

DIFFERENTIATION OF SARCOIDOSIS FROM TUBERCULOSIS USING REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT. *Background:* The clinical and pathological features of sarcoidosis and tuberculosis may mimic each other, and when the caseous necrosis is not seen in tuberculosis tissue, differentiation is not easy. *Objective:* This study evaluates the ability of real-time PCR quantification and sets the quantitative value to differentiate sarcoidosis from TB. *Methods:* Formalin-fixed and paraffin-embedded sections of biopsy samples, from 104 patients with sarcoidosis, 31 patients with tuberculosis, and 55 controls with other respiratory diseases (26 with nonspecific lymphadenitis and 29 with emphysema bullae), were collected to amplify insertion sequence IS986 of *Mycobacterium tuberculosis* (MTB) genome by real-time quantitative PCR. The diagnostic performance of qualitative and quantitative analysis of real-time quantitative PCR was assessed by receiver-operating characteristic (ROC) curves. *Results:* MTB DNA was detected in 20 of the 104 sarcoidosis samples and 7 of the 55 control samples, but was detected in all of the 31 tuberculosis samples. The numbers of MTB genomes were 0~4.71x10³ copies per ml in sarcoidosis samples, 1.58x10²~5.43x10⁷ copies per ml in tuberculosis samples and 0~1.02x10³ copies per ml in controls with quantitative analysis. Receiver-operating characteristic (ROC) curves showed that MTB genome quantification had greater diagnostic performance than MTB genome qualification in discriminating patients with sarcoidosis from those with tuberculosis (area under the ROC curves: 0.994 vs 0.904, *P*<0.001). The sensitivity and specificity of qualitative analysis were 100% and 80.8% respectively. At cut-off value of 1.14x10³ copies per ml for MTB genome quantification, the sensitivity was 96.8% and specificity was 98.1%. *Conclusions:* The real time PCR quantification is a valuable test for differentiation between sarcoidosis and tuberculosis, and the MTB genome copies number of 1.14x10³ copies per ml should be preferred as quantitative cutoff value for the differentiation. (*Sarcoidosis Vasc Diffuse Lung Dis* 2008; 25: 93-99)

KEY WORDS: sarcoidosis, tuberculosis, *Mycobacterium tuberculosis*, real-time quantitative PCR, ROC curve

INTRODUCTION

Sarcoidosis is a multisystem noncaseating granulomatous disorder of unknown origin, and is simi-

lar in clinical, histological, and immunologic features to tuberculosis (TB). In clinical situation, it is often difficult to tell sarcoidosis from tuberculosis, especially when the caseous necrosis is not seen and acid-fast staining is negative in biopsy tissue of tuberculosis. The availability of an inexpensive, sensitive and specific test is very important, especially in regions where both diseases are common. Recently, association of MTB with sarcoidosis has been described, but a consensus about if MTB related to sarcoidosis has not been achieved and the value dif-

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ferentiate diagnosis way between sarcoidosis and tuberculosis had not obtained. Wilsher et al using PCR to amplify insertion element IS6110 from fresh tissue samples with sarcoidosis, tuberculosis and controls in 1998. In their study, MTB DNA was not detected in any of the tissue samples from patients with sarcoidosis or other respiratory disease but was found in all four patients with tuberculosis. They considered MTB is unlikely to be a factor in the pathogenesis of sarcoidosis (1). The next year, Ishige et al used PCR to estimate the number of mycobacterial and propionibacterial DNA in lymph nodes. Quantitative PCR was done to amplify segments of 16S ribosomal RNA of *P. acnes* and *P. granulorum* and of insertion sequence 6110 of *M. tuberculosis*. Their results suggest that *propionibacteria* had resided in the sarcoid lesions but MTB had no difference between people with and without tuberculosis (2).

In this study, we amplified insertion sequence IS986 of MTB genome in formalin-fixed and paraffin-embedded samples of sarcoidosis and TB, and analysed the qualitative and quantitative result by ROC curve to evaluate the ability of real-time PCR quantification and set the quantitative value to differentiate sarcoidosis from TB. The MTB-specific insertion sequence IS986 belongs to IS3 family of insertion sequences and shares many characteristics typified by members of this family (3). The element has been widely used as a highly specific probe for detection and typing of *M. tuberculosis* because of the multiple polymorphism observed among different strains of MTB complex and remains very stable in strains maintained in laboratory culture (4). The sequence of IS986 is virtually identical to another described element IS6110 by Thierry et al in 1990(3). In this study, insertion sequence IS986 of MTB and the human β -globin gene was amplified in all tested groups.

MATERIALS AND METHODS

Patients and samples

Formalin-fixed and paraffin-embedded sections of biopsy samples were retrospectively collected from 104 patients with sarcoidosis, 31 patients with tuberculosis, and 55 controls with other respi-

ratory diseases (26 with nonspecific lymphadenitis and 29 with emphysema bullae) in Shanghai Pulmonary Hospital from January 1998 to March 2007. The diagnosis of sarcoidosis was established by the clinical picture, no evidence of current infection by MTB or other organisms known to produce granulomatous disease, and the presence of non-caseating granulomas in biopsy specimens of involved tissues (5). Patients with sarcoidosis were followed for at least 12 months and there was no evidence of MTB infection. The patients with tuberculosis were bacteriologically proved in sputum, bronchoalveolar lavage samples, or tissue samples affected by caseous necrosis. The sarcoidosis and tuberculosis patients all had improvement after treatment with glucocorticoid and antituberculosis drugs respectively. All these subjects were Chinese Han people, they had received BCG vaccination routinely when they were infants and they had no coincidence with other diseases. In the sarcoidosis group, there were 51 patients in stage I, 49 patients in stage II, 3 patients in stage III and 1 patient in stage IV based on the chest radiograph. In this group, 89 samples were taken from lymph nodes, 8 from lung tissues and 7 from subcutaneous nodules. In tuberculosis group, 17 samples were taken from lymph nodes and 14 from lung tissues. In 55 control samples, 26 samples were taken from lymph nodes with nonspecific lymphadenitis and 29 from lung tissues with emphysema bullae. The detail data of these subjects are shown in table 1. The informed consent was obtained from all of the patients.

Preparation of DNA

Six 8- μ m-thick sections cut from paraffin block and put into autoclaved test tubes, were treated with xylene and then with ethanol. The tissue pellets were suspended in 200 μ l lysis buffer composed of 50 Mm Tris-HCl (Ph 8.0), 20 Mm ethylene diamine tetraacetic acid (EDTA), 10 Mm sodium chloride, 1% sodium dodecyl sulphate (SDS), then digested with proteinase K (1.5 mg/ml) at 55 °C overnight. DNA was purified by phenol/chloroform extraction and precipitated with ethanol. Precipitated DNA was washed once with 70% ethanol and dissolved in TE buffer (10 Mm Tris-HCl, 1Mm EDTA, PH8.0). The quantity and quality of DNA was calculated by spectrophotometry.

Table 1. Characteristics of patients and controls

Patient group	No.	Medium age	Mail/femal ratio	PPD test (positive/negative)	Acid-fast bacillus in biopsy tissue	Biospy time (yr) (1998-2002/2003-2007)	MTB cultures in biopsy tissue
Sarcoidosis	104	47.5	35/69	15/74	104 negative	30/74	104 negative
Tuberculosis	31	40	16/15	27/4	31 positive	12/19	31 positive
Control	55	48	32/23	9/31	55 negative	14/41	55 not done

Oligonucleotide primers and TaqMan probe

Primers were used for real-time (TaqMan) PCR quantification to amplify the insertion sequence IS986 of MTB and the human β -globin gene in all tested groups. Primers MT-F (5'-GTCAGCTGTGTGCAGATCGA-3') and MT-R (5'-AAGAATCCGCT GAGCTGAAG-3') were designed to amplify a 207-bp portion of MTB insertion sequence IS986. Primers BG-F (5'-GCCGGGACCTGACTGACTAC-3') and BG-R (5'-TCTCCTTAATGT-CACGCACGAT-3') were designed to amplify a 101-bp portion of human β -globin gene. TaqMan probes MT-TAQ (5'-ATGAACCGGGTAATTAGCGTG-3') and BG-TAQ (5'-CATGAAGATCCTCACC-GAGCGCG-3') were designed to hybridize with the PCR product of MTB and human β -globin DNA respectively. These probes were labeled with 6-carboxyfluorescein on the 5'-end and 6-carboxytetramethylrhodamine (TAMRA) on the 3' end.

TaqMan PCR

PCR was done in 50 μ l of a mixture containing 5 μ l of DNA sample, 0.5 μ l of primer, 0.5 μ l of the probe, 0.5 μ l of the four deoxynucleotides, 1 μ l Taq DNA Polymerase, 32 μ l ddH₂O and 10 μ l 5 μ PCR buffer. Amplification and detection were done with ABI 7300 sequence detection system. PCR conditions were 10 cycles of 93°C for 2 minutes, 93°C for 45 seconds, 55°C for 1 minute followed by 30 Cycles of 93°C for 45seconds, 55°C for 1 minute. Amplified products were determined by continuous monitoring of fluorescence. After data collection, the cycle threshold (Ct) number was calculated by determining the point at which the fluorescence exceeded an arbitrary lower limit, chosen to cover the range of readings given by all standards in the exponential phase. Therefore the Ct value reflected the overall quantity of target copies in samples. Each specimen

was run in triplicate and considered positive only if at least two of the three results exceeded the threshold. Serial dilution of MTB standard DNA as a standard curve (10 copies to 10⁷ copies) for quantitative analysis, as well as internal controls of β -globin gene and negative controls without bacterial DNA were included in every run.

Statistical analysis

The proportion of positive values obtained from each group was compared using Fisher's exact test. Median values were calculated for each group, and quantitative results were analyzed by the Mann-Whitney U test. Statistical significance was established at a *p* value of <0.05. We used SPSS13.0 for statistical analysis.

To assess and compare the diagnostic accuracy of real-time PCR qualification and quantification for discriminating sarcoidosis patients from tuberculosis patients, ROC curves (6) were plotted and the areas under the curves (AUC) were calculated for comparison. ROC curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1-specificity) at various cutoff points of the tests. An AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. Cutoff values of real-time PCR quantification for differential diagnosis were selected from experimental data as the values that maximized the Youden index (Youden index = sensitivity+ specificity-1). When comparing the AUC of two data from qualification and quantification, Z test was used according to the formula

$$Z = \frac{|AUC_1 - AUC_2|}{\sqrt{SE_1^2 + SE_2^2}}$$

Statistical significance was established at a *p* value of <0.05.

RESULTS

Reference curves

The 207-bp fragments were obtained by amplification of MTB standard DNA. DNA dilution started at 10^7 copies per ml and extended through three sets of 10-fold dilutions to give 10 copies per ml. The three series of samples were then amplified (from 10 to 10^7 copies per ml) in the same run, and the data collected was used to generate a linear-log regression plot (Fig. 1). Similar result was obtained from the human β -globin standard DNA which was further used as an internal control for each run.

Sensitivity of PCR

The sensitivity of the assay was determined by detecting standard samples of MTB DNA which ranged from 10^7 to 10 copies per ml. Ct values (y axis) were plotted against the log of input MTB DNA number (x axis) (Fig. 1), producing a linear slope. The lowest concentration of genomes detected was 10^2 copies per ml, therefore the assay sensitivity was estimated at 10^2 copies per ml.

Specificity of PCR

DNAs from other respiratory bacteria were tested using the assay: *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *M.gordonae*, *M.Vaccae*, *M.avium*, *timothy bacillus*, and *M.fortuitum*. As expected, no cross-

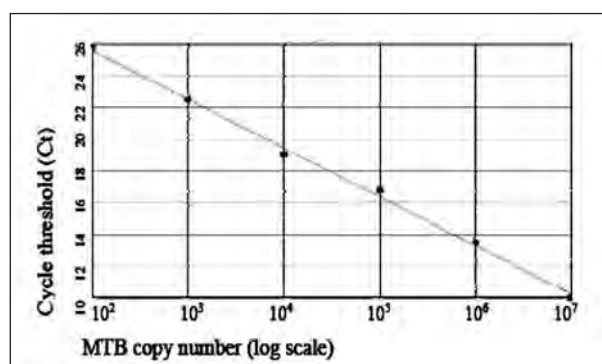


Fig. 1. Standard curve for MTB quantification. Tenfold serial dilutions ranging from 10^7 copies to 10^2 copies of MTB standard DNA were tested in triplicate, and the mean Ct values were plotted against copy number (correlation coefficient 0.997)

reactivity was obtained with any of these bacteria tested.

Qualitation of MTB genome

MTB genome sequences were detected in 20 of 104 (19.2%), 31 of 31 (100%) and 7 of 55 (12.7%) biopsies from patients with sarcoidosis, tuberculosis and controls respectively (Tab. 2). The positive proportion of tuberculosis had statistically significant difference from that of sarcoidosis ($p=0.000$) and controls ($p=0.000$), but there was no statistically significant difference between sarcoidosis and controls ($p=0.377$).

Quantification of MTB genome

For tuberculosis patients, MTB DNA numbers ranged from 1.58×10^2 to 5.43×10^7 copies per ml and the median number was 1.82×10^4 copies per ml, whereas for sarcoidosis patients and controls, the ranges were less extensive (0 to 4.71×10^3 copies per ml in sarcoidosis and 0 to 1.02×10^3 copies per ml in controls), the median numbers were both 0 (Table 2). Overall analysis of the data indicated that the MTB DNA values were not evenly distributed among the three groups (Kruskal-Wallis test; $p=0.000$). The MTB DNA numbers were significantly higher in tuberculosis group than in sarcoidosis and control group respectively (Mann-Whitney U test; $p=0.000$ both), whereas the MTB DNA numbers in sarcoidosis group were not significantly different from those of control group (Mann-Whitney U test; $p=0.361$).

Differential diagnostic performances

As shown by the ROC curve, the ability of MTB genome quantification to differentiate sarcoidosis patients from tuberculosis patients exceeded that of MTB genome qualitation (AUC=0.994 vs

Table 2. MTB genome qualitation and quantification

Group	No. of patients	No. (%) of MTB positive patients (qualitation)	Median copy no. (quantification)
Sarcoidosis	104	20 (19.2)	0
Tuberculosis	31	31 (100)	1.82×10^4
Controls	55	7 (12.7)	0

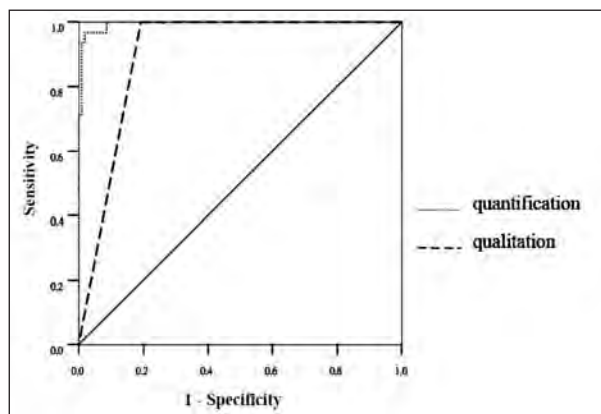


Fig. 2. ROC curve of MTB genome quantification and qualification for discriminating patients with sarcoidosis from tuberculosis. The areas under the curves are 0.994 and 0.904 ($Z=3.555$, $P<0.001$) for quantification and qualification respectively

0.904, $Z=3.555$, $P<0.001$) (Fig. 2). The selected cut-off value for the differential diagnosis was 1.14×10^3 copies per ml for quantitative analysis. The sensitivity and specificity of qualitative and quantitative analysis are shown in table 3 for the differential diagnosis of sarcoidosis and tuberculosis.

PCR in different type of tissues

MTB genome positivity rates of PCR should be evaluated in different type of tissues. In sarcoidosis group, MTB genome sequences were detected in 16 of 89 (18.0%), 3 of 8 (37.5%) and 1 of 7 (14.3%) biopsies from lymph nodes, lung tissues and subcutaneous nodules respectively. There was no statistically significant difference between lymph nodes and lung tissues ($p=0.187$), and no statistically significant difference between lymph nodes and subcutaneous nodules ($p=1.000$). In tuberculosis group, MTB genome positivity rates were both 100% in 17 samples taken from lymph nodes and 14 from lung tissues. In 55 control samples, MTB genome sequences were detected in 2 of 26 (7.69%) and 5 of 29 (17.2%) biopsies from lymph nodes and lung tissues respec-

tively. There was no statistically significant difference between the two types of tissues ($p=0.426$).

PCR in samples from different periods

The samples were classified to two groups according to the biopsy time (1998-2002 yr and 2003-2007 yr). In sarcoidosis group, MTB genome sequences were detected in 8 of 30 (26.7%) samples taken from 1998-2002 yr and in 12 of other 74 (16.2%) samples from 2003 to 2007 yr. There was no statistically significant difference between the two periods ($p=0.221$). MTB DNA numbers ranged from 0 to 1.10×10^3 copies per ml in samples from 1998 to 2002 yr and 0 to 4.71×10^3 copies per ml from 2003 to 2007 yr. The median numbers were both 0. In tuberculosis group, MTB genome positivity rates were both 100% in samples taken from the two different periods. MTB DNA numbers ranged from 1.19×10^3 to 9.72×10^6 copies per ml in 12 samples from 1998 to 2002 yr and 1.58×10^2 to 5.43×10^7 copies per ml in 19 samples from 2003 to 2007 yr. The median numbers were 2.62×10^4 and 1.71×10^4 copies per ml. There was no statistically significant difference between the two periods by t-test ($p=0.585$).

DISCUSSION

Sarcoidosis and tuberculosis are both granulomatous disorders by pathohistology. The differentiation between them is often clinically difficult. Histopathologic definitions of sarcoidosis are inadequate, because sarcoid granulomas are not pathognomonic and cannot be distinguished by microscopic study or histochemistry from granulomas due to other causes. Some TB tissues do not have caseous necrosis and even the distinction between caseation and noncaseation is not absolute.

MTB DNA detection in sarcoidosis samples by traditional PCR has been used for pathogenic study

Table 3. Differential diagnostic performances of MTB genome qualification and quantification in patients with sarcoidosis and tuberculosis

	Cutoff values (copies/ml)	Sensitivity (%)	Specificity (%)	AUC	SE	95% confidence interval for AUC
Qualitation	-	100	80.8	0.904	0.004	0.854-0.953
Quantification	1.14×10^3	96.8	98.1	0.994	0.025	0.986-1.003

of sarcoidosis, but there is no final conclusion about the relationship of MTB and sarcoidosis. Some reports supported that MTB was related to sarcoidosis (7-9) but others did not (1, 10-11). The real-time PCR assay appears to be sensitive, precise, and rapid. Qualitation and quantification of MTB DNA can be obtained within 2 hours, and the method is less laborious than traditional PCR (12). In our study, we evaluated real-time PCR qualitation and quantification of MTB DNA in the two granulomatous disorders and found that sarcoidosis has significantly different MTB DNA measurements from tuberculosis and has no statistically significant difference from controls. This supports the opinion that the MTB is not a factor in the pathogenesis of sarcoidosis. As for its particular antigens participating in the pathogenesis of sarcoidosis or not, it is not yet clear and need further investigation.

The presence of MTB DNA does not only imply active MTB, It can be also seen in patients with prior infection (13, 14). The use of PCR to identify latent tuberculosis has been reported in patients with Crohn's disease (15). In China, latent tuberculosis is common. In our study, 12.7% of control populations and 19.2% of sarcoidosis populations were MTB PCR positive. The differentiating feature of this study was the quantity of MTB DNA.

To our knowledge, different diagnosis between sarcoidosis and tuberculosis had only been reported in several other studies (16-18). Levy H et al. reported that ELISA using mycobacterial sonicates as antigen was a valuable test for the differentiation between sarcoidosis and TB (16). More recently, Tabak L et al. reported that labial biopsy had a high discriminatory value as a diagnostic tool in the differentiation of sarcoidosis from tuberculosis (17), while Peng ZM et al. reported that videomediastinoscopy was an effective procedure for the diagnosis of mediastinal diseases and the examination of CD (4)/CD (8) added more information to the differentiation of tuberculosis from sarcoidosis (18). According to quantitative results in our study, there were 12.7% of control populations and 19.2% of sarcoidosis populations were MTB PCR positive. The specificity of qualitative analysis was 80.8%. To elevate the specificity and provide a valuable way to distinguish sarcoidosis from tuberculosis, we evaluated the ability of real-time PCR quantification and set the quantitative value.

To compare the performance of MTB genome qualitation and quantification by real-time PCR, we used ROC plots, which provide pure indices of accuracy. As shown by the relative positions of the plots in figure 2, the MTB genome quantification exhibits greater observed accuracy than does the MTB genome qualitation for differentiation of sarcoidosis from tuberculosis. Statistical comparison of the AUC shows that the accuracy differences are highly significant ($P < 0.001$). The ROC plot is the most convenient way to quantify the diagnostic accuracy of a test. The AUC values for quantification mean that a randomly selected patient with tuberculosis will have higher copies of MTB genome in biopsy samples than a randomly selected patient with sarcoidosis in 99.4% of the cases. Similarly, the AUC values for qualitation mean that a randomly selected patient with tuberculosis will have positive proportions of MTB genome than a randomly selected patient with sarcoidosis in 90.4% of the cases.

Using ROC curve analysis to assess the diagnostic accuracy of real-time PCR quantification requires the selection of a decision threshold. The large number of subjects included in this study allowed us to calculate with precision the best cutoff values of MTB genome for discriminating patients with sarcoidosis from those with tuberculosis. As both sensitivity and specificity are equally important, we selected cutoff values that maximized the sum of sensitivity and specificity.

According to the quantitative cutoff value of 1.14×10^3 copies per ml, 2 of the 104 cases with sarcoidosis were positive and the numbers of mycobacterial DNA were 1.61×10^3 copies per ml and 4.71×10^3 copies per ml respectively. The positive results found in the 2 patients with sarcoidosis might arise from Bacillus Calmette-Guerin vaccination, died MTB remaining after cured tuberculosis, latent infection or concomitance of sarcoidosis and tuberculosis (19, 20). Only 1 patient with tuberculosis was mycobacterial DNA negative according to the quantitative cutoff value and the number was 1.58×10^2 copies per ml, which might be ascribed to the less volume of caseating granulomas in sample.

There have been reports of immunologic response to mycobacterial proteins in patients with sarcoidosis (21-23). The current method does not exclude another *Mycobacteria* present in the current tissue. Our primers were DNA in the IS986 area.

Drake et al, using a variety of PCR primers, have found evidence of *Mycobacteria* that were not MTB in sarcoidosis tissue samples (22).

In summary, the real-time PCR provides sensitive, precise, and rapid measurement of MTB DNA in formalin-fixed and paraffin-embedded tissue samples. MTB genome quantification by real-time PCR is more accurate than qualitation, and MTB genome copies number of 1.14×10^3 copies per ml should be preferred as quantitative cutoff value for the differentiation between sarcoidosis and TB. The performance of MTB DNA quantification for the differentiation between sarcoidosis and TB in fresh tissue is yet unknown and need more study.

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