Effect of Prolonged Incubation Time on Results of the QuantiFERON TB Gold In-Tube Assay for Diagnosis of Latent Tuberculosis Infection

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Previous reports have shown that the sensitivity of the 6-day lymphocyte stimulation test is much higher than those of commercially available gamma interferon release assays (IGRAs). The aim of this study was to elucidate the effect of prolonged incubation on the results of the QuantiFERON TB Gold in-tube (QFT-GIT) assay. Patients aged 20 years with suspected tuberculosis (TB) were recruited prospectively from 1 May 2009 to 31 December 2010. In addition, healthy volunteers with no history of TB treatment were included as controls. For each participant, three sets of the QFT-GIT assay were performed using 24-, 48-, and 72-h incubation tests, and the results were compared. Thirty-seven patients with suspected pulmonary TB and 33 healthy controls were enrolled in the study. Of the 37 patients with suspected TB, the QFT-GIT assay results were positive for 28 (75.7%) after a 24-h incubation period. After prolonged incubation, the results differed in four (10.8%) of the 37 patients suspected of having TB. Among 27 patients with culture-confirmed TB, the sensitivities of the QFT-GIT assay after the 24-, 48-, and 72-h incubation tests were 85.2%, 81.5%, and 81.5%, respectively. Among the 33 healthy controls, the QFT-GIT assay results were positive in two (6.1%) after a 24-h incubation period. The results changed for two (6.1%) of the 33 healthy controls after prolonged incubation. The specificities of the QFT-GIT assay after 24, 48, and 72 h of incubation were 93.9%, 87.9%, and 90.9%, respectively. Prolonging the incubation time did not increase the sensitivity of the QFT-GIT assay. The manufacturer-recommended incubation time of 16 to 24 h should be respected because prolonged incubation can cause indeterminate or false-positive results.

Recently, gamma interferon (IFN-γ) release assays (IGRAs) have been introduced into clinical practice as an alternative to the traditional tuberculin skin test (TST) (1, 2). Two IGRAs are available commercially, the QuantiFERON TB Gold in-tube (QFT-GIT) assay (Cellestis, Victoria, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Oxford, United Kingdom). A meta-analysis suggested that the QFT-GIT assay may not be as sensitive as the T-SPOT.TB assay (70% for QFT-GIT assay and 90% for T-SPOT.TB assay) (3), but the QFT-GIT assay is more convenient because it does not require mononuclear cell separation.

Since the adoption of the QFT-GIT assay in routine clinical practice, various procedural modifications have been investigated to improve its sensitivity. Incubation without delay (4, 5) and incubation at 39°C rather than 37°C (6) have been reported to increase its sensitivity.

Given that the sensitivity of the 6-day lymphocyte stimulation test is much higher than that of IGRA (7), we postulated that prolonged incubation would increase the sensitivity of the QFT-GIT assay for diagnosing Mycobacterium tuberculosis infection. In addition, in routine clinical practice in South Korea, sampling for the QFT-GIT assay is performed between Monday and Thursday and not on Friday. Because the assay measures the release of IFN-γ from whole-blood samples after a 16- to 24-h incubation period, the postincubation process had to be performed on weekends if samples were drawn on Friday.

In this context, we examined the effect of prolonged incubation on the results of the QFT-GIT assay.

MATERIALS AND METHODS

Participants. Patients aged ≥20 years with suspected tuberculosis (TB) were recruited prospectively from 1 May 2009 to 31 December 2010 at Seoul National University Hospital and Kwandong University Myongji Hospital. In addition, 33 healthy volunteers with neither a history of TB treatment nor contact with active TB patients were recruited as controls. Written informed consent was obtained from all participants, and the study protocol was approved by the ethics review committee and institutional review board at each center.

Determination of sample size. We expected that the sensitivity of the QFT-GIT assay would improve from 70% to 80% by prolonging the incubation period from 24 h to 48 or 72 h. To show this change, a required minimum sample size of 27 was estimated based on a one-tailed test with a 95% confidence interval (CI), a type II error of 0.20, and a power of 0.80.

QuantiFERON TB Gold in-tube assay. From each participant, 9 ml of whole blood was drawn and put directly into three sets of QFT-GIT blood collection tubes, 1 ml each for the nil control tube, TB antigen tube (6-kDa early secretory antigenic target protein [ESAT-6], cyan fluorescent protein 10 [CFP-10], or TB7.7), and mitogen control tube. All tubes were incubated within 6 h of sampling. The first, second, and third sets of tubes were processed and analyzed after the 24-, 48-, and 72-h incubation tests, respectively. IFN-γ levels were measured using a QFT-GIT enzyme-linked immunosorbent assay (ELISA) kit. Samples from the same patient were measured in the same ELISA plate. Samples with a TB antigen value minus the nil value of ≥0.35 IU/ml and ≥25% of the nil value, as determined by the manufacturer’s software, were considered positive.

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Statistical analysis. The change in IFN-γ levels with prolonged incubation was analyzed using a repeated-measures nonparametric test. A P value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL).

RESULTS

Participants. Thirty-seven patients with suspected TB were enrolled in the study (median age, 66 years; age range, 27 to 90 years; 21 [56.8%] males). Mycobacterium bovis Bacillus Calmette-Guérin (BCG) scars were found in 12 patients (32.4%), and seven (18.9%) had a history of TB treatment. In 27 (73.0%) of the 37 patients, pulmonary TB was confirmed by culture of M. tuberculosis.

Thirty-three healthy volunteers were recruited for comparison as controls (median age, 28 years; age range, 23 to 42 years; 19 [57.6%] males). BCG scars were found in 25 (75.6%) participants. None of the volunteers had a history of TB treatment.

Baseline results of the QFT-GIT assay. After 24 h of incubation, QFT-GIT assay results were positive for 28 (75.7%) of the 37 patients. Three patients showed indeterminate results. Among the 27 culture-confirmed TB patients, 23 (85.2%) yielded positive assay results. The median IFN-γ level (value from stimulation of TB antigen minus value from nil stimulation) was 2.19 IU/ml (range, 0.01 to 10.00 IU/ml) (Fig. 1).

Two of the 33 healthy volunteers had positive QFT-GIT assay results and 31 had negative results. The median IFN-γ level was 0.00 IU/ml (range, −0.13 to 10.00 IU/ml) (Fig. 1).

Change in QFT-GIT assay results after prolonged incubation. Patients with suspected TB. The changes in QFT-GIT assay results according to incubation duration are shown in Table 1. The results for four patients (10.8%) differed between the 24-h and longer incubation times.

In two patients with negative results after 24 h of incubation (IFN-γ levels of 0.01 and 0.11 IU/ml), the results were indeterminate after the 48- and 72-h incubation periods because of the differences in the IFN-γ levels after mitogen and nil stimulation (0.05 and 0.28 IU/ml, respectively, at 48 h, and 0.23 and 0.46 IU/ml, respectively, at 72 h).

In one patient with an initial positive result (IFN-γ level, 0.49 IU/ml), the result was still positive after a 48-h incubation period (IFN-γ level, 0.53 IU/ml) but was negative after a 72-h incubation period (IFN-γ level, 0.30 IU/ml). In another patient with an initial positive result (IFN-γ level, 0.53 IU/ml), the result was indeterminate after a 48-h incubation period because the difference between the IFN-γ levels after mitogen and nil stimulation was 0.27 IU/ml. After 72 h of incubation, the result was positive again (IFN-γ level, 0.51 IU/ml).

The IFN-γ level did not change between the 24-h (median, 2.19 IU/ml) and 48-h incubation times (median, 2.60 IU/ml), although the IFN-γ level decreased between the 48-h and 72-h incubation
times (median, 1.88 IU/ml; \( P = 0.003 \)) (Fig. 1). The difference between IFN-\( \gamma \) levels after mitogen and nil stimulation did not change with prolonged incubation (\( P = 0.140 \)).

**Healthy volunteers.** The results of two of the 33 healthy volunteers differed with prolonged incubation (Table 1). The initial results of both subjects were negative, with IFN-\( \gamma \) (TB antigen value minus the nil value) levels of 0.30 and 0.28 IU/ml, respectively. In the first volunteer, the result was positive after both the 48-h (IFN-\( \gamma \) level, 0.67 IU/ml) and 72-h incubation periods (IFN-\( \gamma \) level, 0.46 IU/ml). In the other volunteer, the result was positive after a 48-h incubation period (IFN-\( \gamma \) level, 1.47 IU/ml) and negative after a 72-h incubation period (IFN-\( \gamma \) level, −0.45 IU/ml).

The IFN-\( \gamma \) level increased between the 24-h and 48-h incubation periods (\( P = 0.034 \)) but the IFN-\( \gamma \) level did not change between the 48-h and 72-h incubation periods (\( P = 0.715 \)) (Fig. 1). The difference between the IFN-\( \gamma \) levels after mitogen and nil stimulation did not change after prolonged incubation (\( P = 0.317 \)).

**Sensitivity and specificity of QFT-GIT assay after prolonged incubation.** Among the 27 culture-confirmed TB patients, 23, 22, and 22 yielded positive assay results after the 24-, 48-, and 72-h incubation tests, respectively. As a result, the sensitivities of the QFT-GIT assay after the 24-, 48-, and 72-h incubation tests were 85.2%, 81.5%, and 81.5%, respectively. Among the 33 healthy volunteers, 31, 29, and 30 yielded negative assay results after the 24-, 48-, and 72-h incubation tests, respectively. As a result, the specificities of the QFT-GIT assay after the 24-, 48-, and 72-h incubation tests were 93.9%, 87.9%, and 90.9%, respectively.

**DISCUSSION**

Since the clinical adoption of the QFT-GIT assay, the impacts of various procedural modifications on the assay results have been reported. First, the importance of immediately incubating the tubes at 37°C has been underscored. According to two reports, immediate incubation reduces the incidence of indeterminate results (4, 5). In a study conducted in Zambia, a 24-h delay in the start of incubation produced indeterminate results in five of 109 TB patients (8).

Modifications to the incubation process have been made and evaluated to improve the sensitivities of assays based on IFN-\( \gamma \) measurements. First, because fever augments the proinflammatory immune response *in vivo*, incubation was conducted at 39°C rather than at 37°C (6). In that study, incubation at 39°C increased the IFN-\( \gamma \) level and IFN-\( \gamma \)-inducible protein 10 response to TB antigens in participants with a low initial response. Another study has reported much higher sensitivity for the 6-day lymphocyte stimulation test than for the two commercially available IGRAs (the QFT-GIT and T-SPOT.TB assays) (7).

Meanwhile, it would be very helpful if prolonged incubation did not affect IGRA results. Prolonged incubation could allow for sampling on Fridays and processing on the following Monday. In addition, samples could be incubated for several days during their transport to a central laboratory.

In the present study, we used the QFT-GIT assay using incubation times (48 and 72 h) that are longer than the manufacturer-recommended time and compared the results with those of the standard incubation time (16 to 24 h). With prolonged incubation, the negative or indeterminate baseline results for four of the TB patients did not change to positive results. Instead, changes to positive results occurred in several patients because of the decreased difference between IFN-\( \gamma \) levels after mitogen and nil stimulation. As a result, the sensitivity of the QFT-GIT assay decreased slightly after prolonged incubation.

Conversely, prolonged incubation changed the results from negative to positive in two healthy volunteers. Because these two volunteers had no evidence of a history of TB treatment or contact with TB, these results might be interpreted as false-positive results caused by prolonged incubation. Consequently, the specificity of the QFT-GIT assay also decreased slightly after prolonged incubation.

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**TABLE 1 Results of the QuantiFERON TB Gold in-tube assay according to incubation period**

| Patient group and patient (no. of patients) | Final diagnosis | QFT-GIT results after incubation times of 
<table>
<thead>
<tr>
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<tr>
<td>-------------------------------------------</td>
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<td>---------------------------------</td>
</tr>
<tr>
<td>Patients with suspected TB (37)(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With consistent results (33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–21</td>
<td>TB</td>
<td>Positive  Positive Positive</td>
</tr>
<tr>
<td>22 and 23</td>
<td>TB</td>
<td>Indeterminate Indeterminate Indeterminate</td>
</tr>
<tr>
<td>24–28</td>
<td>TB, not confirmed</td>
<td>Positive Positive Positive</td>
</tr>
<tr>
<td>29–32</td>
<td>TB, not confirmed</td>
<td>Negative Negative Negative</td>
</tr>
<tr>
<td>33</td>
<td>TB, not confirmed</td>
<td>Indeterminate Indeterminate Indeterminate</td>
</tr>
<tr>
<td>With inconsistent results (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>TB</td>
<td>Positive  Positive Negative</td>
</tr>
<tr>
<td>35</td>
<td>TB</td>
<td>Positive  Indeterminate Positive</td>
</tr>
<tr>
<td>36</td>
<td>TB</td>
<td>Negative  Indeterminate Indeterminate</td>
</tr>
<tr>
<td>37</td>
<td>TB</td>
<td>Negative  Indeterminate Indeterminate</td>
</tr>
<tr>
<td>Healthy volunteers (33)</td>
<td></td>
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<tr>
<td>With consistent results (31)</td>
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<tr>
<td>1–29</td>
<td>None</td>
<td>Negative  Negative Negative</td>
</tr>
<tr>
<td>30–31</td>
<td>None</td>
<td>Negative  Positive Positive</td>
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<tr>
<td>With inconsistent results (2)</td>
<td></td>
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</tr>
<tr>
<td>32</td>
<td>None</td>
<td>Negative  Positive Positive</td>
</tr>
<tr>
<td>33</td>
<td>None</td>
<td>Negative  Positive Positive</td>
</tr>
</tbody>
</table>

\( a \) QFT-GIT, QuantiFERON TB Gold in-tube assay.  
\( b \) TB, tuberculosis.
To fully appreciate our results, the limitations of this study should be acknowledged. First, the sensitivity from prolonged incubation in the study was drawn from patients with active TB. In a previous study recruiting participants with mostly remote latent TB infection, the explanation for the increased sensitivity was that prolonged incubation allowed memory T cells to respond, while only effector cells would be able to mount an IFN-γ response in the short-term assay (7). Because the effector cells were still prevalent among patients with active TB, the lack of effect of prolonged incubation periods in our study cannot be generalized.

Second, although the healthy volunteers who participated in our study did not have a history of past TB treatment or contact with active TB patients, they might have been exposed to tuberculous bacilli because the annual incidence of TB in South Korea is 100 out of 100,000 (9). Consequently, the specificities presented in this study might be underestimated.

In conclusion, the use of prolonged incubation times did not increase the sensitivity of the QFT-GIT assay. The recommended incubation time of 16 to 24 h should be respected because prolonged incubation can cause indeterminate or false-positive results.

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REFERENCES