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USEFULNESS OF THE MYCOBACTERIUM TUBERCULOSIS DIRECT ASSAY IN EARLY DIAGNOSIS OF EXTRAPULMONARY TB

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Abstract – The aim of this study was to evaluate the *in situ* detection of living mycobacterium TB rRNA by the mycobacterium TB direct assay (MTD) and its clinical significance in the early diagnosis of extrapulmonary TB. Eighty-six patients were recruited from the Shanghai Public Health Clinical Center from June to November in 2010, having been diagnosed with extrapulmonary TB, including tuberculous peritonitis (n=22), lymphatic TB (n=21), tuberculous meningitis (n=15), HIV-associated TB (n=13), nephroTB (n=9), spinal TB (n=2), cutaneous TB (n=13), parotid TB (n=1), chest wall TB (n=1), intestinal TB (n=1). One hundred and five extrapulmonary specimens, including CSF, puncture fluid, drainage, pleural fluid, urine, secretion, ascites, lymphatic tissue and marrow were collected from the patients. The samples were examined using acid-fast stain, solid culture, liquid culture and MTD in parallel. In MTD, the target segments of MTB rRNA in either cultures or clinical specimens were amplified prior to being qualitatively detected with the hybridization protection assay (**HPA)**. The sensitivities of MTD and acid-fast staining in liquid and solid cultures were 48.6%, 41.9%, 20.0% and 14.3%, respectively. MTD sensitivity was higher than that of the others and its specificity was 100%. We concluded that MTD rRNA detection is an effective, rapid, convenient, sensitive and reliable method for the early diagnosis of extrapulmonary TB.

Key words: Mycobacterium TB; rRNA; MTD; extrapulmonary TB

INTRODUCTION

Mycobacterium TB (MTB*)* is the causative agent of tuberculosis (TB). Successful disease control relies on early identification of suspicious subjects and rapid detection of MTB. Traditional diagnostic methods include smear staining of acid-fast bacilli (AFB) in liquid or solid culture. Culture results are usually not available before 2 to 3 weeks, whereas AFB staining lacks sensitivity and specificity. Infections with MTB often cause pulmonary disease but can also involve extrapulmonary organs such as the skin, bone, kidney, lymph node and marrow (Ashford et al., 2001; Chao et al., 2002). Extrapulmonary TB accounts for 25-30% of total TB and its mortality accounts for 14.1-17.6% of overall TB-caused death (Huang et al., 2000). Diagnosis of extrapulmonary TB is even more difficult due to its complicated clinical manifestation and lack of specific features. Recently, many methods have been developed to meet such needs, and nucleic acid amplification is one of them. Due to their natural ability to recognize one single organism, nucleic acid amplification methods have the potential to decrease dramatically diagnostic duration as well as increase detection specificity. IS*6110* or 16S rRNA genes have been used

as targets in nucleic acid amplification (Watterson et al., 2000). Several kits that use 16S rRNA sequences as probes or amplification primers are commercially available for the identification of mycobacteria (Drobniewski et al., 2000; St Amand et al., 2005; Tottey, 2013; Lazzeri et al.,2012). We selected the quickest methodology for the detection of mycobacteria in both culture and extrapulmonary tissue *in situ* (St Amand et al., 2005) and compared it with other conventional methods, hoping to improve the early diagnosis and treatment of extrapulmonary TB.

MATERIALS AND METHODS

Specimen origins

Eighty-six patients diagnosed with extrapulmonary TB, including tuberculous peritonitis (n=22), lymphatic TB (n=21), tuberculous meningitis (n=15), HIV-associated TB (n=13), nephrotuberculosis (n=9), spinal TB (n=2), cutaneous TB (n=13), parotid TB (n=1), chest wall TB (n=1) and intestinal TB (n=1), were recruited from the Shanghai Public Health Clinical Center from June to November in 2010. 105 extrapulmonary specimens including CSF (n=29), puncture fluid (n=24), drainage (n=15), pleural fluid (n=14), urine (n=9), secretion (n=9), ascites (n=3), lymphatic tissue (n=1) and marrow (n=1) were collected from the patients. Thirty-one subjects with non-TB such as cryptococcal meningitis or liver disease were recruited as negative control. These patients were characterized by definite diagnosis, no history of TB, and on-going TB was ruled out by chest X-ray (CXR) or lab tests. The examined specimens included CSF (n=13), ascites (n=7), pleural fluid (n=4), puncture fluid (n=3), secrete (n=2), drainage (n=1) and urine (n=1).

Instruments and reagents

The MTD kit was purchased from Gen-Probe UK Co Ltd, and the M**TB antigen detection** reagent **kit** (**colloidal gold) was from** Hangzhou Genesis Biodetection & Biocontrol**Ltd**; Acid fast stain (Zhuhai, Beisuo).

MTD detection

Mycobacteria were released from host cells by ultrasonication and heated to denature nucleic acid and untwisted rRNA. The target segment of rRNA was exponentially amplified at 42°**C**. The complementary sequence of amplification products was bound by chemiluminescent-labeled probes and subsequently detected with HPA. Once the RNA:DNA complex was stabilized, successfully hybridized probes were selected and visualized in a chemiluminescent detector. Detection was according to the assay instructions; all batches passed quality control.

Other methods

Each sample was solid or liquid cultured acid-fast stained according to the Ziehl-Neelsen method. The solid culture followed the L-J medium culture protocol; the liquid culture followed the requirements of BACTEC MGIT 960.

RESULTS

Results of MTB detection in 105 extrapulmonary samples with MTD, culture and smear in parallel, are shown in Table 1. The sensitivity of the four methods was MTD 48.6% (51/105), smear 41.9% (44/105), liquid culture 20.0% (21/105), and solid culture 14.3% (15/105). The sensitivity of MTD was significantly different from that of the liquid and solid culture ($P<0.01$), but the same as that of the smear ($p>0.05$). All liquid culture positive specimens were also MTD positive and the specificity of MTD was 100%. All MTD, culture and smear results in the control group were negative. The results of MTB detection in 105 specimens with MTD, culture and smear are shown in Tables 2-5.

DISCUSSION

Gen-Probe MTD is a molecular diagnostic reagent approved by the FDA and SFDA to identify MTB (Coelho et al., 2008). It is a chemiluminescent-labeled probe capable of detecting MTB rRNA by binding a complementary nucleic acid in cultured or

Table 1. The results of MTB detection in 105 extrapulmonary samples with MTD, culture and smear in parallel

Methods / Diagnosis	MTD Positive	Smear Positive	Solid Culture Positive	Liquid Culture Positive	Number
Tuberculous Peritonitis	13	10	1	3	29
LymphoTB	20	18	11	11	28
Tuberculous Meningitis	7	3	1	3	18
AIDS *	4	6	0	2	15
NephroTB	1	2	1	2 contaminated	9
Spinal TB	2	2	0	1	2
Cutaneous TB	1	1	0	0	1
Parotid TB	1	1	1	1	1
Chest Wall TB	1	1	0	0	1
Intestinal TB	1	0	0	0	1
Total	51	44	15	21	105

*In all 15 AIDS cases, 10 were combined with tuberculous meningitis and 5 with tuberculous peritonitis

Table 2. The results of MTB detection in 105 specimens with MTD, culture and smear

Methods / Diagnosis	MTD Positive	Smear Positive	Solid Culture Positive	Liquid Culture Positive	Number
CSF	12	6	2	6	29
Puncture Fluid	13	12	5	5	23
Drainage	13	12	5	7	15
Pleural Fluid	4	3	0	0	14
Urine	1	2	1	2 contaminated	9
Secrete	3	7	0	1	9
Ascites	3	0	0	0	3
Lymphatic Tissue	2	1	2	2	2
Marrow	0	1	0	0	1
Total	51	44	15	21	105

Results show the contamination rate of urine was highest

non-cultured specimens for diagnostic purpose and is characterized by both high sensitivity and high specificity, which enables it to rapidly differentiate MTB from *mycobacterium avium*, *mycobacterium intracellulare*, *mycobacterium kansasii* and *mycobacterium gordonae*. While its clinical diagnostic significance has been verified (Bergmann et al., 1999), its extrapulmonary application has yet to be elucidated. The present study was focused on the *in situ* detection of MTB rRNA in clinical specimens.

Extrapulmonary TB accounts for 25-30% of all TB. The percentage is 9.7-11.8% in China, and its mortality accounts for 14.1-17.6% of all TB-caused death (Huang et al., 2000). Diagnosis of extrapulmonary TB is difficult due to its complicated clinical manifestation and lack of specific features. At present, clinical examination combined with culture, microscopic examination of direct smear staining and adjuvant chest X-ray is still golden standard diagnostic. All these methods are based on clinical symptoms,

Table 3. The results of 44 smear-positive detected with MTD, culture and smear in parallel

Methods / Diagnosis	MTD Positive	Smear Positive	Solid Culture Positive	Liquid Culture Positive
CSF	4	1	2	6
Puncture Fluid	10	5	5	12
Drainage	12	5	7	12
Pleural Fluid	2	0	0	3
Urine	1	1	1 contaminated	2
Lymphatic Tissue	1	1	1	1
Secrete	3	0	1	7
Marrow	0	0	0	1
Total	33	13	16	44

Table 4. The results of 61 smear-negative detected with MTD, culture and smear in parallel

Methods / Diagnosis	MTD Positive	Smear Positive	Solid Culture Positive	Liquid Culture Positive
CSF	8	1	4	23
Puncture Fluid	3	0	0	11
Drainage	1	0	0	3
Pleural Fluid	2	0	0	11
Urine	0	0	1 contaminated	7
Ascites	3	0	0	3
Lymphatic Tissue	1	1	1	1
Secrete	0	0	0	2
Total	18	2	5	61

Table 5. The results of 51 MTD-positive detected with MTD, culture and smear in parallel

Methods / Diagnosis	MTD Positive	Smear Positive	Solid Culture Positive	Liquid Culture Positive
CSF	4	2	6	12
Puncture Fluid	10	3	5	13
Drainage	12	5	7	13
Pleural Fluid	2	0	0	4
Ascites	0	0	0	3
Urine	1	1	1 contaminated	1
Lymphatic Tissue	1	2	2	2
Secrete	3	0	1	3
Total	33	13	21	51

which limit their ability to detect sub-clinical infection. The direct smear is convenient, rapid and inexpensive, and it is still the diagnostic of choice, but its clinical application is limited by factors such as: 1) a low positive rate, approximately 10% (0-87%), less than 10^4/ml is not detectable; 2) poor specificity, as all species of mycobacterium can be positively stained, and another test is necessary to confirm this; 3) inability to discern living from dead cells; 4) limited (Hooja et al., 2011). In this study, 11 smear-positive cases were MTD negative, and possible causes included 1) false positive smear test of non-tuberculous mycobacteria; 2) 7 out of those 11 were follow-up patients who had received treatment, and these false positives could have resulted from killed bacteria; 3) mishandling of specimens, existence of amplification inhibitor or incomplete digestion. There was one case where the smear was positive but the first MTD was negative; MTD turned positive the second time after another around of digestion. Limited quantities of specimens or templates could interfere with the sensitivity. We also found there 18 smear-negative cases were MTD-positive, indicating that the smear test was influenced by multiple factors.

In a study of CSF collected from 132 tuberculous meningitis patients, Thwaites et al. (2004) showed that a relatively large volume of CSF (>6 ml) was an independent factor determining its ability to detect acid-fast bacilli, and that an average of 30 min microscopic examination as well as appropriate repeats could improve the detection rate. Additionally, the length of disease course, neutrophil ratio, lactic acid level and CSF/serum glucose ratio were all reported to be associated with acid-fast bacilli detection. The culture possesses a mildly increased detection rate and might be able to guide clinical treatment by providing drug sensitivity data, however, it requires at least 10^3~10^4 bacilli/ml to be positively detected, which makes it more difficult to culture, especially bacilli with defective cell walls or compromised by chemotherapy. Considering the lengthy growth period (approx 3-8 weeks), which could be reduced to 3-10 days by an advanced Bactec culture system together with gene probe technology, its low positive rate (as low as 20-30%), and its inability to differentiate MTB and nontuberculous mycobacteria, culture is obviously not a favorable tool for the diagnosis of MTB infection. To improve the clinical efficacy of anti-TB medicines, the development of a rapid species identification assay is warranted (Tomioka et al., 2006). Contamination is another issue, especially of urine samples. Due to the existence of normal flora, the contamination rate of liquid culture is still high in spite of decontamination procedures. In this study, 2 of a total of 9 samples were contaminated, of which 1 was detected positive with MTD, indicating MTD was preferred to avoid potential contamination of urine samples and increase detection sensitivity simultaneously. The positive rates of the smear test and culture in the detection of extrapulmonary TB are low because of the limited quantity of bacteria.

In this study, all 21 liquid culture positive specimens were MTD positive as well. The MTD assay can detect MTB rRNA in cultured or non-cultured specimens. Thirty-one extrapulmonary specimens were examined with MTD, culture and smear in parallel, and all results turned out to be negative, implying 100% specificity of MTD in the detection of extrapulmonary TB. The samples were TMA amplified prior to HPA for qualitative analysis of MTB rRNA, and the whole process only took 2.5-3.5 hours. Since rRNA is believed to be detectable only in live bacteria due to its quick degradation after the death of bacteria, the contribution of dead bacteria will be ruled out in a positive outcome (Kahlisch et al., 2012). Because of its fragile single strand structure and vulnerability to degrade, a false positive or cross contamination are much less likely. The application of MTD in the detection of MTB in sputum samples has been repeatedly verified. In this study, we selected 11 specimens to be tested with MTD, TB DNA, culture and smear in parallel, finding 6 smear positives, including 2 cases positive for all four tests and 1 positive for all except MTD, which was later proved to be nontuberculous mycobacteria. 48.6% sensitivity and 100% specificity were identified in this study with a positive rate higher than that of culture. MTD is a rapid, sensitive and specific test of extrapulmonary TB. The quality of clinical specimens is essential for the accuracy of the test, and a correct way for speci-

men collection and a sufficient sample may improve detection rate (Havlir et al., 1999). For those patients with combined HIV infection, the lack of extrapulmonary symptoms at the end stage due to compromised lymphocyte-mediated immune response may interfere with the diagnosis and prognosis of the disease (Maher et al., 2005). Among all 15 AIDS samples, MTD was positive in 4 cases including 3 smear positive and 1 smear negative; 6 cases were positive for smear including 3 MTD negative cases, and the sensitivity of MTD in detection of HIV infection-related extrapulmonary TB was 33.3% (Watterson et al., 2000; Thwaites et al., 2004).

A rapid, accurate diagnostic is essential for TB prevention. We demonstrated that MTD carried the most important clinical significance in the diagnosis of extrapulmonary TB with its ability to detect MTB rRNA *in situ*. With the development of molecular biotechnology, it has become possible to establish a diagnosis of TB within one day, which could help with early diagnosis and the initiation of treatment at the early stage of the disease.

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