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Value of Mycobacterium Tuberculosis Direct Assay in Early Diagnosis of Extrapulmonary Tuberculosis.

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Abstract

The objective is to evaluate in situ detection of living mycobacterium tuberculosis rRNA using Mycobacterium Tuberculosis Direct Assay (MTD) and its clinical significance in early diagnosis of extrapulmonary tuberculosis. 86 patients having been diagnosed to have extrapulmonary tuberculosis including tuberculous peritonitis (n=22), lymphatic tuberculosis (n=21), tuberculous meningitis (n=15), HIV-associated tuberculosis (n=13), nephrotuberculosis (n=9), spinal tuberculosis (n=2), cutaneous tuberculosis (n=13), parotid tuberculosis (n=1), chest wall tuberculosis (n=1), intestinal tuberculosis (n=1) were recruited from Shanghai Public Health Clinical Center from June to November in 2010, 105 extrapulmonary specimens including CSF, puncture fluid, drainage, pleural fluid, urine, secrete, ascites, lymphatic tissue and marrow were collected from those 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 be qualitatively detected with hybridization protection assay (HPA**). The sensitivity of MTD, acid fast stain, liquid culture and solid culture was 48.6%, 41.9%, 20.0% and 14.3% respectively. MTD's sensitivity was higher than the others and its specificity was 100%. MTD rRNA detection is an effective, rapid, convenient, sensitive and reliable method in early diagnosis of extrapulmonary TB.**

Keywords: Mycobacterium tuberculosis; rRNA; MTD; Extrapulmonary TB.

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Introduction

Tuberculosis (TB) is still a global public health problem and mycobacterium tuberculosis (MTB) is the causative agent. Successful disease control relies on early identification of suspicious subjects and rapid detection of MTB. Traditional diagnostic methods include smears staining of acid-fast bacilli (AFB), liquid culture or solid culture. Nevertheless, culture results are usually not available prior to 2 to 3 weeks, whereas AFB staining lacks sensitivity and specificity. *Mycobacterium tuberculosis* infections often cause pulmonary disease but also can involve extrapulmonary organs such as skin, bone, kidney, lymph node and marrow [1, 2]. Extrapulmonary tuberculosis accounts for 25%-30% of total tuberculosis and its mortality accounts for 14.1%-17.6% of overall tuberculosis-caused death [3]. Diagnosis of extrapulmonary diagnosis is even more difficult due to its complicated clinical manifestation and lack of specific feather. Recently many methods have been developed to meet such needs, and nucleic acid amplification method is one of them. Due to their natural ability to recognize one single organism, nucleic acid amplification methods have the potential to dramatically decrease diagnostic duration as well as increase detection specificity, and IS*6110* or 16S rRNA genes were reported to be two of the targets in nucleic acid amplification method [4]. Several kits that use 16S rRNA sequences as probes or amplification primers are commercially available for molecular identification of mycobacteria [5-8], among which we selected the quickest methodology for the detection of mycobacteria in both culture and extrapulmonary tissue in situ [7] and compared it with other conventional methods in parallel, hoping to improve early diagnosis and treatment of extrapulmonary tuberculosis.

Materials and methods

Specimen origins

86 patients were diagnosed to have extrapulmonary tuberculosis including tuberculous peritonitis (n=22), lymphatic tuberculosis (n=21), tuberculous meningitis (n=15),

HIV-associated tuberculosis (n=13), nephrotuberculosis (n=9), spinal tuberculosis (n=2), cutaneous tuberculosis (n=13), parotid tuberculosis (n=1), chest wall tuberculosis (n=1), intestinal tuberculosis (n=1) were recruited from 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), secrete (n=9), ascites (n=3), lymphatic tissue (n=1) and marrow (n=1) were collected from those patients. 31 subjects with non-tuberculosis such as cryptococcus meningitis or liver disease were recruited as negative control. Those patients were characterized by definite diagnosis, no history of tuberculosis, and on-going tuberculosis was ruled out by 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).

Instrument and reagents

Olympus inverted microscope (Type: CKX41); GEN-PROBE LEADER 50i、INCUBATORS (Biomerieux (French) Co Ltd); -BATCTEC MGIT 960 System (Becton Dickinson Co Ltd). Middlebrook 7H9 medium powder (American Difico Co Ltd); OADC and PANDA were purchased from Becton Dickinson Co Ltd; MTD kit was purchased from Gen-Probe UK Co Ltd; mycobacterium tuberculosis antigen detection reagent kit (colloidal gold) was from Hangzhou Genesis Biodetection & BiocontrolLtd; Acid fast stain (Zhuhai, Beisuo).

MTD detection

MTD could be divided into two sections, amplification and detection, both occurred in a same tube. Initially, mycobacteria were released from host cells by ultrasonication. Heated to denature nucleic acid and untwisted rRNA. The target segment of rRNA was exponentially TMA amplified under a constant temperature of 42 °C. The complementary sequence of amplification products was bound by the chemiluminescence 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 performance and results reading strictly followed assay instruction, and all batches passed quality control. The whole process only took 2.5-3.5 hours.

Other methods

The deposit of each sample collected from prior procedure was acid-fast stained, solid cultured or liquid cultured. Acid fast stain followed Ziehl-Neelsen method, solid culture followed L-J medium culture protocol, and liquid culture followed the requirement of BACTEC MGIT 960.

Results

Results of MTB detection in 105 extrapulmonary samples with MTD, culture and smear in parallel, as shown in Table 1.

The sensitivity of 4 methods was: MTD:48.6% (51/105), smear:41.9% (44/105), liquid culture: 20.0% (21/105), solid culture:14.3% (15/105). The sensitivity of MTD was significantly different from that of liquid/solid culture ($P<0.01$), but not different from that of smear ($p>0.05$). All liquid culture positive specimens were also MTD positive, and the specificity of MTD was 100%. All of MTD, culture and smear results in control group were negative.

The results of MTB detection in 105 specimens with MTD, culture and smear in parallel were shown in table 2-5.

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
Lymphotuberculosis	20	18	11	11	28
Tuberculous Meningitis	7	3	1	3	18
AIDS *	4	6	0	2	15
Nephrotuberculosis	1	2	1	2 contaminated	9
Spinal Tuberculosis	2	2	0	1	2
Cutaneous Tuberculosis	1	1	0	0	1
Parotid Tuberculosis	1	1	1	1	1
Chest Wall Tuberculosis	1	1	0	0	1
Intestinal Tuberculosis	1	0	0	0	1
Total	51	44	15	21	105

**In all 15 AIDS cases, 10 was combined with Tuberculous Meningitis and 5 was combined 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
Pucture 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 showed: the contamination rate of urine was highest

***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
Pucture 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 nagetive 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
Pucture 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
Pucture 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

Discussion

GEN-PROBE MTD is a molecular diagnostic reagent that has been approved by FDA and SFDA to identify MTB [9]. It is a chemiluminescence labeled probe capable of detecting MTB rRNA by binding a complementary nucleic acid in cultured or non-cultured specimens for diagnostic purpose and is characterized by both high sensitivity and high specificity which enable it to rapidly differentiate MTB from mycobacterium avium, mycobacterium intracellulare, mycobacterium kansasii and gordon mycobacteria. Being a reliable modality to detect MTB in sputum specimens bronchial specimens (e.g. bronchial lavage fluids or bronchial aspirates) or tracheal aspirate sediment specimens, its clinical diagnostic significance has been reportedly verified [10], nevertheless, its extrapulmonary application yet to be elucidated. This study is focused on in situ detection of MTB rRNA in clinical specimens, hoping to improve the early diagnosis and treatment of extrapulmonary tuberculosis.

It has been reported that extrapulmonary tuberculosis accounts for 25%-30% of total tuberculosis, this ratio is 9.7%-11.8% in China, and its mortality accounts for 14.1%-17.6% of all tuberculosis-caused death [3]. Diagnosis of extrapulmonary diagnosis is difficult due to its complicated clinical manifestation and lack of specific feather. Presently, clinical examination combined with culture, microscopic examination of direct smear staining and adjuvant chest X-ray is still golden standard diagnostic. All those methods are based on clinical symptoms, which limit its ability to detect sub-clinical infection.

Direct smear is convenient, rapid and inexpensive, and it is still the diagnostic of choice, but its clinical application is limited by variable factors as followed: 1) Low positive rate, approximately 10% (0-87%), less than 10^4/ml is not detectable; 2) Poor specificity, all species of mycobacterium could be positively stained, and another test is necessitated to confirm it; 3) Unable to tell live from dead; 4) Limited by the principle [11], a break-through development seems impossible. 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 those false positive could result from killed bacteria; 3) Mishandling of specimens, existence of amplification inhibitor or incomplete digestion; There was one case whose smear was positive but first time MTD was negative, and MTD turned positive second time after another around of digestion; Limited quantities of specimens or templates could interfere with the sensitivity. We also found there were 18 smear negative cases were MTD positive, indicating smear test was influenced by multiple factors.

In a study of CSF collected from 132 tuberculous meningitis patients, Thwaites et al showed a relative big volume CSF (>6mL) was an independent factor determining its ability to detect acid-fast bacilli, and an average 30 minutes microscopic examination duration as well as appropriate repeat could improve detection rate [12]. Additionally, 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 rate.

Culture possesses a mildly increased detection rate and might be able to guide clinical treatment by providing drug sensitivity data, but it requires at least 10^3~10^4 bacilli/ml to be positively detected, which makes it more difficult to culture, especially for those bacilli of small quantity, defect cell wall, or compromised by chemotherapy. Considering lengthy growth period, approx 3-8 weeks, which could be reduced to 3-10 days by using advanced Bactec culture system together with gene probe technology, its low positive rate, most 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 clinical efficacy of anti-TB medicines, development of a rapid species identification assay is warranted [13].

Contamination is another issue, especially urine sample, due to existence of normal flora, contamination rate of liquid culture is still high in spite of decontamination procedure. In this study, 2 of a total of 9 samples was contaminated, among which, 1 was detected positive with MTD, indicating MTD was preferred to avoid potential contamination of urine sample and increase detection sensitivity simultaneously. The positive rates of smear test and culture in detection of extrapulmonary tuberculosis are low because of limited quantity of bacteria.

In this study, all 21 liquid culture positive specimens were MTD positive as well. MTD assay is able to detect MTB rRNA in cultured or non-cultured specimens. We selected 2 liquid culture positive cases to test again with MTD again which were also positive, with a similar RLU level. 31 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 detection of extrapulmonary tuberculosis.

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 death of bacteria, contribution of dead bacteria will be ruled out in a positive outcome [14]. 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 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 positive rate higher than that of culture.

MTD is a rapid, sensitive and specific test of extrapulmonary tuberculosis. The quality of clinical specimen is essential for the accuracy of the test, and a correct way for specimen collection and sufficient sample may improve detection rate [15]. For those patients combined with HIV infection, lack of extrapulmonary symptom at the end stage due to compromised lymphocyte mediated immune response may interfere with the diagnosis and prognosis of the disease [16]. 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 tuberculosis was 33.3% [4, 12].

A rapid, accurate diagnostic is essential for tuberculosis prevention. We demonstrated MTD carried the most important clinical significance in diagnosis of extrapulmonary tuberculosis with its ability to in situ detect MTB rRNA. With the development of clinical diseases, we must upgrade ourselves from bacteria level to molecular level, from traditional methods to modern methods to study and explore from all directions. With the development of molecular biotechnology, it has become possible to establish a diagnosis of tuberculosis within one day, which could help with early diagnosis and initiation of the treatment at the early stage of the disease, so as to control its spreading, and administration of wrong medicine based on a misdiagnosis, and reduce medical cost accordingly. Clinical experience should be combined with new technologies to facilitate rapid and accurate diagnosis as well as standardized treatment of tuberculosis.

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