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THE USE OF INTERFERON-GAMMA RELEASING ASSAYS (IGRA) TO IMPROVE THE DETECTION OF TUBERCULOSIS IN CAPTIVE-BRED NONHUMAN PRIMATES

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INTRODUCTION

Tuberculosis (TB) is an important zoonotic disease that is caused by the pathogenic species of *Mycobacterium*. The pathogenic species belonging to the *Mycobacterium tuberculosis* complex (which includes *M. tuberculosis* & *M. bovis*) are the most important cause of TB in captive non-human primates (NHPs) in biomedical facilities (Lerche N.W., et al. 2008). The detection of TB in NHP colonies is traditionally done using the tuberculin skin test (TST) which utilizes purified protein derivatives (PPD) preparations made from *M. tuberculosis* and *M. bovis* or/and *M. avium*. In NHPs, TST is usually done by injecting PPD intradermally into one eyelid, observing the injection site at 24, 48 and 72 hours for delayed hypersensitivity reactions and grading the severity of these reactions on a scale of 0 to 5 where 3 to 5 is interpreted as TB infection. These readouts are usually subjective and liable to false interpretations.

Therefore, there is a need to develop and optimize a laboratory-based method to supplement TST in TB detection. The QuantiFERON-TB Gold Plus (QFT) test (Qiagen, Germantown, MD, USA), one of the commercially available blood tests commonly referred to as interferon-gamma release assay (IGRA) tests, is an approved TB test for use in humans. Some of the major advantages of IGRA tests is their enhanced ability to diagnose latent TB infections, and reduced complicity with previous vaccination or exposure to *M. bovis* Baccille Calmette-Guérin (BCG) (Venkatappa T.K., et al. 2019). This study explored the use of QFT and our in-house IGRA tests for TB detection in African green monkeys (AGMs). The overall aim was to establish an optimized IGRA test that can be used in conjunction with the conventional TST to improve our TB detection and surveillance in our NHPs.

METHODOLOGY

Animals: During a routine TST screening of our captive-bred African green monkeys (AGMs; *Chlorocebus aethiops*), peripheral blood samples were obtained from 45 monkeys for laboratory testing by QFT test (Fig. 1), which was modified as indicated below to optimize the quality assurance outcome of the positive controls. Of the 45 monkeys, 15 of them and 51 other AGMs were tested using an in-house TB IFN- γ ELISPOT test six months earlier.

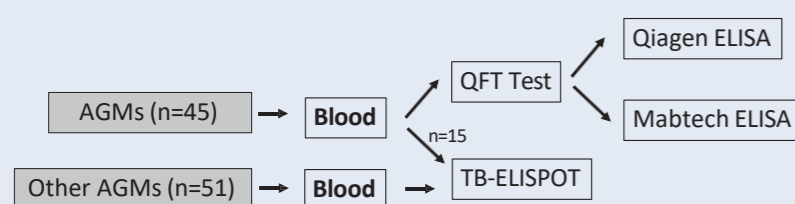


Fig. 1: Experimental design: TST was performed by an intradermal injection of 0.1 mL (3,000 IU PPD from *M. bovis*) into one eyelid per animal. Peripheral blood was obtained by venipuncture of the femoral vein. Blood for ELISPOT was collected in heparin tubes while blood for QFT test was collected directly into Qiagen blood collection tubes according to manufacturer's instructions. Qiagen and Mabtech ELISA kits were utilized in the QFT tests.

QFT tests: QuantiFERON-TB Gold-Plus™ tubes were used to collect blood according to manufacturer's instructions. Before incubation was started, the mitogen control tubes were randomly grouped into 3 and concanavalin A (Con A) added as shown in Fig. 2 to supplement the phytohemagglutinin (PHA) mitogen already contained in the control tubes. Plasma was harvested after 24-hr incubation for batch analysis by ELISA. **IFN- γ ELISA kits:** IFN- γ in the plasma samples was quantified using two commercial kits, the Qiagen and the Mabtech ELISA kits, according to manufacturer's guidelines. The Qiagen kit defined readings >0.5 IU/mL for TB1 and TB2 as positive for TB infection.

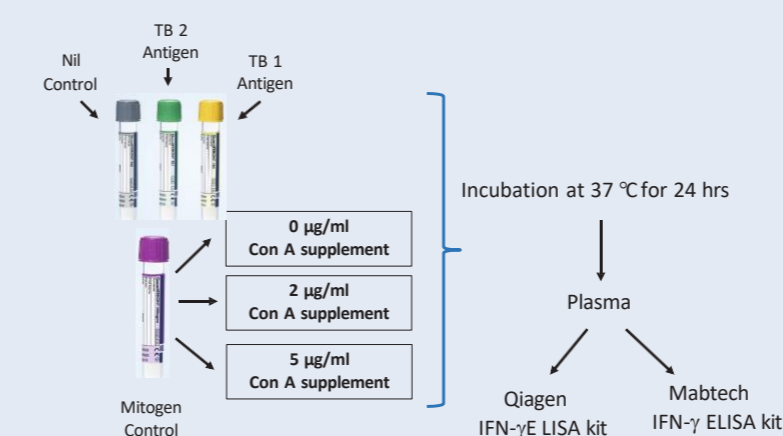


Fig. 2: Optimization of mitogen control. Qiagen's QFT-TB Gold Plus blood collection sets were used. Each set comprised of 4 tubes as follows: Nil or 'No antigen', TB1 antigen, TB2 antigen, and mitogen control tubes. The mitogen control tube contained PHA as the stimulant (which is a less effective polyclonal T-cell stimulator than Con A for AGMs cells). The mitogen control tubes were randomly divided into three groups (n=15, each) for supplementation with either 5 or 2 µg/mL of Con A mitogen or without supplementation during the 24-hr incubation at 37 °C together with the other tubes. After incubation, plasma was harvested from the whole blood and the IFN- γ quantified using commercial ELISA kits obtained from Qiagen and Mabtech according to manufacturer's instructions.

TB IFN- γ ELISPOT: Freshly isolated peripheral blood mononuclear cells (PBMC) from whole heparinized blood were used in an in-house IFN- γ ELISPOT assay that we previously developed (Chege et al., 2008). The PBMC were stimulated with BCG lysate, *M. tuberculosis* 6-kDa early secretory antigenic target (ESAT-6) and culture filtrate protein 10 (CFP-10) peptides (2 µg/mL). The assay positive control used Con A (1 µg/mL).

Data analysis: GraphPad Prism software was used for graphical and statistical analyses.

RESULTS

Supplementation with 5 µg/mL Con A significantly increased the quantities of IFN- γ in the mitogen controls for both ELISA kits

- The Qiagen kit detected 3 of 45 samples as positive for TB antigens but only one of them corresponded to a reading >5 pg/mL in the Mabtech kit suggesting possible concordance in 1 of the 3 positive samples.
- There was a positive correlation in the amounts of IFN- γ measured in the mitogen control samples between the Qiagen and Mabtech ELISA kits (Fig. 3).
- Supplementation of the mitogen control tubes with 5 µg/mL, but not with 2 µg/mL Con A, significantly increased the amount of IFN- γ produced in the QFT tests for both ELISA kits (Fig. 4).

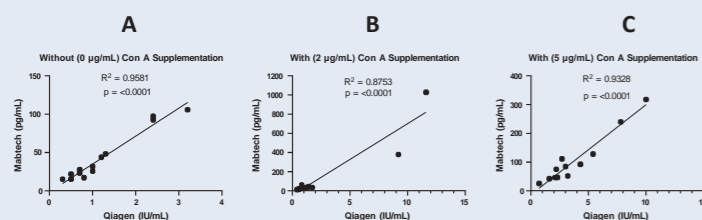


Fig. 3: Positive correlation. Qiagen and Mabtech ELISA kits were used to measure IFN- γ in the plasma harvested from the mitogen control tubes in which no Con A (A) or either 2 µg/mL (B) or 5 µg/mL (C) Con A was added before the 24-hr incubation of the whole blood at 37 °C. The line graphs show positive correlation between the two kits indicating comparable degree of detection.

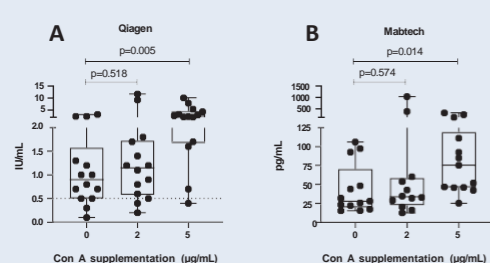


Fig. 4: Amounts of IFN- γ produced in mitogen controls. The amounts of IFN- γ cytokine measured in the mitogen control tubes were compared for tubes in which nil (no Con A supplementation), 2 µg/mL or 5 µg/mL Con A was added. The solid circles in the bar graphs show individual measurements for the Qiagen (A) and Mabtech (B) kits with the box and whiskers indicating the interquartile ranges and the medians shown as horizontal lines. Statistical comparisons were performed using a nonparametric t test. The p values are shown.

An IFN- γ ELISPOT test can distinguish non-pathogenic and pathogenic mycobacterial infections.

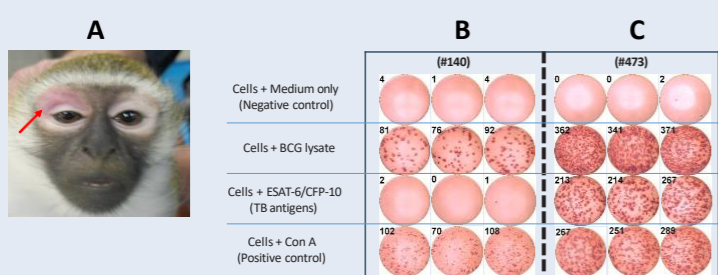


Fig. 5: Representative outcomes of positive TST and TB IFN- γ ELISPOT tests. (A): 24 hours after a TST was performed. The reddening and swelling of the upper right eyelid (red arrow) represents a grade 3 on the scale of severity (interpreted as TST positive; a tentative TB diagnosis). (B) and (C): show images of spots (referred to as spot-forming units, SFU) of a BCG responder or non-pathogenic mycobacterial infection (B) and a TB-infected AGM (C). The SFU is calculated from triplicate wells and normalized as SFU per million cells. A cut-off value of 50 SFU/million cells is normally set for positive responses. The negative control must be <50 SFU/million while the positive control must be >100 SFU/million.

Testing using an IFN- γ ELISPOT test accurately detects TB infection with a potential for early TB detection and distinguishing of non-TB responses.

- IFN- γ ELISPOT test detected responses in 14 of 66 AGMs that were tested (Table 1).
- Of the 14 responders, 8 had responses to BCG lysate antigens only while 6 responded positively to ESAT-6 + CFP-10 antigens as well as BCG lysate (except 1 AGM) (Tables 2 & 3).
- Majority of the 14 IFN- γ ELISPOT responders had TST scale of severity from 3 to 5 (representative: Fig. 5).
- Of the 14 IFN- γ ELISPOT positive AGMs, 3 were confirmed as TB-infected by a 6-week mycobacterial culture of the lung tissue (obtained at necropsy and culture done by an external pathology lab).

Table 1

IFN- γ ELISPOT Test Outcome	No. Tested
Negative	52
Positive	14
Total	66

Table 2

IFN- γ ELISPOT Positive Responses	No. Tested
BCG lysate only	8
ESAT-6/CFP-10	6
Total	14

Table 3

Animal ID	IFN- γ ELISPOT Responses (SFU/million cells)		TB Confirmation	
Group	ID #s	BCG Lysate	ESAT-6 + CFP-10	Culture
BCG responders (n=8)	140	415	0 (<50)	N/A
	247	145	0 (<50)	N/A
	311	220	0 (<50)	N/A
	343	88	2 (<50)	N/A
	371	807	27 (<50)	Negative
	407	143	32 (<50)	N/A
	415	278	17 (<50)	N/A
	419	497	5 (<50)	N/A
TB-infected (n=6)	3	1547	1183	Positive
	243	1033	187	N/A
	428	748	728	Positive
	464	1432	507	N/A
	473	1795	1156	Positive
	477	40 (<50)	112	N/A

N/A: Not available

Tables 1-3: Summary of IFN- γ ELISPOT test outcomes. Table 1: shows the summary of IFN- γ ELISPOT outcomes; Table 2: shows the summary of positive IFN- γ ELISPOT responses categorized as responses to BCG lysate only and to ESAT-6 + CFP-10 (TB antigens) and BCG lysate; and Table 3: shows the magnitudes of positive responses to BCG lysate and ESAT-6 + CFP-10 antigens. The ELISPOT responses are reported as net SFU/million PBMC (after subtraction of background (i.e. Negative control) responses. Cut-off for positive responses: 50 SFU/million PBMC).

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KEY FINDINGS & CONCLUSION

- Supplementation of mitogen controls with Con A increases the production of IFN- γ thereby improving the quality assurance outcomes and validity of QuantiFERON-TB Gold Plus in detecting TB in captive-bred NHPs.
- TB IFN- γ ELISPOT has potential in being developed into a reliable confirmatory test for TB diagnosis in NHPs.
- TB IFN- γ ELISPOT test can include non-pathogenic mycobacterial antigens to expand TB diagnosis and enable it to distinguish TB from non-pathogenic mycobacterial infections in NHPs.
- Both QFT and TB IFN- γ ELISPOT are suitable supplementary laboratory blood tests for TST to improve TB diagnosis in NHPs.