting or conversion), and not merely random variation (40).

1.63 Effect of age and gender on TST reactivity

Current literature has shown that increasing age is an important factor for TST positivity. Also, the male gender has been shown to be associated with TST positivity. This may be related to increasing exposure among males as age increases, owing to social factors such as employment and migration. In a study conducted in South Africa to study the effect of age and sex on in-vivo and in-vitro immune response to PPD and other mycobacterial antigens in a cohort of children and young adults (mean age 14 years) it was shown that age and sex did not impact the

development of TST indurations measured 48-72 hours after administration of PPD (2TU), but increasing age was associated with higher interferon-gamma assay positivity. This may be due to BCG influencing TST response; while the interferon-gamma assays are not affected by prior BCG exposure (41). In a study conducted in Israel on pediatric hospital health care workers (HCW's), increasing age was shown to significantly impact TST positivity (42). A study conducted in Zambian and South African communities to determine the risk factors associated with both positivity in TST and Quantiferon TB Gold In Tube (QFT) assays showed that increasing age was significantly associated with positivity for both these assays (43).

A study in Ethiopia to determine the prevalence of latent TB among apparently healthy adults in a pastoral community showed that the prevalence of latent TB infection (LTBI) was significantly higher in males compared to females, using a cutoff of 10mm or greater with a 2 TU tuberculin (44). The same was not true with the QFT assay (44). A retrospective study was conducted in Greece in 6 to 7 year- old, non-BCG immunized school children to determine the trends in TST positivity over 17 years. Factors that might be associated with TST positivity- gender, nationality (native vs. foreign -born) and place of residence (urban vs. semi-urban vs. rural) were analyzed, and the results showed that being foreign –born and living in semi-urban or rural areas was associated with a positive TST response. However there was no difference in responses between males or females in relation to these factors and TST positivity (45).

The demographic and socio-economic characteristics of a population have important implications both at level of the individual, as well as at the community level in determining the prevalence of TB of that population.

1.64 Effect of prior BCG vaccination

BCG, on account of the similarity of its antigens to tuberculin has been shown to produce false positive tuberculin reactions. Factors such as age at vaccination, type and dosage of vaccine, number and size of BCG scars, period of PPD testing following BCG vaccination, as well as nutritional status of the individual, may all influence the TST response.
In a study to determine cytokine response to BCG vaccination administered to newborns at two different ages- at birth and 2 months, Akkoc T *et al* showed that the interferon –gamma response

in those vaccinated at birth was significantly higher than in those who were vaccinated at 2 months of age. A TST with 0.1ml PPD conducted in both groups at 8 months of age showed no difference in response however (46) . Another study by Sakha K *et al* to determine the immunogenicity of neonatal BCG vaccination in children at the age of 7-8 years in Iran (all of whom had a BCG scar) by testing with 5 TU PPD showed that in nearly 95.3% of the subjects, TST reaction was <5mm. Also, those children with larger BCG scars (5 mm or more) showed TST reactions greater than 5mm. None of the children had a positive TST reaction (≥10mm) (47). Saito M *et al* conducted a study among 6-26 year old subjects residing in a shanty-town of Peru to study the effect of multiple BCG vaccinations since birth (48). They showed that greater the number of BCG scars, higher the chance of a positive TST (≥ 10mm). Those with more numbers of BCG scars also had larger TST reactions (48). A study conducted in infants by Roth A *et al* to study BCG vaccination technique, PPD reaction and BCG scarring in a cohort of children born in Guinea- Bissau using 3 different strains of BCG (Type 1- Pasteur Merieux, France; type 2- Intervax Biologicals L.T.D, Canada; and type 3-Staten Serum Institut , Denmark) showed that the type 1 strain of the BCG vaccine produced larger TST reactions than type 3(49) . Also, larger post-vaccination wheals (indicative of higher dosage of vaccine) were more likely to result in greater numbers of PPD reactions and BCG scars (49). However, the route of BCG vaccination (either intradermal or subcutaneous) had no bearing on the TST reaction, although the intradermal route had produced better BCG scarring (49). Another study conducted in Peru to study BCG scar formation and TST reactivity in infants showed that in those children with visible scars, TST reaction was between 5-9 mm, and had no effect on TST reactions that were equal to or greater than 10mm, which were all associated with a history of contact (50). Hizel K *et al* conducted a study in Turkey to study the effect of age and prior BCG vaccination on TST reactivity in adults belonging to two groups- medical students and elderly people in a retirement home (51). They demonstrated that as age increased, TST positivity also increased, but BCG vaccination did not affect TST positivity (51) . In a study conducted among hospital employees in New York to investigate a rise in TST conversion among them, Horowitz HW *et al* found that recent BCG vaccination was associated with nearly 71% of the TST conversions (52). They noted, however, that the mean sizes of such reactions were smaller than those conversions caused by recent infection with M.tb (recent exposure) in the non-BCG vaccinated group (52).

In India, BCG is given routinely as part of the Expanded Program on Immunization (EPI), and has coverage of about 87% (WHO UNICEF, India 2011). Our study also showed reported vaccine coverage of nearly 85%. From the discussion above, it may be that prior BCG vaccination affects TST positivity, and also the size of TST reactions. However, is likely that reactions over 10mm are true infections due to M.tb especially in high prevalence settings, where the risk of transmission remains high. Hence, prior vaccination should not hinder management of those who are TST positive, especially in those who are at high risk of acquiring infection, namely close contacts and immunosuppressed persons.

1.65 Effect of malnutrition on TST reactivity

Malnutrition is known to affect cell-mediated immunity and subsequently the tuberculin response. Chandra .R.K studied the effect of malnutrition on peripheral blood lymphocytes and cell-mediated immunity using the reagents phytohaemagglutinin (PHA) and dinitrochlorobenzene (DNCB) respectively in 15 children diagnosed as malnourished by the Boston growth standards, with ten healthy children suitably matched for age and gender (53). It was seen that both rosette formation by peripheral blood lymphocytes and delayed hypersensitivity development were impaired in the malnourished subjects (53) . When the children were assessed 6-16 weeks later after correction of the nutritional deficit, the number of rosette-forming cells increased, and the response to mitogen stimulation also was enhanced (53). Sinha DP *et al* had conducted a study in children aged 2-6.5 years diagnosed as malnourished by the Harvard growth standards at a rural setting in West Bengal, India to study the effect of malnutrition on BCG vaccination (54). All children who developed < 5mm response to 5TU were given 0.1ml of BCG, and the TST was repeated 6-8 weeks later. It was seen that severe protein deficiency (kwashiorkor type) malnutrition impaired the tuberculin response post BCG vaccination as compared with the response in children who were well nourished. However, severe caloric malnutrition (marasmic type) had no bearing on the repeat TST response (54). In a study to assess factors associated with TST positivity and anergy using tuberculin (5TU), tetanus and candida antigens in disadvantaged communities in Peru, it was shown that protein malnutrition selectively suppressed TST reactivity (positive reaction measured by ≥ 10mm), but had no effect on tetanus or candida antigen reactivity (positive

reaction measured by ≥ 5mm) (55). Ganapathy KT *et al* studied the effect of malnutrition-Quetlet Index for age group 1-9 years, classified as normal or undernourished (PEM) in a group of unvaccinated children aged 1-9 years and found that malnutrition did not affect the tuberculin response (56). Another study by Chadha VK *et al* conducted in children aged between 5-8 years to study the prevalence of under-nutrition and its effect on tuberculin response demonstrated that there was no association between the two factors (57) .

The prevalence of malnutrition (estimated as the number of underweight children as compared to an international reference standard) measured in children 0-3 years is nearly 46% in India, according to the National Family Health Survey -3 conducted in 2005-06. Nearly 30% of adults have a BMI of < 18.5 (33% women and 28% men). The prevalence of anemia among women aged 15-49 years is almost 56%, and has shown a worsening trend since the last survey. Other determinants of nutritional status such as prevalence of anemia, infestation with helminthes and micronutrient deficiencies during adolescence, might affect response to tuberculin. The extent to which the tuberculin response is affected by the interplay of the above mentioned factors in this age-group needs to be addressed.

1.66 Effect of HIV –AIDS on TST reactivity

TB is a common opportunistic infection in persons infected with HIV-AIDS. Assessing tuberculin reactivity in such persons is a challenge, as multiple factors such as immune status ($CD4^+$ T -cell counts); previous PPD positivity, BCG vaccination, active TB disease or other co-infections may confound the reaction. Heubner RE *et al* conducted a study in HIV infected individuals to determine the prevalence of delayed hypersensitivity (DTH) anergy and the usefulness of two-step tuberculin testing in them (58). They found that anergy was associated with decreasing $CD4^+$ T -cell counts, at levels of 200-499 cells/mm^3 and < 200cells/mm^3. Two-step TST testing of the 103 individuals who mounted a negative response initially (< 5mm) resulted in positive response (boosting) defined by reactions > 5mm only in 7 persons, of whom only one was initially anergic. All the 7 had higher $CD4^+$ counts (532± 218) compared to the other non-boosters (268± 282) (58) . This was also true from another study by Markowitz N *et al*, who conducted a cross-sectional study among HIV seropositive and seronegative persons in order to determine the prevalence and predictors of PPD reactivity and skin test anergy in both

these groups (59).They found that TST reactivity decreased as the $CD4^+$ T-cell counts decreased, which was statistically significant below a level of 400 cells / mm^3 (59). As the CD4 count decreased, prevalence of anergy increased. This was also significant for counts less than $400/mm^3$ (59). In a study to evaluate the prevalence of tuberculin skin test reactivity, anergy and HIV infection in hospitalized patients admitted at an acute care facility, Janis E *et al* showed that increasing age, hypertension and male gender were significantly associated with positive TST reactions (mean induration 19mm, range 10-34mm) (60). Of those who were HIV seropositive, none had reactions $\geq$ 5mm, and the frequency of anergy was strongly associated with decreasing levels of CD 4^+ T cells ranging from < 200 to 500 cells/mm^3 in the HIV seropositive patients (60).

Although there is no cut-off for $CD4^+$ T -cell counts below which negative tuberculin reactions or anergy can be predicted, apart from the above mentioned factors, the fact remains that the tuberculin response may be confounded by other factors in the host, such as malnutrition, other high-risk behaviors and co-infections (especially in HIV infected individuals), various immune-suppressive conditions or co-morbidities, or simply procedural errors. However, data have shown that TST responses may remain negative in HIV infected individuals with a history of TB contact. In a setting of high TB prevalence, where the risk of exposure remains high, the usefulness of the TST in identifying infected individuals needs to be assessed.

1.67 The role of genetics in TB

Genetic factors may also contribute to the variation in tuberculin skin test response and susceptibility to TB disease. A study carried out in South Africa among 128 families including 350 siblings, in order to determine the gene loci that would have an impact on TST reactivity, showed that one gene at chromosomal position 11p14 was involved in either response or non-response to TST (61). The non-response may be explained by an innate resistance to TB; mediated by a T-cell independent mechanism. The other locus was mapped at chromosomal position 5p15, which probably controlled the intensity of delayed hypersensitivity and in turn the degree of response to TST (61).

Another study conducted in Ghana among TB patients and TST positive/negative controls, to determine the frequency of four IL-10 (interleukin 10) promoter variants and their significance in

HIV negative TB patients showed that one variant, which resulted in low levels of IL-10 (46.9pg/ml) was more frequent in TB cases and TST positive controls, compared to TST negative controls (5) . This may imply Interleukin 10 has a suppressive effect on T-cell activity. Cox RA *et al* conducted a study among 51 Mexican-American TB patients, and 54 healthy TST positive/negative controls to determine whether susceptibility and/or immune response to TST was related to HLA (human leukocyte antigen) phenotype frequencies of Class I or II (62). Although no difference between the two classes was found, there was a decreased frequency of HLA-DR3 among cases compared to TST positives, which may imply greater susceptibility to develop disease following infection. Also, an increased frequency of HLA-DR7 was seen among TST negative controls compared to those who were positive (62). Of the 31 patients who were separated into high responders (10.27 ± 0.1 mm), N=14, and low responders (8.6 ±0.2mm), N=17; the former showed increased frequency of HLA-A9 and B40; while the latter showed increased frequency of HLAB14 and DR1 phenotypes. These, however, did not reach statistical significance (62).

1.7 Frequency distribution of the TST response

The tuberculin skin test is widely used as a tool to detect TB infection and also as an adjunct to diagnose TB disease in neonates and young children (38). However, it is subject to limitations. The test requires recall of the immune system to respond to another antigenic challenge; this response may, however, have faded over time. However, when an individual does respond with a positive reaction, there is no way of knowing if this is a consequence of previous exposure with BCG vaccination, non-tuberculous mycobacteria (NTM) or recent infection with *Mycobacterium tuberculosis* (M.tb).

From a large scale tuberculin survey conducted by the WHO Tuberculosis research office (TRO) in different regions of the world, including Denmark, USA, India, Sudan, Philippines and Viet Nam, among school children aged 5-19 years and TB patients; it was shown that the distribution of reactions to tuberculin (5TU) seemed to follow a normal frequency curve, with a mode at 16 mm (26). It was also seen that this curve was constituted on the left-hand side by reactions with a mode at 6-7mm (represented by those uninfected with M.tb); on the right by 11-12mm (represented by the reactions formed by those persons infected with M.tb)- this was similar

among both adult TB patients as well as children infected with M.tb (26). The intermediate region consisted of reactions ranging from 6-12mm. This region tended to merge with the reactions on the right hand of the curve; this part of the frequency distribution seemed to vary the most with respect to the different regions across the world (26). From another survey among 1-24 year old subjects in India to test tuberculin sensitivity between human and avian tuberculins, it was shown that the human tuberculin was more effective in bringing out reactions among those who were M.tb infected; while the avian tuberculin for those subjects showing non-specific kind of response (27) . Although it has been shown that increasing doses of tuberculin may increase the reaction size (non-linear dose-dependency), the size still remains within the range observed for non-specific reactions (reactions slightly skewed to the left). It was later established that this pattern is produced by infection with non-tuberculous mycobacteria (28).

1.8 Determining cut-off values for a positive or negative TST reaction

The cut-off values for determining whether the TST is positive or negative have been arbitrarily defined – values 10mm or greater being considered positive (for immune-competent individuals) and 5mm or greater for those who are immune-compromised (8). These values have been arrived at, based on tuberculin surveys in different regions of the world conducted by the WHO and others, which have demonstrated that most reactions due to other mycobacteria- BCG or environmental; are less than 12 mm (26). Reactions due to M.tb have been shown to be an all or none response- either the individual does not respond or shows a small reaction; or will respond with reactions ranging between12-16, or greater (26). These values have be dealt with caution, in the context of whether the study was conducted in low or high prevalence populations, the subjects' risk of being infected, or the presence of HIV and other immuno-suppressive condition(s).

The accurate reading of the test assumes importance when making a clinical decision as to whether or not the individual concerned requires chemotherapy for latent TB and/or active disease. This is particularly difficult to assess when the readings are close to cut-off values. Menzies D in his review mentions that during repeat tuberculin testing, chance variation of

reading a TST reaction is less than 6mm in 95% of subjects, which means any increase in TST response of 6mm or greater during repeated testing should be assumed to be a true biological phenomenon (either conversion or boosting) and not random variation in response (40) . Ayub A *et al* showed that a cut-off of 5 mm to define a positive reaction was appropriate for those with highest risk of acquiring infection (such as health care workers (HCW's) and recent close contacts of infectious cases) and 15mm for those with lower risk (63).They also mentioned that a person would have tuberculin 'converted', if within a two-year period of testing, the tuberculin reaction increased by 10 mm or more (63) . Tissot F *et al*, conducted a study in a BCG vaccinated, low risk population to determine the cut-off value for TST response beyond which BCG vaccination had no influence on (64) . They showed that there was a strong influence of BCG vaccination on TST size when the TST response was ≤ 18mm, and age of the individuals was less than 40 years. This was true even after adjusting for other factors that predicted tuberculin positivity, namely origin from a country with moderate to high TB prevalence, history of contact, history of LTBI or having a high number or prior TST's (64). Agrawal SV suggested that in children who were BCG immunized, a 15mm cut-off was ideal up to 5 years following immunization, and a 10 mm cut-off there after (65) .

Chan PC *et al* conducted a study to determine the cut-off values for TST using 2 TU in children aged between 3 months and 14 years who had received BCG vaccination (and had BCG scars) during their neonatal period, and compared them with two other groups- one, which participants had no BCG scars and the other wherein participants had a BCG scar and were also contact-positive (66). They noted that the effect of BCG immunization diminished by the age of seven; a cut-off of 10mm was ideal at this age to determine positive reactions, for those who were at high risk of infection (66) . Bugiani M *et al*, in a study conducted in adult BCG vaccinated subjects (age at vaccination ranging between 16-39 years) to determine the cut-offs for previously vaccinated subjects and concluded that a cut-off of 10mm is ideal to consider individuals as truly infected, as the effect of BCG vaccination seemed to diminish 3-5 years afterward (67). A cut-off of 15 mm was considered as a strongly positive result, independent of the effect of the vaccine (67).

1.9 Two-step tuberculin testing

Two-step tuberculin testing is performed in individuals who develop a negative or sub-optimal response to the initial TST. It is generally repeated within 1-4 weeks of the initial test. The results of this second test are considered as the baseline value for further serial testing. The consequence of a two-step test may be either of two biological phenomena- *boosting*, which is the phenomenon of increased tuberculin response upon re-testing in the absence of new infection, similar to the anamnestic immunological response(40), or *conversion* , which implies development of a new delayed hypersensitivity type of reaction following a negative initial two-step test. These phenomena may be seen as a result of infection with either M.tb, non-tuberculous mycobacteria (NTM) or BCG vaccination (40).
The booster phenomenon is maximal if the second (repeat) TST is given between 1-4 weeks of the first test (40). Various criteria are used to define boosting; however, the most commonly used criterion is a reaction size of ≥ 10mm with an increment of 6mm or more over the initial test. These criteria were chosen based on the fact that biologic variations as well as those due to administration and reading result in a standard deviation of less than 3 mm on repeat testing. Hence chance variation should result in less than 6 mm in at least 95% of subjects (40). It is imperative to identify such persons who do not react initially, but would respond to a second antigenic challenge; otherwise they may be misclassified as converters when tuberculin tested anytime later.

The prevalence of boosting varies among populations, ranging from 0 per cent to nearly 31% (68).This variation may be explained by differences in background prevalence of TB in the population and probability of cross-reaction with non tuberculous mycobacteria or BCG.

Conversion following a negative two-step reaction is most effectively identified when undertaken 3 -8 weeks after suspected exposure, as that is the time needed for the delayed hypersensitivity response to develop following infection (40).

Another term may require mention here- *reversion,* which refers to the phenomenon of negative response on serial testing following an initial positive reaction (40). It is more common in the

elderly, although there are reported data that have documented reversion even in younger individuals.

The results of a two-step TST may be used as the baseline reference for further serial testing. Those who develop a positive response to the second test are said to have developed a booster response; and are evaluated for LTBI – active TB is ruled out, and the individual is put on isoniazid preventive therapy (IPT). In India, isoniazid preventive therapy is offered to all household contacts younger than 6 years of age, and in HIV positive persons who are suspected to harbor latent TB infection (69).

Those who develop a negative response to the second test are followed-up for risk of development of TB disease at a later point in time, which is greatest within two years following exposure. Any positive test following this baseline test is said to be a conversion, and individuals are again treated for LTBI, after ruling out active TB disease. However, for those individuals with a suspected exposure during the initial test, a single TST, or a repeat TST 3-8 weeks later may be recommended, as this would be the time required for an individual to develop delayed hypersensitivity; which would be recognized as a conversion.

As mentioned earlier, two-step testing is used in serial testing of healthcare workers (HCW's) and other high risk groups such as day-care workers, prison inmates and those in other institutional settings, as they run the risk of on-going exposure. The prevalence of boosting among HCW's varies widely, ranging from 0-10% or even higher (68). This may be linked to differences in types of patients catered to by them; the infectiousness of the cases, duration of exposure or years of work in such settings. Silva VMC *et al* had shown in their study among medical students that greater years of working at the hospital was linked to both initial positive as well as boosted reactions (70) . This may imply cumulative exposure as years of service increased. On the contrary, some studies have shown no such relation, or even greater prevalence among those with lesser patient contact (71, 72). This finding may indicate greater non-occupational community exposure. Other studies that have been conducted among health care workers to study boosting of TST response have shown that being foreign-born and BCG vaccinated is associated with the booster response (73-75) This in turn may be linked to varying

background prevalence of TB, NTM sensitization or BCG vaccination in the native countries. However, Fihmi S *et al,* in a study among staff at a geriatric hospital to determine the prevalence of positive skin tests including boosting after a two-step test, showed that boosting was not associated with either BCG or foreign birth (76). A baseline value will thus be established, which would be used as reference to detect any conversion at a later point in time. Contacts of infectious cases would benefit from this method of testing as well. A history of contact with an infectious case is strongly related to the occurrence of tuberculin conversion (7) ; highlighting the value of a repeat TST in such individuals in whom for procedural reasons or factors linked to anergy, may produce sub-optimal responses to the initial TST. Many other factors also have been linked to such conversion- male gender, especially after adolescence, which may reflect differences in exposure; increasing age or proximity to the index case (7). Ochs CW conducted tuberculin survey in Navy recruits, and since there were no known cases of active TB then, it was presumed they were similar to the general population. The conversions observed among these recruits were probably due to infection with atypical or non-tuberculous mycobacteria (77). A study conducted in internationally adopted children in an American hospital to determine the usefulness of repeat tuberculin testing within 3 months of an initial TST showed that there were at least 20% more cases who were diagnosed as having LTBI on repeat testing (78). It was also shown that malnutrition had a bearing on tuberculin response- those who had an initial positive TST were better nourished than those who had a negative response. Also, those who converted after the repeat TST were more likely to be better nourished than those who remained negative (78). Children with evidence of BCG vaccination were more likely to be positive both on the initial as well as repeat TST. None of the children tested had a diagnosis of active TB disease (78). This highlights the importance of interpreting the TST response in the context of the population under study; potential confounders including malnutrition, BCG vaccination, NTM sensitization; and HIV infection will have to be taken into consideration.

1.91 Experience with two-step testing

In a survey of TB screening practices in the United States of America, wherein most states had a tuberculin screening policy for high risk groups, it was shown that two-step testing was useful in areas with high background rates of non-mycobacterial infection (NTM) (79). This might be due

to the false-positivity of TST reactions caused by prior NTM sensitization; which two-step testing would probably help eliminate. Salles CG *et al* conducted a retrospective two-step TST survey among contacts aged ≥ 12 years in Rio de Janeiro, Brazil to determine whether enhanced reactions following two-step TST were boosting or conversion. Since none of them had developed TB disease two years following this repeat testing, they may have merely boosted, and not converted, they concluded (80). However, the number of tested persons being small, it would have been difficult to determine whether this was boosting or conversion (80). Another study conducted among health-care workers (HCW's) in a Canadian hospital to study the predictors of positive TST reactions 2 years following a negative 2-step TST showed that being foreign –born and BCG vaccinated resulted in positive TST responses, despite two negative reactions and the absence of contact during the intervening period (81). However, this was a low-prevalence population and contact history was negative, hence the influence of BCG may be stronger in such settings. Narain R concluded from tuberculin surveys conducted in India that the repeat TST showed a higher or 'positive' result in those who had initial reactions that were intermediate, or *non-specific* (82). In his study, Narain R showed that rates of conversion from a negative to a positive reaction following the second test were higher in areas where non-specific reactions to tuberculin was prevalent, and it was seen to increase with age (82). But exposure to M.tb was seen among a minimal number of the persons studied; and hence the increase in reaction may have been due to a boosting of the first response.

1.92 Two-step tuberculin testing – what can we learn?

Two-step tuberculin testing may thus be used in settings where serial testing will be undertaken, in order to measure on-going TB exposure. It may also be used to identify potential boosters- (i) those individuals who develop false-positive reactions due to remote mycobacterial exposure and whose immunity may have waned (ii) those who do not respond initially due to anergy from malnourished states or HIV and other immunosuppressive conditions or (iii) due to procedural variations. All along, one has to keep in mind the predictive value of a positive test- it would obviously be higher in high prevalence settings. Yet, specificity may be compromised in such a setting, although the sensitivity might be higher. Although targeted screening of high-risk groups would seem logical, there are at least one or more confounders of the tuberculin response such as

prior BCG vaccination, sensitization with non tuberculous mycobacteria (NTM); prior M.tb infection or factors producing immunosuppression , including operational factors, which might affect the interpretation of the results. Hence, two-step testing of a general population which is susceptible to high risk of transmission of infection may be justified as well.

IV. RATIONALE

The tuberculin skin test is the most widely used tool to detect TB infection. Individuals classified as having LTBI would be managed according to local guidelines. Individuals who fail to react to the initial test, from anergy due to malnutrition, HIV or factors linked to procedural variations? Malnutrition is a significant problem in our population. The prevalence of malnutrition (estimated as the number of underweight children as compared to an international reference standard) in children 0-3 years is nearly 46% in India, according to the National Family Health Survey -3 conducted in 2005-06. Nearly 30% of adults have a BMI of < 18.5(33% women and 28% men). The prevalence of anemia among women aged 15-49 years is almost 56%, which has shown a worsening trend since the last survey.

The state of Andhra Pradesh, where the present study was conducted is among the six states in India classified as 'high prevalence' for HIV (range 0.5% to 1.4%, currently estimated at around 0.9%). The prevalence of HIV among young adults aged15-24 years is much less compared to the older age groups. Given these two problems, and with malnutrition contributing more than HIV infection, there is a possibility that individuals may be anergic; and may not respond adequately to a tuberculin test. A second test within the next 1-4 weeks may help to identify such individuals. Not only would such testing help identify those individuals who would potentially boost their response to a second test, it also helps to delineate such persons during further serial testing, thus preventing their misclassification as converters during subsequent testing.

India is ranked 17th among the 22 high TB burden countries of the world in terms of estimated new cases occurring annually [RNTCP, India 2011]. The rate of infection is nearly 168/1, 00,000, with an ARTI of 1.5%. It is estimated that nearly 40% of the country's people are infected with MTb [RNTCP India, 2011].

Adolescents in India constitute nearly 20% of the total population. They form an important group for TB transmission- social and economic constraints lead to employment-seeking and migration, which increases their chance of exposure to TB. It has been shown that the force of infection, which is function of the probability of an effective encounter with an infectious TB case and is a measure of recent transmission; is maximum in this age group (83). Also, the force of infection has been shown to increase with age (83). These data indicate the importance of studying TB in adolescents in a high TB prevalence setting. In addition, adolescents are likely to be the target population for post-exposure TB vaccines in high prevalence countries. Because the prevalence and incidence of TB infection as well as disease are important to evaluate in adolescents, for the reasons mentioned above, we evaluated a two -step TST in participants with a sub-optimal response to the first TST, in order to determine the prevalence of boosting in a community setting with a high prevalence of underweight, and to better delineate the socio-demographic and clinical associations of an enhanced response to the second TST with the ultimate aim of determining whether this approach is of value in epidemiological studies.

V. OBJECTIVES:

2.1 General Objective:

To evaluate the usefulness of conducting the two-step TST in adolescents who do not respond or show sub-optimal responses to a 2 TU TST in a high TB prevalence setting.

2.2 Specific Objectives:

1. To determine the proportion of individuals who develop a sub-optimal response (< 5mm) following an initial TST and would therefore undergo a two-step TST, and to describe their characteristics
2. To determine the proportion of individuals who show enhanced responses to the second TST
3. To determine the factors that are associated with enhanced response to the second test

VI. MATERIALS and METHODS

3.1 Study design and setting

This work is a sub-study of a prospective cohort study conducted between February 2007 and April 2010 in adolescents aged between 11 to 18 years in Palamaner Taluk, Andhra Pradesh, South India, in order to determine the 2- year incidence of TB in the study population. As part of the study, all subjects who were enrolled underwent a tuberculin skin test at baseline, as outlined below.

3.2 Study population

The eligible study population included 12,388 adolescents who attended high schools/junior colleges in Palamaner Taluk, South India based on documented enrollment in all the school and colleges in the study area. The response rate was 53.6% (enrolled subjects/eligible population x100).

3.3 Inclusion Criteria

i. Male and female adolescent volunteers 12 -18 years of age and attending high schools or junior colleges in Palamaner Taluk

ii. Informed consent from parent/guardian and assent from the subjects or consent from the subject if he/she is 18 years or older

3.4 Exclusion Criteria

i. Plan for the family to move from the study area in the next two years

ii. Unable to attend follow-up session for reading of the tuberculin skin test

3.5 Baseline Evaluation

6643 participants, including 3441 boys and 3202 girls were recruited after consent for the purpose of the study. The response rate was 53.6%. Demographic, clinical and anthropometric data were collected routinely for all participants at baseline (Figure1)

3.51 Demographic characteristics

Demographic characteristics included age (source-school records), religion, caste, highest level of education attained by the subjects' parents. Parental occupation along with total household income was recorded. The type of house, the kind of fuel used for cooking and presence of an electrical connection at home was also noted.

3.52 Clinical history

Clinical history including information on BCG immunization(as reported), history of current or past TB disease, history of close contact with an adult with proven tuberculosis, current signs of TB disease, and history of hospitalization within the last six months and/or diagnosis of acute or chronic disease was recorded.

History regarding presence of symptoms- cough, fever, reported weight loss, hemoptysis or night sweats as indicators of TB disease, was considered positive if either the parent(s) or subjects answered 'yes'. Presence/absence of the BCG scar was also recorded.

3.53 Anthropometric assessment

Anthropometric assessment included height which was measured in centimeters using a stadiometer calibrated to the nearest millimeter. Weight of each participant was recorded in kilograms using a digital weighing machine calibrated to the nearest 0.1 kilogram (Bhasheen Health product Pvt. Limited, Jalandhar, India). BMI for age (a marker of nutritional status), was computed as z scores using WHO ANTHRO software (version 3.2.2) with the 2007 WHO guidelines as reference for the 5-19 age group. The cutoffs of the z scores used to categorize nutritional status were; (> +1 SD) overweight, (> + 2 SD) obese, (< - 2SD) thinness and (< -3SD) severe thinness.

Up to 29 ml of blood sample was drawn from each of the subjects for hematological, nutritional and TB immunological assays.

3.54 Tuberculin Skin Testing

The tuberculin skin test was administered as part of the baseline investigation (N= 6608). 2 TU of RT 23 (SPAN DIAGNOSTICS,INDIA) was administered using the Mantoux technique- 0.1 ml was injected intradermally into the volar aspect of the left forearm. Test reactions were read 48-96 hours later by measuring the largest transverse diameter of the reaction perpendicular to the fore-arm using the ball-pen technique. Field workers and staff nurses were specifically trained to read the tuberculin reactions. These were overseen by the field staff, study-coordinators or medical officers, and staff were retrained if any discrepancies were noted. A cut-off value of less than 5 mm was used to define sub-optimal response to the initial TST.

3.55 Follow-up

Those who developed sub-optimal responses as described above, were scheduled to receive a repeat TST within the next 1-4 weeks. Two cut-off values were used to define enhanced response following the two-step TST- an absolute value of 10 mm or greater with an increase of 6 millimeter or more over the initial reaction, or an increment of 6mm or more over the initial reaction.

3.6 DATA MANAGEMENT and ANALYSIS

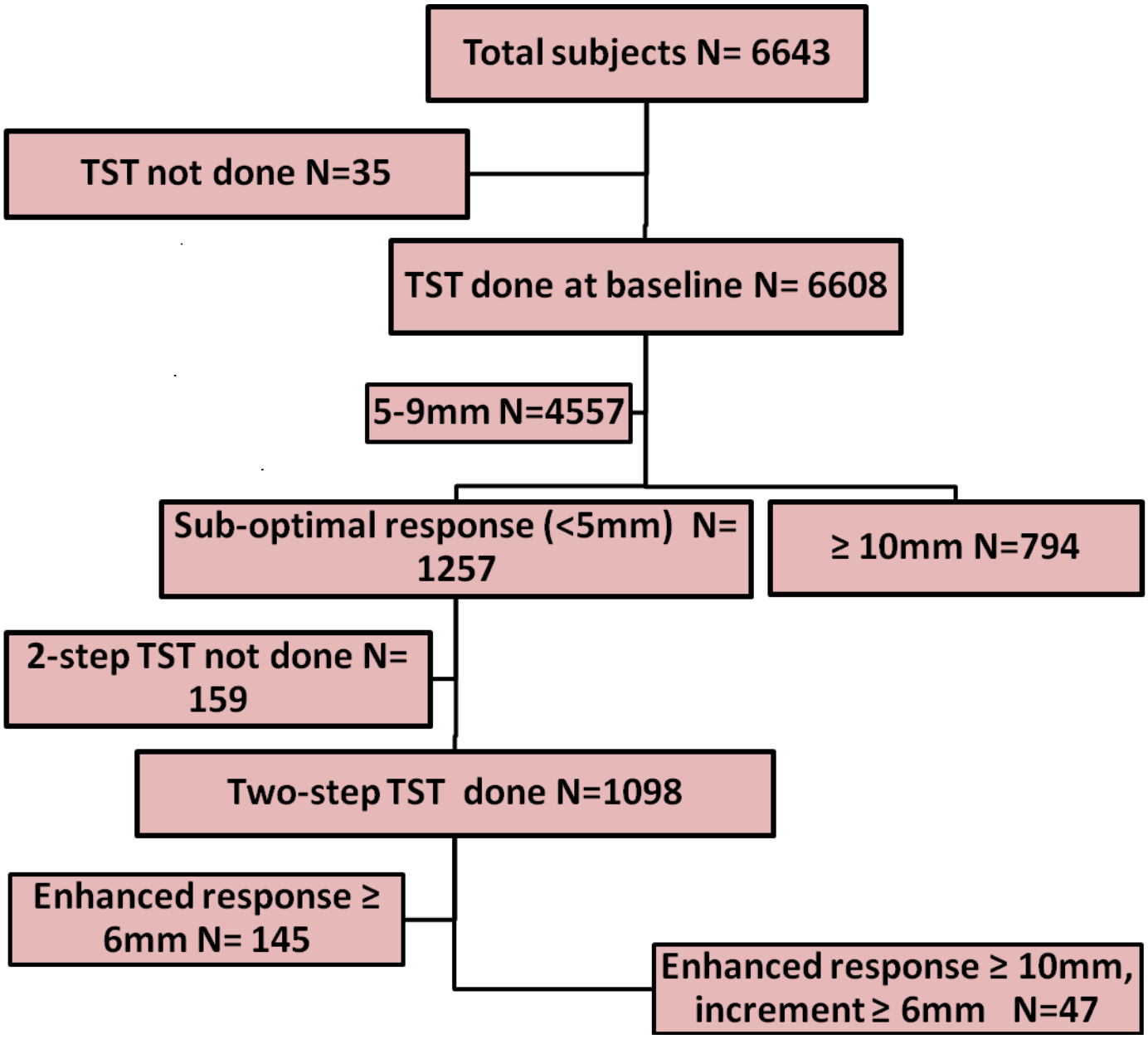
Data collected as part of baseline investigation were abstracted on to customized data acquisition software- double data entry was performed- STATISTICAL PACKAGE for SOCIAL SCIENCES (SPSS) version 18.0, SPSS inc. Chicago, Illinois was used for analysis of the data. Descriptive statistics were reported as numbers and percentages. The Chi-square test was used to test the association between the categorical variables- gender, age, socio-economic status, BCG status, BMI for age, and exposure to TB, in relation to a sub optimal response to the initial TST and enhanced or persistent sub-normal responses to the second step TST. Multivariate logistic regression was conducted on the variables that were significant in the univariate analysis, to investigate the association between TST response and socio-demographic and clinical variables. Results of this model were reported as odds ratios with 95% confidence intervals.BMI for age was computed using WHO – ANTHRO software (version 3.2.2), which was used as a marker for nutritional status. Level of significance was set at 5%.

3.7 Ethics and regulatory considerations

Written assent and consent from the parents or guardians was obtained during enrollment of the participants. The protocol and informed consent forms were reviewed and approved by the Institutional Review Board (IRB), St. John's Research Institute, Bangalore, India and the Independent Ethics Committee (IEC) of the AERAS Global TB Vaccine Foundation. The protocol was also approved by the Ministry of Health Screening Committee, Indian Council of Medical Research, Government of India. The studies were conducted after meeting with state and local education and health authorities.

Figure1. Flowchart- Baseline and two-step tuberculin testing

(continued to next page)



A total of 6643 adolescent subjects aged between 11-18 years participated in this cohort study (mean age= 13.5 years, SD ±1.4). The male/ female ratio was 1.1:1 (51.5 % vs. 48.2%). 6608/6643(99.4%) participants received a TST at baseline. Of these, 1257/6608(19 %) developed a sub-optimal response (< 5mm), among whom non-responders (0 mm) were

601(9.1%). Two-step testing was done for 1098/1257(87.3%) participants, who were included in the analysis. 47/1098(4.3%) developed a positive response defined by reactions ≥ 10mm, with an increment of ≥ 6mm over the initial test. With the sole criterion of an increase of ≥ 6mm used to define an enhanced response, 145 (13.2%) developed such a response. Among the 1098, 126 had undergone reversion - repeat TST value lesser than initial TST; hence were excluded from the analysis. For the 159/1257, two-step testing was not undertaken for the following reasons – participants reporting sick (9/159), refusal for the second test (31/159), refusal due to Board Exams at school (4/159) or non- availability during reading of the test (89/159). For 26 participants, the TST was repeated beyond the window-period of 4 weeks, hence was not considered for the analysis.

VII RESULTS

4.1 Population characteristics

Of the total 6608 adolescents who were tuberculin tested during baseline, 1257(19%) had a sub-optimal response. Among them, 627/1257 (49.8%) were males, while females constituted 50.2% (630/1257). 5674/6643 (85.4%) had reported a history of BCG immunization, and 62.6% (4161/6643) had visible scars. 2007/6643(30.2%) of the subjects were underweight. *TABLE* 1 compares the socio-demographic and clinical characteristics of those participants who had a sub-optimal response with the rest of the participants.

4.2 Association of sub-optimal tuberculin response with socio-demographic and clinical characteristics

Sub-optimal response was more likely to develop in those who were younger (11-12 year olds) {OR 2.01 (1.34-3.04), p value < 0.01} compared to those who were 17-18 years of age. When structural characteristics of the house, such as type of wall was used as a marker for socio-economic status, subjects living in houses made of brick were less likely to develop a sub-optimal response than those whose houses were made of packed mud , thatch or other materials {OR 0.77(0.66-0.89), p value < 0.01). Presence of a BCG scar was less likely to be associated with sub-optimal TST response {OR 0.75(0.66-0.85), p value < 0.01} compared to those subjects who had no measurable scars. Those who were underweight were more likely to develop a sub-

optimal response {OR 1.21(1.06-1.38), p value <0.01}. Subjects who reported weight loss as a symptom were more likely to develop sub-optimal response {OR 3.43(1.50-7.76), p value < 0.01}.

Multivariate regression analysis was performed with factors that were significantly associated with a sub-optimal response in the univariate analysis. The results of this analysis also showed that younger participants -11 to 12 year olds, and 13-14 year olds {Adjusted OR 1.96(1.31-2.93) and 1.48(0.99-2.19), p value < 0.01} respectively were more likely to develop sub-optimal response as compared to the older subjects. Those who belonged to higher socio-economic status (using literary status of the mother and structural characteristics of the house as indicators) were less likely to develop sub-optimal response {Adjusted OR 0.77(0.66-0.89)}, so also those with visible BCG scars {Adjusted OR 0.74(0.65-0.85)}. Children who were underweight were more likely to respond sub-optimally to an initial TST {Adjusted OR 1.22(1.06-1.39)}.

TABLE 2 shows that 47/1098 (4.3%) of the participants developed enhanced responses of ≥ 10mm with an increment of 6mm or more following a repeat TST. A history of contact, though not statistically significant at the univariate level, was significantly associated with an enhanced TST response after adjusting for clinical and socio-demographic characteristics in the multivariate logistic regression model {Adjusted OR 7.28(1.31-40.9)}.

TABLE 3 shows that 145/1098 (13.2%) developed enhanced responses to a repeat TST using an increment of ≥ 6 mm as the sole criterion. A history of contact as well as type of fuel used for cooking (wood compared to other types) was associated with an enhanced response. Even after adjusting for the other factors, the multivariate analysis showed that those with a contact history were more than 5 times likely to develop an enhanced TST response { AOR 5.86 (1.15-29.72), p value 0.02}.

TABLE 4 shows the association of TB exposure and TST positivity. It was shown that those with a history of exposure had 2.5 times greater odds of being TST positive, compared to those who had no history of exposure {OR 2.56 (1.15-5.54), p value < 0.01}.

(Please refer to corresponding tables provided in pages 54-65)

VIII DISCUSSION

5.1 Study findings

We present here a sub-analysis of a prospective cohort study among adolescents in order to evaluate the usefulness of two-step tuberculin testing in a high TB prevalence population. Enhanced response both at initial (≥ 10mm) and repeat testing (≥ 10mm, with an increment of ≥ 6mm or only an increase of ≥ 6mm) was associated with a history of exposure to TB. A sub-optimal TST response (< 5mm) to the initial TST was associated with younger age, under-nutrition, absence of a BCG scar and lower socio-economic class.

5.2 The Booster phenomenon

The booster phenomenon is commonly related to two-step testing and varies with the prevalence of TB in the population, the extent of BCG vaccination and the prevalence non-tuberculous mycobacterial infection. Our study population showed a prevalence of boosting of 4.3%.

5.21 BCG immunization increases the likelihood of boosting

In our study, booster responses were more likely to occur among those with a visible BCG scar, although this association did not reach statistical significance. Studies have shown that the presence of a BCG scar is associated with increased TST positivity (47, 48, 84). However, other factors related to BCG vaccination such as the dose administered, type of vaccine, the number of vaccinations, the number of scars as well as the age at vaccination may influence the relationship between BCG immunization and tuberculin response (47-49, 52, 58, 73, 85). It has been observed that the effect of BCG is minimal when TST reactions are ≥ 10mm (47, 48, 50, 84). In contrast to the evidence provided above, studies on boosting by Silva VMC *et al* (70) and Srour-Fihmi S *et al* (76) have shown that being BCG vaccinated had no effect on the booster response.

Many of the above mentioned studies have dealt with populations that had no documented TB exposure, or active TB disease. Contact with an infectious case may still override the effect of BCG on TST reactivity, especially in high prevalence settings. The effect of BCG immunization may be greater in lower prevalence populations. However, this needs to be further examined.

5.22 Increasing age is associated with boosting

Increasing age has been associated with the booster phenomenon (68, 73). This association of increasing age with boosting may reflect either greater or cumulative exposure among older individuals, and also diminishing immune capacity in the elderly, owing to which elderly individuals may not respond to the initial TST. In contrast, a study among children aged 6 months to 14 years of age found that the booster response was highest among those aged 6 months to 6 years, who also had BCG scars (86) .This may be explained by the cross-reaction due to BCG vaccination at this young age. However, the older subjects did not show such boosting; regardless of their BCG scar status (86) . This supports the notion that BCG immunity wanes with time. Our study among adolescents within a relatively small age range demonstrated an increased likelihood of boosting with increasing age. The force of infection is said to be maximum at around 15 years of age, increasing up to about 19 years, and this may be the reason for the boosting seen at this age, probably representing remote infection with M.tb. Also, social factors such as migration and employment may account for the increasing latent TB infection seen in this age group.

5.23 Sensitization with non-tuberculous mycobacteria

The booster phenomenon may also be linked to prior exposure to non-tuberculous mycobacteria (65, 66). The prevalence of NTM in India varies from 0.5-8.6% (87). Boosting is commonly seen in populations in tropical climatic conditions, where background sensitization with non-tuberculous mycobacteria is high (26, 29). Only 3(0.1%) of those with an enhanced response following repeat testing had prior sensitization with non-tuberculous mycobacteria, which did not reach statistical significance.

5.24 Prior M.tb infection

Remote exposure to M.tb has also been associated with the booster phenomenon. Of the 841 positive reactors in our study population (Table 4), 10 (1.2%) had a documented history of prior contact. This history of prior contact is most certainly an underestimate since community level exposures are likely to be high in a high TB disease prevalence setting. Of those with a history of

contact, only one developed a boosted response, when the criterion for defining a booster response as ≥ 10mm, with an increment of ≥ 6mm was used. This number is too small to conclude that remote infection produces boosting. However, when only an increment of ≥ 6 mm was used, 3 persons had a history of contact, and significantly higher odds of displaying boosting {AOR 5.86 (1.15-29.72)}. This may imply that using a smaller cut-off and thus increasing sensitivity (albeit at the cost of decreasing specificity), more number of boosters may be identified. Annual tuberculin testing is not warranted in casual contacts and low-risk groups as the chances of being truly infected with M.tb may be minimal, but the two-step test might be useful to separate the persons who show boosted responses from those at higher risk (household contacts, HIV infected, HCW) who are more likely to develop true conversions due to recent M.tb exposure.

5.3 Estimating the annual risk of tuberculosis infection (ARTI)

The annual risk of tuberculosis infection can be estimated from tuberculin surveys conducted among school-children, which gives an approximate estimate of the risk of transmission of TB in the community. Initially conducted among unvaccinated children, data showed that there were no differences between the ones who were or were not BCG vaccinated and since BCG vaccination is almost universal in most high prevalence settings now, it is increasingly difficult to separate the two. The ARTI is calculated using the formula-

$R= 1-(1-P)^{1/a}$, where 'R' is the ARTI estimated at mid-year when the study was conducted, 'P' is the prevalence of TB infection, and 'a' is the mean age of the sample plus 0.5.

In our study sample, P was estimated as 4.3%, and the mean age of the sample was 13.5 years. The ARTI thus estimated was 1%, using the conventional cut-off of 10mm as a positive TST response.

In order to evaluate the potential effect of boosting on the calculated ARTI we used the cut-offs for boosting described earlier. The re-calculated ARTI which included initial responders plus those who displayed boosting was still estimated at 1%. This was less than the national average of 1.5%, the last estimate being in 2006. This may reflect diminished TB transmission in the

general community, which in turn may reflect upon better TB control in terms of case-finding and treatment.

TST sensitivity may be increased by lowering the cut-off for determining a positive response. However, there is danger of under-estimation, owing to factors such as HIV and malnutrition which may produce false- negative responses. Although the state of Andhra Pradesh is among the six high HIV prevalence regions in the country (HIV prevalence 0.5-1.5%) there has been a decline over the years, the current prevalence is 0.9% (2009). We did not measure HIV status in our study population, but existing data have shown that the prevalence among 15-24 year old individuals is lower than the older age groups, and this is unlikely to have been a confounder in our study. The high prevalence of malnutrition in our sample population may result in under-estimation of infection, but the two-step testing procedure might help identify such individuals who are likely to respond to a second test and boost their immune response.

Data have shown that larger initial tuberculin reactions are likely to show boosting (68, 73, 85). TST reactions due to prior infection with M.tb are said to be an all or none phenomenon, that is, those with infection show responses that are significantly larger and tend to cluster on the right hand side of the distribution, while those uninfected frequently show small reactions. The intermediate zone consists of reactors who are infected with other mycobacteria- either BCG or NTM. By conducting two-step testing, there may be a slight skewing of the response to the right, as the responses that tend to be larger than 10mm will be accounted for by boosting. Only a few reactions in our study were associated with a history of prior contact, hence the enhanced response following repeat testing may be attributable to boosting.

5.4 Cost- benefits of two-step testing

A two-step test entails that the individual with an initial negative response return for a second testing, and again for reading of the results 48-72 hours later. This may prove expensive in resource-limited settings, due to the extra costs in terms of transport, loss of work-time, and material costs. However, the costs of wrongly classifying an individual as a converter, when in fact the response was merely a booster reaction may be substantial, as this requires further

evaluation in terms of a chest X-ray, preventive chemotherapy, and medical evaluation. Also, intangible costs such as drug side-effects, and patient distress cannot be accounted for accurately. Targeted tuberculin testing of high-risk groups may be a more feasible approach- however; no literature exists so far to correctly identify those individuals who would potentially show a boosted response to two-step testing. Factors such as older age, and those who are at risk of occupational exposure, as well as casual contacts may be candidates for two-step testing; but there are data showing boosting even in younger age groups (86, 88) including our study sample. Also, response among HCW's is variable, and no risk factors can accurately be assigned to be responsible for boosting. Hence, the benefits of testing an entire population to identify a few potential boosters may be expensive; nevertheless, given the benefits of identifying them preventing their misclassification, two-step testing of populations may still be applied. In a study among hospital and clinic employees (ages 24-44 years), Le CT showed that despite a small number of boosters that were identified (6/1521; 0.4%) among those tested, it was an additional 62 USD per person for the two-step test, and was feasible to perform. Hence it was suggested that 2-step testing would be cost-effective when applied as a pre-employment examination (88) .

Direct costs of tuberculin testing in our study, South India

SL. No.	Material description	Quantity	Unit Price (INR)
01	TST Vial (PPD-2 TU)†	1 Vial	95.00
02	TST Syringe(PPD Syringe)	1 Syringe	4.50
03	Cotton rolls†	1 roll	30.00
04	Rubber gloves	1 pair	10.00
	Transport †		
01	Tata Sumo Jeep (four-wheeler)	1km	6.50
02	TVS Mopeds(two-wheeler)	1km	1.50
			Total ~ 72 INR†

† Considering an average of 25 persons tested per visit and approximately 200 kilometers travelled by four-wheeler

Additional cost incurred in two-step testing

Cost of test (material cost) per subject	**72 INR**
No. of subjects re-tested	**1098**
Total cost	**79,056 INR**

The cost of conducting a single tuberculin test is approximately 1.5 USD per person for a single test. By having to re-test an additional 1098 subjects, an increase of about 0.8% in the budgetary allotment would be required. In addition, re-testing required approximately 90 days (1098/25x2- assuming that 25 people are tested /read on a single day) to complete with the attendant personnel costs.

Nevertheless, two-step testing may still be used in TB endemic settings, where the risk of transmission remains high and malnutrition is highly prevalent. Although only a few individuals with boosting have been identified in our study (0.7%), they have been separated from being misclassified as converters.

6.1 Sub-optimal response

Sub-optimal response, defined by a reaction size of less than 5mm was seen in nearly 19% of the study subjects.

6.11 Malnutrition and its effect on cell-mediated immune response

Malnutrition is a significant problem in our community. In our study, we used BMI for age as an index of nutritional status, and found that nearly 30% of them were undernourished (WHO 2007 standards). This was significantly associated with sub-optimal TST responses. Data have shown

that there is significant impairment of cell-mediated immune response in malnourished states. Consequently, tuberculin response may be affected, producing false-negative reactions. Pelly TF *et al* had shown that protein energy malnutrition produced anergy specifically to tuberculin, but did not affect delayed hypersensitivity to either tetanus or candida antigens, the latter being used to test for anergy (55). Kielmann AA et *al* conducted a study in a rural population of pre-school children in Punjab, India and demonstrated that there was a step-wise improvement in tuberculin reactivity as the nutritional status improved, in children who were < 65% below the median as per Harvard Growth Standards (89). However, several studies have demonstrated that mild to moderate degrees of malnutrition may not affect response to PPD (56, 57, 90, 91). But other factors such as HIV infection, anemia, infestation with helminthes or concurrent illnesses may have confounded this effect, hence have to be dealt with caution. The two-step test may be useful in such settings, helping to identify those who might potentially boost their immune response to the second test.

Among those who showed enhanced response following the second TST, 17/47(36.2%) were underweight; two step testing helped identify these individuals.

6.12 Age and TST response

Increasing age is known to result in a positive TST response; this probably reflects cumulative exposure as age increases. Results from our analysis also reconcile this finding- the younger age groups were more likely to show a sub-optimal response compared to the older age-groups.

6.13 Socio-economic determinants of TB infection and disease

TB is a disease that runs a protracted course, from the time of acquiring infection to subsequent disease development. During this time, interaction between social and environmental factors along with host characteristics may determine the course of the illness. TB is known to disproportionately affect the socially and economically disadvantaged. Most of the incident cases occurring today are from the low and middle-income countries.

In our study, we used maternal literacy status and structural characteristics of the house to determine the socio-economic status of the subjects. It was observed that belonging to lower

socio-economic class was significantly associated with sub-optimal response to the initial TST in the individuals. This was true even after adjusting for the other variables. It may follow that individuals of lower socio-economic class are also more malnourished, and from our discussion above we know that this affects the tuberculin response.

6.14 BCG vaccination and TST response

BCG vaccination has long been known to affect tuberculin response, owing to sharing of similar antigens with tuberculin. BCG scarring following vaccination is variable, and its presence may be considered a reasonable estimate of prior vaccination. In our study population, nearly 85% reported being BCG vaccinated, of whom 62.6% had recognizable scars. Absence of a BCG scar was significantly associated with sub-optimal responses in the subjects. There was no difference with regard to BCG immunization status. Our vaccination policy advocates BCG vaccination during infancy, and revaccination with BCG is uncommon in our setting, hence the influence of BCG on TST response may be considered negligible, provided the risk of exposure can be assumed to be minimal.

6.15 Prior M.tb exposure and TST response

Contact with an infectious case of TB has also been associated with tuberculin positivity. Greenaway C *et al*, in a casual contact investigation study in a high TB prevalence population suggested that boosting may have been responsible for the positive TST reactions following two-step testing of that population (92) . In a hospital based cross-sectional TST survey in Malaysia , it was shown that HCW's employed in medical wards (where the likelihood of direct exposure to active cases was higher) compared to those in surgical or orthopedic wards had significantly higher TST responses (93) . This remained after controlling for prior BCG vaccination, which can produce false positive tuberculin reactions. Those HCW's of Asian ethnicity showed a higher rate of conversion, and also had higher rates of baseline tuberculin positivity (93). This was also true from another hospital based study in New York, which showed that HCW's who worked in areas with high risk of patient contact had greater risk of conversion (94). As reported

earlier, HCW's are a high risk group, who would benefit from two-step testing, as prevalence of boosting has been reported to be as high as 50% in this population.

In our study, initial positive tuberculin reactions defined by a reaction size of $\geq$ 10mm were seen in 794 (12 %) of those who underwent baseline tuberculin testing .Of them, 9 (1.15%) reported a history of prior contact. Those who had a history of exposure had at least 2.5 times greater odds of having a positive TST response (p value < 0.01). This shows that a history of exposure to an infectious case is an important risk factor for TST positivity. By conducting the two-step TST, an additional 47 infected cases (4.3%) were detected in the study population. However, only one of these subjects had had a history of contact with an infectious case. This number is too small to conclude that contact was associated with boosting. The prevalence of boosting may vary depending on the risk of prior mycobacterial exposure, as well as potential ongoing risk, such as in HCW's. In a general population such as ours with substantial risk of non-occupational and community transmission, two-step testing might be a valuable tool to detect persons infected with M.tb, by separating those who have boosted reactions due to prior mycobacterial exposure from those with recent conversion.

7.1 Internal validity: Internal validity is the degree to which the findings are true to the study itself, after accounting for potential confounders. Although there were 35 subjects missing for baseline investigation, and another 159 missing for repeat TST testing, these numbers are small and are unlikely to have affected the validity of our study. Reported data were used for obtaining information on clinical and demographic characteristics. Also, anthropometry and TST administration/reading was performed by different field personnel. But this may have only been a source of random error, not systematic error.

7.2 External validity: External validity refers to how well the study can be generalized to other cases. These study data can be applied to rural adolescent populations in India. In addition it may have applicability to other populations where the background TB rates are high, BCG vaccination is universal, and HIV prevalence is low. In settings where HIV prevalence is also high, the interpretation of two-step testing may be performed with caution.

IX LIMITATIONS

Only school- going children have been included in this analysis. In doing so we may have excluded a disproportionate number of low socio-economic children. Including them would have made our study more representative of the population studied – however the logistics involved would have placed a considerable burden on the conduct of the study.

X CONCLUSION

Two-step tuberculin testing may be a valuable tool to detect TB infection in resource-limited settings. It helps to identify potential boosters who might not have reacted initially owing to factors such as malnutrition; or developed false positive response due to prior mycobacterial exposure. These persons may be separated from those who might be truly infected with M.Tb, and thus prevent them from undergoing further serial testing and be misclassified.

XI TABLES (continued to next page)

TABLE 1 Association of sub-optimal TST response (< 5 millimeter to an initial TST) with socio-demographic and clinical characteristics

VARIABLES	<5mm (N=1257)	≥5mm (N=5351)	P value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
GENDER					
Male	627(18.4)	2787(81.6)	0.16	0.92(0.81-1.04)	0.89(0.79-1.02)
Female®	630(19.7)	2564(80.3)			
AGE-GROUP					
11-12	421(23.7)	1357(76.3)		2.01(1.34-3.04)	1.96(1.31-2.93)
13-14	659(18.8)	2839(81.2)	<0.01	1.51(1.01-2.26)	1.48(0.99-2.19)
15-16	146(13.3)	954(86.7)		0.99(0.64-1.54)	0.97(0.63-1.48)
17-18®	31(13.4)	201(86.6)		1.00	1.00
MOTHER'S EDUCATION*					
Illiterate	644(20.2)	2546(79.8)	0.02	1.16(1.02-1.31)	
Others®	612(17.9)	2799(82.1)			
FATHER'S EDUCATION*					
Illiterate	347(20.1)	1377(79.9)	0.16	1.10(0.96-1.27)	
Others®	903(18.6)	3953(81.4)			
RELIGION					
Hindu	1108(18.9)	4743(81.1)	0.62	0.95(0.78-1.16)	
Others®	149(19.7)	608(80.3)			
CASTE					
Dalit/Harijan	212(17.2)	1018(82.8)	0.08	0.86(0.73-1.02)	

Others®	1045(19.4)	4333(80.6)			
TYPE OF WALLS* Brick Others®	 951(18.2) 306(22.4)	 4287(81.8) 1062(77.6)	 <0.01	 0.77(0.66-0.89)	 0.77(0.66-0.89)
TYPE OF FUEL Wood Others®	 1087(19.2) 170(18.0)	 4578(80.8) 773(82.0)	 0.40	 1.08(0.90-1.30)	
BCG IMMUNIZATION* Yes No®	 1061(18.8) 167(20.0)	 4588(81.2) 668(80.0)	 0.40	 0.93(0.77-1.11)	
BCG SCAR Present Absent®	 718(17.3) 539(21.9)	 3424(82.7) 1927(78.1)	 <0.01	 0.75(0.66-0.85)	 0.74(0.65-0.85)
BMI Thin/Severe thin Normal or Overweight/Obese®	 422(21.1) 835(18.1)	 1575(78.9) 3776(81.9)	 <0.01	 1.21(1.06-1.38)	 1.22(1.06-1.39)
h/o Contact Yes No®	 7(18.9) 1250(19.0)	 30(81.1) 5321(81.0)	 0.99	 0.99(0.40-2.37)	 0.97(0.42-2.22)
h/o Past TB Yes No®	 2(22.2) 1255(19.0)	 7(77.8) 5344(81.0)	 0.81	 1.22†	 1.54(0.30-7.77)
SYMPTOMS‡					

Cough					
Yes	11(27.5)	29(72.5)	0.17	1.62(0.76-3.39)	
No®	1246(19.0)	5322(81.0)			
Fever					
Yes	4(22.2)	14(77.8)	0.73	1.22(0.34-3.95)	
No®	1253(19.0)	5337(81.0)			
Weight loss					
Yes	12(44.4)	15(55.6)	0.01	3.43(1.50-7.76)	
No®	1245(18.9)	5336(81.1)			
Night sweats					
Yes	5(55.6)	4(44.4)	<0.01	5.34(1.25-23.61)	
No®	1252(19.0)	5347(81.0)			
Hemoptysis					
Yes	3(42.9)	4(57.1)	0.11	3.20(0.57-16.83)	
No®*	1254(19.0)	5347(81.0)			

*Because data were not available on some individuals total numbers are lower than the number of subjects who participated in the study

BCG- Bacille Calmette Guerin

BMI- Body Mass Index for Age

CI- Confidence Interval

®- reference group

‡ Considered positive if either parent/subject answered ‘yes’

† Confidence limits not reported due to small numbers

Multivariate analysis: adjusted for age, gender, type of walls of house (surrogate of socio-economic status), BCG immunization and BCG scar, contact (surrogate of exposure to TB), BMI (surrogate of nutritional status)

TABLE 2 Factors associated with enhanced responses of ≥ 10mm with an increment of ≥ 6 mm following a two-step TST

VARIABLES	≥10mm (N=47)	0-9mm (N= 1051)	P value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
GENDER					
Male	18(3.4)	515(96.6)	0.15	0.65(0.34-1.22)	0.61(0.32-1.15)
Female®	29(5.1)	536(94.9)			
AGE-GROUP					
11-12	16(4.2)	368(95.8)		0.78(0.10-16.67)	0.73(0.90-5.94)
13-14	24(4.1)	560(95.9)	0.93	0.77(0.10-16.13)	0.72(0.91-5.71)
15-16	6(5.4)	105(94.6)		1.03(0.11-24.02)	1.03(0.12-9.26)
17-18®	1(5.3)	18(94.7)		1.00	1.00
MOTHER'S EDUCATION*	23(4.2)	528(95.8)	0.8	0.95(0.51-1.76)	
Illiterate	24(4.4)	522(95.6)			
Others®					
FATHER'S EDUCATION*					
Illiterate	17(5.7)	282(94.3)	0.17	1.53(0.80-2.93)	
Others®	30(3.8)	762(96.2)			
RELIGION*					
Hindu	38(3.9)	925(96.1)	0.14	0.58(0.26-1.31)	
Others®	9(6.7)	126(93.3)			
CASTE					
Dalit/Harijan	6(3.4)	169(96.6)	0.54	0.76(0.29-1.91)	
Others®	41(4.4)	882(95.6)			

TYPE OF WALLS					
Brick	39(4.7)	791(95.3)	0.22	1.60(0.71-3.76)	1.74(0.76-3.98)
Others®	8(3.0)	260(97.0)			
TYPE OF FUEL					
Wood	38(4.0)	907(96.0)	0.29	0.67(0.30-1.52)	
Others®	9(5.9)	144(94.1)			
BCG IMMUNIZATION*					
Yes	39(4.2)	898(95.8)	0.89	0.94(0.37-2.52)	0.72(0.28-1.85)
No®	6(4.4)	130(95.6)			
BCG SCAR					
Present	30(4.8)	599(95.2)	0.35	1.33(0.70-2.55)	1.60(0.82-3.14)
Absent®	17(3.6)	452(96.4)			
BMI					
Thin/Severe thin	17(4.6)	351(95.4)	0.69	1.13(0.59-2.15)	1.33(0.71-2.51)
Normal or Overweight/Obese®	30(4.1)	700(95.9)			
h/o Contact					
Yes	1(14.3)	6(85.7)	0.19	3.79†	
No®	46(4.2)	1045(95.8)			
h/o Past TB					
Yes	0(0)	2(100.0)	0.76	*not reported due	
No®	47(4.3)	1049(95.7)		to small numbers	
SYMPTOMS‡					
Cough					
Yes	1(10.0)	9(90.0)	0.37	2.52†	
No®	46(4.2)	1042(95.8)			

Fever					
Yes	1(25.0)	3(75.0)	0.04	7.59†	
No®	46(4.2)	1048(95.8)			
Weight loss					
Yes	1(8.3)	11(91.7)	0.49	2.06†	
No®	46(4.2)	1040(95.8)			
Night sweats					
Yes	0(0)	5(100.0)	0.64	*not reported due	
No®	47(4.3)	1046(95.7)		to small numbers	
Hemoptysis					
Yes	0(0)	2(100.0)	0.76	*not reported due	
No®	47(4.3)	1049(95.7)		to small numbers	

* Because data were not available on some individuals total numbers are lower than the number of subjects who participated in the study

BCG- Bacille Calmette Guerin

BMI- Body Mass Index for Age

CI- Confidence Interval

®- reference group

‡ considered positive if either parent/subject answered yes

† Confidence limits not reported due to small numbers

Multivariate analysis: adjusted for age, gender, type of walls of house (surrogate of socio-economic status), BCG immunization and BCG scar, contact (surrogate of exposure to TB), BMI for age (surrogate of nutritional status).

TABLE 3 Factors associated with enhanced responses of ≥ 6mm following a two-step TST

VARIABLES	≥6mm (N=145)	0-5mm (N= 827)	P value	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
GENDER					
Male	69(14.8)	396(85.2)	0.95	0.99(0.68-1.43)	1.01(0.70-1.46)
Female®	76(15.0)	431(85.0)			
AGE-GROUP					
11-12	50(14.5)	294(85.5)		1.19(0.25-7.83)	1.11(0.24-5.1)
13-14	76(14.7)	442(85.3)	0.83	1.20(0.25-7.83)	1.09(0.24-4.97)
15-16	17(18.1)	77(81.9)		1.55(0.29-10.86)	1.43(0.29-6.99)
17-18®	2(12.5)	14(87.5)		1.00	1.00
MOTHER'S EDUCATION*					
Illiterate	74(15.3)	410(84.7)	0.76	1.06(0.73-1.53)	
Others®	71(14.6)	416(85.4)			
FATHER'S EDUCATION*					
Illiterate	45(16.9)	221(83.1)	0.28	1.23(0.82-1.84)	
Others®	99(14.2)	600(85.8)			
RELIGION					
Hindu	119(14.1)	726(85.9)	0.06	0.64(0.39-1.05)	
Others®	26(20.5)	101(79.5)			
CASTE					
Dalit/Harijan	22(14.8)	127(85.2)	0.96	0.99(0.58-1.65)	
Others®	123(14.9)	700(85.1)			
TYPE OF WALLS					

Brick	110(14.9)	626(85.1)	0.97	1.01(0.66-1.56)	0.98(0.64-1.51)
Others®	35(14.8)	201(85.2)			
TYPE OF FUEL					
Wood	112(13.4)	725(86.6)	<0.01	0.48(0.30-0.76)	
Others®	33(24.4)	102(75.6)			
BCG IMMUNIZATION*					
Yes	130(15.7)	696(84.3)	0.08	1.73(0.90-3.40)	1.65(0.86-3.15)
No®	12(9.8)	111(90.2)			
BCG SCAR					
Present	89(15.7)	478(84.3)	0.42	1.16(0.80-1.69)	1.09(0.74-1.60)
Absent®	56(13.8)	349(86.2)			
BMI					
Thin/Severe thin					
Normal or	46(14.4)	274(85.6)	0.74	0.94(0.63-1.39)	0.95(0.64-1.40)
Overweight/Obese®	99(15.2)	553(84.8)			
h/o Contact					
Yes	3(50.0)	3(50.0)	0.02	5.80(0.93-36.31)	5.86(1.15-29.72)
No®	142(14.7)	824(85.3)			
h/o Past TB					
Yes	0(0)	2(100.0)	0.55	*not reported due to	
No®	145(14.9)	825 (85.1)		small numbers	
SYMPTOMS					
Cough					
Yes	1(16.7)	5(83.3)	0.90	1.14†	
No®	144(14.9)	822(85.1)			
Fever					
Yes	1(33.3)	2(66.7)	0.37	2.86†	

No®	144(14.9)	825(85.1)			
Weight loss					
Yes	1(8.3)	11(91.7)	0.52	0.52(0.02-3.88)	
No®	144(15.0)	816(85.0)			
Night sweats					
Yes	0(0)	5(100.0)	0.35	*not reported due to	
No®	145(15.0)	822(85.0)		small numbers	
Hemoptysis					
Yes	0(0)	1(100.0)	0.67	*not reported due to	
No®	145(14.9)	826(85.1)		small numbers	

* Because data were not available on some individuals total numbers are lower than the number of subjects who participated in the study

BCG- Bacille Calmette Guerin

BMI- Body Mass Index for Age

CI- Confidence Interval

®- reference group

‡ Considered positive if either parent/subject answered ‘yes’

† Confidence limits not reported due to small numbers

Multivariate analysis: adjusted for age, gender, type of walls of house (surrogate of socio-economic status), BCG immunization and BCG scar, contact (surrogate of exposure to TB), BMI for age (surrogate of nutritional status).

TABLE 4 **shows the proportion of individuals with enhanced responses using a cut-off of ≥ 10mm at either baseline or following two-step testing, associated with a history of exposure.**

TB EXPOSURE	**TST RESPONSE Of ≥ 10 mm in the initial test and following the two-step test**		**TOTAL**	**Unadjusted OR (95% CI)**	**P value**
	POSITIVE	**NEGATIVE**			
YES	10(27%)	27(73%)	37	2.56(1.15-5.54)	<0.01
NO	831(12.6%)	5740(87.4%)	6571		
TOTAL	841	5767	6608		

XII APPENDIX

SOURCE DOCUMENTS

Enter # recorded on page 3 only if child is enrolled in study.
PID # ___ ___ -- ___ ___ ___

EPI-002-IN
Adolescent TB Study

INFORMED CONSENT PROCESS

Date of consent discussion: __ __ / __ __ / __ __ __ __ (dd mm yyyy)

Village name	**Parent's name**	**Address and Contact details**
_____		
School/College name	**Adolescent's Details** **Reported Age:**_____ _____**Yrs** **Initials:**_____ _____ _____	Land line:_____ Mobile:_____ E-mail:_____

Was ADEQUATE TIME given for parent/ 18 year old adolescent's consideration, questions, and answers?	☐ YES		☐ NO
Does parent/ 18 year old adolescent express understanding of the PURPOSE of the study?	☐ YES		☐ NO
Does parent/18 year old adolescent express understanding that participation is VOLUNTARY?	☐ YES		☐ NO
Does parent/ 18 year old adolescent express understanding WHAT PARTICIPATION INVOLVES?	☐ YES		☐ NO
Does parent/18 year old adolescent express understanding of the difference between ACTIVE and PASSIVE follow-up?	☐ YES		☐ NO
Does Parent know that their child will also be discussed **separately** about the study and their participation? (Check NA in the case of 18 old adolescent)	☐ NA	☐ YES	☐ NO
Does parent/18 year old adolescent give permission to review the school and or other records for an official date of birth?	☐ YES		☐ NO
Was Informed Consent signed? If YES, date consent signed: __ __ / __ __ / __ __ __ __ (dd mm yyyy)	☐ YES		☐ NO
If NO, record the reason			
Record approximate length of time taken to complete Informed Consent Process.			

Name of study team member who conducted consent process

Signature of study team member who conducted consent process *Date*

Study Team Member Code
☐☐☐

INFORMED ASSENT PROCESS
(Only if adolescent is less than 18years of age)

Date of assent discussion: __ __ / __ __ / __ __ __ __ (dd / mm / yyyy) Or ☐ NA

Version of the consent used:

☐ Original/ same language as consent form *(for the age group between 15-17)*
☐ Simplified version of the consent *(for the age group between 12-14)*

Was ADEQUATE TIME given for adolescent's consideration, questions, and answers?	☐ YES	☐ NO
Does adolescent express understanding of the PURPOSE of the study?	☐ YES	☐ NO
Does adolescent express understanding that participation is VOLUNTARY?	☐ YES	☐ NO
Does adolescent express understanding WHAT PARTICIPATION INVOLVES?	☐ YES	☐ NO
Does adolescent express understanding of the difference between ACTIVE and PASSIVE follow-up?	☐ YES	☐ NO
Is adolescent interested to participate?	☐ YES	☐ NO
Was Informed Assent signed? If YES, date assent signed: __ __ / __ __ / __ __ __ __ (dd / mm / yyyy)	☐ YES	☐ NO
If NO, record the reason		
Record approximate length of time taken to complete Informed assent Process.		

_____
Name of study team member who conducted assent process

Signature of study team member who conducted assent process *Date*

Study Team Member Code
☐☐☐

SOURCE DOCUMENTS

EPI-002-IN
Adolescent TB Study

PARTICIPANT INITIALS: ____ ____ ____ **PID#:**____ ____ - ____ ____ ____

SCREENING and ENROLLMENT

Date of screening/enrollment (Study Day 0): __ __ / __ __ / __ __ __ __ (dd mm yyyy)		
Adolescent's date of birth __ __ / __ __ / __ __ __ __ (dd mm yyyy)	**Adolescent's Gender M or F** _____	**Name of the School/College** **Grade(7 to 12):**_____ _____
Was copy of signed consent given to parent or 18 year old adolescent?	☐ YES	☐ NO
Was copy of signed assent given to adolescent?	☐ NA ☐ YES	☐ NO
Does family plan to live within study area for next 2 years?	☐ YES	☐ NO
Is adolescent able to attend follow-up visit for reading Mantoux test?	☐ YES	☐ NO
Does adolescent meet all eligibility criteria?	☐ YES	☐ NO
If **YES**, record sequentially assigned **PID #** ____ ____ - ____ ____ ____ Surveillance group assigned to: ❒ ACTIVE ❒ PASSIVE		
If NO, do not assign a PID #; Record reason not enrolled:		

Give parent completed Study Participant ID Card. Review the contact information noted on the card with them. Tell parent and or participant to bring the PID card and show when ever they contact the physician or the study staff.

The adolescent is considered enrolled in the study when the PID # is assigned. From this point forward, use the initials and the PID # as identifiers of the adolescent.

_____

Name of study team member who conducted screening and enrollment process

Signature of study team member who conducted screening and enrollment process *Date*

PARTICIPANT INITIALS: ___ ___ ___ **PID#:**___ ___ - ___ ___ ___

STUDY DAY 0

Socio-economic factors:

Religion:

- ❑ Hindu
- ❑ Muslim
- ❑ Christian
- ❑ Sikh
- ❑ Jain
- ❑ Buddhist
- ❑ Other; specify: _____
- ❑ Not answered

Caste:

- ❑ Dalit/Harijan
- ❑ Others
- ❑ Not answered

Highest level of education attained:

Mother:

- ❑ Illiterate
- ❑ Primary
- ❑ Secondary
- ❑ High school
- ❑ Higher secondary
- ❑ College
- ❑ Not answered

Father:

- ❑ Illiterate
- ❑ Primary
- ❑ Secondary
- ❑ High school
- ❑ Higher secondary
- ❑ College
- ❑ Not answered

Occupation:

Mother: _____ Or, ❑ Not answered

Father: _____ Or, ❑ Not answered

Completed by Headquarters ❑❑ ❑❑

What is the total monthly household income? _____ Rupees Or, ❑ Not answered

STUDY DAY 0 (continued)

What are the walls of the house made of?

- ❑ Packed mud
- ❑ Stone
- ❑ Bamboo
- ❑ Thatch
- ❑ Wood
- ❒ Brick
- ❑ Other; specify: _____
- ❑ Not answered

Does the house have an electricity connection?

- ❑ Yes
- ❑ No
- ❑ Not answered

Which type of fuel is used as major cooking fuel?

- ❑ LPG
- ❑ Electricity
- ❑ Wood
- ❑ Kerosene
- ❑ Coal
- ❑ Agricultural residue
- ❑ Others, Specify_____

Study Team Member Code

❑❑❑

PARTICIPANT INITIALS: ____ ____ ____ **PID#:**____ ____ - ____ ____ ____

STUDY DAY 0(continued)

Details of BCG Immunization:
Was participant immunized with **BCG**?

- ❑ Yes
- ❑ No
- ❑ Not known
- ❑ Not answered

History of TB disease *Any YES answer either from the parent or from the participant has to be considered as positive*	Parent interview Study Team Member Code ☐☐☐	Participant interview Study Team Member Code ☐☐☐
Did your child/you have TB disease in the past	❑ Yes, indicate the age of the child____ ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Is your child/ Are you currently taking TB medications ***If* YES *Mantoux should not be applied***	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
History of **positive TB contact** (for more than 8 hrs/week)	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Symptoms <u>for more than two weeks</u> Unexplained Cough	❑ Yes ❑ No	❑ Yes ❑ No
Unexplained Weight loss	❑ Yes ❑ No	❑ Yes ❑ No
Unexplained Fever	❑ Yes ❑ No	❑ Yes ❑ No
Unexplained Night sweats	❑ Yes ❑ No	❑ Yes ❑ No
Haemoptysis(Blood seen in mucous when cough)	❑ Yes ❑ No	❑ Yes ❑ No
Hospital admission(in the past six months for any illness)	❑ Yes ❑ No	❑ Yes ❑ No

PARTICIPANT INITIALS: ____ ____ ____ **PID#:**____ ____ - ____ ____ ____

STUDY DAY 0 (continued)

Diagnosis of any acute or chronic diseases in the past	Parent interview	Participant interview
Gastro enteritis	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Pneumonia	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Meningitis	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Asthma	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Diabetes	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Anemia	❑ Yes ❑ No ❑ Not known	❑ Yes ❑ No ❑ Not known
Others	❑ Yes ❑ No If **YES** specify: _____	❑ Yes ❑ No If **YES** specify:
If any of the above answers is YES then check YES otherwise check NO	❑ Yes ❑ No	❑ Yes ❑ No

Name of family doctor: _____ Or, ❑ None

Location: _____

PARTICIPANT INITIALS: ____ ____ ____ **PID#:**____ ____ - ____ ____ ____

STUDY DAY 0 (continued)

Physical measurements

Weight: ___ ___ ___. ___ Kg

Height: ___ ___ ___. ___ cm

Presence of BCG scar

Whether **BCG** scar is present?

- ❑ Yes
- ❑ No

Blood Draw for Research of Immunology of TB

Draw the blood before applying the Mantoux. Blood should be collected and labeled as per instruction. ***Not more than two trails*** *should be made for the blood draw.*

Date of blood draw:___ ___/ ___ ___/ ___ ___ ___ ___
dd mm yyyy

Time of blood draw *(use 24 hour clock)*: ___ ___ : ___ ___
hh mm

Number of CPT tubes used for blood collection:

- ❑ 1
- ❑ 2
- ❑ 3

If the number of tubes is less than 3 fill the Protocol Deviation Form. CPT tubes should be kept in the cool box to maintain temperature at 2-8°C and transport to the Lab within 4 hours from the time of collection.

Blood collected for Serum preparation:

- ❑ Yes
- ❑ No

Study Team Member Code ☐☐☐

Hemoglobin concentration:

____ ____ - ____ g/dL

Study Team Member Code ☐☐☐

PARTICIPANT INITIALS: ____ ____ ____ **PID#:**____ ____ - ____ ____ ____

STUDY DAY 0(Continued)

Tuberculin skin test: *(If the adolescent is taking TB medication then check NA. Test will be read within 2 – 4 days after application. Test should be applied after the blood draw.)*

Date applied: __ __ / __ __ / __ __ __ __ (d d / m m / y y y y) Or ❑ NA

Study Team Member Code ☐☐☐

Time applied *(use 24 hour clock)*: __ __ : __ __ (h h : m m)

Date read: __ __ / __ __ / __ __ __ __ (d d / m m / y y y y)

Study Team Member Code ☐☐☐

Reading time *(use 24 hour clock)*: __ __ : __ __ (h h : m m)

Result *(Record widest transverse measurement in whole number)*: ___ ___ mm

If the Tuberculin skin test reaction is less than 5mm apply the second test 1-4 weeks after the first test was applied to the opposite arm. The larger will be used as baseline value.

Name ***Signature***

Repeat Tuberculin skin test *(If the first test result is less than 5mm repeat the test 1-4 weeks after the first test was applied to the opposite arm)*

Date applied: __ __ / __ __ / __ __ __ __ (d d / m m / y y y y) Or ❑ NA

Study Team Member Code ☐☐☐

Time applied(*(use 24 hour clock)*: __ __ : __ __ (h h : m m)

Date read: __ __ / __ __ / __ __ __ __ (d d / m m / y y y y)

Study Team Member Code ☐☐☐

Reading time *(use 24 hour clock)*: __ __ : __ __ (h h : m m)

Result *(Record widest transverse measurement in whole number)*: ___ ___ mm

The larger result will be used as baseline value.

Name ***Signature***

Baseline *(write the first tuberculin skin test result as the baseline value if it is more than 5mm otherwise compare first and second test results; use the larger result as baseline value.)*

Baseline value:___ ___ mm

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