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Comparison of Tuberculin Skin Test and QuantiFERON TB Gold *In-tube* Assay for the diagnosis of tuberculosis infection and disease in young children; study conducted as part of the development of a TB vaccine site in Southern India

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy in International Health

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ABBREVIATIONS

ARTI	Annual risk and Rate of Tuberculosis Infection
BCG	Bacillus Calmette-Guerin
CDC	Center for Disease Control and Prevention
CFP-10	Culture Filtrate Protein 10
CVW	Case Verification Ward
ELISA	Enzyme Linked Immunosorbent Assay
EMEA	European Medicines Agency
ESAT-6	Early Secretory Antigenic Target-6
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IGRA	Interferon Gamma Release Assay
LJ	Lowenstein Jensen
MGIT	Mycobacterial Growth Indicator Tube
NALC	N-Acetyl L-Cysteine
NTM	Non Tuberculous Mycobacteria
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
QFT	QuantiFERON TB
QFT-G	QuantiFERON TB Gold
QFT-GIT	QuantiFERON TB Gold *In-Tube*
RCF	Relative Centrifugal Force
RD1	Region of Difference 1
RNTCP	Revised National Tuberculosis Control Programme
T_h	T helper
TB	Tuberculosis
TST	Tuberculin Skin Test
WHO	World Health Organization

SUMMARY

Background

An estimated 35% of tuberculosis (TB) cases worldwide are accounted by India and China alone. Pediatric TB accounts for 11% of TB cases worldwide. The most common type of TB in children is smear negative pulmonary TB. Data from the Revised National Tuberculosis Control Programme (RNTCP) in India indicate that in 2009, the percentage of new smear positive cases was 72% of the total case detection rate of 132/100,000. Of all the new cases detected, 7% were pediatric cases. Forty per cent of the population in India is thought to be infected with *M. tuberculosis* and the population has an estimated annual risk of infection of 1.5%.

Due to difficulty in obtaining a sputum sample in children, *M. tuberculosis* very often goes undetected in smear and culture. In children <5 years, the tuberculin skin test (TST) is widely used as an aid in the diagnosis of TB infection. A decade ago, the QuantiFERON test (QFT), an Interferon Gamma Release Assay (IGRA) was approved by the Food and Drug administration (FDA) and European Medicines Agency (EMEA) and is now used as an aid in the diagnosis of TB infection. Although the TST is known for its limitations related to variations in dose, inter-observer errors related to administration and reading, potential lack of specificity related to non tuberculous mycobacteria (NTM) and Bacillus Calmette-Guerin (BCG) vaccination, it may have a higher sensitivity than the QFT. However, the available literature shows that QFT has a higher specificity and potentially higher predictive value than the TST.

Diagnosis of childhood TB remains a challenge. Although, QFT has shown promising results in few studies, there is limited data on its diagnostic performance in children. One of the limitations of the QFT in TB-endemic areas is that it will not differentiate between latent and active TB. However, in children – especially young children – all TB infection can be considered to be of recent origin and given the shorter duration of progression from infection to disease in these children, they are therefore at 'high risk' of developing TB. The available studies suggest that QFT may therefore prove clinically useful in the evaluation of TB in children and feasible to perform. There is a need to perform more studies in children in India to evaluate different tests in relation to TB infection and disease with a larger sample size in order to overcome the diagnostic challenge that exists in the field of childhood TB. The overall aim

of this study is to contribute to the diagnosis of TB infection and disease in young children in India, a high TB burden country.

Methodology

The present study is nested within a prospective cohort study. BCG vaccinated infants <14 days of age and born to mothers from Palamaner taluk in Andhra Pradesh were enrolled. Informed consent was obtained and information such as age, weight of the infant, parents' demographic characteristics, and socio economic data were collected from clinical records and by interview. Infants were randomly assigned to an active or passive follow up group and followed for 2 years. Infants suspected of having TB based on symptoms, failure to thrive (FTT) and a recent history of contact with TB were referred to the case verification ward (CVW) for diagnostic evaluation of TB. A TST and chest X-Ray was performed. One sample each of a gastric aspirate and induced sputum were collected on two consecutive days which were used for smear microscopy and culture. Mycobacterial cases were confirmed by speciation using Genotype MTBC and CM kit (HAIN) or COBAS TaqMan MTB test kit (Roche). Blood was collected, plasma isolated and QuantiFERON gold *In tube* assay (QFT-GIT) performed.

Results

There were 4,382 children enrolled in the study of which 2,215 were included in the active surveillance group and 2,167 in the passive surveillance group. Seven hundred and forty six participants were referred to the CVW of which 53 came for a second visit, three for a third visit and one for a fourth visit during the two year period of follow up. In 709 participants both TST and QFT-GIT were performed. The percentage of children who were TST positive was 10.2% (n=72/709) while 5.8% were QFT-GIT positive (n=41/709).There were a total of four cases with definite (bacteriologically confirmed) TB; three of which were positive for TST and QFT. The 4th case was negative on culture, TST and QFT-GIT but was positive on smear microscopy. A direct polymerase chain reaction (PCR) on the induced sputum sample was positive for *M. tuberculosis.* There were 9 cases with 'probable' TB, of which one was positive for TST but negative for QFT-GIT and the rest were negative for both. Children with a recent history of contact with a TB case had increased odds of being TST positive (OR 2.11[95% CI 1.07; 4.17], p=0.03), QFT-GIT positive (OR 3.46 [95% CI 1.50; 7.81], p=<0.01) and combined

TST and QFT-GIT positive (OR 7.08[95% CI 2.51; 19.65], p=<0.01) in a univariate analysis. In a multivariate analysis, the association between TST positivity and recent history of contact with TB was not statistically significant (OR 1.58 [95% CI 0.69; 3.63], p=0.27). However, for QFT-GIT positivity the association with contact remained statistically significant in a multivariate analysis (OR 4.36 [95% CI 1.79; 10.54], p=0.01). Children with wasting based on the weight for height Z score (<-2SD) had reduced odds of being TST positive in a univariate analysis (OR 0.31 [95% CI 0.17; 0.56], p=<0.01) as also those with FTT (OR 0.19 [95% CI 0.12; 0.33], p= <0.01]. There was no association between QFT-GIT positivity and nutritional status. The agreement between TST (cut off ≥10mm) and QFT-GIT was fair [κ 0.31 (95% CI 0.19; 0.40), p=<0.01] and TST (cut off ≥5mm) and QFT-GIT was poor [κ 0.09 (95% CI 0.04; 0.11), p=<0.01] for all children with both TST and QFT-GIT tests performed and referred to the CVW for the first time (n=709). The percentage of indeterminate QFT-GIT results was 3.6%.

Conclusion

The incidence of TST positivity was higher than that of QFT-GIT positivity. Due to a lack of a gold standard for the diagnosis of TB infection in children and the low number of bacteriologically confirmed cases, sensitivity and specificity of the tests in relation to TB infection and disease could not be calculated. A recent history of contact with TB was associated with QFT-GIT positivity, but not TST positivity in a multivariate analysis and this association was stronger when both TST and QFT-GIT positivity was considered together. Additional studies to confirm the utility of a two-step approach i.e. QFT-GIT test on those who are TST positive would be relevant. This approach would be strengthened if the likelihood of progression to TB disease was higher for those infants with combined TST / QFT-GIT positives rather than either test alone. TST and not QFT-GIT positivity was reduced in undernourished participants; this is significant given the high prevalence of under nutrition in children of this age group in India. The data suggest that QFT-GIT may be better for the diagnosis of a recent infection as compared to the TST in a setting where under nutrition is high. The overall agreement between TST and QFT-GIT was fair at a cut off of ≥10mm for TST. The number of indeterminate test results in the QFT-GIT was low.

1.0. INTRODUCTION

1.1. History

Tuberculosis (TB) has a long history. Molecular analysis of skeletons from Egyptians have proved the presence of skeletal TB almost 5000 years ago (1). TB was also known as pthisis (meaning wasting away, consumption) a name which appeared in the Greek literature during the 5th century AD (2). The TB epidemic started in Europe around the 17th Century and continued for the next 200 years. It later came to be known as "The Great White Plague" (3)

The natives of North America experienced a major outbreak of TB in 1880 and by 1886 the death rates had increased to 9000 per 100,000 people. TB was rare among the natives of Africa living in small remote villages. However they experienced a high mortality rate when they were exposed to the disease when they came in contact with the Europeans. African slaves were free from the disease on their arrival in America. However, they developed TB and on return to their homeland, the TB mortality rose to 700 per 100,000 (3).

TB was also documented in Asia i.e. India and China as early as 3300 and 2,300 years ago respectively, (2) but only towards the end of the 19th century was a high incidence of the disease found in these two countries (3).

1.2. The organism and its transmission

On 24 March 1882, Robert Koch - a German physician - demonstrated the bacillus which causes TB at the Berlin Physiology Society meeting (4). This bacillus was later called *Mycobacterium tuberculosis*.

TB is transmitted through air (5). TB infection occurs due to the inhalation of infective droplets, which are usually less than 5µm in diameter (6). The risk of infection depends on the duration, probability, proximity to exposure, intensity of an exposure and also on the infectiousness of the source (7, 8).

TB is known to be contagious and if untreated, each infected individual with active TB is capable of infecting 10-15 people on an average every year (5).

1.3. Pathogenesis and Immunology

Infectious individuals transmit the organisms through air via droplet nuclei (9). The larger droplet nuclei which are around 10μm in diameter are carried by air and when inhaled by a healthy individual typically land in the upper respiratory tract but do not progress further due to the action of the mucus and ciliary system of the respiratory tract. The smaller droplet nuclei which are 5μm or lesser in diameter can escape the actions of the mucus and ciliary system of the respiratory tract and manage to penetrate further and land in the bronchioles and the alveoli. The alveolar macrophages then phagocytose the inhaled bacilli. They are, however, unable to kill the bacteria and these bacteria continue replicating within the macrophages. These infected macrophages are then transported to the lymph nodes from where the mycobacteria move to other lymph nodes and organs like the kidney, spinal cord and other organs leading to extra pulmonary tuberculosis (3, 10).

A cell mediated immune response develops after 2-3 weeks of initial infection. The T helper (T_h) cells activate the macrophages which aid in ingesting and digesting the intracellular bacteria while the T cytotoxic (T_c) cells lyze the macrophages resulting in the formation of caseous granulomas with central necrosis (3).

The primary infection site, characterized by a single lesion and involvement of the draining lymph node is called the Ghon complex (3) and the appearance of this in chest radiographs is considered a classic sign of *M. tuberculosis* infection.

1.4. Risk of progression from TB infection to disease in infants and children

Since infants have a less developed immune system as compared to adults and school-children, they have a higher probability of developing a disease following infection. The progression of infection to disease varies with age as shown in Table (a), below. Infants are at a higher risk of progressing to pulmonary TB disease as compared to children between 2-5 yrs of age. Since more than 90% of children progress to disease within 12 months of primary infection, all children below 3 yrs of age and/or immune compromised children can be categorized as a high risk group (11).

In most of the cases, infection is caused by exposure to an infectious pulmonary TB individual within the household (12). Most infections progress to disease within a year following infection. However, some may progress within 2 years. In a healthy infant with no signs or symptoms of TB but suspected to have TB infection, the only evidence is often a

positive tuberculin skin test (TST). In the majority of cases the source of exposure in an infant is the mother (6).

Table (a). Average age specific risk for disease development following primary infection

Age at primary infection (yr)	Pulmonary disease (%)	Miliary or central nervous system TB (%)
< 1	30-40	10-20
1-2	10-20	2-5
2-5	5	0.5
5-10	2	<0.5
>10	10-20	<0.5

Adapted from Marais et al. The natural history of disease of childhood intra-thoracic tuberculosis: a critical review of the prechemotherapy literature. Int J Tuberc Lung Dis 2004;8(4):392–402 (11)

A child infected with TB is vulnerable and can develop the disease at any given point of time. A weak immune system (as in the case of HIV) is the major factor contributing to the progression of infection to disease. However other infections (such as whooping cough and measles) and malnutrition which is common in infants and early childhood in developing countries may also contribute to it (6).

1.5. Epidemiology of childhood TB

TB is also referred as a "*disease of poverty*" and the majority of deaths due to TB occur in developing countries, with more than 50% of deaths occurring in Asia. There were 9.4 million new TB cases worldwide in 2009 of which, an estimated 1.0-2.0 million cases were seen in people with HIV (13). Tuberculosis in children is often neglected since children are often considered ineffective transmitters of the bacillus (14). However the World Health Organization (WHO) reported that "childhood tuberculosis accounts for 11% of the TB cases worldwide and about 1 million children develop TB annually worldwide"(15).

1.5.1. World Scenario

The WHO reports that "most of the estimated cases of TB occurred in Asia (55%) and Africa (30%), with small proportions of cases in the Eastern Mediterranean Region (7%), the European Region (5%) and the Region of America (3%)". An estimated 35% of TB cases worldwide are accounted for by India and China alone (13).

In a study in a high prevalence region in South Africa, of 1,445 neonates who were followed for 2 years, 69.5% had a contact with an infectious source and 11.9% were culture positive for *M. tuberculosis* (12).

In 2007, in the United States (US) 13,000 new cases of TB disease were diagnosed, out of which 820 were children younger than 15 yrs of age. TB rates were 9.5 times higher in the foreign born individuals in the US as compared to those of US origin. Most of the cases occured in people coming from Mexico, Philippines, Vietnam, China and India (14).

In Japan, from the 1970's, there was a drop in the number of newly notified childhood TB cases between 0-14 years of age. However the age group between 0-4 years has consistently shown the highest occurrence since the 1970's. In 2008 the incidence rate of pediatric TB was 0.55 per 100,000 population (16).

1.5.2. Indian scenario

In India, 40% of the population is thought to be infected with TB and the annual risk of infection is 1.5% (17). In 2009, $1/5^{th}$ (21%) of all the TB cases in the world were found in India alone (13).

In 2009, the total population covered by the Revised National Tuberculosis Control Programme (RNTCP) was 1,16,41,00,000. The total annualized case detection rate was 132 per 100,000 population, 624,617 new smear positive cases, 384,113 new smear negative cases and 233,026 new extrapulmonary cases were registered under the RNTCP. The annualized new smear positive case detection rate was 54 per 100,000 population. Out of all new cases detected, 86,532 (7%) were pediatric cases. Cure rate of the new smear positive cases were 85% (18).

1.6. Diagnosis of TB in children

Diagnosis of TB in children remains a challenge. In children, contact with an adult with TB is important (12). Due to difficulty in obtaining sufficient sputum sample in children, the sputum smear microscopy (standard means for diagnosis of TB) and culture (considered gold standard for diagnosis of TB) fail to be used to diagnose TB in children. However the bacteriological yield is higher in children with advanced disease. In low endemic countries, a history of contact with an infectious source accompanied with a positive skin test and abnormal suggestive chest radiograph is used to diagnose TB and has worked well in such settings (19).

In some settings structured diagnostic algorithms are used. In a study in South Africa, structured diagnostic algorithms were compared for their ability in diagnosing childhood tuberculosis and the approaches categorized as binary (positive or negative), hierarchical (definite, probable, possible, unlikely or not TB) and numerical (score >x = TB). The study showed that compared to the binary outcomes, the related hierarchical approach showed better agreement (12).

1.7. The tuberculin skin test

The tuberculin skin test (TST) also known as the Mantoux test is widely used as an aid in the diagnosis of TB. Delayed type hypersensitivity (DTH) reactions are involved in the TST. The DTH is a localized inflammatory reaction which is induced by the cytokines secreted by activated T_h cells on encounter with certain types of antigens. The DTH was first observed by Robert Koch in 1890 when individuals infected with *Mycobacterium tuberculosis*, who were injected intradermally with a filtrate derived from mycobacterial culture, developed a localized inflammatory response. This kind of localized skin reaction was called as a “tuberculin reaction”. Tuberculin is a purified protein derived (PPD) from heat-killed tubercle bacilli (20). Usually, the tuberculin, PPD-RT23, in a 0.1 mL dose, equivalent to 2 TU (tuberculin units), or PPD-S, equivalent to 5 TU, is injected intradermally by trained personnel and is read after 24-48 hrs. A positive reaction is indicated by an induration of ≥10mm, although lower cutoffs may be used in immunocompromised states such as HIV. A positive TST is not diagnostic test for TB disease by itself but only indicates TB infection. A negative test does not always exclude the possibility of having TB (3).

1.7.1. Weaknesses and Strengths

Even though the TST has been used for the diagnosis of TB for more than a century, tuberculin, which is a crude mixture of many antigens, is known to cross-react with NTM and the BCG vaccine and give false positive results (21). A positive result may also result from a past infection with *Mycobacterium tuberculosis.* It is also known for its limitations in accuracy and reliability (22). Moreover, TST reading needs to take place 48-72 hours after the administration of the tuberculin which requires the patient to visit the hospital/place of administration twice. Variability in doses, manufacturers, errors in administration and reading cut off values leads to variable results. In immune-suppressed patients and in malnourished children false negative TST results may occur (23).

One of the major advantages of the TST is low cost - it is less expensive than the new Interferon-γ Release Assays (IGRAs) and in some studies in children, has been shown to have a higher sensitivity than the IGRAs (24, 25). It is also readily available.

1.7.2. TST and TB infection/TB Disease

The TST is routinely used as an aid for the diagnosis of TB infection. In children <5yrs TST is generally used for identifying TB infection (26). TB disease is distinguished from TB infection in that TB disease usually has associated signs and symptoms and abnormal chest X-rays after the infection. In adults, it is easier to differentiate between infection and disease since the disease often occurs as a result of reactivation of dormant bacilli acquired during a previous infection, giving time for a robust immune response to develop. However, in children it is not so well defined as more often initial or primary infection progresses rapidly to disease (6).

1.7.2.1. TST and TB infection

In a contact investigation, individuals with lower levels of recent exposure were more likely to be positive by TST, suggesting that TST may be better at detecting a remote infection which was present before the recent exposure (27) – though an alternate explanation may simply be that these results were false positives.

In children, as in adults, latent TB infection lacks a diagnostic gold standard.(14) Many studies have compared the TST to different IGRAs and indicated that the TST has lower specificity than the IGRA (24, 28, 29).

1.7.2.2. TST and TB disease

In a study involving 28 children aged 4 months to 7 yrs with culture confirmed active TB, TST showed a sensitivity of 100% (24). In another study with 25 children with culture-confirmed active TB, sensitivity for TST was estimated to be 88% at a cut off of ≥10mm and 83% at a cut off of ≥15mm. When children with probable active tuberculosis were included (defined on the basis of epidemiologic, clinical, and radiographic findings in the absence of a positive culture), the sensitivity of TST fell to 71% at a cut off of ≥10mm and 60% at a cut off of ≥15mm (25). In a study in Taiwan the clinical symptoms and demographic characteristics of 103 children were studied and children with probable and confirmed TB were enrolled and TST performed. TST was positive in 69.6 % of the cases with pulmonary TB (probable and confirmed TB) (30).

Due to the low cost, lack of requirement of laboratory equipments like pipettes, enzyme linked immunosorbent (ELISA) reader, computer to analyze the data, extensive training issues and ready availability as compared to the IGRA (31), TST continues to remain the test of choice in many countries (17).

1.7.3. Indian Scenario

In a 15 year follow-up study in Southern India, a randomized control trial which involved 280,000 individuals, showed that TST positivity was significantly associated with the development of TB (32). In a study conducted in Southern India, Gopi *et al* showed that BCG vaccination did not interfere with the tuberculin survey to estimate the annual risk and rate of tuberculosis infection [ARTI] (33). Some studies have shown that BCG induced tuberculin response after vaccination ranges from 1-19mm induration (34, 35). Many factors influence the size of the induration produced in response to the tuberculin after BCG vaccination such as manufacturer of BCG, dose and method of manufacture.

As mentioned earlier, many studies conducted in India (17, 36-38) suggest, that in spite of its limitations in accuracy and specificity, TST may be a useful tool for the diagnosis of TB mainly because of its low cost and ready availability and due to the fact that BCG vaccine in

infancy has limited effect on TST results in later life, including in older children and adults (33).

1.8. The QuantiFERON test

Until 2001, the TST was the only immunological test which was available for the diagnosis of TB infection (39). Many studies (40, 41) led to the recognition of the role of Interferon-γ (IFN- γ) in the regulation of the cell mediated immune response to TB infection which paved the way for the application of the IGRA in the diagnosis of TB infection. The QuantiFERON TB test (QFT) (Cellestis Limited Victoria, Australia) was the first IGRA test which was approved by the Food and Drug Administration (FDA) to be used for the diagnosis of TB infection in 2001 (42). The guidelines for the use of this test was published by the Center for Disease control (CDC) in 2003 (43).This test used an ELISA to measure the IFN-γ released in response to the PPD when compared with the controls. Inspite of using a control for nontuberculous mycobacterial sensitization (*M.avium* antigen) and saline as a negative control, the test showed lower specificity when compared to the TST (44). Genomic analysis of *M. tuberculosis* helped determine the *M. tuberculosis*-specific antigens; early antigenic secretory taget-6 (ESAT-6) and culture filtrate protein -10 (CFP-10). These antigens are known to induce the production of IFN-γ in sensitized T_h cells. These two antigens were absent from the BCG strain and from most NTM and hence test results were not confounded by BCG vaccination and infection with NTM (45). The QuantiFERON TB Gold test (QFT-G) (Cellestis Limited, Victoria, Australia) was the second IGRA test approved by the FDA for the diagnosis of TB infection in the year 2005 (46). The guidelines were published by the CDC in the same year (47). In this test the whole blood was incubated separately with the two antigens (ESAT-6 and CFP-10) and the plasma collected. The amount of IFN-γ released was calculated as the difference in IFN-γ concentration in the plasma from the blood sensitized to the antigens minus IFN-γ concentration in plasma from blood incubated without an antigen (47).

The ESAT-6 and CFP-10 antigens are present in *M. kansasii, M. szulgai* and *M. marinum* which may lead to cross reaction which in turn may lead to false positivity, though studies have shown that human infections with such pathogens seem to induce low levels of IFN-γ (48). As these antigens are recognized by fewer T lymphocytes and often stimulate less IFN-γ compared to PPD, a more sensitive ELISA than the one used in QFT was needed (43).

In 2007, QuantiFERON-Gold In-tube assay [QFT-GIT] became the third IGRA test to be approved by the FDA (49). This test used ESAT-6, CFP10 and an additional antigen, TB 7.7 and the blood was collected in tubes coated with a mixture of the 3 antigens and one negative control and one containing heparin, dextrose and phytohemogluttinin which served as a positive control.

The TB response was calculated as difference in IFN-γ concentration in plasma from blood stimulated with a mixture of the antigens minus the IFN-γ concentration in plasma from blood incubated without antigen (i.e., nil). This test was evaluated in the US and a number of other countries and the interpretation criteria promulgated. Tests with a nil of 0.7–8.0 and a TB response of 25%–50% of nil were interpreted as positive rather than as indeterminate. Also, tests with a nil of 0.7–8.0 and a TB response that is <25% of nil were interpreted as negative, whereas for QFT-G they were interpreted as indeterminate (26, 47).

The T-SPOT.*TB* became the fourth IGRA to be approved by FDA in 2008. In this test, peripheral blood mononuclear cells (PBMCs) are incubated with control materials and two mixtures of peptides from ESAT-6 and CFP-10. An enzyme-linked immunospot assay (ELISPOT) is used to detect increase in the number of cells that secrete IFN-γ (represented as spots in each test well) after stimulation with antigen as compared to the media control (nil) (50). The TST, QFT-GIT and T-SPOT.*TB* showed sensitivities of 95%, 91% and 84% respectively when pooled sensitivity was calculated from three studies (24, 25, 51). The largest study was carried out with culture confirmed active TB in which the estimated sensitivity of TST, QFT-GIT and T-SPOT.*TB* were 94%, 83% and 95% respectively (51).

1.8.1. Strengths and Weaknesses

In general the specificity of QFT is considered to be higher than the TST as the proteins included in the QFT are encoded within the region of difference 1 (RD1) of the *M. tuberculosis* genome and hence are more specific to *M. tuberculosis* than the PPD. These proteins are not shared with the BCG strains and many of the NTM (28). QFT is a quantitative laboratory assay and hence is not subject to the kind of bias and inter-observer errors which are seen in TST which occur during TST injection and reading of the induration (26).

In addition to the kit which is not readily available in most high TB endemic countries, the QFT requires skilled, trained technicians, and lab equipment such as incubator, calibrated pipettes, a micro plate reader and shaker. It is also prone to cross contamination (31). There are

limited published data evaluating IGRA performance in children. Compared to adults the IGRA performance in children is less well understood since there are very few studies which provide separate results for children and adults and even fewer, segregate results by narrow age-categories. Second, the frequency of indeterminate IGRA results in children vary greatly from 0-17% and also vary greatly between different IGRA tests (52-56). Although low IFN-γ is produced in response to mitogen for majority of the indeterminate results, the reason for this low response in children is unclear. It is thought that lack of immunological maturity in young children may lead to the lower IFN-γ produced in response to mitogen. The indeterminate results can also be affected by the differences in the concentration of TB antigen and mitogen used for stimulation and differences in interpretation criteria especially, in case of different IGRA tests. There is also a concern regarding the low sensitivity of the IGRAs when compared to the TST (24, 57).

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1.8.2. QFT and TB infection/TB disease

1.8.2.1. QFT and TB infection

In four studies which involved individuals unlikely to have TB infection, the pooled specificity was calculated. For QFT-GIT pooled specificity was found to be 99% and for TST was found to be 85% (24, 58-60). The low specificity for the TST may be due to false positive TST results following BCG vaccination or exposure to NTM.

1.8.2.2. QFT and TB disease

In a study in Germany which involved 601 individuals with culture confirmed active TB, the QFT-GIT was reported to perform better than the TST (at a cut-off of 5 mm) in predicting subsequent active tuberculosis in untreated contacts. Out of 219 contacts that had an induration of ≥5mm, five progressed to disease whereas out of 41 contacts that had positive QFT-GIT, six progressed to TB disease (61).

In a study in Australia, QFT was positive in 83% cases in individuals with proven active disease, 59% in those treated with TB previously and 80% in those exposed but who were TST negative (41). In two studies involving children with culture confirmed active TB QFT showed a sensitivity of 93% and 80% respectively (24, 25). Many studies have been performed using the QFT in a number of countries in the world and depict very promising results. All of these suggest that the QFT has a higher specificity than the TST (24, 25).

1.8.3. Indian scenario

There are very little data published on QFT studies performed among children in India. In a study in India, 105 children suspected of TB or had a history of contact with TB were admitted to the hospital for the diagnosis of TB (56). The agreement (κ=0.73) between QFT-GIT and TST was good. BCG immunization was not associated with positivity of either of the tests. However, the agreement between the two tests are likely to be variable in different childhood populations (51, 52) with varying socio-demographic and nutritional characteristics.

1.9. Comparison between TST and QFT

The TST and QFT are aids in diagnosing TB infection. These tests can be used to identify individuals who are likely to benefit from a treatment which includes individuals who are or will be at increased risk to TB infection or may progress to active disease if infected (26).

In two reports of a TB contact investigation, greater recent exposure which was measured by infectiousness of the source and duration of exposure was more strongly associated with positive QFT results than the TST. This suggests that QFT may be better than a TST in detecting any recent infection. In the same studies, individuals with a lower amount of exposure were more likely to be TST positive than QFT positive which suggests that TST may be better than QFT in detecting remote infections (27, 62) – or that the TST generated more false positive results. However, in two studies neither of the tests showed any difference in the proportion of children with recent and remote exposure to active TB (63, 64). This may, in part, be due to a small sample size.

In a study including 28 children between 4 months to 7 years of age, the sensitivities for TST and QFT-GIT were 100% and 93% respectively. These children had active TB which was confirmed by culture (24). In a similar study among 25 children the sensitivity of TST at ≥10mm was 88% while that for QFT-GIT was 80%. However, when children with probable TB (which was defined based on the clinical, epidemiologic and radiographic findings but absence of positive culture) were included, the sensitivity for TST at ≥10mm dropped to 71% (25).

In studies comparing the TST to QFT in children, the agreement between TST and QFT is variable and varies between 0.17-0.86.(24, 53-56, 65-71). However, the results cannot be compared across studies since the objectives of the studies were different and hence they followed different inclusion and exclusion criteria and were designed differently. The concentration of tuberculin used was different across the studies and was obtained from different manufacturers. This could lead to variations in the TST results. Some studies used QFT- G (66, 72) while others used QFT-GIT (24, 67) while one used both (73). It is also difficult to pool the sensitivities of the QFT and TST due to these variations. Moreover, the TST and QFT reading vary across different studies, being affected by the source of population, criteria for the interpretation of tests, prevalence of infection and proportion of infections confirmed by smear and culture, estimates of recent and remote exposure, recent TST performed, BCG vaccination and co-infection with other diseases like NTM disease and HIV. A recent meta-analysis by Machingaidze *et al* showed that for diagnosing active TB disease in children, the sensitivity of QFT was reduced in TB high-burden countries as compared to low-burden countries (74). There is little published data from India comparing the TST and QFT in children, moreover, these studies are limited by their small sample sizes (56). Hence, in India we need studies which include a larger sample size of children of the younger age group especially children ≤ 3 yrs as this age group is at a higher risk of progressing to TB disease.

1.10. The challenges faced

The most common type of TB in children is smear negative pulmonary TB. The smear negativity is, in part, due to the fact that it is difficult to obtain sufficient sputum sample from children for smear microscopy. Moreover, cavitating TB is infrequent in children (6).

In India the available literature on IGRAs show that IGRAs have a better specificity than the TST which is carried out routinely in the population. The first study performed to evaluate the QFT tests in India was conducted in rural India by Dogra *et al* (56), but was limited by its small sample size.

Thus, pediatric TB which, until recently, remained a neglected portion in the field of TB continues to be a diagnostic challenge. There is a need to develop and evaluate newer diagnostic tools to improve the detection of cases of TB. IGRAs have shown promising results in some studies (29, 75-77) but, there are very few published data available on their diagnostic performance in children and infants. The studies which are available suggest that the IGRAs

may be feasible and in some cases may also prove clinically useful in the evaluation of TB in children.

2.0. RATIONALE

Pediatric TB continues to remain a diagnostic challenge. Due to the paucibacillary nature of the infection and difficulty in obtaining a sputum sample in children, *M. tuberculosis* often goes undetected in smear and culture in children. Moreover, treatment is started on the suspicion of TB. Thus, in the absence of a gold standard, an alternative test is necessary to diagnose TB infection and TB disease and distinguish the two. The TST is generally used as an aid in diagnosing TB infection in children <5 years of age though it is known to have its limitations. Gopi *et al* (33) have shown that TST was not affected by BCG. QFT-GIT has also been used worldwide to diagnose TB infection. There is only one study from India (56) which compares the TST with QFT in children for the detection of TB infection. A greater number of studies are required to determine the diagnostic utility of the QFT-GIT for the diagnosis of TB infection and disease in children in India. Given the high prevalence of under nutrition in children <3 years of age in India and the fact that there are some data that TST is affected by under nutrition, an alternative test that performs reasonably well in immunocompromised states (including that associated with under nutrition) would be useful. The QFT-GIT has been shown to perform better than the TST in immunocompromised states, including HIV. This study explores the utility of the TST and QFT-GIT in a typical rural population in Southern India, with a relatively high burden of under nutrition.

3.0. OBJECTIVES

3.1. General Objectives

To contribute to the diagnosis of TB infection and disease in young children in India, a high TB burden country.

3.2. Specific Objectives

a) To compare and evaluate the TST and QFT-GIT for the diagnosis of TB infection and disease in young children in Southern India.

b) To determine the extent to which TST / QFT-GIT positivity is associated with demographic, clinical, nutritional and mycobacteriological (other than *M.tuberculosis*) characteristics.

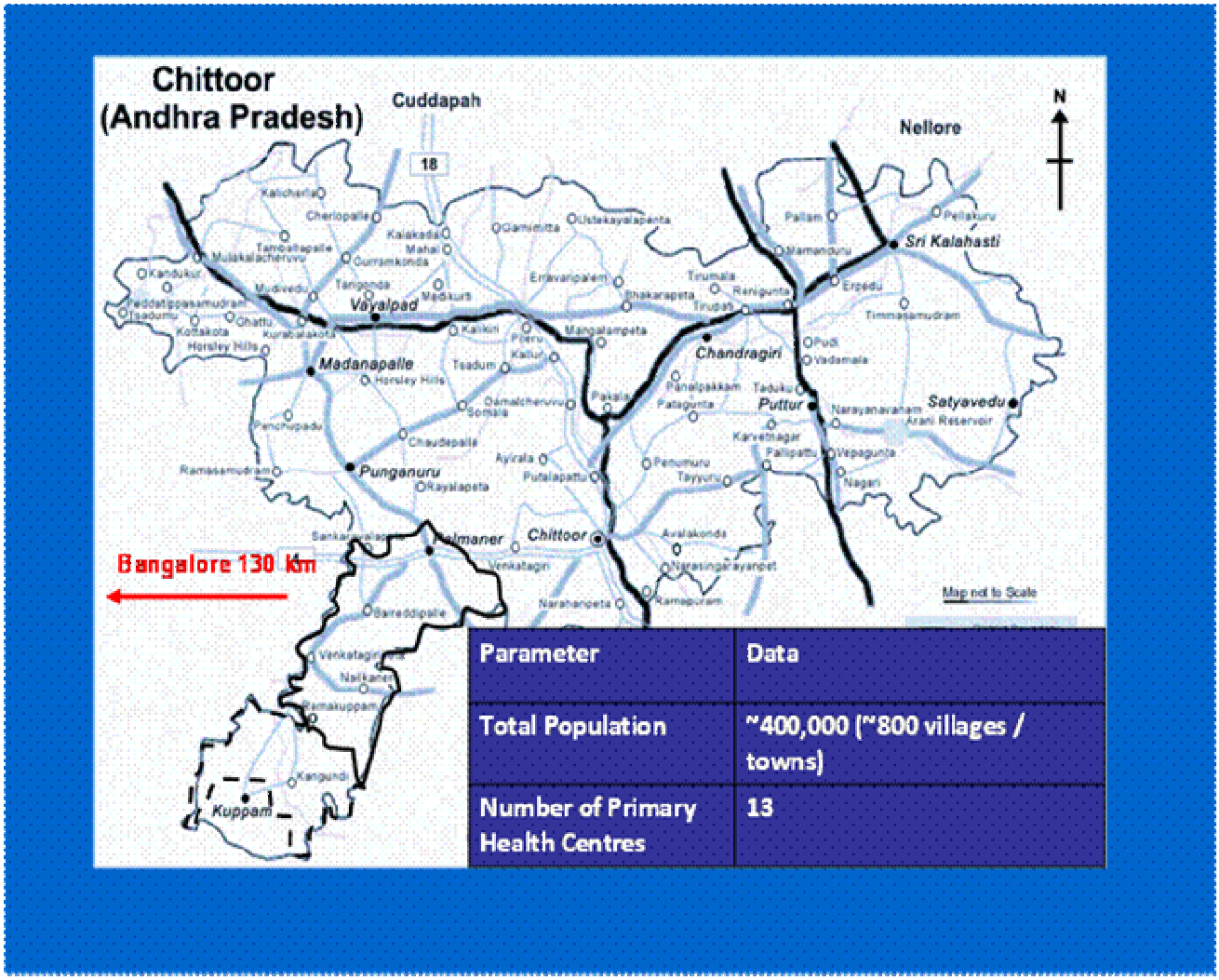
c) To determine the proportion of indeterminate test results in the QFT-GIT test.

4.0. METHODOLOGY

4.1. Study site

Palmaner (Palamaner) taluk is situated in Chitoor district (Figure a), Andhra Pradesh in Southern India. It is located at 3.200^0 N and 72.7500^0E. It is elevated at 683 m above sea level.

Figure a. *Map of study area*



The Palmaner taluk is further divided into smaller units called “mandals”. In our study we included 7 mandals from Palmaner taluk and the neighbouring Kuppam taluk. The 7 mandals comprised of 550 population units. These 550 population units covered a population of 400,000.

4.2. Study design

The present analysis is nested within a prospective cohort study carried out in infants enrolled within 14 days of date of birth and followed up for 2 years in Palamaner taluk in Andhra Pradesh, Southern India.

4.3. Study population

Infants less than 14 days of age and vaccinated with BCG within 72 hours of birth, born to mothers who were permanent residents of Palamaner taluk or in case of migration, who could provide contact information for the purpose of further follow ups were recruited and followed up for 2 years. These infants were further assigned to the active (bimonthly home visit to check for recent contact to TB, symptoms and anthropometry) or passive follow up surveillance group (TB education given but with no scheduled periodic home visits).

4.4. Selection and screening of the participants

The pregnant women participating in the study were identified antenatally, most often in the second trimester by the study staff working in the community and followed up through their pregnancy. They were informed about the study during the post-natal period either while they were still admitted for delivery or at home after they had been discharged. Newborns were eligible for entry into the study if BCG vaccine was administered within 72 hours of birth and parental informed consent obtained within 14 days of birth.

Following informed consent the infants were assigned to active and passive surveillance groups and followed up for two years after birth.

At the time of enrollment the mother of the child was provided a child health card, which helped the mother to track important health information such as vaccinations received. All mothers were asked to contact the study office if the infant or child was diagnosed with TB or if the child developed any symptoms suggestive of TB or a recent exposure to TB disease. Clinicians and others who would know of a death of an infant were asked to contact the study office in case of deaths of infants enrolled in the study. All participants were provided with a Study Identification Card and were asked to show it to their treating physician. The card had the subject study ID number and the study physician contact details.

Children with either symptoms suggestive of TB, failure to thrive (FTT) or contact with a TB case were referred to the case verification ward. Information on the infant's birth weight, date of vaccination, name of the family doctor were obtained from the clinical records.

4.5. Inclusion and exclusion criteria

4.5.1. Inclusion criteria

All the participants had to meet the following criteria at the time of enrollment.

1. Vaccinated with BCG within 72 hours of birth
2. Enrolled within 2 weeks of date of birth

4.5.2. Exclusion criteria

The subjects were not included in the study if they didn't meet the following criteria at the time of enrollment.

1) Parent/guardian declined to provide informed consent
2) The entire family planned to move out of Palamaner taluk area within the next 2 years

4.6. Baseline evaluations

The following information was collected from clinical records and/or by interview

a) Infant's birth (weight, gender)
b) BCG vaccination
c) Demographic characteristics
d) Socio-economic data (parental education, type of house and cooking fuel)

4.7. Diagnosis of TB and case verification

Infants who had symptoms suggestive of TB, FTT (based on any of the following criteria after evaluation of the weight for age growth chart; a)loss of weight or no gain in weight over two consecutive visits, b) Crossing of two centile lines, downwards on growth chart, c) weight consistently remaining below the third percentile line) and/or a recent contact with TB were admitted and further evaluated in a diagnostic ward (Figure b) at Emmaus Swiss Hospital which is referred to as the "Case verification ward" (CVW).

On admission, a TST was performed by a trained health care worker/nurse/doctor. This was read 48 hours later prior to discharge of the infant.

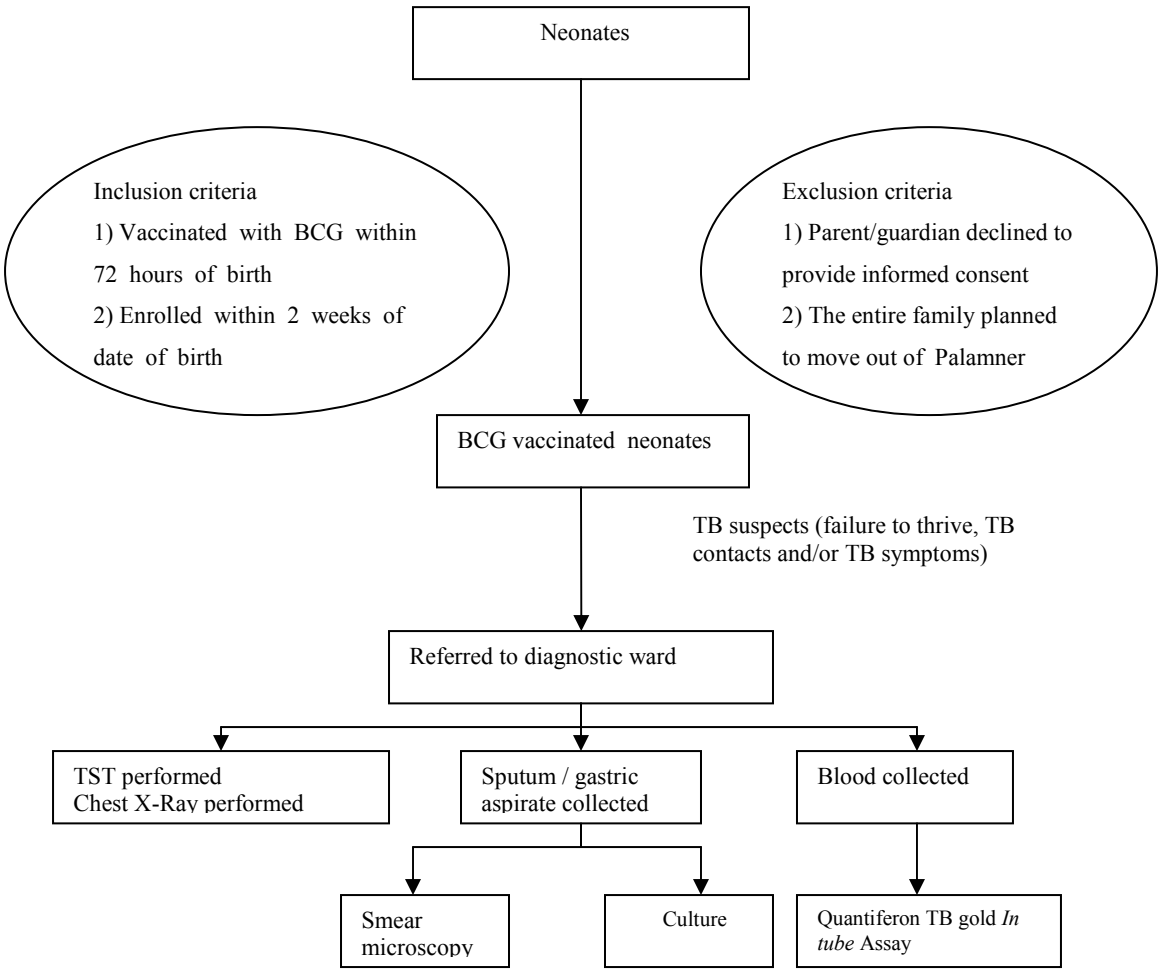
A blood sample was also taken at admission, out of which 3 ml was apportioned to QFT-GIT assay. Blood was drawn for other clinical tests as well. The blood drawn during a diagnostic visit did not exceed 8 ml. A heel prick was done and hemoglobin estimated prior to drawing the blood. The total volume of blood drawn or the decision not to draw blood was based on the treating physician's assessment and the hemoglobin (Hb) report. Blood was not drawn if the Hb was below 10 grams/deciliter. A chest radiograph (AP view) was also obtained during the admission.

The next morning a gastric aspirate wash was collected and induced sputum obtained for smear and culture. The same procedures were repeated on the subsequent day.

The sputum samples were digested and decontaminated using the N-acetyl L-cysteine (NALC)-sodium hydroxide method. The induced sputum and gastric aspirate (collected in 50 ml tubes) were diluted with 0.5% NALC dissolved in sodium hydroxide (4%)-sodium Citrate (2.3%) solution in a ratio of 1:1. The tubes were incubated for 15 min at room temperature with intermittent swirling. At the end of incubation Phosphate buffer saline (PBS [pH 6.8]) was added up to the 45 ml mark on the tube. The tubes were then centrifuged at 3050 relative centrifugal force (*rcf)* for 15 minutes at 4^0C. After centrifugation, the supernatant was discarded and the sediment was resuspended using 1.5ml-2ml of the PBS. 300 µl of the sediment was inoculated onto Lowenstein Jensen (LJ) media and 500 µl into the Mycobacterial Growth Indicator Tube (MGIT) respectively. A smear was also simultaneously prepared on a glass slide for smear microscopy (Figure 2). These slides were heat fixed and stained with auromine O dye, destained using acid alcohol and counterstained with potassium permagnate. The slides were then observed under the fluorescent microscope.

The LJ and MGIT were followed up for 8 weeks. Samples which showed growth on LJ or MGIT were classified as "positive for culture" after confirmation by Ziehl-Neelsen staining. For Zeihl-Neelsen, the heat fixed slides were stained with concentrated carbol-fuchsin filtered through a Whattmann No. 1 filter paper and allowed to stand for 5 minutes. The slides were gently heated using a spirit lamp intermittently. The slides were destained using 25% sulphuric acid for 3 minutes. The slides were then counterstained with methylene blue. After each step the slides were rinsed with distilled water. Once the slides were dry they were observed under the light microscope. A positive and negative control slide was included in every batch

Figure b. Flow chart for the neonatal cohort study



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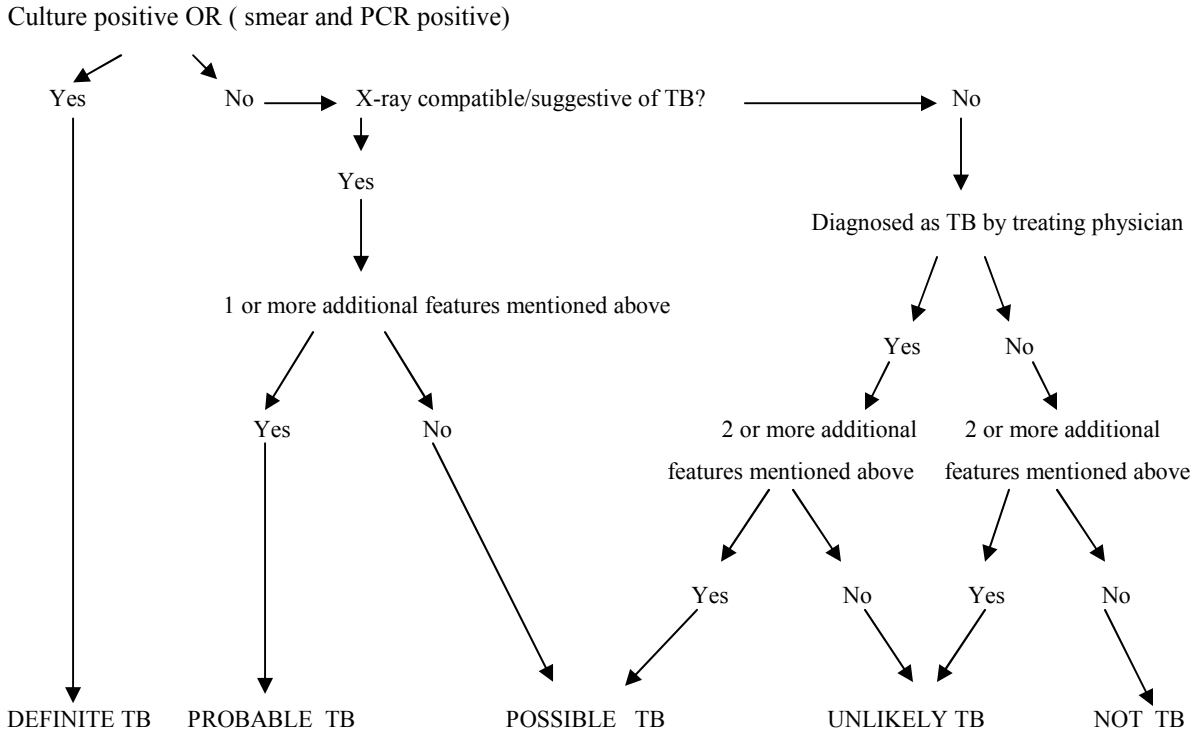
All positive cultures were speciated using the HAIN MTBC kit (78) to exclude NTM and BCG disease. For those who were culture negative but who had x-ray findings suggestive of TB, direct PCR on induced sputum or gastric aspirate samples were done using the Roche real time PCR kit (79). All subjects who were sputum positive were put on treatment.

A diagnostic algorithm was used to classify patients as 'definite' TB, 'probable' TB, 'possible' TB, 'unlikely' TB and 'Not' TB (Figure c).

Figure c. Diagnostic algorithm for TB classification of children referred for a diagnostic workup

1. Mantoux ≥ 10 mm
2. Cough > 2 weeks
3. FTT or recent loss of weight (rLOW)
4. Recent history of contact with a TB case



4.8. Tuberculin skin testing

The TB suspects received a TST of 2 TU (Span Diagnostics Ltd, India) which was administered by a trained health care worker/nurse/doctor. The test was read after 2 days. An induration of ≥10mm was used as a positive cut off for TST.

4.9. Sample collection

4.9.1 Blood

Blood was collected on the day of admission. One ml of blood was collected in each of the TB antigen, nil antigen and mitogen tubes for QFT-GIT respectively.

4.9.2 Gastric aspirate and induced sputum

The gastric aspirate and induced sputum samples were collected on 2 consecutive days, in the fasting state, early in the morning. For the gastric aspirate, an infant feeding tube was inserted into the stomach of the child and the contents were aspirated and collected in a 50 ml tube containing 10% sodium carbonate. This was followed by a stomach wash using normal saline (0.9% sodium chloride). For the induced sputum, nebulization was performed with hypertonic saline and the secretions following cough were collected using an infant mucous extractor into a 20 ml tube.

4.10. Sample transport and receipt

The gastric aspirate and induced sputum samples were transported using ice packs to maintain the temperature at approximately 4^0C. The samples were received by the laboratory personnel. The details namely participant identification number (PID), age, gender and type of sample (gastric aspirate or induced sputum) were entered in a register and a Lab ID was assigned.

4.11. QuantiFERON TB Gold In tube assay

4.11.1. Principle

The QFT-GIT uses specialized blood collection tubes, which are used to collect whole blood. Blood is incubated for 16-24 hours, after which, plasma is harvested and tested for the

presence of IFN-γ produced in response to the peptides of the antigens ESAT-6, CFP-10 and TB 7.7.The assay was performed according to the instructions provided in the kit (31).

4.11.2. Materials provided

1) Nil control, TB antigen and mitogen Control tubes
2) Microplate strips
3) Lyophilized Human IFN-γ standard,
4) Diluent, Lyophilized conjugate (100X concentration)
5) Wash buffer (20X concentration)
6) Enzyme substrate and enzyme stopping solution.

4.11.3. Materials required but not provided

1) 37^0C incubator, calibrated pipettes
2) Microplate shaker, washer and reader

4.11.4. Procedure

4.11.4.1. Sample Collection and Handling

1) 1 ml of blood was collected in each of the three tubes provided with the kit. The tubes supplied were:

a) The nil control tube with no antigens added, serving as a negative control
b) The mitogen tube - which served as a positive control and as an inbuilt control for the correct handling of the blood samples and incubation. It also provided information about the individual's immune status.
c) The TB antigen tube - coated with ESAT-6, CFP-10 and TB 7.7 antigen.

2) The tubes were mixed by shaking vigorously up and down 10 times to ensure that the entire inner surface of the tube had been coated with the blood.

3) The tubes were then transported to the lab and handed over to the lab personnel.

4) The tubes were shaken vigorously again and incubated at 37^0C for 16-24 hours.

5) After incubation the tubes were centrifuged at 2000 *rcf* for 15 minutes.

6) The plasma (supernatant) was then transferred to appropriately labeled vials and stored at

-20^{0}C. They were later shipped from the field laboratory to the laboratory in St. John's Research Institute, Bangalore in cryoshippers where the QFT-GIT assay was performed.

4.11.4.2. Assay protocol

1) The supernatants (plasma) from the QFT-GIT tubes and the reagents, except the conjugate were brought to room temperature (22^{0}C ± 5^{0}C) prior to testing.

2) 28 samples were processed in a batch. The batch number and date of assay was noted down.

3) A sample template was prepared before performing the assay to note and add the samples in the respective wells.

4) The freeze dried kit standards were reconstituted with distilled water as mentioned on the vials.

Reconstitution of the standard to the stated volume produced a solution with a concentration of 8.0 IU/mL

5) Preparation of standards:

a) Four tubes were taken and labeled as S1, S2, S3 and S4.

b) 150µl of the green diluent was added to S1 and 210 µl to S2, S3 and S4 respectively.

c) 150 µl of the kit standard was added to S1 and mixed thoroughly.

d) 70 µl was transferred from S1 to S2 and mixed thoroughly.

e) 70 µl was then transferred from S2 to S3 and mixed thoroughly

f) The green diluent alone in S4 served as the zero standard.

g) Thus S1 contained 4IU/mL, S2 1 IU/mL, S3 0.25 IU/mL and S4 contained 0 IU/mL respectively. The standards were assayed in triplicates.

6) The freeze-dried Conjugate 100X concentrate was reconstituted with distilled water as mentioned on the vial.

7) Working strength conjugate was prepared by diluting the required amount of reconstituted conjugate 100X concentrate in green diluent to obtain a final concentration of 1X and was prepared according to the number of strips used.

8) Prior to the assay, plasma was mixed to ensure the even distribution of IFN-γ.

9) 50 µl of the freshly prepared working strength conjugate was added to the required ELISA wells using a multichannel pipette.

10) 50 µl of the test plasma was added to the appropriate wells using a multichannel pipette according to the template prepared. Finally 50 µl of the standards 1 to 4 were added in triplicates.
11) The conjugate and plasma samples/standards were mixed thoroughly for 1 minute using a microplate shaker.
12) The plates were covered with a lid and incubated at room temperature ($22^0C \pm 5^0C$) for 120 minutes ± 5 minutes.
13) During the incubation period wash buffer, 20X concentrate was diluted to prepare a working solution of final concentration of 1X. At the end of the incubation period the wells were washed with 400 µl each of the wash buffer for 6 cycles with a 5 second soak time.
14) Then 100 µl of the enzyme substrate solution was added to each well and mixed thoroughly using a microplate shaker.
15) The plate was covered with a lid and incubated at room temperature ($22^0C \pm 5^0C$) for 30 minutes in the dark.
16) Following the 30 minute incubation, 50µl of the enzyme stopping solution was added to each well and mixed thoroughly using the microplate shaker.
17) The Optical Density (OD) of each well was measured within 5 minutes of stopping the reaction using the microplate reader fitted with a 450nm filter and a 620nm-650nm reference filter. OD values were used to calculate the results.

4.11.5. Interpretation of results

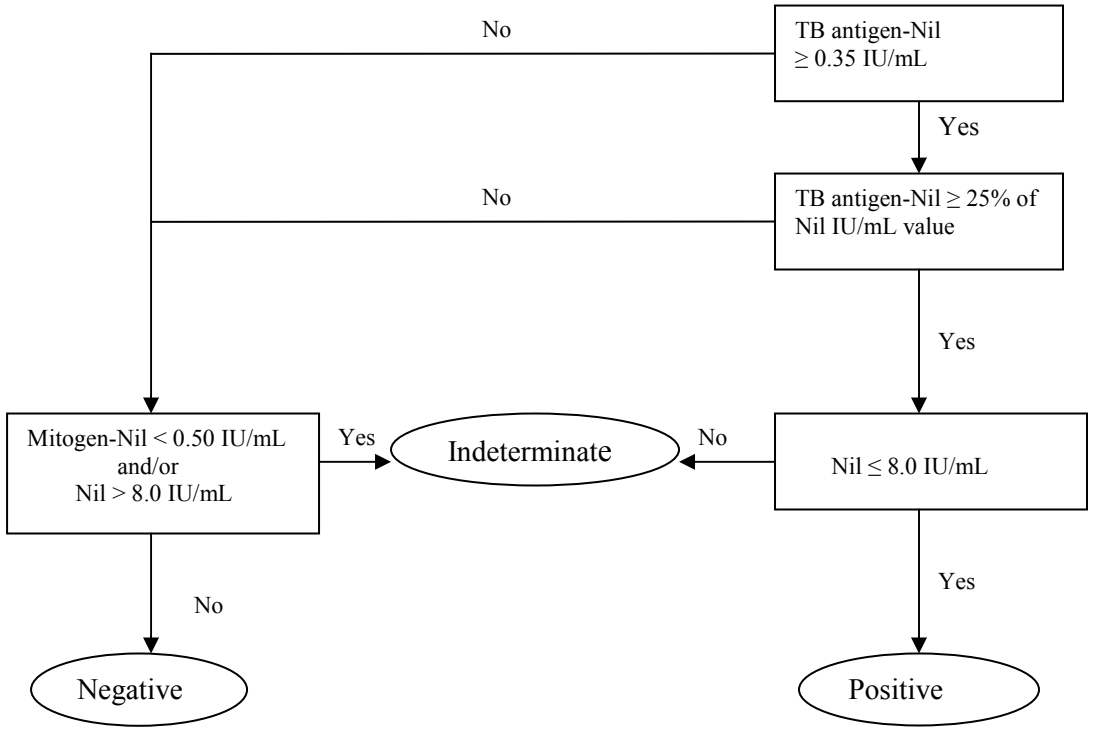
The results were interpreted using the following criteria provided by the manufacturer as shown in Table b and Figure c below.

Table b. Interpretation of results when nil, TB antigen and mitogen tubes are used

Nil (IU/mL)	TB antigen minus Nil (IU/mL)	Mitogen minus Nil (IU/mL)	Quantiferon-TB (IU/mL)	Report/ Interpretation
≤ 8.0	<0.35	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and <25% of nil value	≥ 0.5		
	≥ 0.35 and ≥25% of nil value	Any	Positive	*M. tuberculosis* infection likely
	< 0.35	< 0.5	Indeterminate	Results are indeterminate for TB antigen response
	≥ 0.35 and <25% of nil value	< 0.5		
>8.0	Any	Any		

Adapted from QFT-GIT kit insert (31)

Figure c. Interpretation flow diagram when nil, TB antigen and mitogen tubes were used according to manufacturer's instructions (31)



Adapted from the QFT-GIT kit insert (31)

As mentioned by the manufacturer, diagnosing or excluding TB disease and assessing probability of latent TB infection (LTBI), requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting the results. The standard curve was plotted using the TB QuantiFERON Software provided by Cellestis.

4.12. Data management and analysis

4.12.1. Data entry and documentation

The clinical research forms (CRF's) were used to capture the data generated in the diagnostic process and the laboratory investigations. The medical staff maintained a source document into which all the data pertaining to the study were included which comprised of both the medical report and the laboratory report. A requisition form was maintained by the medical officer and a laboratory data collection form was maintained by the laboratory staff. Good clinical practice (GCP) guidelines were followed for entry of the data into the documents/forms and for rectifying the entered data. The data were simultaneously entered into a database and the hard copies were archived.

4.12.2. Data analysis

Patient information was entered in the EpiInfo software and the data was analyzed using PASW program version 18.0. Binary logistic regression was used for the analysis. The indeterminate QFT results were considered as negative in the statistical analysis. All the binary categorical variables were coded as 0 and 1. The string variables were converted to numerical variables for statistical analysis. Univariate analysis was performed to identify individual factors which were significantly associated with TST and QFT-GIT positivity during the first diagnostic visit and was restricted to those who had both TST and QFT results. In a multivariate model, age, gender, education of the father (surrogate of the socio-economic status), cough>2 weeks (surrogate of symptoms), contact (surrogate of exposure to TB), FTT (surrogate of nutritional status) and isolation of NTM in any gastric and/or induced sputum sample were included. A p-value of <0.05 was considered significant. Kappa value (κ) was used to express the concordance between the QFT-GIT and TST.

4.12.3. Anthropometric data analysis

Taking age and gender into consideration, the differences in measurements were expressed as standard deviation or Z score units. The international nutrition community commonly uses the Z-score since "using Z-scores allows us to identify a fixed point in the distributions of different indices and across different ages and useful summary statistics can be calculated from them" (80).

Z-score is defined as the difference between the value for an individual and the median value of the reference population for the same age or height, divided by the standard deviation of the reference population (80)

This can be written in equation form as:

$$\text{Z-score (or SD-score)} = \frac{\text{(Observed value) – (Median reference value)}}{\text{Standard deviation of reference population}}$$

The cut-off used for Z score was -2 standard deviation. Thus a child with a Z-score <-2 for weight-for-age was considered underweight; a child with a Z-score of <-2 for weight-for-height was considered as wasting and a child with a Z-score of <-2 for height-for-age was considered stunted.

4.13. Confounders

The number of repeated visits to the CVW for the same child could influence the TST outcome. Hence the data was segregated according to the number of visits and analyzed.

Since TST was performed on all the participants at the end of two year follow up period as part of the study design and since all with a TST ≥10mm were referred to the CVW, there were a higher percentage of children of the higher age group with a TST positive result referred to the CVW. Thus, while it is plausible that TST positive results are more common in older children, because of greater exposure to TB, in this study it was also related to the study design.

4.14. Ethical Considerations

The study was conducted according to the Declaration of Helsinki, Protection of human subjects (21 CFR 50), Institutional Review boards (21 CFR 56) and Obligations of Clinical Investigations (21 CFR 312) as well as the Indian Council of Medical Research Guidelines, 2006. The protocol and informed consent form, and all protocol amendments were reviewed and approved by the Institutional Review Board (IRB) and Independent Ethics Committee (IEC) of the AERAS global TB vaccine foundation and St. John's Medical College prior to any procedures being conducted. The study was also approved by the Ministry of Health Screening Committee of the Government of India. Written informed consent was obtained from each subject's legally authorized representative prior to any procedures being conducted.

Confidentiality was maintained by using coded numbers and initials to identify the laboratory specimens, source documents, study reports etc. All records were maintained in a secure archive with access available only to designated study personnel.

5.0. RESULTS

There were 4,382 children enrolled in this study. Of these, 2,215 were included in the active surveillance and 2,167 were included in the passive surveillance group. Seven hundred and forty six participants were referred to the TB CVW as described below: 638 (85.5%) were from the active surveillance group and 108 (14.5%) from the passive surveillance group. Out of the 746, 53 came for a second visit (45-active surveillance, 8– passive surveillance), three (two-active surveillance, one- passive surveillance) for a third visit and one (active surveillance) for a fourth visit.

5.1. Referral to the Case Verification Ward (CVW)

5.1.1. First visit

The children were referred to the CVW based on either symptoms suggestive of TB, failure to thrive (FTT) and/or a recent contact with an index case with TB disease. In our study there were 62.6% (467/746) admitted to the CVW based on FTT alone, 11.9% (89/746) based on symptoms alone and 5.6% (42/746) based on contact alone. 19.3% (144/746) were admitted based on either two of the above-mentioned criteria and 0.6% (4/746) based on all three i.e. FTT, symptoms and contact.

5.1.2. Second visit

During the second visit, 54.7% (29/53) were admitted based on FTT alone, 1.9% (1/53) based on symptoms alone and 5.7% (3/53) based on contact alone. 35.8% (19/53) were admitted based on either two of the above-mentioned criteria and 1.9% (1/53) based on all three.

5.1.3. Third visit

During the third visit, one child was admitted based on FTT, one based on symptoms and one based on FTT and symptoms.

5.1.4. Fourth visit

During the fourth visit, there was only one child referred to the CVW and that was based on both symptoms and contact.

5.2. Characteristics of the participants referred to the CVW

Out of the 746 children referred to the CVW (first visit only), both TST and QFT-GIT was performed on 709 children. The baseline characteristics of these 709 children are given in Table 1. Approximately two thirds (66.3%) of the children were ≥12 months of age. The proportion of parents (mothers; 70.7%, fathers; 81.6%) that were literate (with an education of primary school or greater) was higher as compared to those that were illiterate (mothers; 29.3%, fathers; 18.4%). Nearly half (49.9%) of the children were under weight (WAZ), 43.2% wasting (WHZ) and 26.6% stunted. Data were missing for two children for WHZ and one child for HAZ as anthropometry was not done. Approximately two thirds (76.6%) of the children had FTT. Chest X-ray was negative in 97.7% of the children; 1.7% had abnormal X-rays suggestive of TB and 0.6% had other radiological abnormalities.

5.3. Characteristics of the definite and probable cases of TB

There were totally four definite cases of TB and nine cases of probable TB. Out of these 13 cases, three of those with definite TB were both TST and QFT-GIT positive. In these 3 children, the IFN-γ response to the mitogen was high (> 10 IU/mL) as also to the TB antigen (>8.5 IU/mL). The fourth participant diagnosed as a definite case of TB had a TST of 9mm and

was negative for QFT-GIT with an IFN-γ response to the TB antigen of only 0.26 IU/mL. In addition, 13 participants who were smear negative but culture positive had invalid results with the HAIN-MTBC kit such that the universal control and genus control bands were negative. These samples are being reassessed and may have implications for the identification of other genotypically confirmed TB cases. There was one participant of the 9 diagnosed as 'probable TB' (PID-012-0009) with a TST of 16mm who was negative for QFT-GIT. None of the 'probable TB' cases had IFN-γ responses to the TB antigens that were any where near as high as the three who had 'definite TB' and were QFT-GIT positive (Table 2).

5.4. TST and QFT-GIT results for the definite, probable, unlikely and not TB

Seven hundred and twenty nine participants were classified as 'definite TB', 'probable TB', 'unlikely TB' and 'not TB'. The diagnosis was unavailable for the remaining participants because of incomplete data.

a. Definite TB: There were two cases of definite TB diagnosed during the first visit and two during the second visit. Three of these were negative for smear but showed growth on culture (LJ) and was confirmed by speciation using the HAIN kit. These three were also positive for both TST and QFT-GIT. One case was positive on smear microscopy and was confirmed by speciation using the Roche real time kit. This case was negative for TST and QFT-GIT (Table 3).

b. Probable TB: There were eight cases of probable TB diagnosed during the first visit and one during the second visit. Of these one was positive for TST but negative for QFT-GIT while the rest were negative for both (Table 3).

c. Unlikely TB: There were 126 diagnosed as "unlikely TB" during the first visit and eight during the second visit. Out of the 126, 11 were positive and 85 were negative for both TST and QFT-GIT. 30 were positive for either TST or QFT-GIT. Out of the 8, 2 were positive and 6 were negative for both TST and QFT-GIT. (Table 3)

d. Not TB: 552 were classified as "not TB" during the first visit and 30 during the second visit. Out of the 552, 5 were positive and 509 were negative for both TST and QFT-GIT. Thirty eight were positive for either of the two tests. Out of the 30, 2 were positive and 25 were negative for both TST and QFT-GIT. Three were positive for TST but negative for QFT-GIT (Table 3)

5.5. TST and QFT-GIT positive

In our study 10.2% (n=72/709) were TST positive and 5.8% (n=41/709) were QFT positive.

5.6. Association of the clinical, demographic, nutritional and mycobacteriological (excluding M.tuberculosis) factors with TST and QFT-GIT

5.6.1. TB exposure

5.6.1.1. Association with TST

Children with a recent history of contact with TB (taken as a pre-requisite for TB infection) had significantly increased odds of being TST positive (OR 2.11 [95% CI 1.07; 4.17], p=0.03) in a univariate analysis (Table 4). In a multivariate analysis the association was statistically not significant (OR 1.58 [95% CI 0.69; 3.63], p=0.27) (Table 5).

5.6.1.2. Association with QFT

In a univariate analysis, the odds of children with a recent history of contact with TB being positive for QFT-GIT were increased (OR 3.46 [95% CI 1.50; 7.81], p=<0.01) (Table 4) and remained so in a multivariate analysis (OR 4.36 [95% CI 1.79; 10.54], p=0.01) (Table 5).

The odds of children with recent history of contact with TB being positive for combined TST and QFT-GIT was increased (OR 7.08 [95% CI 2.51; 19.65], p=<0.01) (Figure 1)

5.6.2. Demographic and Nutritional factors

5.6.2.1. Association with TST

In a univariate analysis children with wasting based on the weight for height Z score (OR 0.31 [95% CI 0.17; 0.56], p= <0.01) and whose fathers were illiterate (OR 0.37 [95% CI 0.15; 0.88], p=0.03) had reduced odds of being TST positive (Table 4) The odds of being TST positive were also reduced for children who had FTT (OR 0.19 [95% CI 0.12; 0.33], p= <0.01] (Table 4).

In a multivariate analysis, the odds of children with FTT (OR 0.14 [95% CI 0.07; 0.26], p= <0.01) being TST positive was reduced (Table 5).

5.6.2.2. Association with QFT-GIT

The FTT (OR1.09 [95% CI 0.49; 2.52], p=0.82) was not significantly associated with QFT-GIT positivity in a univariate (Table 4) or multivariate analysis FTT (OR 1.58 [95% CI 0.64; 3.87], p=0.33) (Table 5). None of the demographic factors were associated with QFT-GIT positivity in a univariate (Table 4) or in a multivariate (Table 5) analysis.

5.6.3. NTM

5.6.3.1. Association with TST

The odds of children with atleast one NTM isolated from culture (OR 0.47 [95% CI 0.24; 0.92], p=0.03) being TST positive was reduced (Table 4). This association continued to be significant in a multivariate analysis (OR 0.44 [95% CI 0.21; 0.93], p=0.03) (Table 5).

5.6.3.2. Association with QFT-GIT

The odds of children with atleast one NTM isolated from culture (OR 1.20 [95% CI 0.56; 2.54], p=0.61) being QFT-GIT positive was not significant (Table 4). This association was also not significant in a multivariate analysis (OR 1.21 [95% CI 0.58; 2.52], p=0.62) (Table 5).

5.7. Agreement between QFT-GIT and TST

The agreement between TST (cut off ≥10mm) and QFT-GIT was fair [κ 0.31(95% CI 0.19; 0.40), p= <0.01] when all participants were included in the analysis. At a cut off of ≥ 5mm for TST poor agreement was seen [κ 0.09(95% CI 0.04; 0.11), p=<0.01]. When the analysis was restricted to those with a history of contact with TB, the agreement between TST (cut off ≥10mm) and QFT-GIT was good [κ 0.68(95% CI 0.39; 0.82), p= <0.01] and at a cut off of ≥ 5mm for TST was fair [κ 0.23 (95% CI 0.08; 0.22), p=0.01] (Table 6)

5.8. Characteristics of children with Indeterminate QFT-GIT results

There were 26 participants with an indeterminate QFT-GIT result accounting for 3.6% (26/718) of the total QFT-GITs performed. When these were compared with children who had either a positive or negative QFT-GIT, the group with indeterminate test results had a higher percentage of children who were <1 year of age (84.6% Vs. 30.7%; p<0.001) and had cough >2 weeks (42.3% Vs 15.4%; p=<0.01). The reason for an indeterminate test result was due to low IFN-γ produced in response to mitogen in 24/26 (92.3%) or a high value of IFN- γ produced in the nil tube in 2/26 (7.7%).

5.9. Association of age with IFN-γ produced in response to mitogen

The IFN-γ produced in response to mitogen (phytohemagluttinin) at a cut off of ≥5 IU/ml were significantly related to age; increasing age was associated with higher values (Figure 2).

6.0 DISCUSSION

6.1. Discussion based on results

Without an accurate diagnostic test it is very difficult to measure the precise burden of childhood TB. As mentioned earlier, unlike the case of adolescents, where culture serves as a gold standard for the diagnosis of TB, it may not be the same scenario in children < 5 years of age (6). Treatment is often initiated on the suspicion of TB. Thus in the absence of a gold standard, TST serves as the only test widely used as an aid in the diagnosis or rather screening of childhood tuberculosis. However this test has its own limitations (3).

In our study we compared the TST with the QFT-GIT in young children. TST and QFT-GIT were positive for 75% (3/4) of the definite cases of TB. These three cases had a high IFN-γ produced in response to mitogen and TB antigen. Two probable cases of TB had indeterminate QFT results due to low IFN-γ produced in response to mitogen and TB antigen. However, in the rest of the participants even though the IFN-γ produced in response to mitogen was high, the IFN-γ produced in response to TB antigen was low. One of the issues in childhood TB is determining the clinical relevance of 'probable' TB cases. While the algorithm

that we used was decided *a priori*, at the start of the study, there have been several publications, some fairly recently, that have indicated that there is poor agreement between clinical scoring methods (12, 81). Further analyses are possible with other clinical scores, but the overriding issue is still the absence of a gold standard for smear and culture negative TB (81). Although our data on the IFN-γ responses to the TB antigen in bacteriologically confirmed TB is on a small sample, the particularly high IFN-γ responses to the TB antigen in 3 of 4 definite TB cases, suggests that further studies may be able to define a cutoff for TB disease which can supplement the current clinical scores in the diagnosis of bacteriologically negative TB.

Exposure *to M. tuberculosis* represented by a recent history of contact with TB (a pre-requisite for TB infection) showed an association with TST (OR 2.11 [95% CI 1.07; 4.17], p=0.03) in a univariate analysis. This association was higher for QFT-GIT (OR 3.46 [95% CI 1.50; 7.81], p=<0.01). This is similar to the results found by Arend *et al* (27) where QFT-GIT was more strongly associated with recent exposure to *M. tuberculosis* than TST. Thus children who were exposed to an infectious source were more likely to be QFT-GIT positive than TST positive. In a multivariate analysis the association between history of TB contact and TST (OR 1.58 [95% CI 0.69; 3.63], p=0.27) was lost, however the association with QFT-GIT (OR 4.36 [95% CI 1.79; 10.54], p=0.01) continued to be strong. This suggests that the association between TST and exposure to *M. tuberculosis* i.e recent history of contact with an infectious source may be affected by other factors like nutritional status. The association with a recent history of contact with TB was stronger for children who were positive for combined TST and QFT-GIT. This shows that a recent history of contact with TB is strongly associated with TST and QFT and even more strongly with positivity to combined TST and QFT-GIT (Figure 1). The stronger association between exposure *to M.tuberculosis* and combined TST and QFT-GIT positivity also suggests that a two step approach i.e. QFT-GIT test on those who have a TST positive may have some utility. When we compare the association of history of contact with TB with TST, QFT-GIT and combined TST and QFT-GIT (Figure 1), we see that the confidence intervals overlap. Thus, even though there is an increased odds in children with a history of contact with TB being positive for combined TST and QFT-GIT, the overlap in the CI may suggest that the number of positives for TST, QFT-GIT and combined TST and QFT-GIT with a recent history of contact with TB may be small. Thus, future prospective studies evaluating the two tests with more number of children with history of contact with TB may be

able to bring out the association between history of contact with TB and TST, QFT-GIT and combined TST and QFT-GIT.

Malnourished, under-weight and immuno-compromised children may give false negative TST results (23). In our study children who had FTT had reduced odds of being TST positive in a univariate (OR 0.19 [95% CI 0.12; 0.33], p= <0.01) and multivariate (OR 0.14 [95% CI 0.07; 0.26], p=<0.01) analysis. 68.5% (13/19) of the children who had a history of contact and FTT were negative for TST. This may be due to the fact that children with an FTT may have suppressed immune responsiveness. Even if they do have an TB infection, they may not be able to produce enough chemokines to cause a hypersensitivity reaction in the skin and hence may not produce any induration in the TST. Thus, it may be that as in the case of HIV (82), QFT-GIT which is unaffected by nutritional status, may have a specific role in populations with a high burden of under nutrition.

At a TST cut off of ≥10mm there was fair agreement (κ= 0.31) with QFT-GIT which was better than at a TST cut off of ≥ 5mm (κ=0.09) where the agreement was poor. When we considered children with a history of contact, the agreement between the two tests at a TST cut off of ≥10mm was good (κ=0.68), suggesting that both the tests perform well in children with a history of exposure to TB. In another study by Dogra *et al*,(56) based on hospitalized children in rural India good agreement (κ=0.73) was found between the TST (cut off ≥ 10 mm) and QFT-GIT. Although these two studies were conducted in India and on a pediatric population, they cannot be directly compared as the one by Dogra *et al* is a hospital-based study with a sample size of 105. Nonetheless, it is encouraging that the results are somewhat comparable.

Age is known to be related to response in the TST (3). However, in our study we could not relate the TST to age since the protocol required all infants with a TST ≥ 10mm to be referred into the case verification ward leading to a bias in the presentation of TST results and this was done only at study close out (i.e. 2 years) which might be expected to lead to a greater number of people being TST positive at 2 years than at earlier ages compared to those who received a TST only after referral into the CVW. Thus, while it is plausible that increasing age is associated with a positive TST, our study design does not allow us to specifically test this association.

In our study, children with NTM were less likely to be TST positive suggesting that NTM may not have had an effect on the TST. This observation, is similar to that seen in another study in Gambia which showed that during early life, TST is not affected by NTM

(83). Atleast one NTM isolated on culture did not have association with QFT-GIT positive. The antigens used in QFT-GIT i.e ESAT-6, CFP-10 and TB 7.7, are present in three NTM, namely *M. kansasii, M. szulgai* and *M. marinum*. In our study, we isolated *M. kansasii* from three children all of whom had a negative QFT. The significance of QFT positivity and NTM isolation in clinical samples needs to be further explored in children. While diagnostic criteria for NTM disease in children are not clearly defined, available criteria (84) suggest that the children in this study who had NTM's isolated were unlikely to fulfill criteria for NTM disease and that these were likely, commensals.

Children are thought to have a weak immune system at birth and as they grow, the immune system develops (85). This is in agreement with the IFN-γ produced in response to mitogen recorded in the QFT-GIT assay, where with increasing age the IFN- γ produced in response to mitogen also increased (0-5 months [69.2%], 6-11 months [72.4%], 12-17 months [86.1%] and ≥18 months [96.4%]). The numbers of children with indeterminate results were low (3.6%). When compared to the children with either QFT-GIT positive or negative, the indeterminate group had a higher percentage of children (84.6% Vs. 30.7%; $p<0.01$) from the younger age group (<1 year) suggesting that since the younger children are thought to have a weaker immune system which leads to a lower IFN-γ produced in response to mitogen, they will tend to have more indeterminate results rather than a positive or negative QFT-GIT result. However more number of studies evaluating the QFT-GIT test in children <1 year of age are required to enumerate this.

6.2 Methodological issues

6.2.1. Validity

Internal validity: "Internal validity is the degree to which the results of a study are correct for the sample of patients being studied" (80).

External validity: "External validity is the degree to which the results of an observation hold true in other settings" (80).

6.2.1.1. Internal Validity

A number of factors could have affected the internal validity of this study. Different staff personnel administered the tuberculin to the children and measured the TST reading.

There could have been variation in the injection of the tuberculin and could lead to a reader bias which could affect the TST reading. The height and weight measurements were also measured by different staff personnel. Similar to the tuberculin administration, different staff personnel collected the gastric aspirate and induced sputum. This could lead to variations in the amount and quality of gastric aspirate collected. However, standard operating procedures (SOPs) were used for all field, clinical and laboratory procedures. Equipment was calibrated regularly and the training of staff was extensive and well documented. Missing data were minimal.

The contamination of the LJ (11.9%) and MGIT (1st inoculation- 26.5%, reinoculation I-12.3% and reinoculation II 2-7.4%) used for the growth of the *M. tuberculosis* could theoretically have led to a reduced number of isolates of *M. tuberculosis*/NTM. However, a high percentage of NTMs were isolated (approximately in 26% of participants) and since NTMs are fragile organisms it would suggest that the probability of not detecting MTB was minimal.

6.2.1.2. External validity

This study has a high external validity for rural populations in India with similar background TB rates; the enrollment and follow up of eligible births were high, loss to follow up during the two year period was minimal (98/4382).

7.0. CONCLUSION

TST positivity was higher than QFT-GIT positivity. Positive TST and QFT-GIT was significantly associated with a recent history of TB contact; this association was stronger when both TST and QFT-GIT were used in unison. However the association between TB exposure and TST was significantly affected by under nutrition. The concordance between the TST and QFT-GIT in BCG vaccinated children was fair. The number of indeterminate QFT results was low (3.6%). Thus QFT-GIT could be compared with the TST for diagnosis of TB infection in young children. Due to a lack of gold standard to compare the two tests with and the low number of culture confirmed cases we could not calculate the sensitivity or specificity of the tests in relation to TB disease. More number of studies on children with larger number of

culture confirmed cases need to be carried out to evaluate both the tests in relation to TB disease in children.

8.0 LIMITATIONS

The numbers of bacteriologically confirmed cases of TB were low in this population and thus we were unable to determine the sensitivity or specificity of the tests in relation to TB disease. While recent exposure to *M. tuberculosis* was captured, the gradient of exposure was not; this would have been useful to assess in relation to TST and QFT-GIT. The diagnosis of 'probable TB' was done using a single algorithm – this could be extended using other clinical scores that utilize the parameters that were captured for this study – however, the absence of a gold standard would continue to be a concern in the interpretation of 'probable TB'.

9.0 FURTHER RESEARCH

Lessons from this study suggest that the following areas may be important to pursue:

1. The evaluation of combined TST /QFT-GIT in the evaluation of progression of infection to disease.
2. The evaluation of combined TST / QFT-GIT in high risk populations (e.g. contacts of bacteriologically confirmed TB) would allow for the greater likelihood of identifying larger numbers of bacteriologically confirmed TB in children (a limitation of this study).
3. The determination of an IFN-γ response to TB antigen cutoff in the QFT-GIT to supplement the existing scores for the diagnosis of 'probable TB'.

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11.0 TABLES

Table 1. Baseline characteristics of the participants referred to the CVW the first time and on whom both TST and QFT were performed. (n=709)

	n (%)
Age (n=709)	
0-5 months	65 (9.2%)
6-11 months	174 (24.5%)
12-17 months	245 (34.6%)
≥ 18 months	225 (31.7%)
Gender (n=709)	
Male	372 (52.5%)
Female	337 (47.5%)
Mother education (n=709)	
Illiterate	208 (29.3%)
Others	501 (70.7%)
Father education (n=707)*	
Illiterate	130 (18.4%)
Others	577 (81.6%)
Wall (n=709)	
Bricks	540 (76.2%)
Others	169 (23.8%)
Cooking fuel (n=709)	
Wood	638 (90.0%)
Others	71 (10.0%)
Cough ≥2 weeks (n=709)	
Yes	129 (18.2%)
No	580 (81.8%)
Contact (n=709)	
Yes	67 (9.4%)
No	642 (90.6%)
X-ray (n=709)	
Abnormal-TB	12 (1.7%)
Abnormal-Not TB	4 (0.6%)
Negative	693 (97.7%)

Table 1 (Continued). Baseline characteristics of the participants referred to the CVW the first time and on whom both TST and QFT were performed. (n=709)

	n (%)
FTT (n=709)	
Yes	543 (76.6%)
No	166 (23.4%)
WHZ (n=707)*	
<-2(wasting)	305 (43.2%)
≥ 2 (normal)	402 (56.8%)
HAZ (n=708)*	
<-2(stunted)	188 (26.6%)
≥ 2 (normal)	520 (73.4%)
WAZ (n=709)	
<-2(under weight)	354 (49.9%)
≥ 2 (normal)	355 (50.1%)

*- data missing for the remaining

WAZ – Weight for age z score

WHZ – Weight for height z score

HAZ- Height for age z score

(cut-off used for Z score is -2 standard deviation)

FTT – Failure to thrive based on any of the following criteria after evaluation of the weight for age growth chart:

1). Loss of weight or no gain in weight over two consecutive visits

2). Crossing of two centile lines, downwards on growth chart

3). Weight consistently remaining below the third percentile line

X-ray- At least 2 out of 3 radiologists reported chest x-rays considered for "Abnormal- suggestive of TB" and "Abnormal-Not TB".

Table 2. Characteristics of the definite and probable cases of TB

PID	Visit to the CVW	Age (months)	Gender	DefiniteTB/ Probable TB	TST (mm)	QFT-GIT	Mitogen values	TB antigen values	Nil values
039-0009	1st	18	M	Definite	9	Negative	>10	0.26	0.15
144-0006	2nd	26	M	Definite	20	Positive	>10	>10	0.4
307-0008	2nd	9	M	Definite	10	Positive	>10	8.73	0.32
315-0016	1st	28	M	Definite	13	Positive	>10	>10	0.25
006-0004	1st	10	F	Probable	6	Indeterminate	0.59	0.09	0.1
012-0009	1st	24	F	Probable	16	Negative	>10	0.38	0.15
107-0001	1st	18	F	Probable	0	Negative	>10	0.07	0.08
148-0005	1st	15	M	Probable	4	Negative	>10	0.27	0.18
157-0004	1st	8	M	Probable	5	Indeterminate	0.31	0.17	0.29
207-0002	2nd	14	M	Probable	4	Negative	>10	0.36	0.96
279-0009	1st	10	F	Probable	2	Negative	1.31	0.15	0.13
376-0005	1st	16	F	Probable	4	Negative	3.98	0.04	0.06
407-0005	1st	13	F	Probable	5	Negative	>10	0.09	0.08

PID – Participant Identification
CVW – Case verification ward
TB – Tuberculosis
TST – Tuberculin skin test
QFT-GIT - QuantiFERON Gold *In-tube* assay
Nil - without antigens added, serving as a negative control for QFT-GIT
Mitogen - serving as a positive control for QFT-GIT. It also provided information about the individual's immune status
TB antigen – mixture of ESAT-6, CFP-10 and TB 7.7 antigen

Table 3. Tuberculin skin test and QuantiFERON TB Gold *In-tube* assay results for the definite, probable, unlikely and Not TB (first and second visit)

		n	1st visit TST ≥10mm	1st visit TST <10mm	n	2nd visit TST ≥10mm	2nd visit TST <10mm
Definite TB		2			2		
QFT-GIT	Positive		2	0		1	0
	Negative		0	0		0	1
Probable TB		8			1		
QFT-GIT	Positive		0	0		0	0
	Negative		1	7		0	1
Unlikely TB		126			8		
QFT-GIT	Positive		11	3		2	0
	Negative		27	85		0	6
Not TB		552			30		
QFT-GIT	Positive		5	18		2	0
	Negative		20	509		3	25

TST – Tuberculin skin test
QFT-GIT - QuantiFERON Gold *In-tube* assay

Table 4. Univariate associations of demographic, clinical, nutritional and mycobacteriological factors (excluding *M. tuberculosis*) with the tuberculin skin test and QuantiFERON TB Gold *In-tube* assay (first visit).

	n	TST		Unadjusted OR 95% CI	QFT-GIT		Unadjusted OR 95% CI
		≥10mm n (%)	<10mm n (%)		Positive n (%)	Negative n (%)	
Age	709						
0-5 months		4 (5.6)	61(9.6)	0.23 (0.08;0.66)**	6(14.6)	59(8.8)	0.99 (0.34; 2.74)
6-11 months		6 (8.3)	168(26.4)	0.13 (0.05; 0.29)**	6(14.6)	168(25.2)	0.35 (0.12; 0.93)**
12-17 months		12(16.7)	233(36.6)	0.18 (0.09; 0.34)**	8(19.6)	237(35.5)	0.33 (0.13; 0.80)**
≥18 months[1]		50(69.4)	175(27.4)	1.0	21(51.2)	204(30.5)	1.0
Gender	709						
Male		42(58.3)	330(51.8)	1.30 (0.79; 2.13)	24(58.5)	348(52.1)	1.30 (0.66; 2.58)
Female[1]		30(41.7)	307(48.2)	1.0	17(41.5)	320(47.9)	1.0
Mother education	709						
Illiterate		19(26.4)	189(29.7)	0.85 (0.49; 1.47)	14(34.1)	194(29.0)	1.27 (0.62; 2.58)
Others[1]		53(73.6)	448(70.3)	1.0	27(65.9)	474(71.0)	1.0
Father education	707*						
Illiterate		6 (8.3)	124(19.5)	0.37 (0.15; 0.88)**	9(22.0)	121(18.2)	1.27 (0.55; 2.86)
Others[1]		66(91.7)	511(80.5)	1.0	32(78.0)	545(81.8)	1.0
Wall	709						
Bricks		53(73.6)	487(76.5)	0.85 (0.49; 1.49)	32(78.0)	508(76.1)	1.12 (0.50; 2.58)
Others[1]		19(26.4)	150(23.5)	1.0	9(22.0)	160(23.9)	1.0
Cooking fuel	709						
Wood		63(87.5)	575(90.3)	0.75 (0.35; 1.59)	36(87.8)	602(90.1)	0.79 (0.28; 2.37)
Others[1]		9 (12.5)	62(9.7)	1.0	5(12.2)	66(9.9)	1.0
Cough ≥2 weeks	709						
Yes		8(11.1)	121(19.0)	0.53 (0.24; 1.14)	4(9.8)	125(18.7)	0.47 (0.14; 1.41)
No[1]		64(88.9)	516(81.0)	1.0	37(90.2)	543(81.3)	1.0

Table 4 (Continued). Univariate associations of demographic, clinical, nutritional and mycobacteriological factors (excluding *M. tuberculosis*) with the tuberculin skin test and QuantiFERON TB Gold *In-tube* assay (first visit)

	n	TST		Unadjusted OR 95% CI	QFT-GIT		Unadjusted OR 95% CI
		≥10mm n (%)	<10mm n (%)		Positive n (%)	Negative n (%)	
Contact	709						
Yes		12(16.7)	55(8.6)	2.11 (1.07; 4.17)**	10(24.4)	57(8.5)	3.46 (1.50; 7.81)**
No[1]		60(83.3)	582(91.4)	1.0	31(75.6)	611(91.5)	1.0
FTT	709						
Yes		32(44.4)	511(80.2)	0.19 (0.12; 0.33)**	32(78.0)	511(76.5)	1.09 (0.49; 2.52)
No[1]		40(55.6)	126(19.8)	1.0	9(22.0)	157(23.5)	1.0
WHZ	707*						
<-2(wasting)		15(20.8)	290(45.7)	0.31 (0.17;0.56)**	18(43.9)	287(43.1)	1.03 (0.52; 2.04)
≥ 2 (normal)[1]		57(79.2)	345(54.3)	1.0	23(56.1)	379(56.9)	1.0
HAZ	708*						
<-2(stunted)		22(30.6)	166(26.1)	1.24 (0.73; 2.12)	14(34.1)	174(26.1)	1.47 (0.71; 2.99)
≥ 2 (normal)[1]		50(69.4)	470(73.9)	1.0	27(65.9)	493(73.9)	1.0
WAZ	709						
<-2(under weight)		30(41.7)	324(50.9)	0.69 (0.42; 1.13)	24(58.5)	330(49.4)	1.45 (0.73; 2.87)
≥ 2 (normal)[1]		42(58.3)	313(49.1)	1.0	17(41.5)	338(50.6)	1.0
NTM	707[a]						
Atleast 1 positive		11(15.7)	181(28.4)	0.47 (0.24; 0.92)**	12(30.8)	180(26.9)	1.20 (0.56; 2.54)
Negative[1]		59 (84.3)	456(71.6)	1.0	27(69.2)	488(73.1	1.0

* - data missing for the remaining

[a] – culture confirmed MTB cases excluded

**-p values <0.05

[1]-reference group

WAZ – Weight for age Z score **, WHZ** – Weight for height Z score**. HAZ**- Height for age Z score (cut-off used for Z scores is -2 standard deviation)

TST- Tuberculin skin test, **QFT-GIT**-QuantiFERON Gold *In-tube* assay, **OR**-Odds ratio, **CI**- Confidence interval

FTT – Failure to thrive

Table 5. Multivariate associations of demographic, clinical, nutritional and mycobacteriological factors (excluding *M. tuberculosis*) with the tuberculin skin test and QuantiFERON TB Gold *In-tube* assay (first visit)

	n	TST		Adjusted OR 95% CI	QFT-GIT		Adjusted OR 95% CI
		≥10mm n (%)	<10mm n (%)		Positive n (%)	Negative n (%)	
Age	709						
0-5 months		4 (5.6)	61(9.6)	0.23 (0.11; 0.46)**	6(14.6)	59(8.8)	0.96 (0.34; 2.77)
6-11 months		6 (8.3)	168(26.4)	0.09 (0.04; 0.26)**	6(14.6)	168(25.2)	0.31 (0.11;0.86)
12-17 months		12(16.7)	233(36.6)	0.11 (0.04; 0.37)**	8(19.6)	237(35.5)	0.34 (0.15; 0.81)
>= 18 months[1]		50(69.4)	175(27.4)	1.0	21(51.2)	204(30.5)	1.0
Gender	709						
Male		42(58.3)	330(51.8)	1.38 (0.78; 2.44)	24(58.5)	348(52.1)	1.31 (0.66; 2.55)
Female[1]		30(41.7)	307(48.2)	1.0	17(41.5)	320(47.9)	1.0
Father education							
Illiterate	707*	6 (8.3)	124(19.5)	0.41 (0.16; 1.05)	9(22.0)	121(18.2)	1.26 (0.56; 2.82)
Others[1]		66(91.7)	511(80.5)	1.0	32(78.0)	545(81.8)	1.0
Cough ≥2 weeks	709						
Yes		8(11.1)	121(19.0)	0.47 (0.19; 1.19)	4(9.8)	125(18.7)	0.68 (0.22; 2.14)
No[1]		64(88.9)	516(81.0)	1.0	37(90.2)	543(81.3)	1.0

Table 5 (Continued). Multivariate associations of demographic, clinical, nutritional and mycobacteriological factors (excluding *M. tuberculosis*) with the tuberculin skin test and QuantiFERON TB Gold *In-tube* assay (first visit)

	n	TST		Adjusted OR 95% CI	QFT-GIT		Adjusted OR 95% CI
		≥10mm n (%)	<10mm n (%)		Positive n (%)	Negative n (%)	
Contact	709						
Yes		12(16.7)	55(8.6)	1.58 (0.69; 3.63)	10(24.4)	57(8.5)	4.36 (1.79; 10.54)**
No[1]		60(83.3)	582(91.4)	1.0	31(75.6)	611(91.5)	1.0
FTT	709						
Yes		32(44.4)	511(80.2)	0.14 (0.07; 0.26)**	32(78.0)	511(76.5)	1.58 (0.64; 3.87)
No[1]		40(55.6)	126(19.8)	1.0	9(22.0)	157(23.5)	1.0
NTM	707[a]						
Atleast 1 positive		11(15.7)	181(28.4)	0.44 (0.21; 0.91)**	12(30.8)	180(26.9)	1.21 (0.58; 2.52)
Negative[1]		59 (84.3)	456(71.6)	1.0	27(69.2)	488(73.1	1.0

* - data missing for the remaining

**-p values <0.05

[1]-reference group

[a] - culture confirmed MTB excluded

WAZ – Weight for age Z score

WHZ – Weight for height Z score

HAZ- Height for age Z score

(cut-off used for Z scores is -2 standard deviation)

FTT – Failure to thrive

TST- Tuberculin skin test

QFT-GIT-QuantiFERON Gold *In-tube* assay

OR-Odds ratio

CI- Confidence interval

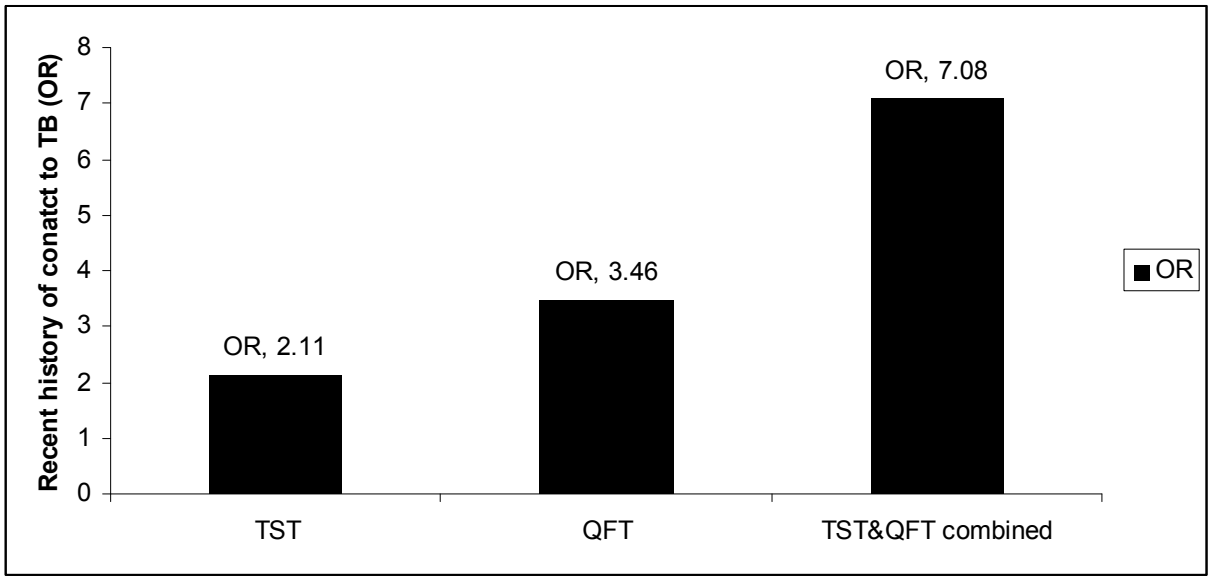
Multivariate analysis: adjusted for age, gender, education of the father (surrogate of the socio-economic status), cough>2 weeks (surrogate of symptoms), contact (surrogate of exposure to TB), FTT (surrogate of nutritional status) and NTM.

Table 6. Overall agreement between Tuberculin skin test and QuantiFERON TB Gold *In-tube* assay (First visit)

	QFT-GIT Positive n (%)	QFT-GIT Negative n (%)	Kappa (95% CI)	p value
TST n=709				
≥10mm	20(48.8)	52(7.8)	0.31 (0.19; 0.40)	<0.01
< 10mm	21(51.2)	616(92.2)	Fair	
With history of contact **TST n=67**				
≥10mm	8(80.0)	4(7.0)	0.68 (0.39; 0.82)	<0.01
< 10mm	2(20.0)	53(93.0)	Good	
	QFT-GIT Positive n (%)	**QFT-GIT Negative n (%)**		
TST n=709				
≥5mm	32(78.8)	285(42.7)	0.09 (0.04; 0.11)	<0.01
< 5mm	9(22.0)	383(57.3)	Poor	
With history of contact **TST n=67**				
≥5mm	10(100.0)	29(50.9)	0.23(0.08; 0.22)	0.01
< 5mm	0(0.0)	28(49.1)	Fair	

TST – Tuberculin skin test
QFT-GIT - QuantiFERON Gold *In-tube* assay

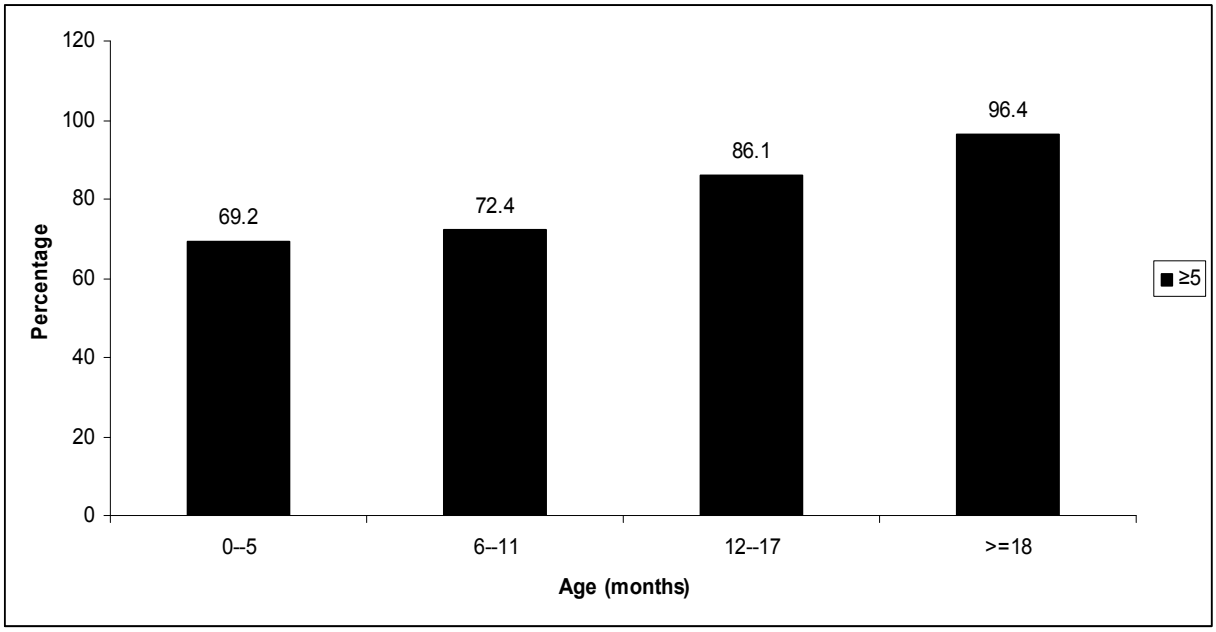
Figure 1. Comparison of the association between TST, QFT-GIT and combined TST and QFT-GIT with recent history of contact with TB



*95% CI; TST – (1.07; 4.17), QFT- (1.50; 7.81) and Combined TST and QFT-2.51; 19.65)

OR – Odds ratio
CI - Confidence interval
TST – Tuberculin skin test
QFT-GIT - QuantiFERON Gold *In-tube* assay

Figure 2. Association of age with IFN-γ produced in response to mitogen.



*y – axis: Percentage of children with ≥5 IU/mL of IFN- γ in response to mitogen

12.0 APPENDICES
12.1. CASE VERIFICATION WARD VISIT SOURCE DOCUMENT

CASE VERIFICATION WARD DIAGNOSTIC VISIT
(Unscheduled)

Date Case Verification Ward Admission Informed Consent signed : __ __ / __ __ / __ __ __ __
d d m m y y y y

Date of admission to Case Verification Ward: __ __ / __ __ / __ __ __ __
d d m m y y y y

Referral from *(check one)*: ❒Self-referral

❒ Active follow-up visit

❒ Passive surveillance *(check one)*:

❒ Hospital/primary health centre/private practitioner

❒ Microscopy centre; ❒ Emmaus Swiss Hospital
❒ Other

❒ Field study team *(between scheduled visits for Active group, or passive surveillance for Passive group)*

Was the child diagnosed with TB prior to this admission to the Case Verification Ward? ❒ Yes ❒ No

If child has history of symptoms and/or TB + contact and/or physician diagnosed/suspects TB, child is eligible for admission to CVW. If child is on and Adherent to TB medication for more than 8 weeks,the child is not eligible to be admitted to the CVW.

Study Team Member Code
❒❒❒

Does the child meet admission criteria? ❒ Yes ❒ No

Physical assessment **Date of assessment** __ __ / __ __ / __ __ __ __
(Place leading zero if necessary.) d d m m y y y y

Weight ___ ___ . ___ kg

Height ___ ___ . ___ cm

Temperature ___ ___ °C

Respiratory rate ___ ___ ___ per min

SaO_2 ___ ___ ___ %

Heart rate ___ ___ ___ bpm

Head circumference ___ ___ . ____cm

Mid-arm circumference ___ ___ . ___ cm

CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

Parent interview **Date of interview** __ __ / __ __ / __ __ __ __
d d m m y y y y

Is child experiencing *(per parental report)*:

Recent illness ❒ Yes; specify_____ How many days _____
Treated: ❒ Yes; specify treatment _____
❒ No
❒ No

Recent loss of weight ❒ Yes
❒ No

Loss of appetite ❒ Yes; how many days _____
❒ No

Cough ❒ Yes; how many days _____
Treated: ❒ Yes; specify treatment _____
❒ No
❒ No

Fever ❒ Yes; how many days _____
Treated: ❒ Yes; specify treatment
_____
❒ No
❒ No

Has child had a significant exposure (e.g. approximately 8 hours) to an individual on TB treatment?
❒ Yes
❒ No

Was child started on TB therapy prior to admission to Case Verification Ward?
❒ Yes; number of days already on treatment: _____ days
(Record on TB Treatment Record case report form.)
❒ No

Respondent/source of information *(check all that apply)*:

❒ Mother
❒ Father
❒ Guardian
❒ Other; specify: _____

Study Team Member Code
❒❒❒

CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

Brief review of systems: physical exam

HEENT ❒ Normal ❒ Abnormal;
_____

Dermatologic ❒ Normal ❒ Abnormal;
_____

Neurologic ❒ Normal ❒ Abnormal;
_____

Respiratory ❒ Normal ❒ Abnormal;
_____

Cardiovascular ❒ Normal ❒ Abnormal;
_____

Gastrointestinal ❒ Normal ❒ Abnormal;
_____

Urogenital ❒ Normal ❒ Abnormal;
_____

Musculoskeletal ❒ Normal ❒ Abnormal;
_____

Failure to thrive (FTT)? ❒ Yes ❒ No

Study Team Member Code ☐☐☐

_____
Name ***Signature***

Tuberculin skin test *(Test will be read within 2 – 4 days after application. Test should be applied day of admission.)*

Date applied: __ __ / __ __ / __ __ __ __
d d m m y y y y

Study Team Member Code ☐☐☐

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

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

Study Team Member Code ☐☐☐

_____
Name ***Signature***

CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

Chest radiograph *(A-P)* **Date:** __ __ / __ __ / __ __ __ __
d d m m y y y y

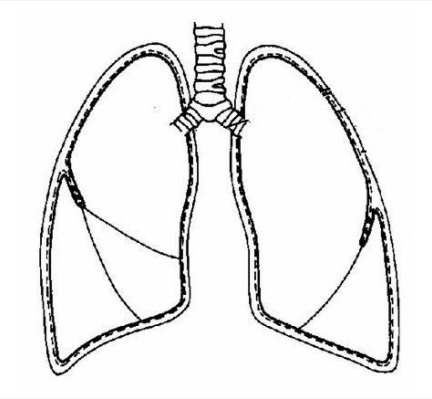
- ❒ Normal
- ❒ Abnormal, TB
- ❒ Abnormal, not TB

Impression

Apex:

Hilar:

Mid-zone:



Right Left

Lower zone:

NOTES:

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Name ***Signature*** ***Date***

Study Team Member Code

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CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

SMEAR

1 of 2: Induced sputum **Date obtained:** __ __ / __ __ / __ __ __ __
(d d / m m / y y y y)

Consistency:
- ❒ Salivary
- ❒ Mucoid
- ❒ Muco purulent
- ❒ Blood stained

Result:
- ❒ Positive; specify if
  - ❒ Scanty
  - ❒ 1+
  - ❒ 2+
  - ❒ 3+
- ❒ Negative

1 of 2: Gastric wash **Date obtained:** __ __ / __ __ / __ __ __ __
(d d / m m / y y y y)

Result:
- ❒ Positive; specify if
  - ❒ Scanty
  - ❒ 1+
  - ❒ 2+
  - ❒ 3+
- ❒ Negative

---

2 of 2: Induced sputum **Date obtained:** __ __ / __ __ / __ __ __ __
(d d / m m / y y y y)

Consistency:
- ❒ Salivary
- ❒ Mucoid
- ❒ Muco purulent
- ❒ Blood stained

Result:
- ❒ Positive; specify if
  - ❒ Scanty
  - ❒ 1+
  - ❒ 2+
  - ❒ 3+
- ❒ Negative

2 of 2: Gastric wash **Date obtained:** __ __ / __ __ / __ __ __ __
(d d / m m / y y y y)

Result:
- ❒ Positive; specify if
  - ❒ Scanty
  - ❒ 1+
  - ❒ 2+
  - ❒ 3+
- ❒ Negative

NOTES:

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Name ***Signature*** ***Date***

CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

CULTURE

1 of 2: Induced Sputum **Date obtained:** __ __ / __ __ / __ __ __ __
dd mm yyyy

- ❒ Positive
- ❒ Negative
- ❒ Contaminated

If culture result is positive, record PCR speciation. Otherwise, check Not applicable.

❒ Not applicable *(Check if culture was negative; Do not complete below.)*

PCR:

MTB	❒ Positive	❒ Negative
MOTT	❒ Positive	❒ Negative
BCG disease	❒ Positive	❒ Negative

If culture result is positive, record drug sensitivities. Otherwise, check not applicable.

❒ Not applicable (*Check if culture was negative or contaminated; Do not complete below.)*

Drug sensitivities:

Streptomycin	❒ Sensitive	❒ Resistant
Isoniazide	❒ Sensitive	❒ Resistant
Rifampicin	❒ Sensitive	❒ Resistant
Ethambutol	❒ Sensitive	❒ Resistant

NOTES:

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Name ***Signature*** ***Date***

CASE VERIFICATION WARD DIAGNOSTIC VISIT (continued)
(Unscheduled)

CULTURE

1 of 2: Gastric Wash **Date obtained:** __ __ / __ __ / __ __ __ __
(d d / m m / y y y y)

- ❒ Positive
- ❒ Negative
- ❒ Contaminated

If culture result is positive, record PCR speciation. Otherwise, check not applicable.

❒ Not applicable (*Check if culture was negative; Do not complete below.)*

PCR:

MTB	❒ Positive	❒ Negative
MOTT	❒ Positive	❒ Negative
BCG disease	❒ Positive	❒ Negative

If culture result is positive, record drug sensitivities. Otherwise, check not applicable.

❒ Not applicable (*Check if culture was negative or contaminated; Do not complete below.)*

Drug sensitivities:

Streptomycin	❒ Sensitive	❒ Resistant
Isoniazide	❒ Sensitive	❒ Resistant
Rifampicin	❒ Sensitive	❒ Resistant
Ethambutol	❒ Sensitive	❒ Resistant

NOTES:

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Name ***Signature*** ***Date***

12.2. SUBJECT INFORMATION AND CONSENT FORM

SUBJECT INFORMATION AND CONSENT FORM

ADMISSION TO TB CASE VERIFICATION WARD AND DIAGNOSTIC WORK-UP

EMMAUS SWISS LEPROSY PROJECT / ST. JOHN'S INSTITUTE OF POPULATION HEALTH AND CLINICAL RESEARCH

Study Number: EPI-003-IN

Study Title:
A Prospective Epidemiological Study of TB Disease in Neonates in Palamaner Taluk, South India to Determine the Incidence of Pulmonary Tuberculosis and the Impact of Surveillance Methodology on the Measurement

Introduction

- We want you to know that your child's participation in this study is optional.
- You may choose not to have your child take part in this study.
- You can decide later to take your child out of the study.
- You will lose no benefits if your child does not take part in the study.

Before you decide whether or not to allow your child to participate in the study please take as much time as you need to ask any questions and to discuss the study with members of the study team, family members, friends or others.

Who is the sponsor of the study?
Aeras Global TB Vaccine Foundation, USA, is the sponsor of the study. This is a research study being done by the Emmaus Swiss Leprosy Project, Palamaner and the St. John's Institute of Population Health and Clinical Research, Bangalore, in partnership with the Aeras Global TB Vaccine Foundation, USA. The person in charge of the study is Professor Mario Vaz, Head of Population Health, St. John's Institute of Population Health and Clinical Research, Bangalore. There are no costs to you for any of the processes outlined below if you decide to allow your child to participate.

Why are you asking my child to take part in this study?
Your child is already enrolled in the research study of TB disease in this community, and the study doctors want to find out if your child has TB.

As part of this research study, if your child has symptoms of TB or has been exposed to someone who has TB, the study doctors will examine your child and perform tests to see if your child has TB. You are being asked to give your permission to have your child admitted to the special TB diagnostic ward that has been set up for this study in the Emmaus Swiss Hospital in order for these tests to be done.

What is the purpose of this research study?
The primary purpose of this study is to find out how many infants and children get TB disease during the first two years of life in this community. The diagnosis of TB in infants and children is very difficult. Your child can have TB but have no symptoms. However, the symptoms of TB are very much like other diseases. The best way to diagnose TB is to grow the germ that causes TB. The

doctors doing this study have built a special ward at Emmaus Swiss Hospital to diagnose TB in infants and children.

The decision to enroll your child in the study is voluntary, and you can decide to stop your child's participation (in other words, withdraw your child) at any time. If you decide you want to withdraw your child from the study, you should inform a study team member in order for them to have accurate records about the research.

I**f you decide not to enroll your child in the study** you should know that standard tests and health care are available through the government health services and private practitioners.

What does my child have to do in this study?
You may either bring your child to the TB diagnostic ward because you think your child has symptoms of TB, or another doctor may refer your child to the study doctor for follow-up at the diagnostic ward. When you arrive, the study doctor will ask you some questions about your child's health and any symptoms of TB they have, and whether or not your child has been exposed to someone who has TB. The study doctor will then decide if your child needs to be admitted for further testing.

If your child is admitted to the TB diagnostic ward, you will be able to stay in the ward overnight with your child. Your child will need to be admitted for about 2 nights. Your child will be examined and a chest radiograph (chest X-ray) will be taken. Your child will also be given a TB skin test. The TB skin test is called a Mantoux and is done by injecting a very small amount of substance under the skin with a needle on the forearm. It will form a small bump right after the injection that will last for about 10 minutes. About a day or two later, it may form a pimple that looks like a mosquito bite if your child has a positive reaction to the test (meaning, your child may have been exposed to TB.) A study team member will "read" the test by looking at your child's arm where the injection was made about two to three days after the test was done.

The study doctor and nurse will need to get a sputum sample from your child in order to test it for the TB germ. Sputum is the mucus matter that is coughed up from your child's lower airways. In order to help your child cough up the sputum, a light mist or vaporized spray of liquid material will be given to your child to breathe in. This is called a nebulizer. The nebulizer will help your child cough up sputum. It may be necessary to pat your child on the back to help with coughing. This procedure is called sputum induction. The sputum induction procedure will be done again the next day in order for the doctors to have two samples of sputum.

The study doctor and nurse will also need to get a sample of your child's gastric fluid (liquid) in order to test it for the TB germ. Gastric fluid is the liquid that is found in your child's stomach. In order to remove some of the fluid from your baby's stomach, a thin soft flexible tube will be placed in your child's mouth and then passed further into the stomach. Some of the gastric fluid will then be gently sucked out. This procedure is called gastric aspirate. The gastric aspirate procedure will be done again the next day in order for the doctors to have two samples of gastric fluid.

The study doctor and nurse will also need to get a blood sample from your child. This sample will be taken from the forearm of your child and the total volume that is taken is 6 ml (a little more than 1 teaspoon full). Before taking the blood, the doctor / nurse will prick the heel with a needle to get a couple of drops of blood to test for haemoglobin. This test will tell the doctor whether your child is producing blood well. If the value in this test is low, the doctor may decide to reduce the blood sample or not to take the blood sample at all.

What happens after the testing is done in the TB diagnostic ward?

After your child has had the second sputum induction and the second gastric aspirate, and after the TB skin test has been read, your child will be discharged from the ward. Transportation home will be provided. All of the results of the tests performed may not be available before your child is discharged from the TB diagnostic ward. Some of the results may not be known for up to 8 week. However, the study doctor will provide you with a letter to explain the results of the tests that are known at the time and will follow up with another letter later if your child has TB. Your child's doctor, if you have specified one, will also receive a copy of all of the results that are sent to you.

What happens if my child has TB?
If your child is diagnosed with TB before being discharged from the diagnostic ward, the study doctors will either start your child on anti-TB medication or will give you a one-week supply of anti-TB medication. You should start your child on the medication immediately after leaving the diagnostic ward. The medication provided will follow treatment guidelines of the tuberculosis control program of the Indian government health services.

If at any time your child is diagnosed with TB, your child's doctor and the District TB Officer will be notified to ensure that your child receives the necessary follow-up care and medication. Treatment for TB disease takes at least six months.

Why would my child not get these tests?
If your child has a history of wheezing, the sputum induction will not be done. There may be other reasons this test or other tests might not be done. The doctor will discuss these reasons with you.

Can my child get hurt by being in the study?
Your child may experience a brief burning sensation immediately after injection of the TB skin test. A blister, ulceration (break in the skin), fever, or lymph node swelling may occur if the child is sensitive to the substance, but this is very rare.

Your child may also experience a brief burning sensation when the prick on the heel is done and the blood sample from the forearm is collected. Sometimes there may be a bruise where the blood has been collected. The skin may appear darker and this usually disappears after a few days.

The chest X-ray is the most common radiologic procedure. The chest X-ray will expose your child very briefly (often less than one-half second) to a very small amount of radiation. Young children are more sensitive to the risks of radiation, but experts consider the benefits of the chest X-ray to be much greater than any potential risks from the brief, small radiation exposure your child will undergo.

Sputum induction is not a routine procedure. However, this procedure has been used in a similar TB diagnostic ward in South Africa for more than three years with over 1,600 children. None of them had a severe reaction. A doctor from this study visited the ward in South Africa and learned the procedure and has since done these tests on other infants, here in Palamaner. Since this is not a routine procedure your child will be closely monitored for adverse reactions such as spasms that narrow the child's airways similar to asthma. Sputum induction may cause your child some minor brief discomfort. Your child may also experience shortness of breath, a feeling of tightness in the chest, coughing, gasping or wheezing. If this happens, your child will be treated with drugs to stop the spasms and open the airways. To prevent this from happening, the doctors may exclude your child from sputum induction if your child has a history of asthma or symptoms similar to asthma.

The gastric aspirate procedure is routinely performed in hospitals. There is a small chance that the tube will enter the airway instead of the stomach. There is also a small chance that some of the stomach fluids may enter your child's lung which could later lead to pneumonia. If either of these happen, your child may cough, gasp, and have trouble breathing. This is reversed by taking the tube out.

Are there any benefits to being in the study?
The special TB diagnostic ward set up for this research study uses tests that are not available in the public health clinics in India. The major benefit to your child is that there will be a very complete diagnosis done for TB.

Mothers' and infants will receive all meals while in the TB diagnostic ward at Emmaus Swill Hospital. Transportation will be provided to and from Emauss Hospital for the TB diagnosis. No other form of payment is available.

What if my child gets hurt while in the study?
If your child gets hurt as a direct result of being in the study the doctors and nurses working on the study will ensure your child gets treatment for any pain or injuries that result from taking part in the study. The researchers will not pay money to you if your child gets hurt in the study

How will my child's information be kept private?
Except for the consent form, your child will be identified by a study code on all study records, and not by name. You will receive a copy of this consent form. Every effort will be made to keep your child's study records strictly confidential. The researchers will be allowed to see your child's records. Your child's records must also be made available for review upon request by regulatory agencies that oversee this research.

Who can I contact if I have questions?
The Institute Ethics Review Board of St. John's Medical College, Bangalore, and the Research in Human Subjects/Institutional Review Board of the Aeras Foundation, which reviews the ethics of all studies with human subjects, have approved this study.

If you ever have questions or concerns about this study, your rights, or if you are injured as a result of being in this study you should contact:

St. John's Medical College, Research Ethics Committee
Name: Professor Karuna R Kumar (Convener)
Address: St. John's Medical College, Bangalore 560034
Phone: 080-22065057

Principal Investigator:
Professor Mario Vaz, Population Health Group, St. John's Institute of Population Health and Clinical Research, St. John's National Academy of Health Sciences, Bangalore 560034, India. Telephone 080-22065059, Fax 080-25532037 email: mariovaz@iphcr.res.in

Operational Manager:
Dr. AJW Jacob, Director, Emmaus Swiss Leprosy Project, LS Farm, PO Palamaner 517408, Chittoor Dt. A.P. Telephone 08579-252255

INFORMED CONSENT: Admission to Case Verification Ward and Diagnostic Work-up

Study Number: EPI-003-IN
Study Title:
A Prospective Epidemiological Study of TB Disease in Neonates in Palamaner Taluk, South India to Determine the Incidence of Pulmonary Tuberculosis and the Impact of Surveillance Methodology on the Measurement

I have read and discussed this consent form. My questions have been answered. I freely agree for my child participate and be examined for tuberculosis.

Child's Name _____

_____

Parent/Guardian's Name *(please print)* | Parent/Guardian's Signature or Thumb Print | Date

_____

Name of study personnel taking consent *(please print)* | Signature | Date

Interpreter:

☐ Interpreter used OR ☐ Interpreter not used

Witness:

☐ Witness used OR ☐ Witness not used

_____

Name (please print) | Signature | Date

Please indicate whether witness is:

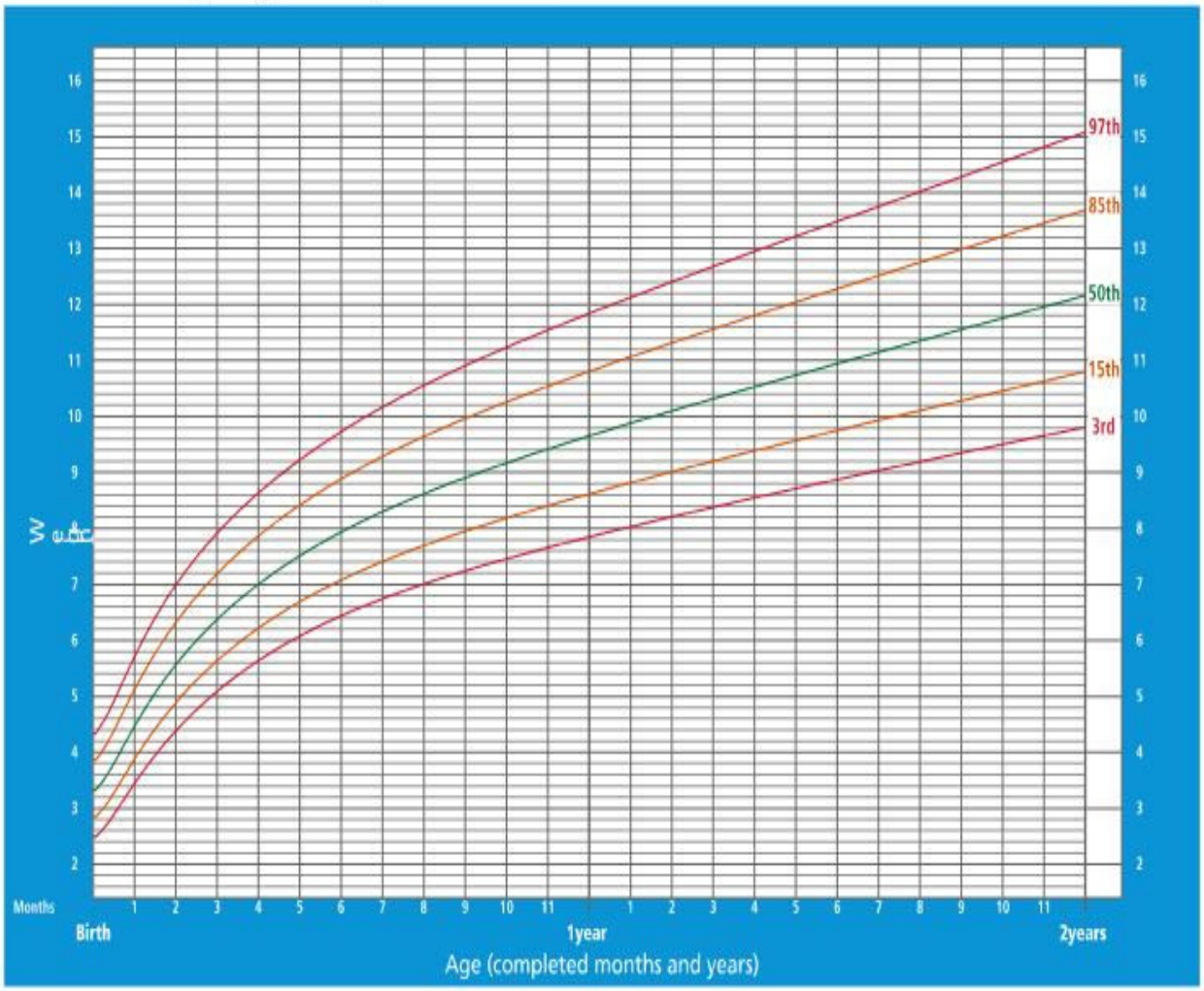
☐ Study personnel OR ☐ Independent / Impartial person

Note: The subject must be given a copy of this consent form. A signed copy must be filed in the subject's medical record at the Study Offices.

Weight-for-age BOYS

Birth to 2 years (percentiles)

World Health Organization

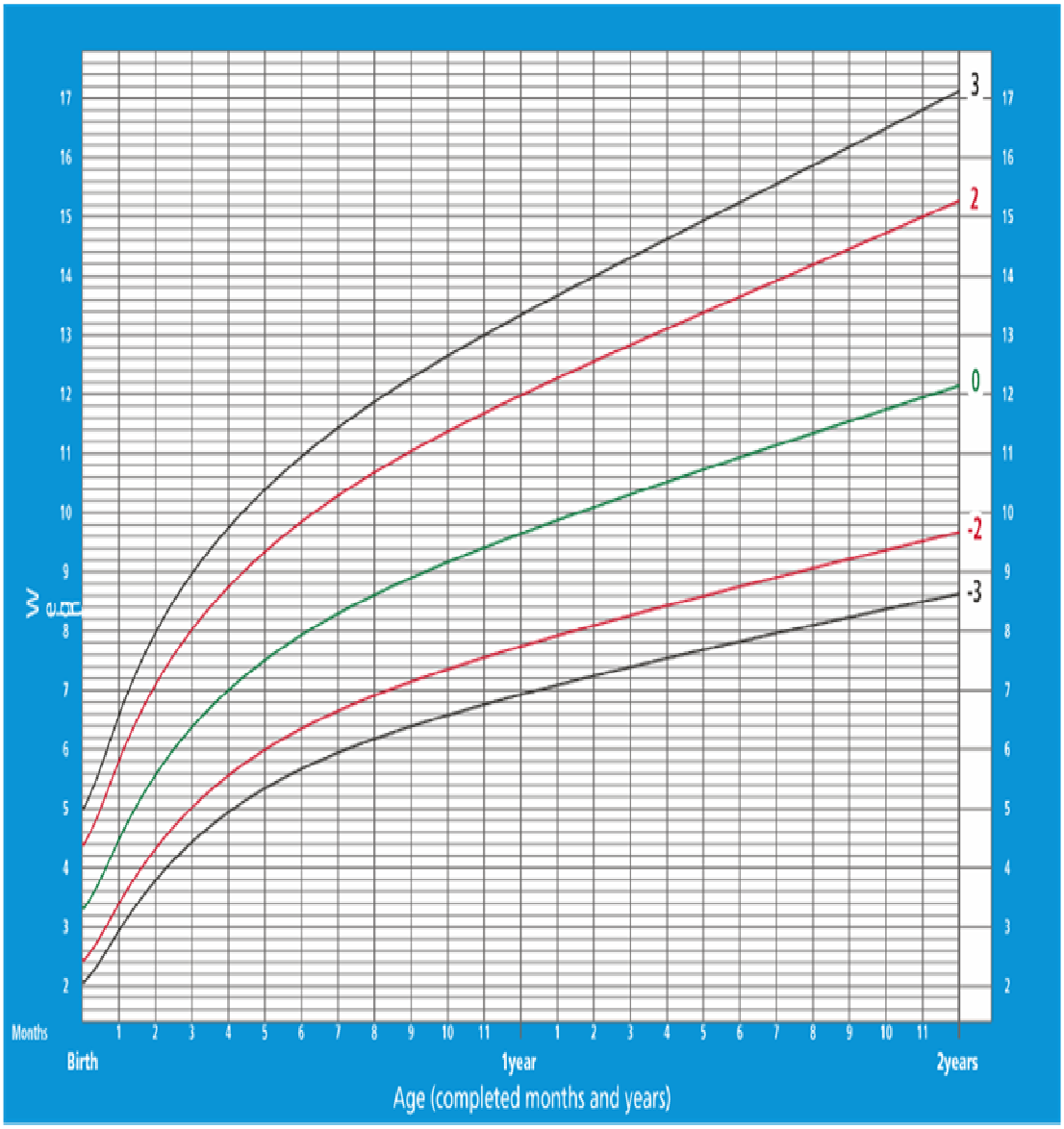


WHO Child Growth Standards

Weight-for-age BOYS

World Health Organization

Birth to 2 years (z-scores)

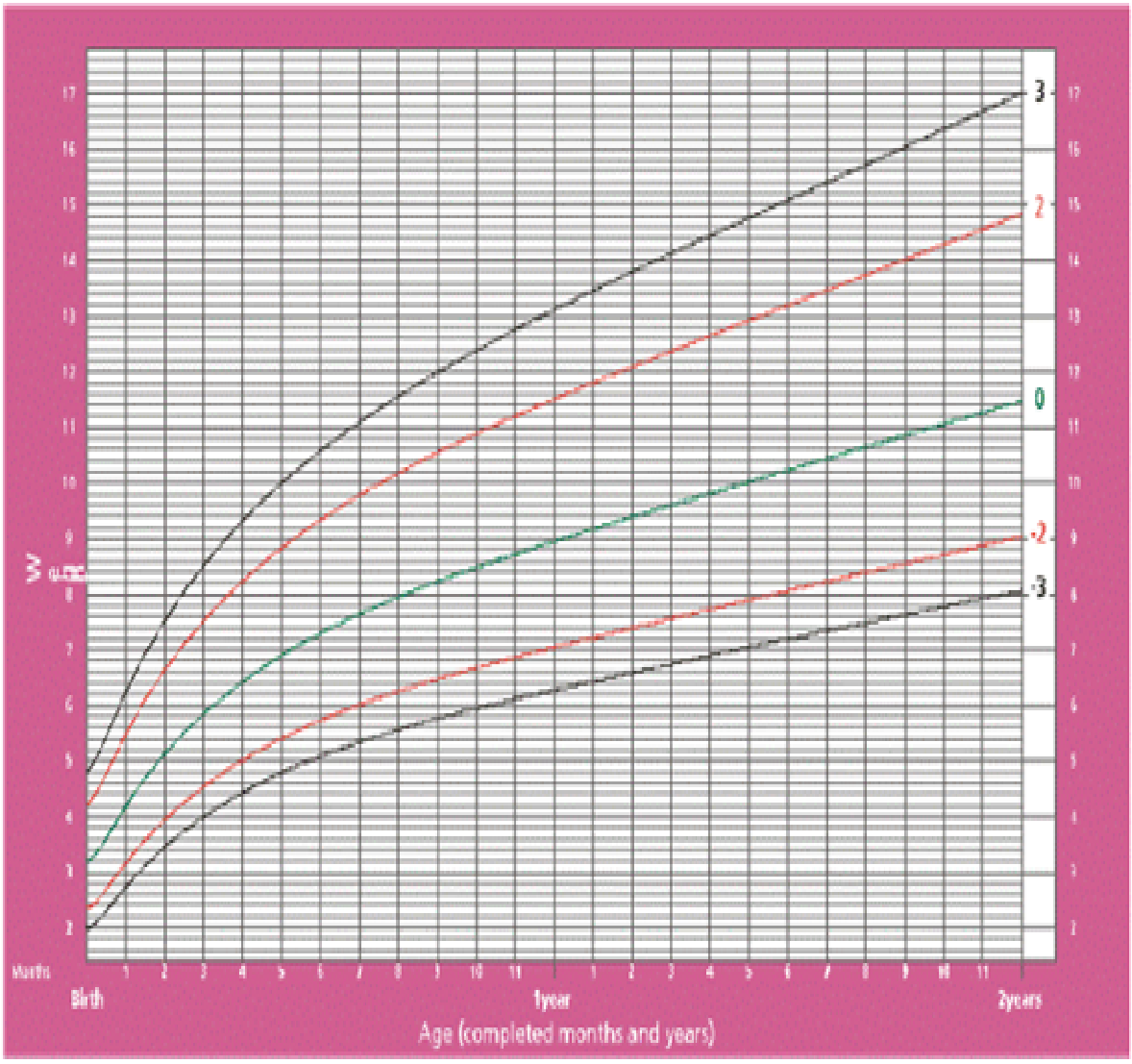


WHO Child Growth Standards

Weight-for-age GIRLS

Birth to 2 years (z-scores)

World Health Organization

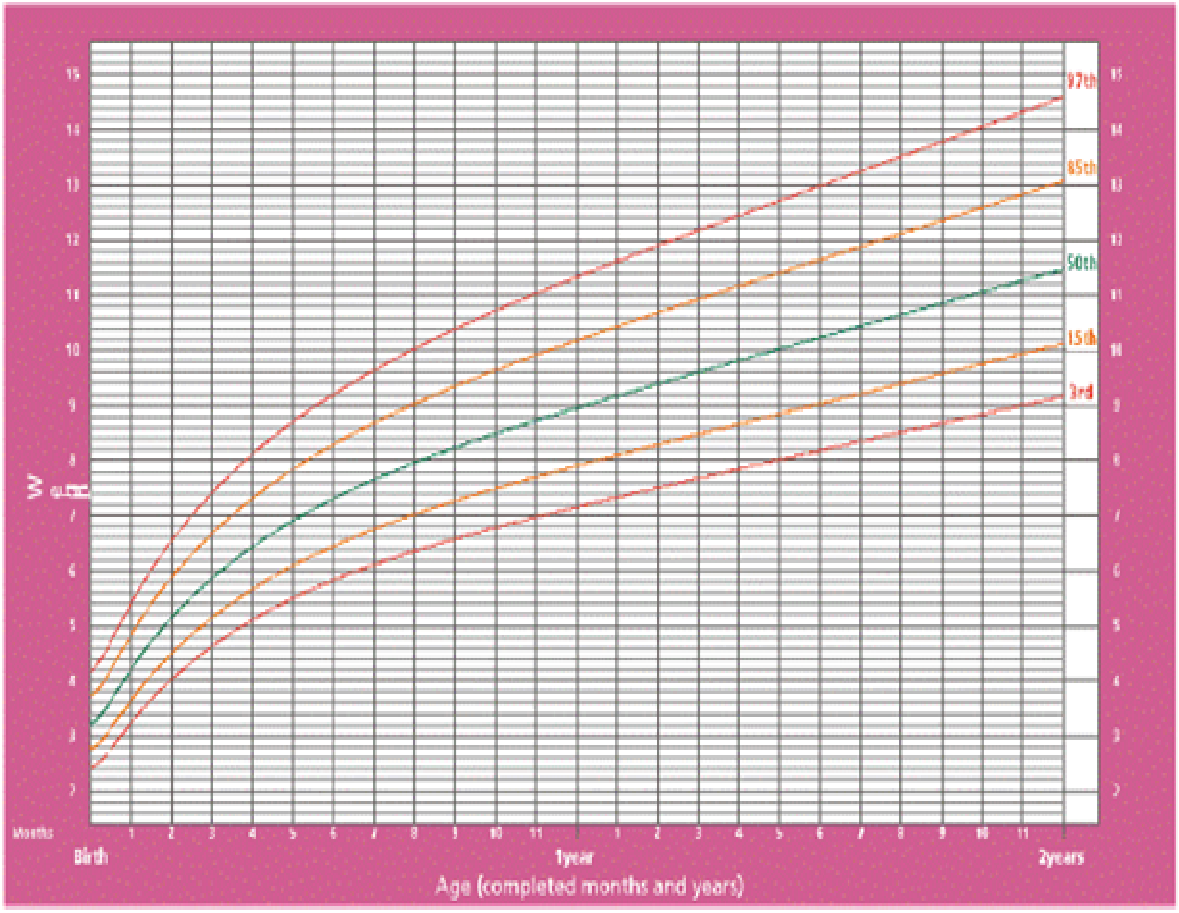


WHO Child Growth Standards

Weight-for-age GIRLS

World Health Organization

Birth to 2 years (percentiles)



WHO Child Growth Standards