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SHORT COMMUNICATION

Detection of *Mycobacterium tuberculosis* in Nonhuman Primates by qPCR

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ABSTRACT

Simple and highly sensitive methods to monitor infection or exposure to *Mycobacterium tuberculosis* in nonhuman primates (NHPs) are unavailable except for the tedious tuberculin skin test (TST). The study focused on establishing quantitative real-time PCR (qPCR) as the method for direct and non-invasive detection of *M. tuberculosis* in peripheral blood of NHPs *in vitro*. We investigated the rates of *M. tuberculosis* in three different NHP colonies. All the TST-positive samples were confirmed as infected except for three samples with CT values above 40, which were deemed negative. Four TST-negative samples were shown to be positive using CT. In the three different colonies, the detection rates of *M. tuberculosis* in the peripheral blood were 10, 19.5 and 24%, respectively. The results suggested that qPCR was an auxiliary method with speed, accuracy and simplicity, which facilitate the screening and breeding of specific pathogen-free (SPF) or higher grade simians, and for disease surveillance programs.

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INTRODUCTION

Nonhuman primates (NHPs) are widely used as models to investigate human disease and pharmacological mechanisms due to similar physiological and genetic characteristics (Engel *et al.*, 2013). However, they are prone to microbial infection such as simian immunodeficiency virus, simian retrovirus and tuberculosis, which may jeopardize animal health, affect the study results, and pose a risk to personnel (Ayouba *et al.*, 2013). Tuberculosis is one of the leading causes of death globally and is transmitted widely in captive monkeys following contact with infected humans, domestic and wild animals (Mahmood *et al*., 2014).

Traditionally, the tuberculin skin test (TST) used for detection of *Mycobacterium tuberculosis* is a tedious and subjective procedure with low sensitivity (Engel *et al.*, 2013). Immunological cross-reactivity with various mycobacterial species leads to false positive or negative results due to the latent period following infection or immunosuppression, respectively, in progressive tuberculosis (Lerche *et al.*, 2008). Currently, the new generation of diagnostic tests comprises interferon gamma (IFN-γ) release assays and acid fast stain of sputum samples for diagnosis of *M. tuberculosis*. However, the results suggest that the IFN-γ responses to tuberculin antigen may not be reliable in cynomolgus macaques and that another cut-off was needed to interpret the test (Kim *et al.*, 2009). Therefore, the foregoing methods do not address the diagnostic needs of NHPs, which is a major challenge in both laboratory and field studies. Several studies investigated *M. tuberculosis* in peripheral blood of humans using PCR, with conflicting results. Studies suggest that the common PCR may be an appropriate tool for detection of *M. tuberculosis* in the peripheral blood (Dubey *et al.*, 2013). Other studies proposed a contrary view (Hajiabdolbaghi *et al.*, 2014). Therefore, we evaluated the role of qPCR for direct and non-invasive detection of *M. tuberculosis* in the peripheral blood of NHPs for the first time *in vitro*. We identified *M. tuberculosis* in three different NHP colonies for disease surveillance.

MATERIALS AND METHODS

Sample collection and DNA extraction: Twelve TST-positive EDTA-blood samples were collected during 2012-2013 from simians infected with *M. tuberculosis* to determine the choice of whole blood or plasma for detection of *M. tuberculosis.* They were divided into two aliquots with similar volume. One aliquot was centrifuged for 10 min at 2500×*g* for plasma collection, and the other aliquot was used to extract DNA directly. Compared with

qPCR, 110 samples, which were positive or negative by TST, were collected during 2013-2014, and DNA was extracted from the plasma using the method described above. Similarly, 116 plasma samples (30 from Guangdong AA Biotechnology Cooperation, 49 from Sichuan BB Biotechnology Cooperation, and 37 from Guangdong CC Biotechnology Cooperation) were collected randomly from three different colonies for analysis of *M. tuberculosis* prevalence in simians during March and April of 2015. All the DNAs in whole blood or plasma were extracted using the HiPure Blood DNA Kits (Magen Company, Guangzhou) following the manufacturer's protocol, dissolved in TE buffer and stored at -20ºC.

Real-time PCR: We selected the IS6110 sequence, a DNA sequence specific for *M. tuberculosis*, to design the primers and probe, as shown in Table 1 (Dubey *et al.*, 2013). We constructed the plasmid with the target sequence and determined the concentration with the Nanodrop to obtain the standard curve with ten-fold serial dilutions using ABI7500 machine. The cycle threshold (CT) values were expressed as the mean and standard deviation. The coefficient of variation was calculated as the percentage of the CT standard deviation divided by its mean. The qPCR volume and conditions for IS6110 DNA amplification followed the instructions, and each experiment was performed in triplicate. Finally, all the clinical samples were detected using the same program. The amplicons with high CT values were sequenced by Sangong cooperation (Shang Hai).

Table 1: Primers and probe for detection of *M. tuberculosis*

Primer name	Sequence(5'-3')	Amplicon
Primer-F	GCCAACTACGGTGTTTACGG	101bp
Primer-R	AGTTTGGTCATCAGCCGTTC	
Primer-P	FAM-CAAAGTGTGGCTAACCCTGAACC GTGAG-BHQ1	

RESULTS AND DISCUSSION

Standard Curve: Three independent, 10-fold serial dilutions of the plasmid served as templates to generate standard curves using the TaqMan®-based qPCR assays. As shown in Fig. 1, the linear equation was Y=-3.32 X+40.38, with the y-intercepts indicating that the CT value of detection sensitivity was 40.38. The y-intercept value corresponded to the CT value for a single target molecule and values of ~40 indicated excellent sensitivity, which was unmatched by TST, ELISA, common PCR and nested PCR (Lerche *et al.*, 2008). The value of coefficient of correlation (R^2), which served as linearity indicator of the CT values in the standard curves, was 0.996.

CT values of whole blood and plasma: The CT values of whole blood and plasma treated similarly varied as shown in Table 2. The plasma CT values were three times less than in whole blood, which suggested that the plasma contained more copies of target DNA. The result indicated that the plasma was more appropriate for DNA detection with minimal interference by RBC (Londin *et al.*, 2011). In the samples GD008 and GD012, the two repeats were detected only once, because the extracted DNA level was close to the detection limit, which further confirmed that the detection of target DNA in the plasma reduced the probability of false-negative results.

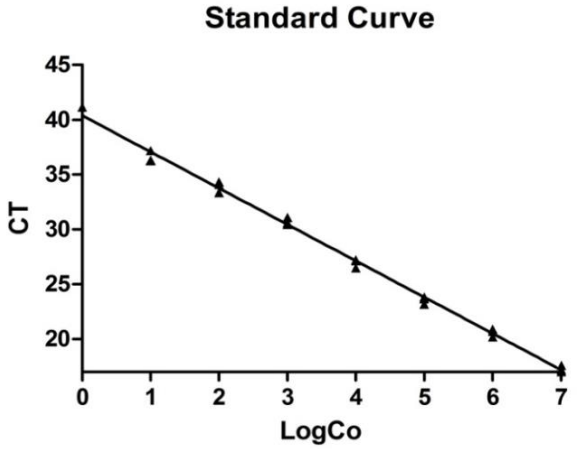

Fig. 1: Regression analysis based on CT values obtained using TaqMan-based qPCR.

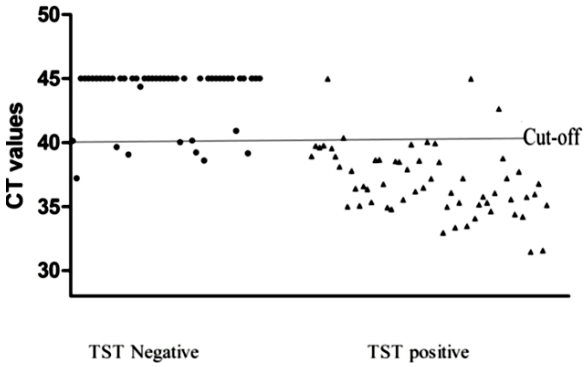

Fig. 2: Distribution of CT values in known TST-positive and negative samples. A CT value with 40 was recognized as the cut-off.

Table 2: CT values in different samples

No.	Sample name	CT values (different sample types)	
		Whole blood	Plasma
1	GD001	34.50/35.44	31.55/31.54
2	GD002	35.51/35.63	32.94/33.70
3	GD003	33.39/35.39	30.86/32.91
4	GD004	32.58/34.16	32.48/33.29
5	GD005	36.99/38.44	32.37/31.63
6	GD006	39.93/34.51	35.1034.13
7	GD007	33.85/33.11	33.79/34.17
8	GD008	39.86/ND[1]	33.81/36.18
9	GD009	35.40/34.89	30.69/30.61
10	GD010	36.02/35.54	31.25/30.42
11	GD011	26.15/27.02	23.45/23.83
12	GD012	ND/42.64	33.68/34.30

ND: Not determined.

Table 3: CT values in TST-positive/-negative samples

Samples	T values		
	CT<40	CT>40	ND
TST-positive	57	3*	1
TST-negative	4*	7*	38

Note: Because the CT cut-off value was approximately 40, we segregated all the CT values into three levels: CT<40, CT>40 and ND (not detected). * denotes sequenced amplicons.

Table 4: CT values in the three colonies

Farm name	CT values		
	CT<40	CT>40	ND
AA	3	0	27
BB	7	1	41
CC	9	0	28

Note: AA, BB and CC represent three different private corporations.

CT values in known TST-positive and -negative samples: As shown in Fig. 2 and Table 3, all the TST-positive samples, had CT values varying from 32 to 40, which corresponded to 1×10^3 copies, with one copy of *M. tuberculosis* DNA from peripheral blood except in one sample that had no CT value and two that had CT values of 40.03 and 40.39. In the TST-negative samples, 38 showed no CT values, and 11 had different CT values ranging from 37.21 to 44.36. The figure was based on sample CT values of 45 or less. The amplicons in the samples with TST-negative CT values and CT values above 40 in TST-positive samples were sequenced. The results showed that the amplicons in TST-negative samples with CT values of 37.21, 39.06, 38.58, and 39.23 represented target sequences of *M. tuberculosis* DNA, while other amplicons and special amplicons in TST-positive samples were sequenced unsuccessfully. The results suggested that the extremely low levels of *M. tuberculosis* DNA in peripheral blood were detected with high sensitivity using qPCR and eliminated the false negatives associated with TST (Engel *et al.*, 2013).

Detection of *M. tuberculosis* in three colonies: The results showed that the CT values of the three colonies also varied greatly (Table 4). In AA feeding farm, the number of CT values below 40 was three. Other colonies had no CT values. In BB, seven out of 49 samples had CT values below 40, and one had CT value of 41.5, which was sequenced unsuccessfully. In CC, all the nine samples had CT values below 40. Therefore, the detection rates of three different NHP colonies were 10, 19.5 and 24%, respectively, which reflected the characteristics of the NHP colonies and their management. The results suggested that the AA farms had the lowest proportion of qPCR-positive samples contrary to the TST-negative data provided by the breeders, which further suggested that the traditional TST method had limitations (Engel *et al.*, 2013). Therefore, breeding of SPF or higher grade simians requires high-quality experimental models of NHP's real-time PCR is undoubtedly an effective tool to determine the *M. tuberculosis* infection in simians.

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Authors' contribution: ZHL and SHL diagnosed the samples and wrote the report for this manuscript. WY and JW performed the molecular biology examination. FGM collected the samples RH and YZ provided guidance on the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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