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ORIGINAL ARTICLE

Effect of micronutrient deficiency on QuantiFERON-TB Gold In-Tube test and tuberculin skin test in diagnosis of childhood intrathoracic tuberculosis

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BACKGROUND/OBJECTIVES: Data on performance of QuantiFERON-TB Gold In-Tube test (QFT) and tuberculin skin test (TST) in children with active tuberculosis from high burden countries in the context of micronutrient deficiency are scarce. The objective of this study was to evaluate the effect of micronutrient deficiency on the performance of TST and QFT in children with intrathoracic tuberculosis.

SUBJECTS/METHODS: Children with probable intrathoracic tuberculosis underwent TST, QFT, gastric lavages and induced sputum examination for AFB (Acid-Fast Bacilli) smear and culture. Zinc, copper, ferritin and vitamin D were measured on stored serum samples. The study used cross-sectional data at initiation of anti-tubercular therapy.

RESULTS: Three hundred and sixty-two children (median age 115.5 months (interquartile range: 73, 144), 200 (55.3%) girls) were enrolled in the study. Microbiological confirmation of tuberculosis could be obtained in 128 patients. TST and QFT were positive in 337 (93%) and 297 (82%) children, respectively. Performance of both the tests was unaffected by weight-for-age and height-for-age '*z*-scores' or by serum copper levels. TST was not affected by serum zinc and ferritin levels. Children with negative QFT results had lower mean serum zinc level ($P = 0.01$) and higher ferritin levels ($P = 0.007$) as compared to those with positive test. Higher proportion of children with positive TST were vitamin D deficient/insufficient ($P = 0.003$).

CONCLUSION: Micronutrient status, especially serum levels of zinc, may influence the performance of QFT in children with intrathoracic tuberculosis. Considering the high prevalence of zinc deficiency in developing countries, QFT should be used cautiously for diagnosing tuberculosis.

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Keywords: pediatric tuberculosis; tuberculin skin test; interferon gamma release assay; zinc deficiency; vitamin D status

INTRODUCTION

Childhood tuberculosis (TB) is a common cause of morbidity in developing countries. It is a challenging disease, difficult to diagnose, as the gold standard diagnostic test of demonstration of *Mycobacterium tuberculosis* (MTB) is cumbersome and has a low sensitivity in children. Diagnosis is usually clinico-radiological, aided by evidence of infection with *M. tuberculosis* in the form of positive tuberculin skin test (TST) or interferon gamma release assays (IGRA) such as the QuantiFERON-TB Gold In-Tube test (QFT) and the enzyme-linked immunosorbent spot (ELISPOT) test. Both TST and IGRAs have advantages and drawbacks. TST is affected by the strength of the purified protein derivative used, a need for two visits (one for the application of test and another for reading of the test), subjectivity in measurement of induration, prior BCG (Bacillus Calmette–Guérin) vaccination and infection with atypical mycobacteria.[1] Advantages of IGRAs described in literature include: one time test, no subjectivity in reading and no effect of prior BCG and infection with atypical mycobacteria. Several reports in adults suggest that IGRAs are more sensitive and specific for the diagnosis of TB infection.[2–4] But there are limited data on its utility for diagnosis of TB infection in children. Moreover, most reports are from low burden settings.[5]

Both TST and IGRAs are tests that rely on an intact cell-mediated response to MTB and on unerring functioning of T helper type 1 (Th1) cells.[6,7] There is established evidence that micronutrients, especially zinc and vitamin D, affect the functioning of Th1 subset of cells, thus compromising the immunity against MTB.[8,9] Therefore, it is expected that deficiency of these micronutrients, which are widely prevalent in the developing world, may affect the performance of TST and IGRAs. None of the published reports have studied the influence of micronutrient levels on the performance of IGRA and TST. We report results of a study evaluating the effect of micronutrient deficiency on the performance of TST and QFT in children with intrathoracic TB in a high burden setting.

MATERIALS AND METHODS

The study was carried out at two tertiary care hospitals in New Delhi, India. A total of 362 children, age 6 months to 15 years, were enrolled as part of a

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larger randomized controlled trial to study the role of micronutrient supplementation in children with newly diagnosed intrathoracic TB. Eligible children were enrolled after screening from the outpatient departments and TB clinics of the two hospitals. None of the subjects were receiving any micronutrient supplements at baseline. Children with known HIV infection were not included in the trial. Children were not tested for HIV infection. However, the samples of the children enrolled in the randomized controlled trial were tested for HIV (unlinked, anonymous); the prevalence was 1%.

Diagnosis of intrathoracic tuberculosis was based on clinico-radiological criteria as recommended by the Indian Academy of Pediatrics.[10] Children presenting to our hospitals with cough and/or fever of more than 2 weeks or recent weight loss or history of contact with an adult TB patient in the past 2 years and where TB was considered in the differential diagnosis were subjected to chest X-ray and if required, given a course of antibiotics and re-assessed after 2 weeks with a repeat chest X-ray examination. In the presence of persistent radiological abnormalities, with non-resolution of clinical symptoms and no alternative cause for symptoms and radiological findings, a diagnosis of probable intrathoracic TB was made.

Clinical details including presenting complaints, physical examination and nutritional status were recorded. Height-for-age and weight-for-age *z*-scores were computed using the nutritional anthropometry module of Epi Info version 5 software (Centres for Disease Control and Prevention, Atlanta, GA, USA), based on the National Centre for Health Statistics (NCHS)-WHO reference curves.[11] Children were divided into three categories on the basis of weight-for-age and height-for-age '*z*' scores: $\geqslant -2$ *z*-score; < -2 to $\geqslant -3$ *z*-score and < -3 *z*-score. A frontal/postero-anterior and lateral chest X-ray were read by two pediatricians blinded to the clinical details of the patient. If the findings differed, a third pediatrician was asked to report on the chest X-ray and the majority view was accepted. The radiographic abnormalities were classified as primary complex (lymph node with small alveolar shadow), progressive disease (consolidation, cavity, miliary tuberculosis and multiple mediastinal adenopathy with or without parenchymal lesion) or pleural effusion.[6]

At the initial assessment, all children with probable intrathoracic tuberculosis were subjected to TST and QFT. TST was performed by a trained nurse using five TU purified protein derivative (Span Diagnostics Ltd., Surat, India), injected intradermally using 26 G disposable needle. The test result was measured by same nurse after 48–72 h. The induration in the horizontal axis was measured by a non-stretchable measuring tape. Periodic evaluation of the expertise of the nurse was done to maintain quality control. Induration of 10 mm or more was considered as a positive TST. For the QFT test (Cellestis Ltd., Carnegie, Australia), 3 ml blood was taken using a disposable needle and syringe. One milliliter of blood was directly transferred in each of three tubes provided by the manufacturer: nil control containing only heparin, mitogen control containing phytohemagglutinin and the antigen tube containing *M. tuberculosis*-specific antigens ESAT-6, TB7.7 and culture filtrate protein 10. The test was performed as per the manufacturer's recommendations.[7] The amount of interferon gamma (IFN-γ) in the nil control tube was subtracted to calculate the amount of IFN-γ released in response to *M. tuberculosis*-specific antigens or mitogen. The result was considered positive when the amount of IFN-γ released in response to TB-specific antigens was $\geqslant$0.35 IU/ml and 25% more than the nil control value. The test result was considered to be indeterminate for TB antigen responsiveness if the IFN-γ level was $<$0.35 IU/ml in the TB antigen well and the mitogen minus nil was $<$0.5 IU/ml or if the nil value was $>$8.0 IU/ml. The results of the study comparing the performance of TST and QFT have been reported separately.[12]

Ambulatory gastric lavages and induced sputum samples were collected early morning on two consecutive days for smear by Ziehl–Neelsen staining and mycobacterial culture by BACTEC-MGIT-960 (Becton Dickinson Microbiology System, Sparks, NV, USA) culture system on two consecutive days. Before starting antitubercular therapy, $\sim$3 ml of blood was collected in plain micronutrient-free tubes (Becton Dickinson, Sparks, NV, USA) for micronutrient estimation. Serum was separated within 1 h and stored at $-20\,^{\circ}$C till further analysis. All serum samples were given a unique identification code so that the laboratory personnel estimating the micronutrient levels were blinded to the patients' identity and clinical details.

Children were administered daily antitubercular therapy as per the categories recommended by the Revised National Tuberculosis Control Programme (RNTCP).[13]

Measurement of micronutrients

Estimations of serum zinc, copper, ferritin and vitamin D were carried out on stored serum samples after completion of the randomized controlled trial. Serum zinc and copper concentrations were analyzed in samples diluted 1:3, using a flame furnace atomic absorption spectrophotometer (GBC Avanta, Dandenong, Victoria, Australia) and calibration curves prepared from zinc and copper standards (Merck, Darmstadt, Germany). Validity of assay was checked using SERONORM (Sero AS, Billingstad, Norway). Limits of detection for zinc were 10–160 μg/dl; for copper 40–320 μg/dl. For outliers above the limits of detection, sample was further diluted, reassayed and concentration calculated accordingly. Ferritin was estimated using an ELISA-based kit (ORGENTEC Diagnostika, Mainz, Germany). The lower limit of detection for ferritin was 0.1 ng/ml. For quality control, pooled sera with known values were used. Interassay variability was $<$10%. Children were considered as zinc deficient if serum zinc levels were $<$65 μg/dl.[14] Copper deficiency was defined as serum level less than 80 μg/dl.[15]

25-hydroxyvitamin D [25(OH)D] levels, which is considered to be the best indicator of vitamin D status of the body, were measured from the stored sera by the chemiluminescence immunoassay technology using Liaison 310600 system (DiaSorin Inc., Stillwater, MN, USA) following the manufacturer's protocol.[16] The Liaison 25 (OH) Vitamin D assay is a direct competitive chemiluminescence immunoassay for quantitative determination of total 25-OH vitamin D in the serum. The analytical measurement range for the DiaSorin LIAISON 25(OH) Vitamin D Total Assay is 4–150 ng/ml. The lowest reportable value is 4 ng/ml, based on an interassay precision that approximates 20% coefficient of variation. According to serum 25(OH)D levels, children were classified as vitamin D deficient/insufficient (serum 25 (OH) D level $<$20 ng/ml) or sufficient ($\geqslant$20 ng/ml).[17–19]

Ethical considerations

The study was approved by Institutional review board of both study sites (All India Institute of Medical Sciences, New Delhi and Kalawati Saran Children Hospital, New Delhi, India) and the trial was registered at clinicaltrials.gov (NCT 00801606). Written informed consent was obtained from parents/caretakers of each child.

Data handling and analysis

Data were analyzed using STATA software, version 9 (Stata Corp, College Station, TX, USA). Student's 't'-test was used to compare normally distributed continuous variables, whereas Wilcoxon ranksum test was used if the distribution of variable was not normal. χ^2 or exact test was used for categorical variables as appropriate. In case of serum 25(OH)D levels, all values reported as less than the lower limit of quantification ($<$4 ng/ml) were considered as 4 ng/ml for the purpose of analysis. We performed logistic regression analysis for association between QFT or TST test positivity with serum zinc, copper and 25(OH)D levels, weight-for-age 'z' score, height-for-age 'z' score, age and type of tuberculosis disease; these covariates were chosen as nutritional factors, age and the type of disease may affect the cell-mediated immunity and therefore, the results of TST and QFT.

RESULTS

Three hundred and sixty-two children between 6 months and 15 years of age with probable intrathoracic tuberculosis were enrolled in this study. The median age of study subjects was 115.5 months (interquartile range: 73, 144); 200 (55.3%) of them were girls. Clinical and demographic details of these children are listed in Table 1. X-ray film of chest findings was suggestive of primary pulmonary complex in 108 (30%), progressive disease in 204 (56%) and pleural effusion in 50 (14%) children. Overall AFB (Acid-Fast Bacilli) positivity by smear/detection of MTB by culture of sputum or gastric lavage was 35% (128 patients). Non-tubercular mycobacteria were identified by PCR in 15 (4.1%) children.

TST was positive in 337 (93%) children, with mean ($\pm$s.d.) induration of TST being 17.5 $\pm$ 6.5 mm. An induration of $<$10 mm was recorded in 25 (7%), 10 to $<$15 mm in 59 (16%), 15 to $<$20 mm in 147 (41%) and $\geqslant$20 mm in 131 (36%) patients. The

Table 1. Demographic and clinical characteristics of 362 children enrolled

Characteristics	*Values*
Median age in months (IQR)	115.5 (73, 144)
Girls	200 (55.3%)
Received BCG	268 (74%)
History of contact with adult pulmonary tuberculosis case	133 (37%)
Weight-for-age z-score < -3	138 (38%)
Height-for-age z-score < -3	47 (13%)
Chest X-ray findings	
Primary complex	108 (30%)
Progressive disease	204 (56%)
Pleural effusion	50 (14%)
AFB positive and/or MTB culture positive	128 (35%)

Abbreviations: AFB, acid-fast bacilli; BCG, Bacillus Calmette–Guérin; IQR, interquartile range; MTB, *Mycobacterium tuberculosis*. Values are n (%) unless specified.

sensitivity of TST in culture confirmed cases of tuberculosis was 90.5% (95% confidence interval, CI: 84.1–94.5%).

QFT was positive in 297 (82%) children. Seven (2%) children had an indeterminate QFT test result. These seven children have been considered as negative tests for further analysis. The sensitivity of QFT in culture confirmed cases of tuberculosis was 82.6% (95% CI: 74.9–88.4).

Serum zinc level was measured in 357 children, mean level being 66.5 (s.d.: 23.4) μg/dl. One hundred ninety-eight (55.5%) children had zinc levels below the cutoff of 65 μg/dl. Though serum zinc levels did not affect the TST positivity ($P = 0.49$), the mean serum zinc level was significantly lower in children with a negative QFT results (60.1 vs 67.9 μg/dl, $P = 0.02$). Children who were deficient in zinc (serum levels <65 μg/dl) had significantly more negative QFT results than the children who had sufficient zinc (21.7% vs 13.2%, $P = 0.04$) (Table 2).

Among other micronutrients tested, serum copper levels, measured in 354 children, did not affect the performance of either QFT or TST. Serum 25(OH)D was measured in 248 children. Median 25(OH)D was 8 ng/ml (interquartile range: 5, 12). One hundred and eighty-six (70%) children were vitamin D deficient (<12 ng/ml), 55 (20.7%) were insufficient (12–19 ng/ml) and 25 (9.4%) were vitamin D sufficient ($\geqslant 20$ ng/ml). Children who were vitamin D deficient/insufficient were more likely to have a positive TST ($P = 0.003$) as compared with children who were vitamin D sufficient (Table 2). Although QFT was not affected by serum 25(OH)D levels, a trend for a positive QFT being more often in vitamin D-deficient children was observed ($P = 0.09$) (Table 2).

Ferritin levels were documented in 360 children. The median ferritin level for the whole cohort of children was 51.3 ng/ml (interquartile range: 20.5, 110.8). Children with a negative QFT had a significantly higher mean serum ferritin level (77.9 vs 42.9 ng/ml); $P = 0.007$ (Table 2).

On logistic regression analysis, we observed statistically significant association between TST positivity and 25(OH)D levels (odds ratio: 0.94 (95% CI: 0.89, 0.99); $P = 0.038$), age (odds ratio: 1.02 (95% CI: 1.002, 1.03); $P = 0.02$) and type of tuberculosis disease ($P = 0.024$). For QFT positivity, we observed statistically significant association with serum zinc levels (odds ratio: 1.02 (95% CI: 1.003, 1.042); $P = 0.022$).

DISCUSSION

There is lot of interest in determining the utility of IGRAs in diagnosing latent and active tuberculosis in adult tuberculosis patients and contacts. Performance of these assays in children has not been well studied, particularly in high TB endemic regions. None of the published studies have evaluated the association of micronutrient levels with QFT and TST results. Micronutrients such as zinc and vitamin D have an important role to play in immunity against mycobacterial diseases.[20] They may as well influence the performance of the tests based on body's response of cell-mediated immunity such as IGRA and TST; this issue needs to be evaluated in the scenario of micronutrient deficiency being increasingly reported from developing countries.

In this study, we compared the results of QFT and TST at baseline, as part of a randomized controlled trial on micronutrient supplementation in children with probable intrathoracic tuberculosis. Existing studies indicate that both TST and QFT might be rendered negative or indeterminate by malnutrition.[21,22] But we observed that neither TST nor QFT has any significant relation with weight-for-age, height-for-age or weight-for-height of the children. Interestingly, we found that the mean serum zinc level was lower in patients with a negative QFT result, and children with zinc deficiency were more likely to have negative QFT test reports. Such an observation has not been reported earlier in pediatric population. *In vivo* and *in vitro* studies on zinc depletion and supplementation have shown that zinc-deficient states led to an decreased release of cytokines including interferon-gamma[8], and supplementation of zinc triggered the release of interferon-gamma by the upregulation of natural killer cells and probably a Th2/Th1 shift.[23,24] Earlier animal model studies have shown that zinc deficiency led to decreased tuberculin hypersensitivity in guinea pigs.[20] A study on Brazilian children showed that zinc supplementation, leading to a higher serum zinc level, was associated with more TST-positive cases than placebo.[25] Furthermore, a Peruvian study investigating the effect of topical application of zinc cream on TST induration concluded that low plasma zinc levels were predictive of negative TST.[26] On the other hand, a study designed to determine the appropriateness of TST as a screening tool in hemodialysis patients did not find any association of TST positivity with the zinc status of patients.[27] The TST results were not affected by the serum zinc concentration in our study population. This finding is significant in the context of high TB endemic, developing countries where a large majority of the children suffer from zinc deficiency. In India, the overall prevalence of zinc deficiency ranges from 33.8% in preschool children,[28] 43.8% among under-five children[29] to 57.1% in school children.[30] Globally, the prevalence of zinc deficiency is estimated to be about 31%.[31] More than half (55.5%) of the children we studied were zinc deficient. In such a scenario, it might be more appropriate to rely on the TST rather than the QFT as an adjunct to diagnosis of TB in children.

We also documented higher ferritin levels in children with negative QFT results. Performance of TST was not affected by ferritin levels. Relationship of QFT with blood ferritin levels has not been studied. A study on adult chronic renal failure patients documented an inverse relation between TST and ferritin levels.[32] A study on children with familial hemophagocytic lymphohistiocytosis observed that serum IFN-γ was not detected in infants with hyperferritinemia.[33] This might explain our findings of higher ferritin levels in children with a negative QFT. But the exact mechanism leading to this change; that is, an inverse relation of ferritin and gamma interferon levels needs further evaluation.

Although the levels of zinc, copper or ferritin did not influence TST, it was observed that 25(OH)D levels were more often reduced in children with positive TST. Vitamin D acts as an immunomodulatory agent having a complex role in immunity against MTB; promoting antimicrobial agents such as cathelicidin and/or β defensin and downregulating Th1 cytokine-producing cells at the same time.[9] Many studies have supported the theory that vitamin D-deficient individuals have a higher chance of contracting

Table 2. Relation of TST and QFT with serum zinc, copper, ferritin and 25(OH)D (vitamin D) levels

	TST positive	*TST negative*	P-*value*	*QFT positive*	*QFT negative*	P-*value*
Mean (s.d.) serum zinc levels in μg/dl, $N=357$	66.2 (23.6) $N=332$	69.6 (20.2) $N=25$	0.49[a]	67.9 (23.7) $N=293$	60.1 (20.7) $N=64$	0.01[a]
Serum zinc level[b]						
<65 μg/dl, $N=198$	188 (56.6%)	10 (40%)	0.1[c]	155 (52.9%)	43 (67.2%)	0.04[c]
$\geqslant 65$ μg/dl, $N=159$	144 (43.7%)	15 (60%)		138 (47.1%)	21(32.8%)	
Mean (s.d.) serum copper levels in μg/dl, $N=354$	143.2 (35.4) $N=329$	149.2 (53.8) $N=25$	0.42[a]	143.8 (37.4) $N=291$	142.6 (34.5) $N=63$	0.82[a]
Serum copper level[b]			0.45[d]			0.205[d]
<80 μg/dl, $N=8$	7 (2.13)	1 (4)		8 (2.75)	0	
$\geqslant 80$ μg/dl, $N=346$	322 (97.87)	24 (96)		283 (97.25)	63 (100)	
Median (IQR) serum 25(OH)D levels in ng/ml, $N=248$	8 (5–12) $N=231$	14 (8–20) $N=17$	0.003[e]	8 (5–12) $N=204$	8(6–15.5) $N=44$	0.19[e]
Serum 25(OH)D[b] *levels,* $N=248$			0.003[d]			0.09[d]
<20 ng/ml, $N=225$	213 (92.2%)	12 (70.6%)		188 (92.2%)	37 (84.1%)	
$\geqslant 20$ ng/ml, $N=23$	18 (7.8%)	5 (29.4%)		16 (7.8%)	7 (15.9%)	
Median (IQR) serum ferritin levels in ng/ml, $N=360$	51.5 (20.7–110.4) $N=335$	48.6 (17.02–152.7) $N=25$	0.77[e]	42.97 (19.8–104.1) $N=295$	77.92 (30.4–156.7) $N=65$	0.007[e]

Abbreviations: IQR, interquartile range; QFT, QuantiFERON-TB Gold In-Tube test; s.d., standard deviation; TST, tuberculin skin test; 25(OH)D, 25-hydroxyvitamin D. [a]Significance assessed using 't' test. [b]The figures in parentheses are the column percentages. [c]Significance assessed using the χ^2 test. [d]Significance assessed using the Fisher's exact text. [e]Significance assessed using the Wilcoxon ranksum test.

tubercular infection and developing disease.[34,35] A recent Spanish study found a significant inverse relation between vitamin D status and TST conversion in contacts of TB patients and all cases that showed a TST conversion were deficient in vitamin D.[36] However, it has been observed, in patients on chronic hemodialysis that TST positivity was not influenced by vitamin D therapy.[27] Animal model studies in guinea pigs showed that there was an adverse effect of vitamin D deficiency on tuberculin reaction.[20] These contradictory reports might be underlining the very complex inter-twining pathways by which vitamin D levels affect immunity against MTB. It was somewhat unexpected that 25(OH)D levels did not have a significant effect on QFT results in our study, although there was a trend toward more children with QFT-positive results being deficient in vitamin D. *In vitro* studies have documented that the presence of $1\alpha,25(OH)_2D$ ameliorated the production of IFN-γ in response to stimulation by MTB antigens.[37] A study that looked into the influence of seasonality and vitamin D status on cytokine release observed that increased vitamin D_3, as in summer months, was associated with decreased release of IFN-γ.[38] On the contrary, studies on patients with chronic kidney disease showed that QFT results were not influenced by vitamin D levels.[39] In addition, in another study probing into the factors associated with the performance of the whole-blood-based IGRA did not find an effect of vitamin D deficiency on QFT results.[40]

CONCLUSION

Micronutrient statuses, especially the serum levels of zinc, seem to influence the performance of QFT in children with probable intrathoracic tuberculosis. Considering the high prevalence of zinc deficiency in developing countries, QFT should be used with caution for the diagnosis of tuberculosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX
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