QuantiFERON-TB Gold Plus with Chemiluminescence Immunoassay: Do We Need a Higher Cutoff?

Quantiferon-TB Gold Plus by CLIA: Do we need a higher cut-off?

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Running head: Higher cut-off for QFT-Plus by CLIA?

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ABSTRACT

Quantiferon-TB Gold Plus (QFT-Plus) is the most widely used interferon-γ release assay (IGRA) for the diagnosis of latent tuberculosis infection (LTBI). The aim of this study was to compare QFT-Plus results by enzyme-linked immunoassay (ELISA) on the SKYLAB system with those obtained with chemiluminescence immunoassay (CLIA) on the LIAISON XL analyzer.

Agreement between the two assays was evaluated on 419 QFT-Plus blood samples, and was found to be substantial (75.4%): higher agreement was found for positive (95.4%) and negative (80.4%) results, while most discordances were due to ELISA-indeterminate/CLIA-determinate results.

According to the Italian Clinical Microbiologist Association recommendations, in samples (n=79) with a borderline result in ELISA (0.20-0.70 IU/ml), CLIA median values statistically increased (from 0.29 to 0.59 IU/ml for TB1 and from 0.32 to 0.60 IU/ml for TB2), but remained in the borderline range.

Linear regression analysis indicated a substantial correlation between ELISA and CLIA for antigen tubes TB1 (Pearson's r=0.8666) and TB2 (Pearson's r=0.8728), but CLIA produced higher values than ELISA. ROC analysis showed that the optimal cut-off value in CLIA was 0.45 IU/ml for TB1 and 0.46 IU/ml for TB2.

In conclusion, automated QFT-Plus with CLIA is comparable to QFT-Plus performed by ELISA. Within the linearity range of the test, CLIA detects higher quantitative values than ELISA, resulting in a higher number of determinate results, and the conversion of samples that were close to the cut-off into positive borderline results. A higher cut-off for QFT-CLIA needs to be defined, based on clinical diagnostic criteria.

INTRODUCTION

Latent tuberculosis infection (LTBI) is defined as a state of persistent immune response to *Mycobacterium tuberculosis* complex (MTBc) without clinically manifested evidence of active tuberculosis (TB) disease. About 1.7 billion people worldwide are estimated to have LTBI and 5–10% of them are at risk of developing active TB during their lifetime (1-5).

Two tests are available for the identification of LTBI: the tuberculin skin test (TST) and interferon gamma (IFN-γ) release assays (IGRAs). These are indirect markers of MTBc exposure and indicate a cellular immune response to MTBc. One IGRA, Quantiferon-TB Gold Plus (QFT-Plus, Qiagen) measures IFN-γ released by T-cells following stimulation by MTBc-specific antigens (6). QFT-Plus contains two MTBc-specific antigen tubes, called TB1 and TB2: TB1 contains ESAT-6- and CFP-10-derived long peptides, designed to elicit cell-mediated immune responses from CD4+T-helper lymphocytes; TB2 contains the same long peptides as TB1, in addition to shorter peptides able to stimulate CD8 T-cells (6-8). As CD8+ T-cell response seems to play a role in the early phase of MTBc infection and in reactivation from LTBI, the QFT-Plus test might be useful in identifying recent and remote LTBI, facilitating the decision to start LTBI treatment (9,10). IFN-γ detection with QFT-Plus assay is almost exclusively performed with enzyme-linked immunosorbent assay (ELISA), which has some disadvantages in clinical laboratories, such as labour-intense and time-consuming steps and requiring standard serial dilutions for each microplate.

Recently, new chemiluminescence immunoassays (CLIA) have been developed to detect IFN-γ in human plasma samples. However, to date only few studies have been published comparing QFT-Plus and the previous version QFT-TB Gold In-Tube by ELISA to CLIA (11-13). Among these, the study with the most relevant sample size (341 samples) reports a high degree of agreement (99.1%) between the two methods, using the AdvaSure I3 platform for CLIA (13).

A new fully-automated CLIA detection system to measure IFN-γ in human plasma has recently been developed on the LIAISON XL analyzer (DiaSorin, Italy). CLIA repeatability and reproducibility on this platform were studied by Brantestig, who found that the imprecision of the method is within an

acceptable range and analysis of linearity showed acceptable recovery (12). Furthermore, a recent paper by De Maertaelere et al. conducted on 92 samples showed that CLIA gave significantly higher values for TB1 and TB2 than ELISA (11).

The aim of this study was the head-to-head comparison of IFN-γ detection by ELISA on SKYLAB system and CLIA on the LIAISON XL analyzer in a large number of plasma samples. Furthermore, we compared quantitative IFN-γ responses to MTBc antigens (TB1 and TB2) and Mitogen detected by both methods.

MATERIAL AND METHODS

Samples

In this study, 419 clinical specimens which had been submitted to the Microbiology Unit of S. Orsola-Malpighi University Hospital (Bologna, Italy) for QFT-Plus test by ELISA were also analysed by CLIA.

Sample selection was based on the 3 categories of ELISA QFT-Plus results (positive, negative, indeterminate) according to the manufacturer's cut-off and having a sufficient volume to perform CLIA. Furthermore, an additional category of samples defined borderline was included according to Italian Clinical Microbiologist Association recommendations. Sample size for each category was chosen to ensure a large enough number to perform the analysis on ELISA indeterminate and borderline results since they could be greatly influenced by different methods of measurement.

Samples were anonymized with an alphanumerical code according to the ELISA qualitative result.

Clinical data were not collected for this study. Informed consent was not required as the data were analysed anonymously. The study was conducted in accordance with the Declaration of Helsinki.

Quantiferon-TB Gold Plus (QFT-Plus)

QFT-Plus samples (Qiagen, Germany) were analyzed by ELISA on SKYLAB automated system (DASIT, Italy) and by CLIA on the LIAISON XL instrument (DiaSorin, Italy), according to the standard procedures recommended by the manufacturers (6,14).

The clinical samples were handled according to the standard procedure for QFT-Plus assay, i.e. incubation at 37°C for 16–24 h within 16 hours of sampling, followed by centrifugation at 2700 g at room temperature for 15 minutes, and IFN-γ detection by ELISA. For this study selected samples were promptly frozen at -20°C after ELISA to assure IFN-γ stability (15,16). Before CLIA testing, frozen samples (range 1-101 days) were re-centrifuged at 2700g for 15 minutes to sediment the fibrin clots that can form during storage.

In accordance to the manufacturer's interpretation, positive results were defined as background (Nil)-corrected MTBc antigens (TB1 and/or TB2) values of ≥0.35 IU IFN-γ/ml; if the Nil-corrected Mitogen value was <0.50 IFN-γ IU/ml and/or if the Nil value was >8.0 IFN-γ IU/ml the test was considered indeterminate.

Furthermore, according to the Italian Clinical Microbiologist Association recommendations, the category borderline was defined as Nil-corrected MTBc antigens (TB1 and/or TB2) values within the range 0.20-0.70 IFN-γ IU/ml (17).

Statistical analysis

Cohen's κ statistics were used to assess agreement between ELISA and CLIA QFT Plus results as well as agreement between TB1 and TB2 results for each assay.

The Mann-Whitney test was used to compare medians of Nil-corrected IFN-γ responses to TB1 and TB2 and Mitogen. Since the ELISA QFT-Plus test cannot accurately determine IFN-γ values >10 IU/ml, a value of 10 IU/ml was attributed to plateau values in all the analyses by convention, as already adopted in the literature (18).

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Samples with TB1 and TB2 IFN-γ levels within the analytical range of each assay (<10 IU/ml), excluding indeterminate results, were used to assess the correlation between ELISA and CLIA. Correlation was expressed by Pearson's correlation coefficient (r). For this group, the optimal cut-off values of CLIA for TB1 and TB2 were determined from receiver-operator characteristic (ROC) curve analysis assuming the positive result of the ELISA method as true LTBI or TB.

Statistical analysis was performed using GraphPad Prism version 8.0.1 (USA). Statistical significance was set at $p<0.05$.

RESULTS

Agreement between ELISA and CLIA QFT-Plus results

A total of 419 QFT-TB Plus samples analyzed by ELISA were included in this study with the following results: 153 (36.5%) positive, 168 (40.1%) negative, 97 (23.2%) indeterminate due to low Nil-corrected Mitogen value (<0.50 IFN-γ IU/ml) and 1 (0.2%) indeterminate due to high Nil value (>8.0 IFN-γ IU/ml) according to the manufacturer's cut-off. The same QFT-Plus samples were then processed by CLIA and produced the following results: 182 (43.4%) positive, 197 (47.0%) negative, 34 (8.1%) indeterminate due to low Nil-corrected Mitogen value and 6 (1.5%) indeterminate due to high Nil value.

The comparison of the results obtained by both assays is reported in Table 1. Concordant results were obtained for 316 out of 419 samples (agreement 75.4%, κ=0.61, 95% CI 0.55-0.67). The agreement between TB1 and TB2 results was 95.7% for ELISA (κ=0.91, 95% CI 0.86-0.95), and 95.0% for CLIA (κ=0.90, 95% CI 0.85-0.94).

Of the 103 (24.6%) samples with discordant results, 63 (61.2%) were due to indeterminate ELISA results, which were determinate with CLIA (60 negative and 3 positive). Median mitogen IFN-γ value in these samples was 0.34 IU/ml in ELISA and 0.94 IU/ml in CLIA. In contrast, median mitogen

IFN-γ value in 34 samples which remained indeterminate in CLIA was 0.16 IU/ml in ELISA and 0.27 IU/ml in CLIA, excluding 1 indeterminate case due to high Nil value. These differences were statistically significant ($p<0.0001$).

The discordant cases with a determinate ELISA result were: 2 ELISA-positive/CLIA-negative (both with only 1 MTBc antigen tube positive in ELISA), 5 ELISA-positive/CLIA-indeterminate (all due to high Nil values in ELISA with a median value of 6.78 IU/ml), 33 ELISA-negative/CLIA-positive (median values of 0.21 and 0.24 IU/ml with ELISA statistically lower than median values of 0.49 and 0.51 IU/ml with CLIA for TB1 and TB2, respectively, $p<0.0001$).

Results interpreted according to the Italian Clinical Microbiologist Association recommendations by introducing the category "borderline" (TB1 and/or TB2 values within 0.20-0.70 IFN-γ IU/ml), are reported in Table 2. Concordant results were obtained for 295 of the 419 samples (70.4%, $\kappa=0.59$, 95% CI 0.54-0.65). In samples with a borderline result in ELISA (n=79), CLIA median values statistically increased from 0.29 to 0.59 IU/ml for TB1 and from 0.32 to 0.60 IU/ml for TB2 ($p<0.0001$).

Correlation between ELISA and CLIA TB1 and TB2 IFN-γ levels

For MTBc antigen tubes results within the linearity range 0-10 IU/ml and excluding indeterminate results (n=301), linear regression analysis showed that there was substantial correlation between the two tests, both for TB1 (Pearson's r=0.8666) and TB2 (Pearson's r=0.8728) (Figure 1A, 1B). Furthermore, the regression slopes (1.094 for TB1, 1.177 for TB2) and the intercepts (+0.2606 for TB1, +0.2521 for TB2) indicated that CLIA produces significantly higher values both for TB1 and TB2 than ELISA ($p<0.0001$). In fact, in this group median IFN-γ values of MTBc antigen tubes were statistically higher in CLIA than in ELISA both for TB1 (0.42 vs. 0.21 IU/ml, $p=0.0039$) and TB2 (0.40 vs. 0.22 IU/ml, $p=0.0047$).

Area Under the Curve (AUC) results for TB1 and TB2 are reported in Figure 2A and 2B respectively. For TB1 AUC was 0.978 (95% CI 0.962-0.994, <0.0001) and the cut-off value with the maximal sum

of sensitivity and specificity was >0.45 IFN-γ IU/ml (sensitivity 97.6%, specificity 85.9%). Using the manufacturer's suggested cut-off value of 0.35 IFN-γ IU/ml, the results showed a comparable sensitivity 99.2%, but a lower specificity 81.9%.

For TB2 AUC was 0.980 (95% CI 0.964-0.996, <0.0001) and the cut-off value with the maximal sum of sensitivity and specificity was >0.46 IFN-γ IU/ml (sensitivity 99.2%, specificity 88.7%). Using the manufacturer's suggested cut-off value of 0.35 IFN-γ IU/ml, the results showed the same sensitivity 99.2%, but a lower specificity 81.9%.

DISCUSSION

In this study we compared the QFT-Plus routinely performed by ELISA in our laboratory with the automated CLIA performed on the LIAISON XL instrument on a large number of selected samples (n=419). In particular, we focused our analysis on ELISA indeterminate and borderline results since they could be greatly influenced by different methods of measurement.

We found substantial agreement (75.4%) between the assays interpreted according to the manufacturer's cut-off. Most discordant results (61.2%, n=63) were due to indeterminate ELISA which were determinate in CLIA. In literature only a few studies have been published regarding this comparison, on a smaller number of samples, reporting higher agreement between the two tests: De Maertelaere et al. described a population of 92 samples with 4.3% of indeterminate ELISA and found an overall agreement of 95% (11); Brantestig et al. analyzed 125 samples with 8% indeterminate ELISA and found an agreement of 96.8% (12); Kim and colleagues reported an overall agreement of 99.12% on 341 samples (13). The near perfect agreement obtained by Kim et al. was probably due to the lack of indeterminate cases (0.3%) in their sample population. In contrast, in our population indeterminate ELISA due to low Mitogen value (<0.50 IFN-γ IU/ml) accounted for 23.2%.

The high number of indeterminate results selected for this study allowed us to show that ELISA median Mitogen IFN-γ value in samples which converted to a determinate result in CLIA was 0.34 IU/ml, significantly higher than those which remained indeterminate in CLIA (0.16 IU/ml).

Further discordant results (32%, n=33) were ELISA-negative/CLIA-positive, confirming that CLIA detects higher quantitative values than ELISA. However, in this group ELISA median TB1 and TB2 values were close to the cut-off (0.21 and 0.24 IFN-γ IU/ml for TB1 and TB2, respectively), as were the corresponding CLIA values (0.49 and 0.51 IFN-γ IU/ml for TB1 and TB2, respectively).

The Italian Clinical Microbiologist Association recently suggested defining borderline results as TB1 and/or TB2 values within the range 0.20-0.70 IFN-γ IU/ml and recommended re-testing borderline samples. According to this recommendation, agreement between the two assays was moderate (70.4%), lower than the agreement observed when the manufacturer's cut-off was used. Similarly, Brantestig et al. found a lower agreement (88%) using the Swedish National recommendations, that define a broad borderline range (0.20-0.99 IFN-γ IU/ml), than applying the manufacturer's cut-off (96.8%) (12). However, in our study among the ELISA-borderline samples (n=79) the increased CLIA values remained in the borderline range (0.29 vs. 0.59 IU/ml for TB1 and 0.32 vs. 0.60 IU/ml for TB2), suggesting that this range may not need to be revised for CLIA.

Linear regression analysis indicated substantial correlation between ELISA and CLIA despite the two different methods of measurement; however, CLIA produced significantly higher values both for TB1 and TB2 than ELISA. This is in agreement with previous data on CLIA performance in a smaller study population (11). In our opinion, this difference is not due to pre-analytical factors, but rather to the intrinsic chemistry of the assay based on chemiluminescence technology with paramagnetic microparticle solid phase, allowing the detection of very low levels of IFN-γ (13,19).

AUC analysis indicated that cut-off values of 0.45 IU/ml for TB1 and 0.46 IU/ml for TB2 returned the maximal sum of sensitivity and specificity, suggesting the need of a higher cut-off for QFT-Plus with CLIA compared to ELISA.

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The limitation of our study is the lack of clinical data; further studies on a larger sample size with medical records available should be performed to more clearly define the CLIA threshold on the LIAISON XL system.

In conclusion, QFT-Plus performed with CLIA showed substantial agreement with ELISA. The LIAISON XL analyzer has several advantages such as rapid turn-around time, high analytical measurement ranges and good precision. Within the linearity range of the test, CLIA detects higher quantitative values than ELISA, resulting in a higher number of determinate results, and the conversion of negative samples close to the cut-off into positive borderline results. A higher cut-off for QFT-CLIA needs to be defined, based on clinical diagnostic criteria.

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285 **FIGURE LEGENDS**

286

287 **FIG 1: Regression analysis of TB1 (A) and TB2 (B) IFN-γ levels between ELISA and CLIA**
288 **QFT Plus.** Regression line (solid) and 95% confidence intervals (dotted) for the Nil-subtracted
289 antigen tubes, within the range 0-10 IU/ml, are plotted; r=Pearson's correlation coefficient.

290

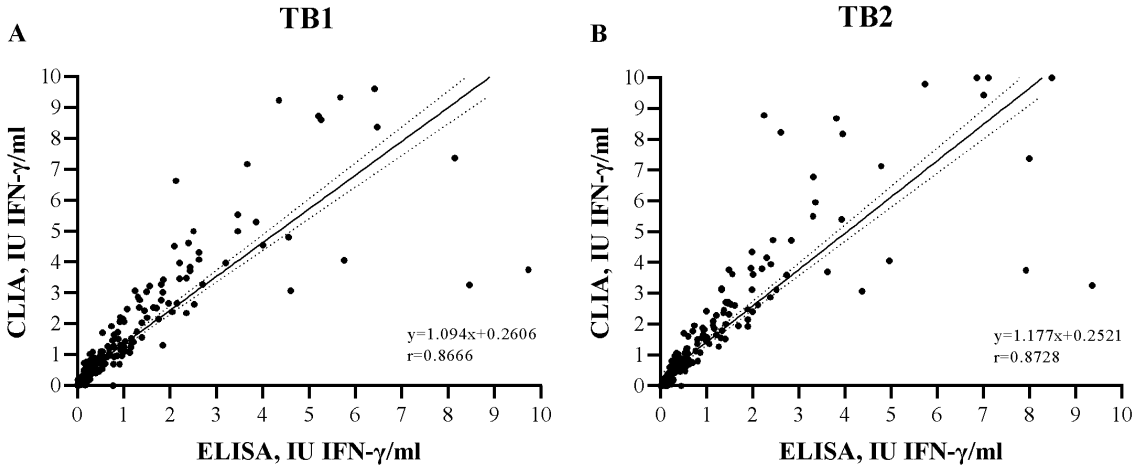
291 **FIG 2: ROC curve of the CLIA QFT-Plus TB1 (A) and TB2 (B) to diagnose latent tuberculosis**
292 **infection.** Sensitivity and specificity according to manufacturer's cut-off and to the cut-off defined
293 by AUC analysis are reported. Infection was assessed based on the results of ELISA QFT-Plus.

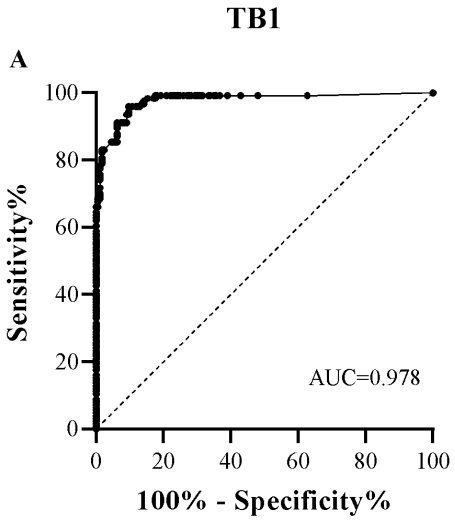
		QFT Plus ELISA			
		Positive, n	Negative, n	Indeterminate, n	Total
QFT Plus CLIA	Positive, n	146	33	3	182
	Negative, n	2	135	60	197
	Indeterminate, n	5	0	35	40
	Total	153	168	98	419
Agreement, %		**95.4**	**80.4**	**35.7**	**75.4**

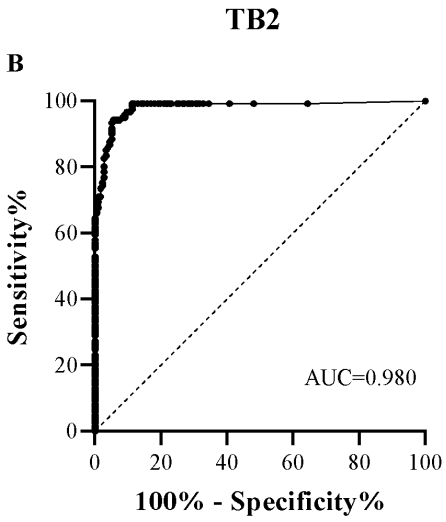
TABLE 1 Results and agreement of QFT Plus assay performed by ELISA and CLIA according to the manufacturer's cut-off.

		QFT Plus ELISA				
		Positive, n	Borderline, n	Negative, n	Indeterminate, n	Total
QFT Plus CLIA	Positive, n	104	41	0	0	145
	Borderline, n	2	34	8	4	48
	Negative, n	1	4	122	59	186
	Indeterminate, n	5	0	0	35	40
	Total	112	79	130	98	419
Agreement, %		**92.8**	**43.0**	**93.8**	**35.7**	**70.4**

TABLE 2 Results and agreement of QFT Plus performed by ELISA and CLIA according to the Italian Clinical Microbiologist Association recommendations.







TB1 cut-off	Sensitivity (95% CI)	Specificity (95% CI)
>0.35	99.2 (95.57-99.96)	81.9 (75.59-86.89)
>0.45	97.6 (93.13-99.34)	85.9 (79.98-90.25)

TB2 cut-off	Sensitivity (95% CI)	Specificity (95% CI)
>0.35	99.2 (95.47-99.46)	81.9 (75.59-86.89)
>0.46	99.2 (95.47-99.96)	88.7 (83.19-92.57)