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Original Article

Diagnostic value of mycobacterium tuberculosis DNA detection by polymerase chain reaction on bone tuberculosis

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Abstract: Bone tuberculosis is one destructive lesion caused by bone invasion of blood-borne mycobacterium tuberculosis that presents in human spine, hip, knee, elbow, hands, and feet. The early diagnosis of bone tuberculosis is difficult because of the lack of medical history, specific signs or bacterial culture in the early stage of the disease and also because of the lack of sensitivity of the parasitological methods available. This study thus investigated the value of polymerase chain reaction (PCR) in diagnosing bone tuberculosis targeting mycobacterium tuberculosis. A total of 115 bone tuberculosis samples were collected in parallel with 95 control samples. PCR was used to test mycobacterium tuberculosis DNA after culture. Enzyme linked immunosorbent assay (ELISA) was applied to test serum tuberculosis mycobacterium specific antibody (TB-SA) levels. There were 92 samples positive for TB-DNA (positive rate = 80.0%). Culture of TB showed 26 positive samples (22.6% positive rate). There were 60 samples were positive for TB-SA (52.5%). PCR showed a significantly higher positive rate than other two methods ($P < 0.05$). PCR approach has 80.0% sensitivity for TB, plus 9.7% specificity and 86.7% accuracy, which were higher than other methods. PCR method has higher sensitivity and specificity in detecting TB DNA. The advantage of the PCR method is that TBSA in samples or TB culture can be more rapidly detected than ELISA.

Keywords: Polymerase chain reaction, bone tuberculosis, DNA, sensitivity, specificity

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by infection of mycobacterium tuberculosis (MT). MT can invade into multiple human body organs, in which lung is the major infection site [1]. Due to its infectious, refractory and severe damage, it has been a major health concern over decades [2]. Although sophisticated treatment is already rather advanced for TB, further advances are still needed due to the new challenges e.g. environmental pollution, weakened immune systems, application of immunosuppressant, and transmission of AIDS [3, 4]. Moreover, higher motility of population and more drug resistant MT strains both increase transmission rate and treatment difficulties [5]. TB is often occurs in young and middle-aged adults, which results in an increase trend in its incidence [6]. With insidious onset, longer incubation time, and slow progression, TB is a chronic infectious disease which is often misdiagnosed [7]. Although the infectious site of TB is mainly in pulmonary tissues, MT can be transmitted to various organs such as skin, meninges, peritoneal, lung and bones via blood borne or lymph circulation [8]. Bone tuberculosis is one destructive lesion caused by blood-borne invasion of MT into bone or joint tissues, and frequently occurs in the spine, hip, knee, feet, elbow, or hand to affect loading of bone/joint tissues and/or limb motility [9]. Bone tuberculosis is often accompanied with low fever, lassitude, night sweat, lower appetite and thinning, plus joint swelling, pain, atrophy and/or dysfunction, causing major issues for the patient [10].

The key for bone tuberculosis includes early diagnosis and timely treatment to alleviate joint structural damage and retard functional damage [11]. However, due to lower specificity in early clinical manifestation, the early diagnosis of bone tuberculosis is relatively difficult and

requires multiple approached combing history, body sings, auxiliary assays and MT culture [12, 13]. These methods, however, have a relative lower sensitivity and specificity [14]. This study thus tested MT DNA using polymerase chain reaction (PCR) assay to investigate its implication for bone tuberculosis diagnosis.

Materials and methods

Research subjects

A total of 115 samples were collected from bone tuberculosis patients who were admitted in Ningbo No. 2 Hospital from January 2015 to August 2016, including 72 males and 43 females (aging between 24-67 years, average age = 34.5 ± 5.2 years). Inclusive criteria: Typical TB pathology change under assay or during surgery; visible MT after bacterial culture; typical clinical symptoms, body signs and imaging features; effective anti-TB treatment. A patient can be diagnosed with any one item of those abovementioned features. Exclusion criteria: Accompanied with other infectious diseases, malignant tumor, severe diabetes, kidney/liver disease, pulmonary fibrosis, bone metabolic disorder, systemic immune disease or any complication of cancer.

Ninety-five non-bone tuberculosis samples were collected, including 65 males and 30 females, with ages between 23 and 69 years (average age = 33.6 ± 6.3 years). There were 25 cases of bone tumors, 47 cases of osteoarthritis and 23 cases of rheumatoid arthritis or traumatic arthritis in the control group. Nil statistical significance was presumed to exist in sex, age or other general information between two groups. This study obtained signed inform consents from all participants and was approved by the Ethics Committee of WuXi Traditional Chinese Medicine Hospital.

Major equipment and reagent

PCR diagnostic kit for MT was purchased from Yilikang Co. (China). Serum TB-specific antigen (TB-SA) assay kit was purchased from Daan gene (China). PCR cycler was purchased from Gene Corp (US). Electrophoresis apparatus was purchased from Liuyi Instrument (China). Labsystem version 1.3.1 microplate reader was purchased from Bio-Rad (US).

PCR detection for MT DNA

Sample collection was performed under sterile conditions. Joint fluid, pus or tissue samples were collected and grinded. After adding lysis buffer, tissues were homogenized and centrifuged. The tissue supernatant was used as the template. Joint fluid samples were repeatedly centrifuged for the purpose of collecting the supernatant. A PCR system was prepared using 1 μl TaqDNA polymerase, 8 μl reaction buffer and 16 μl DNA template. Reaction conditions were: 94°C 30 seconds, 65°C 55 seconds and 72°C 35 seconds for 35 cycles, ended with 72°C elongation for 5 minutes. The 10 μl amplification product was mixed with 3 μl EB, and loaded onto 2% agarose gel for electrophoresis under 100V for 30 min. The product size was 245 bp. Primer sequences for MT DNA were: forward, 5'-TCCGC TGCCA GTCGT CTTCC-3'; reverse, 5'-GTCCT CGCGA GTCTA GGCCA-3'.

MT separation and culture

Following the protocols established by Chinese Association of Anti-tuberculosis, samples were centrifuged at 1500 g for 5 minutes, then the supernatant was discarded. Precipitation was inoculated on the improved Roche culture medium and incubated at 37°C. Negative staining was judged post 8 weeks of bacterial-free growth.

ELISA for serum TB-SA antibody

A total of 5 ml of peripheral blood samples were collected from fasted patients. Blood samples were centrifuged at 2000 rpm for 5 minutes to collect the supernatant, which were tested for TB-SA using ELISA kit. In brief, 50 μL of serially diluted standard was added to each well in a 96-well to create standard curves. Then, 50 μl test samples were then added into test well plots in triplicates. Plates were washed 5 times with recommended Wash Buffer. Liquid was removed completely from last wash. The rinsing procedure was repeated for 5 times and then 50 μl of the enzyme labeling reagent was added into each well. After gentle mixture, the well was incubated for 30 minutes at 37°C. Chromogenic substrates A and B were sequentially added (50 μl each), followed by 37°C dark incubation for 10 minutes. The test plate was then mixed with 50 μl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, optical density (OD)

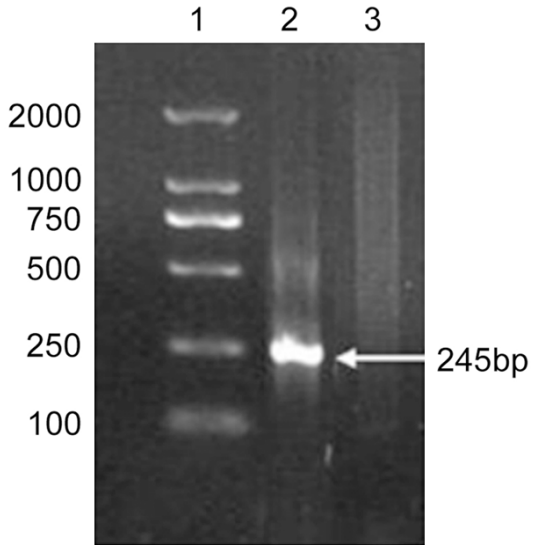

Figure 1. DNA electrophoresis results of PCR assay for MT. Lane 1, DNA marker; Lane 2, bone tuberculosis group; Lane 3, control group.

values at 450 nm wavelengths were measured by a microplate reader within 15 minutes post adding quenching buffer. Positive results were deduced as sample OD/control OD higher than 1.2, while negative results were identified when such ratios were lower than 0.8. Those samples having OD ratios between 0.8 and 1.2 were tested for a second round.

Statistical processing

SPSS 19.0 statistical software was used for analysis. Enumeration data were tested by chi-square method. A statistical significance was defined when $P < 0.05$.

Results

PCR assay for MT DNA

PCR-electrophoresis assay showed amplification band at 245 bp in bone tuberculosis samples, while there was no band showed in control group (**Figure 1**). Bone tuberculosis sample PCR found 92 positive cases of MT positive, while 23 samples were negative for MT (positive rate = 80%). In control samples, we found 5 positive cases of MT while 90 cases were negative (positive rate = 5.3%).

PCR assay for MT DNA and ELISA for TB-SA, in comparison with MT culture

ELISA was used to test for TB-SA antibody, and 60 positive cases was found in bone tuberculosis group (55 negative cases, positive rate = 52.2%). In control group, there were 18 positive cases and 77 negative cases (positive rate = 18.9%). In MT culture, there were 26 positive and 89 negative cases in bone tuberculosis group (positive rate = 22.6%), and 31 positive and 64 negative cases in control group (positive rate = 32.6%). PCR assay thus had significantly higher positive rate of MT DNA than that of ELISA or bacterial culture approach. ELISA also had higher positive rate of bone tuberculosis than bacterial culture. In control group, DNA positive rate was significantly than ELISA or bacterial culture approach, whilst ELISA had lower positive rate than culture method ($P < 0.05$, **Table 1**).

Analysis of sensitivity and specificity

Sensitivity and specificity analysis was performed among the PCR assays for MT DNA, ELISA for TB-SA antibody and MT culture. Results demonstrated that the PCR approach had 80.0% sensitivity, 94.7% specificity and 86.7% accuracy respectively. ELISA for TB-SA antibody had 52.2% sensitivity, 81.1% specificity and 65.2% accuracy respectively. Bacterial culture had 80.0% sensitivity, 67.4% specificity and 42.9% accuracy respectively. The PCR approach displayed significant advantages over the other two approaches ($P < 0.05$, **Table 2**).

Comparison of time consuming among three methods

The lengths of time required for positive results of PCR, ELISA, and bacterial culture were determined. The PCR assay needed significantly shorter time in terms of consuming than ELISA for TB-SA or MT bacterial culture, whilst ELISA consumed less time than bacterial culture ($P < 0.05$, **Figure 2**). These results indicated that shorter lengths of time consuming of PCR assay for MT DNA, benefiting quick identification of results.

Discussion

Bone tuberculosis has insidious onset which progress slowly with the lack of typical symptoms at early stage. Imaging methods such as X-ray thus may not be able to identify the existence of early lesions. Although CT has relatively higher resolution for bone tissues, it has low sensitivity for detecting inflammatory change of synovial tissues and joint cavity fluids.

Table 1. Comparison of PCR for MT DNA, ELISA for TB-SA and MT culture

Approach	Bone tuberculosis			Control		
	Positive cases	Negative	Rate (%)	Positive cases	Negative	Rate (%)
PCR for MT DNA	92	23	80.0*,#	5	90	5.3*,#
ELISA for TB-SA antibody	60	55	52.2*	18	77	18.9*
MT culture	26	89	22.6	31	64	32.6

Note: *, P < 0.05 compared to MT culture; #, P < 0.05 compared to ELISA.

Table 2. Sensitivity and specificity analysis among PCR, ELISA, and culture

Test method	Sensitivity	Specificity	Accuracy
PCR for MT DNA	80.0*,#	94.7*,#	86.7*,#
ELISA for TB-SA antibody	52.2*	81.1*	65.2*
MT culture	22.6	67.4	42.9

Note: *, P < 0.05 compared to MT culture; #, P < 0.05 compared to ELISA.

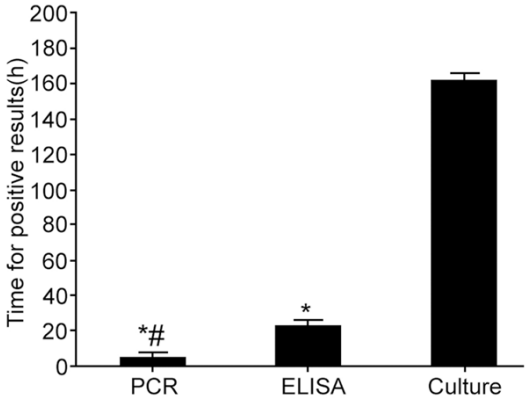

Figure 2. Comparison of time consumption among the three approaches. *, P < 0.05 compared to MT culture; #, P < 0.05 compared to ELISA.

Therefore, a comprehensive diagnosis including clinical symptoms and lab results such as erythrocyte sedimentation are necessary for the diagnosis. The difficulty of forming a diagnosis frequently leads to disease progression and in some cases, severe inflammatory injury and dysfunction of bone-joint structure. The research for early and fast diagnostic technique important for prevention and diagnosis of bone tuberculosis [15, 16].

Although current research reports MT culture is one of the most common method for diagnosis of bone tuberculosis, there is still lots of room to improve including the inconvenience of stringent nutrient requirement, slow progress and long time consuming procedures which may require 2-8 weeks to obtain visible bacterial colonies with only viable MT strains [17]. Moreover, the fact that MT culture can be easily distorted by biological features of MT and treatment side effects, high standard of test condition and technique, and cross-contamination effects all lead to low positive rate and difficulty for promotion [18]. This study utilized improved culture medium to improve the onset of positive results but still requires longer time with lower bone tuberculosis positive rate. TB-SA is one of the specific protein in tuberculosis patient's serum. Post secreting out of cells, TB-SA may induce body immune response which would enhance further production of anti-TB-SA antibody. As TB-SA only exists in pathogenic MT rather than other microbes or non-pathogenic MT, we can utilize serum TB-SA antibody to determine MT infection [19, 20]. This study demonstrated that ELISA for serum TB-SA antibody improved both sensitivity and specificity for bone tuberculosis diagnosis, with a better outcome than MT culture.

PCR approach utilized DNA template for *in vitro* amplification of large amounts of DNA replicates, as it can amplify tracing amounts of DNA to more than 10^6-fold. This approach can also detect one single DNA fragment of MT in the sample, which contribute to rather high sensitivity and specificity, providing novel references for pathology assay and differential diagnosis of bone tuberculosis [21, 22]. This study utilized PCR approach for amplifying 245 bp length DNA fragment as the template, significantly elevating positive rate of bone tuberculosis assay and the test sensitivity and specificity, with less time and higher accuracy. Therefore, the PCR approach presents clinical importance for early diagnosis of bone tuberculosis.

PCR approach for MT DNA has numerous advantages in terms of high sensitivity, specificity, rapidness, and ease of manipulation, all

of which are advantageous over ELISA for TB-SA antibody and MT culture approaches. PCR assay thus provides a novel method for early, rapid and accurate diagnosis of bone tuberculosis, and has important clinical implication.

Disclosure of conflict of interest

None.

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