Commentary

Serial testing of *Mycobacterium tuberculosis* infection in Chinese village doctors by QuantiFERON-TB Gold Plus, QuantiFERON-TB Gold in-Tube and T-SPOT.TB

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SUMMARY

Objectives: To evaluate the performance of QuantiFERON-TB Gold Plus (QFT-Plus) on *Mycobacterium tuberculosis* (MTB) infection test among registered village doctors from China.

Methods: MTB infection of the registered village doctors in Zhongmu County were tested using QFT-Plus and two other interferon-gamma release assays (IGRAs) in parallel: QuantiFERON-TB Gold In-Tube (QFT) and T-SPOT.TB (T-SPOT). Retests were carried out for baseline positives at 3 and 6 months later, respectively.

Results: A total of 616 village doctors were included in the baseline examination. The positivity of QFT, QFT-Plus and T-SPOT was 27.91% (168/602), 31.22% (187/599) and 27.70% (169/610), respectively. The concordance between QFT and QFT-Plus was 94.81% (Kappa coefficient: 0.87) and between T-SPOT and QFT-Plus was 88.93% (Kappa coefficient: 0.73). Reversions were frequently observed for all three assays. With respect to QFT-Plus, the quantitative results of reversions in the serial testing were mostly distributed in an "uncertain range" zone (0.2-0.7 IU/mL). Similar patterns of distribution were observed for QFT and T-SPOT as well.

Conclusion: Village doctors should gain more attention as an at-risk group for TB infection control in rural China. Our results support, by means of serial testing, a good agreement between QFT-Plus and QFT in Chinese population.

Introduction

Latent tuberculosis infection (LTBI) management in high-risk groups is a new priority action for the End TB Strategy.[1] Interferon-gamma release assays (IGRAs) and tuberculin skin test (TST) have been recommended for testing *Mycobacterium tuberculosis* (MTB) infection.[2,3] Our findings of a population-based multicenter cohort study indicated the prevalence of LTBI, estimated by one commercial IGRAs (QuantiFERON-TB Gold In-Tube, QFT), in the general rural population ranged between 13.5% and 19.8% in study sites with varied tuberculosis (TB) epidemic situations.[4] Moreover, our results showed the performance of TST was influenced by multiple factors including BCG vaccination and aging, and it was not suitable for monitoring infection status by serial testing.[5] In practice, IGRAs showed limitations in sensitivity and consistency as well. The sensitivity of QFT and T-SPOT.TB (T-SPOT), another available IGRA, was reported to be approximately 80%, which limited their using as a rule out test for active TB.[6] In addition, several reports have proposed caution in interpreting QFT results close to the assay cut-off, where assay variability is more likely associated with inconsistent results observed in serial testing.[7] Therefore, improvements are needed for MTB infection diagnosis in order to more accurately identify individuals with recent TB infection for subsequent intervention.

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Recently, QuantiFERON-TB Gold Plus (QFT-Plus), as a new generation of QFT, was developed with two TB-specific antigens tubes (TB1 and TB2). TB1 tube induces a specific CD4+ T-cells response, and TB2 was designed to induce interferon-γ (IFN-γ) production by both CD4+ and CD8+ T-cells. It has been reported that QFT-Plus has increased sensitivity compared to QFT with some capability to identify recent infection among contacts.[8,9] However, the performance of QFT-Plus has not been evaluated in Chinese populations. Given the lack of a golden standard for MTB infection testing, we compared the performance of QFT-Plus with the other available IGRAs, QFT and T-SPOT.TB (T-SPOT), by means of serial testing in a specific population.[10] Village doctors are considered at high risk of TB exposure as they are involved in early case finding, referral and clinical management in TB control system in China. However, MTB infection status of village doctors has not been widely investigated despite of the well-known occupational exposure. Therefore, aiming to evaluate the performance of QFT-Plus and to clarify MTB infection burden in village doctors, a prospective study was conducted to examine the prevalence and persistence of MTB infection by serial testing of QFT, QFT-Plus and T-SPOT in parallel.

Materials and methods

Study design and participants

The study aims to evaluate the prevalence of MTB infection and to track the persistence of infection by serial testing of QFT, QFT-Plus and T-SPOT in village doctors in China. The baseline examination was conducted in April 2017 and baseline positives for any test were retested 3 and 6 months later.

All of the village doctors registered in Zhongmu county, Henan Province of China were included in the study. The inclusion criteria included: licensed doctor worked in the local village clinic or community health center; being able to complete the investigations and tests during the study duration; and voluntarily signed the informed consent form. The exclusion criteria included: with current active TB or self-reported prior TB; pregnant or lactating women or women preparing for pregnancy.

Ethical review

The study protocol was approved by the ethics committees of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (No: 2016-I2M-1-013).

Baseline survey

For each study participant, socio-demographic information was collected by a standardized questionnaire administered by trained interviewers. Data collected included age, gender, educational level, self-reported history of close contact with TB patients, smoking status (never smoked or ever smoked), current alcohol drinking status (never used or ever used) and history of type 2 diabetes mellitus (T2DM) (self-reported and/or fasting blood glucose higher than 7.0 mmol/L at baseline examination). Digital chest radiography (CXR) was performed on each study participant to exclude individuals with active TB. Height, weight and the presence of BCG scars were checked as well.

Venous blood was collected in a single tube for QFT, QFT-Plus, T-SPOT. All of the three IGRAs were performed in strict compliance with the recommendations of the manufacturer. A cutoff value of 0.35 IU/mL was used for QFT and QFT-Plus. Positive results for T-SPOT were defined by counting the Spot-forming cells with a cut-off value of $\geq$ 6 spots using blood less than 8 h from blood draw. In addition, blood biochemical examinations were performed for each participant as well.

Serial testing

For baseline positives for each of the three tests, retesting was conducted twice at 3 and 6 months after baseline survey, to determine the persistence of the positive results. Individuals with baseline negative results for all three tests were not retested.

Statistical analysis

Continuous variables were presented by means of median value and interquartile range (IQR). Body mass index (BMI) was calculated as weight over height squared (kg/m^2) and was further categorized as underweight ($<$ 18.5 kg/m^2), normal weight (18.5 to 24.0 kg/m^2), overweight (24.0 to 28.0 kg/m^2) or obese ($\geq$ 28.0 kg/m^2). Baseline positives were grouped by the serial testing results as persistent positive (being positive for both retests), persistent reversed (being negative for both retests) and unstable (being negative for any one retest).

Pearson's Chi-squared test was used to compare the distribution of categorical variables in the study participants. To identify potential factors related to MTB infection (defined by being positive for all three assays), variables showing significant relations in univariate analysis were included in unconditional multiple logistic regression analyses, and the associations were presented by means of odds ratio (OR) and 95% confidence interval (CI). The agreement between the assays was evaluated using the Cohen's kappa coefficient. Data were analyzed using Statistical Analysis System (SAS 9.2; SAS Institute Inc., NC, USA) and GraphPad Prism version 5.0.

Results

There were 625 of 653 registered village doctors participated in the baseline survey with a response rate of 95.71%. After excluding 9 participants due to incomplete data, 616 participants were included in this study. Table 1 shows major characteristics of the study participants and the baseline results of three assays (QFT, QFT-Plus, T-SPOT). Overall, 68.34% (421/616) of the participants were male and three quarters (77.60%) were older than 40 years with a median age of 47 years (IQR: 41-56 years). 29.06% (179/616) participants reported ever smoking and 25.49% (157/616) reported current alcohol drinking. The prevalence of diabetes mellitus type 2 (T2DM) was 8.97% (56/616) and of overweight or obese with (BMI $\geq$ 24.0 kg/m^2) was 70.13% (432/616). About two thirds of the participants (64.45%, 397/616) reported a history of close contact with TB patients. At baseline, the positivity of QFT, QFT-Plus and T-SPOT were 27.91% (168/602), 31.22% (187/599) and 27.70% (169/610), respectively. No indeterminate result was reported for all three assays.

As for the association analysis (Table 2), MTB infection was defined to be positive for all three assays. Only age was found to be significantly associated with the risk of MTB infection in our study population. The findings were not changed when alternatively defining MTB infection by being positive for any of the three tests (Supplementary Table 1).

The agreements across the three assays at baseline were summarized in Table 3. The concordance between QFT and QFT-Plus was 94.81% with a Kappa coefficient of 0.87 (95% CI: 0.83-0.92) and between T-SPOT and QFT-Plus was 88.93% with a Kappa coefficient of 0.73 (95% CI: 0.67-0.79). When using an alternative definition of QFT-Plus positivity (being positive for both TB1 and TB2), the concordance between QFT and QFT-Plus was 96.01% with a Kappa coefficient of 0.90 (95% CI: 0.86-0.94) and between T-SPOT and QFT-Plus was 90.33% with a Kappa coefficient of 0.76 (95% CI: 0.70-0.81).

Fig. 1 shows the retesting results of QFT, QFT-Plus and T-SPOT among their baseline positives. T-SPOT showed the highest

Table 1
Characteristics of the study population.

	n	%
Age (years)		
20–29	40	6.49
30–39	98	15.91
40–49	234	37.99
50–59	113	18.34
≥ 60	131	21.27
Gender		
Male	421	68.34
Female	195	31.66
Highest education level		
Middle school or lower	41	6.65
High school	436	70.78
College or higher	139	22.56
Smoking		
Never smoked	437	70.94
Ever smoked	179	29.06
Current alcohol drinking		
No	459	74.51
Yes	157	25.49
BCG scars		
Absent	128	20.78
Present	488	79.22
BMI (kg/m²)		
< 18.5	12	1.95
18.5-24.0	172	27.92
24.0-28.0	277	44.97
≥ 28.0	155	25.16
T2DM*		
No	568	91.03
Yes	56	8.97
Have close contact with TB patients		
No	397	64.45
Yes	219	35.55
QFT test		
Negative	434	72.09
Positive	168	27.91
QFT-Plus test		
Negative	412	68.78
Positive	187	31.22
T-SPOT test		
Negative	441	72.30
Positive	169	27.70

Abbreviation: BCG, Bacillus Calmette–Guérin; BMI, body mass index; IQR, interquartile range; QFT, QuantiFERON-TB Gold In-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; T2DM, type 2 diabetes mellitus; TB, tuberculosis; T-SPOT, T-SPOT.TB.

* Self-reported and/or fast blood glucose higher than 7.0 mmol/L at baseline.

Table 2
Identification of potential factors associated with tuberculosis infection.

	TB infection rate* n/N (%)	p for χ² test	Adjusted OR (95% CI)
Gender		0.002	
Female	28/195 (14.36)		Reference
Male	106/421 (25.18)		1.36 (0.81–2.29)
Age (years)		<0.001	
20–40	12/138 (8.70)		Reference
40–50	48/234 (20.51)		2.47 (1.25–4.88)
≥ 50	74/244 (30.33)		3.81 (1.93–7.51)
Highest education level		0.069	
Middle school or lower	4/41 (9.76)		
High school	104/436 (23.85)		
College or higher	26/139 (18.71)		
Smoking		0.030	
Never smoked	85/437 (19.45)		Reference
Ever smoked	49/179 (27.37)		1.13 (0.72–1.77)
Current alcohol drinking		0.079	
No	92/459 (20.04)		
Yes	42/157 (26.75)		
BCG scars		0.839	
Absent	27/128 (21.09)		
Present	107/488 (21.93)		
BMI (kg/m²)		0.359	
< 18.5	1/12 (8.33)		
18.5-24.0	34/172 (19.77)		
24.0-28.0	68/277 (24.55)		
≥ 28.0	31/155 (20.00)		
T2DM†		0.048	
No	116/560 (20.71)		Reference
Yes	18/56 (32.14)		1.36 (0.74–2.51)
Have close contact with TB patients		0.458	
No	90/397 (22.67)		
Yes	44/219 (20.09)		

Abbreviation: BCG, Bacillus Calmette–Guérin; BMI, body mass index; CI, confidence interval; IQR, interquartile range; OR, odds ratio; T2DM, type 2 diabetes mellitus; TB, tuberculosis.

* TB infection was defined as be positive for all three tests (QFT, QFT-Plus, T-SPOT).

† With self-reported history of diabetes and/or fast blood glucose higher than 7.0 mmol/L at baseline test.

persistent positive rate (88.41%, 122/138) as compared to QFT (79.41%, 108/136) and QFT-Plus (77.92%, 120/154) ($p = 0.021$). Study participants were classified into 3 groups according to the persistence of the results from the retesting: persistent positive, persistent reversion and unstable group. With respect to QFT-Plus, the majority of the quantitative values of reversions in serial testing were distributed in an "uncertain range" zone (0.2-0.7 IU/mL) as shown in Fig. 2. Similar patterns of distribution were observed for QFT and T-SPOT as well (Supplementary Figure 1 and Figure 2). Multiple comparisons were conducted among the three groups with respect of the baseline TB1, TB2 and TB2-TB1 values. The baseline IFN-γ values of TB1 and TB2 were dramatically higher in persistent positive group than in unstable group ($p = 0.003$) and in persistent reversion group ($p = 0.001$). However, the TB2-TB1 results of the persistent reversion group showed a marginally significant higher baseline IFN-γ level as compared to persistent positives.

When stratified the retests results of QFT-Plus by baseline results of TB1 and TB2, 3.01% (4/133) of TB1+/TB2+, 87.50% (7/8) of TB1+/TB2- and 69.23% (9/13) of TB1-/TB2+ were persistent reversed, respectively (Supplementary Table 2).

Results of 6-month retest for study participants with discordant baseline results (QFT-/QFT-Plus+ and T-SPOT-/QFT-Plus+) were showed in Supplementary Figure 3. Among 25 participants with baseline QFT-/QFT-Plus+, 19 (76.00%) were QFT-/QFT-Plus- and 4 (16%) were QFT+/QFT-Plus+ in retest. Among 39 participants with baseline T-SPOT-/QFT-Plus+, 18 (46.15%) were T-SPOT-/QFT-Plus- and 12 (30.77%) were T-SPOT+/QFT-Plus+. Original quantitative values of serial tests for study participants with discordant baseline results across QFT, QFT-Plus and T-SPOT were reported in Supplementary Tables 3–6.

Discussion

To our knowledge, this is the first study to evaluate the performance of QFT-Plus in a Chinese population by means of serial testing as compared with QFT and T-SPOT. We found village doctors have a markedly higher prevalence of MTB infection as compared to local general population. Reversions occurred for all three assays during retests, QFT-Plus showed higher positivity at baseline test and higher reversions in serial tests. Majority of the reversion might be explained by the biological variability and technical limitations around the cut point, but it could not be excluded the possibility of infection clearance.

China established rural primary healthcare system in 1960–70s, village clinics, township health centers and county hospitals are the foundation of the three-level health service system.[11] By the end of 2013, China had 1.08 million registered village doctors who serve the bottom-tier and provide basic medical services to

Table 3
Agreement between baseline results of QFT-Plus, QFT and T-SPOT.

QFT-Plus positivity was defined by positive for TB1 or TB2

Total	QFT-Plus-/ QFT-*n* (%)	QFT-Plus+/ QFT+*n* (%)	QFT-Plus-/ QFT+*n* (%)	QFT-Plus+/ QFT-*n* (%)	Kappa (95% CI)	Concordant(%)
597	407 (68.17)	159 (26.63)	5 (0.84)	26 (4.36)	0.87 (0.83-0.92)	94.81
	QFT-Plus-/T-SPOT-*n* (%)	QFT-Plus+/T-SPOT+*n* (%)	QFT-Plus+/T-SPOT-*n* (%)	QFT-Plus-/T-SPOT+*n* (%)		
594	388 (65.32)	140 (23.57)	44 (7.41)	22 (3.70)	0.73 (0.67-0.79)	88.93

QFT-Plus positivity was alternatively defined by positive for both TB1 and TB2

Total	QFT-Plus-/ QFT-*n* (%)	QFT-Plus+/ QFT+*n* (%)	QFT-Plus-/ QFT+*n* (%)	QFT-Plus+/ QFT-*n* (%)	Kappa (95% CI)	Concordant(%)
597	422 (70.69)	154 (25.80)	11 (1.84)	10 (1.67)	0.91 (0.88–0.95)	96.48
	QFT-Plus-/T-SPOT-*n* (%)	QFT-Plus+/T-SPOT+*n* (%)	QFT-Plus+/T-SPOT-*n* (%)	QFT-Plus-/T-SPOT+*n* (%)		
594	406 (68.35)	136 (22.90)	26 (4.38)	26 (4.38)	0.78 (0.72–0.84)	91.25

Abbreviation: CI, confidence interval; QFT, QuantiFERON-TB Gold In-Tube; QFT-Plus, QuantiFERON-TB Gold Plus; T-SPOT, T-SPOT.TB.
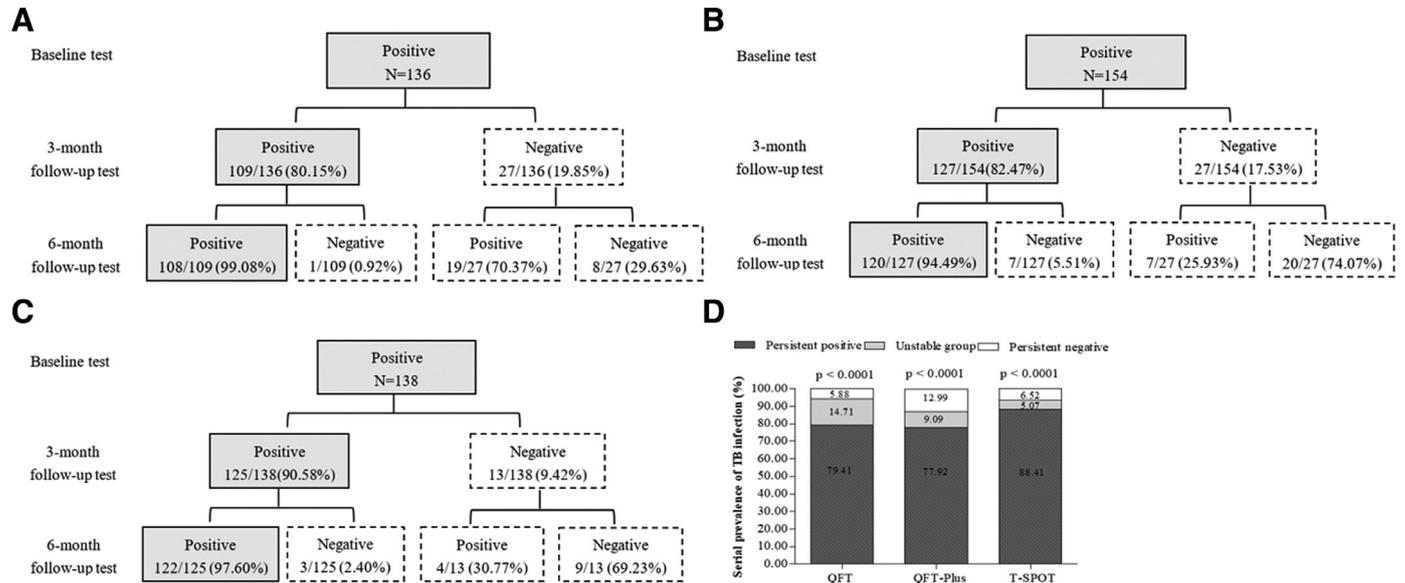

Fig. 1. Results of serial QFT, QFT-Plus and T-SPOT tests for baseline positives. Retests were performed for baseline positives after 3 months and 6 months for QFT (A), QFT-Plus (B) and T-SPOT (C), respectively. Study participants were then grouped by retests results as persistent positive (being positive for both retests), persistent reversed (being negative for both retests) and unstable (being negative for any one retested). (A) Among 136 baseline QFT positives, 108 participants had persistent positive results and 8 had persistent reversion results in the follow-up tests. (B) Among 154 baseline QFT-Plus positives, 120 participants were persistent positive in the follow-up tests and 34 showed negative results in any of the follow-up tests. (C) Among 138 baseline T-SPOT positives, 122 participants were persistent positive in the follow-up tests and 16 showed negative results in any of the follow-up tests. (D) The proportion of persistent positivity for T-SPOT (88.41%) was the highest among three tests (comparison among the groups by the Kruskal–Wallis test, $p = 0.021$).

rural residents. In the present study, the prevalence of MTB infection among village doctors was higher than that in the general population of the study site (QFT: 27.91% vs 15.52%),[4] with occupational exposure being the likely explanation.[12,13] However, the association of infection with close contact with active TB patients was not observed in our study possibly because the majority of the study participants reported a history of close contact (64.45%), limiting the power of our study to disclose the relation between the exposure and the infection status. Alternatively, exposure to active TB was unknown and underreported.

Reversion occurred during retests for all three assays in the present study. Host immunological status and analytical variability have been suggested influence IGRAs repeatability.[14,15] Results close to the cutoff value more frequently fluctuate as a cause of conversion or reversion.[7,16] Therefore, standard operational protocols are needed for laboratory internal quality assurance to minimize potential systematic bias.[17-19] In addition to technical variability, the impact of the individual's host immune response and biological variability could also explain reversion. However without a gold standard of latent MTB infection, it is difficult to determine the valid infection status for fluctuating results, especially when close to the cut point. Hence, several investigators have suggested the introduction of a zone of uncertainty between 0.2 and 0.7 IU/mL for QFT.[7,12] Our results are consistent with such findings that most study participants with reversions had a baseline results around the cutoff value for all three assays. Still, prospective studies are needed to clarify the infection status of the individuals with such assay variability and their risk for developing active disease.

It has been reported that QFT-Plus showed improved sensitivity to identify MTB infection especially recent infection,[20] but several studies from low TB epidemic countries reported a similar sensitivity of QFT-Plus when compared to QFT.[21-25] The suggested advantage of QFT-Plus are the added CD8 antigens as a biomarker for intracellular TB burden and in LTBI, more likely to improve performance in patients recently exposed to TB and from regions with higher risk of infection transmission. We reported the annual QFT conversion rate was 1.5% in the general rural population, which might be higher in village doctors.[5,26] Therefore, we anticipated a different performance of QFT-Plus in this highly exposed healthcare worker population. Indeed, our results showed that in compared with QFT and T-SPOT, QFT-Plus showed higher positivity at baseline. However, 12.99% of baseline QFT-Plus positives reverted to negative when retested and the rate of reversion was significantly higher than QFT (5.88%) and T-SPOT (6.52%). This raises a
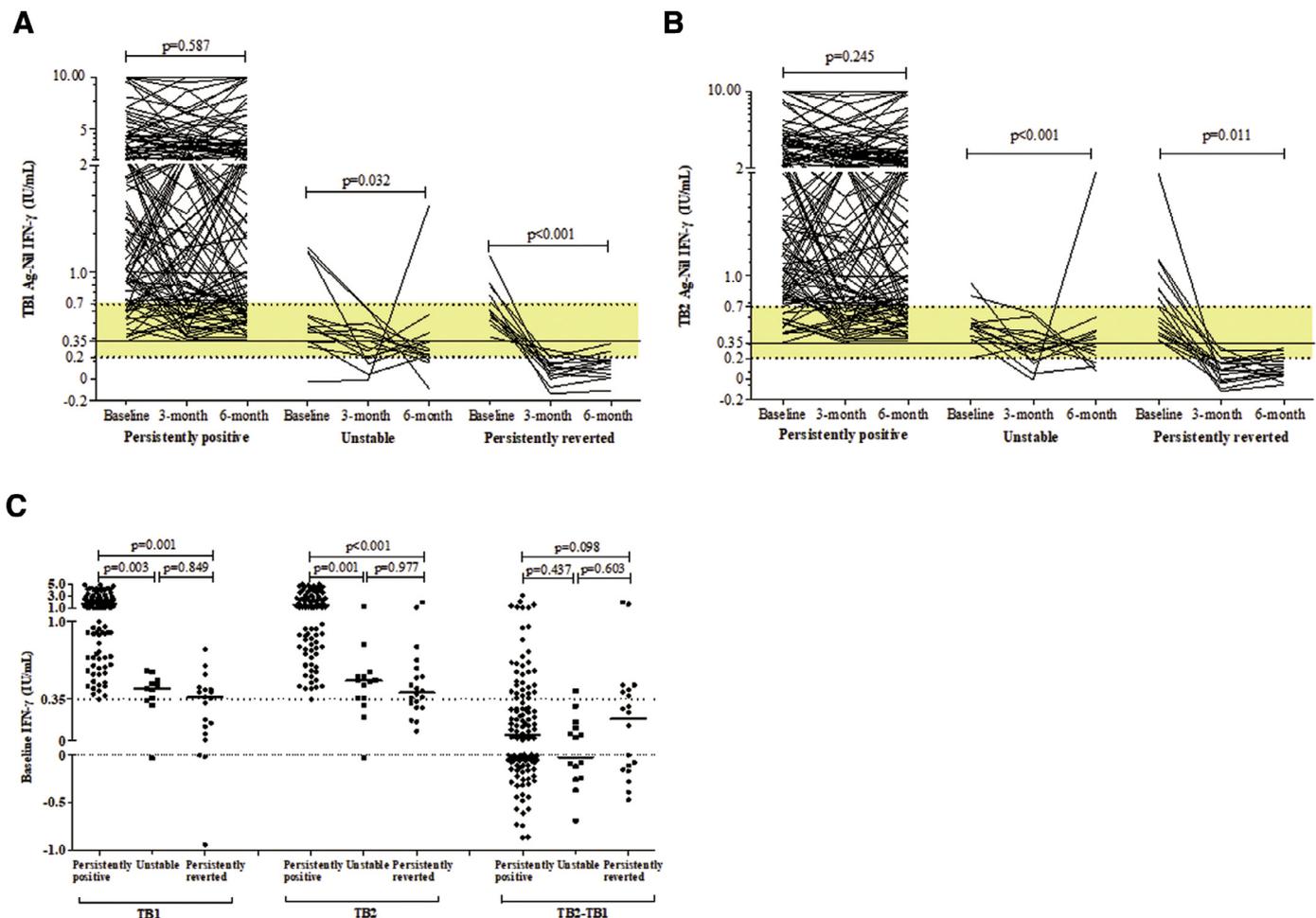
Fig. 2. Distribution of QuantiFERON-TB Gold Plus (QFT-Plus) quantitative values in serial tests for baseline positives. According to the retest results, participants with baseline QFT-Plus+ (i.e. TB1+/TB2+, TB1-/TB2+, TB1+/TB2-) were classified into three groups: persistent positive (being positive for both retests), persistent reversed (being negative for both retests) and unstable (being negative for any one retested). The distribution patterns of quantitative values of IFN-γ level in all three serial tests were shown for TB1 (A) and TB2 (B) for these three groups respectively (the differences were tested by Wilcoxon test). Baseline quantitative values of TB1, TB2 and TB2-TB1 were compared between the three groups (C). As compared to persistent positives, persistent reversion group showed significantly lower baseline IFN-γ level for TB1 and TB2 but marginally higher baseline IFN-γ level for TB2-TB1 (the differences were tested by the Kruskal–Wallis test).

question whether the higher QFT-Plus baseline positivity attributed to improved sensitivity or not.

Based on the QFT-Plus product information, TB1 contains long peptides eliciting a CD4+ T-cells response whereas TB2, beside the same long peptides, contains additional short peptides specific for the CD8+ T-cells. Several studies have described that CD8+ T-cells play a unique function in the recognition and containment of intracellular infection with MTB, an increase of the CD8 T-cells responses has been observed to be associates with MTB load.[27–29] Additionally, a strong CD8+ T-cells response has been identified as well among recently exposed contacts of patients with active TB.[28,30] Observational studies found the difference between the two antigen tubes (TB2-TB1), used as an indirect estimate of specific CD8+ T-cell activation, is associated with factors indicating increased MTB exposure, suggesting a possible role in identifying individuals with recent infection who at greater risk of progression to active TB.[17] In our study, based on retesting results, we classified baseline positives into three groups: persistent positive, persistent reversed and unstable. Infection clearance caused test reversion could not be completely excluded for those with persistent reversion. Consistently, individuals with persistent reversion were found with significant lower TB1 and TB2 but marginally significant higher baseline TB2-TB1 as compared to those persistent positives. It suggests indirectly that individuals

with persistent reversion might be recently infected at baseline with subsequent infection clearance during the short term before retesting. Therefore, as compared to QFT and T-SPOT, the highest baseline positivity for QFT-Plus might be explained at least partly by improved sensitivity to recent infection which under higher risk of clearance in the short term.

In addition, baseline TB1+/TB2- might be false QFT-Plus positive because most of them (87.50%, 7/8) got persistent reversion in the following retests as shown in Supplementary Table 2. When using an alternative positivity definition for QFT-Plus as being positive for both TB1 and TB2, the agreements between QFT-Plus and QFT/T-SPOT were improved. Therefore, the results of TB1 and TB2 might provide more information to clarify host infection status.

When interpreting the results of the present study, several limitations should be kept in mind. First, as an observational study we only provided indirect evidence to support the potentially improved sensitivity of QFT-Plus. Longitudinal studies are needed to confirm QFT-Plus has the capability to identify recent infections supported by recorded exposure and identify those at risk of infection clearance or developing active disease. Second, limited sample size makes evaluation of the factors associated with discordance between assays and serial tests difficult. Third, the intervals between retests were non-standardized which varied from several weeks to several months in previous studies. Re-exposure and

new infection related conversion could not be excluded especially in high-risk populations, which might lead to misclassification of the study participants. Fourth, individuals who were negative by all 3 assays were not retested in our study which limited us to examine variability of results for them. However, such retests will be conducted in yearly follow-up investigation in order to evaluate the conversion of the assays.

In conclusion, village doctors, as an occupationally exposed population, should gain more attention for TB infection control in rural China. Test reversion occurred for all three assays, which might be mostly explained by the testing variability for the results around the cutoff point but infection clearance could not be excluded completely. Our results support the use of QFT-Plus as a replacement for QFT in Chinese population given the good agreement between the two assays.

Funding

Conflicts of interest

No conflict of interest was declared.

Author contributions

L.G designed the study. HR.Z, HN.X, SG.P, ZS.L, DK.W, JX.W, XF.C, XW.L, MF.L, BX.F and Q.J were in charge of data management. HR.Z, HN.X, SG.P, QT.Yand L.G did data analysis and wrote the report. Q.J commented on the report and improved English writing. ZS.L, DK.W, JX.W, XF.C, XW.L, MF.L, MX.Z and BX.F organized investigations at the study sites. All authors contributed to review and revision and have seen and approved the final version of manuscript.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.01.008.

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