Detection of *Mycobacterium tuberculosis* Complex in Formalin-Fixed, Paraffin-Embedded Tissue Specimens with Necrotizing Granulomatous Inflammation by Strand Displacement Amplification

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Rapid, reliable diagnosis of tuberculosis is essential to initiate correct treatment, avoid severe complications, and prevent transmission. Conventional microbiological methods may not be an option if samples are formalin-fixed and paraffin-embedded (FFPE) for histopathological examination. With the demonstration of necrotizing granulomatous inflammation, tuberculosis becomes an important differential diagnosis, although it was not initially suspected. Following paraffin extraction, BDProbeTec ET strand displacement amplification for detection of *Mycobacterium tuberculosis* complex (MTC) was applied to 47 prospectively and 19 retrospectively collected FFPE samples from various sources with granulomatous inflammation and results were compared to tuberculosis notification. Of the prospective samples, 20 were from patients who were notified as having tuberculosis and the assay was positive in 18 (90%). Specificity was 100%. For 27 of the patients with prospectively collected FFPE specimens, culture was performed on a specimen collected at a later date from the same location. Culture revealed MTC in 14 and nontuberculous mycobacteria in four. BDProbeTec ET was positive in 13 (92.8%) of the patients with positive MTC culture and negative in the remaining. The sensitivity and specificity in 19 archival samples was 40% and 100%, respectively, compared to notification data. The assay provided rapid, correct diagnosis on different sources of FFPE samples collected prospectively and therefore offers an important supplementary method for patients where tuberculosis was not initially suspected. (*J Mol Diagn* 2004, 6:231–235)

Tuberculosis (TB) is an increasing health problem worldwide, with up to one-third of the world's population infected with *Mycobacterium tuberculosis* complex (MTC) as estimated by the World Health Organization. Approximately eight million people develop clinical active disease and two million people die every year due to TB although diagnostic methods and effective treatment are available.¹

Until the late 1980s, microbiological diagnosis of TB relied solely on microscopy and culture. Microscopy is fast and inexpensive but exhibits low sensitivity. Although highly specific for acid-fast bacilli, it does not distinguish between the 100 different species of mycobacteria.² Culture offers better sensitivity, but the time required to obtain positive results varies from 2 to 6 weeks due to the slow growth of mycobacteria.³ Introduction of nucleic acid amplification methods has improved the microbiological diagnosis of TB significantly during the last decade. Direct application of these methods combines rapidity with specific detection of MTC and improved sensitivity when compared to microscopy.

Despite the fact that TB is a highly prevalent infectious disease, the clinical diagnosis remains a true challenge due to its varying localization, appearance, and symptoms.^{4–10} Initially, TB may not have been considered and samples may only have been sent for histopathological examination. Such samples are formalin-fixed and paraffin-embedded (FFPE) making microbiological examination, apart from low-sensitive, non-specific microscopy, impossible. If histopathology raises suspicion of mycobacterial infection eg, due to the presence of granulomatous inflammation, it may warrant repeated sample collection. Repeated invasive procedures are both inconvenient, expensive, and can be impossible, thus it is desirable to optimize diagnostic methods applicable on FFPE samples.

New molecular methods are currently being used for diagnostic purposes to detect eg, malignancy and infectious diseases.^{11–13} Furthermore, Rish and co-workers¹⁴ used a PCR method on FFPE tissue from mice experi-

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mentally infected with the H37Rv strain of *M. tuberculosis* and were able to detect as few as nine bacteria in a 5- μ m section of tissue. However, to obtain optimal DNA from such samples, several purification and preparation steps were needed. Previous studies have described the use of PCR on human FFPE specimens for detection of MTC DNA with promising results, but almost all of them used in-house PCR methods.^{15–19} The BDProbeTec ET Direct Detection assay (Becton Dickinson, Sparks, MD, USA) is commercially available for the detection of MTC in clinical specimens. This assay is based on simultaneous strand displacement amplification of MTC-specific IS6110 target DNA of 95 bp and real-time detection by fluorescent-labeled probes. The assay has recently been evaluated in both respiratory and non-respiratory specimens with promising results.^{20–23} The aim of this study was to investigate the performance of the assay in FFPE tissue specimens to evaluate its suitability for detection of MTC in clinical routine specimens and in stored specimens.

Materials and Methods

The study was carried out at the International Reference Laboratory of Mycobacteriology at Statens Serum Institut (IRLM), Copenhagen which is the only microbiological diagnostic laboratory for mycobacterial culture and species identification in Denmark.

Specimens

Forty-seven prospectively collected FFPE tissue specimens (22 lymph node, nine lung, five liver, three subcutaneous tissues, two bowel, two bone, one spleen, one peritoneum, one kidney, and one testis) from 43 patients received continuously from clinical pathology or clinical microbiology departments in Denmark and from Malmö University Hospital in Sweden were included together with 19 retrospectively collected (1998 to 2000) FFPE tissue specimens [10 lymph node, four peritoneum, three gastrointestinal (stomach, bowel, and appendix), one lung, and one tuba uterina] from 17 patients received from the archives of the pathology department of Hvidovre University Hospital. All specimens showed necrotizing granulomatous inflammation on histopathological examination.

Processing

Available (ranging from one to five) 5- μ m sections of the FFPE specimens were deparaffinized as previously described²⁴ with the following modifications: two ml of xylene (Bie and Berntsen A.D., Rodovre, Denmark) was used and the incubation time was 1 hour. The xylene was removed by pipette and the procedure was repeated three times after which the pellet was air-dried. FFPE samples from a patient, who was notified as having TB, was used as a positive control in each deparaffinizing procedure. The pellet was resuspended with 1 ml of distilled water and two drops of the suspension was used

for microscopy slides. These smears were stained with auramine-rhodamine fluorochrome and examined with fluorescence microscopy at $\times 200$ magnification. The number of acid-fast bacilli (AFB) seen in the whole slide was registered.

BDProbeTec ET Direct Detection Assay

Five hundred μ l of the pellet suspension was analyzed according to the manufacturer's instructions, as described in detail elsewhere.²¹ In brief, the suspension was centrifuged with sample wash buffer to remove possible inhibitors, after which the pellet was heated at 105°C for 30 minutes and then resuspended in 100 μ l of sample lysis buffer containing potassium hydroxide. The mixture was sonicated for 45 minutes at 65°C and 600 μ l of sample neutralization buffer was added. Positive and negative controls were included in each lysis procedure. After lysis, 150 μ l of samples and controls were incubated with the oligonucleotides, dNTP, and detector probes in the priming microwells. Subsequently, the samples and controls were transferred into the preheated amplification microwells containing restriction enzyme, DNA polymerase, and dNTPs. Finally, the microwells were sealed and placed into the BDProbeTec ET instrument.

The results were obtained as metric other than acceleration (MOTA), a measurement of the area under the relative fluorescent unit curve. Samples with MOTA values ≥ 3400 were positive for MTC DNA according to the product insert. If the internal amplification control value was > 5000 and the MTC MOTA value was < 3400 , the specimen was negative for MTC DNA. If the internal amplification control MOTA value was < 5000 and the MTC MOTA value was < 3400 , the reaction was inhibited and the result was considered inconclusive.

Precautions to Avoid Contamination

Biological safety cabinets class 2 decontaminated with household bleach, gloves, and aerosol-resistant filter tips were used in all steps to avoid amplicon contamination from prior reactions.

Gold Standard

The results obtained by the BDProbeTec ET system were compared to the final clinical diagnosis retrieved from the national TB registry in both countries and to results obtained by culture where available. For 27 of the patients with prospectively collected FFPE specimens, culture was performed on samples collected at a later date within the same clinical course and from the same location. After possible decontamination on non-sterile specimens with 1.5% NaOH-NALC, the sediment was inoculated onto Löwenstein-Jensen slants (SSI Diagnostica, Denmark) and in BACTEC Mycobacteria Growth Indicator Tube 960 liquid media (Becton Dickinson) in IRLM. Presumed mycobacterial growth was confirmed by Ziehl-Neelsen staining. If no growth was observed after 8 weeks of incubation, the specimen was reported as cul-

Table 1. Detection of *Mycobacterium tuberculosis* Complex in Various Sources of 66 FFPE Tissue Specimens by BDProbeTec ET Compared to TB Notifications

Type of specimens <i>n</i> = 66	Notified with TB <i>n</i> = 35	BDProbeTec ET positive <i>n</i> = 24 (68.5%)
Lymph node (32)	17	12 (70.5%)
Lung (10)	3	2 (66.7%)
Gastrointestinal tissues (5)	5	3 (60%)
Liver (5)	2	2 (100%)
Peritoneum (5)	5	2 (40%)
Subcutaneous tissues (3)	0	0
Bone (2)	0	0
Genital (2)	1	1 (100%)
Kidney (1)	1	1 (100%)
Spleen (1)	1	1 (100%)

ture negative. Species identification was performed by the Inno-LiPA Mycobacteria assay as described elsewhere.²⁵ In Malmö University Hospital culture was done in Löwenstein-Jensen slants and in BACTEC 12B liquid medium (Becton Dickinson) while identification of mycobacteria was performed by DNA probes (AccuProbe, San Diego, CA).

Results

Altogether, 66 FFPE samples from various locations from 60 patients with necrotizing granulomatous inflammation were analyzed by the BDProbeTec ET assay for detection of MTC DNA. Results were compared to TB notifications from the national TB registers in Denmark and Sweden (Table 1).

The prospective part of the study involved 47 FFPE tissue specimens from 43 patients. Of these, 20 specimens were from patients who were notified as having clinical TB. The BDProbeTec ET was positive for MTC DNA in 18 and negative in the remaining 29 specimens (Table 2). The two specimens in which BDProbeTec ET was false negative were lymph nodes from different patients. There were three more specimens (lymph node, bronchoalveolar lavage, and pus from lymph node) from one of these patients and they all yielded negative culture. Two additional lymph node biopsies were received

from the other patient and only one became culture positive. Culture was performed on specimens collected at a later date from 27 of these patients. Culture revealed MTC in 14 patients and BDProbeTec ET was positive for MTC DNA in 13 of these 14 patients. The assay was negative in the specimens from patients with culture positive for nontuberculous mycobacteria (NTM) (two *Mycobacterium avium*, one *M. marinum*, and one *M. xenopi*) and in all the remaining patients. The overall sensitivity and specificity of the BDProbeTec ET were 90% and 100%, respectively, when compared to national TB notification data, and 92.8% and 100%, respectively, when compared to culture alone.

The retrospective aspect of the study involved 19 FFPE tissue samples from 17 patients. The results obtained by the BDProbeTec ET assay are shown in Table 2. Fifteen specimens were from patients having clinical TB, and BDProbeTec ET was positive for MTC in only six. However, the assay yielded high MOTA value close to the cutoff value of 3400 for two of nine false-negative samples. These two false-negative specimens were re-tested and the MOTA values remained unchanged. The assay was negative in the remaining samples. Culture results were available for 15 patients, but it had not been performed on tissue corresponding to the FFPE tissue samples and direct comparison with the BDProbeTec ET results was thus impossible. The overall sensitivity and specificity of the BDProbeTec ET in the archival samples were 40% and 100%, respectively, when compared to national TB notification data.

AFB were demonstrated in eight samples with Ziehl-Neelsen staining by the pathologist whereas it was demonstrated in 18 (12 prospectively and six retrospectively collected samples) with auramine-rhodamine fluorochrome staining after paraffin extraction. Fifteen of these 18 specimens were from patients with TB and the remaining three were from patients with NTM infection.

The positive control for the deparaffinizing procedure remained positive by BDProbeTec ET in each run. None of the positive and negative controls for the lysis procedure yielded discrepant results. We achieved conclusive results in all specimens revealing no inhibition. The time for analysis was 1 working day (8 hours on average) including the paraffin extraction step.

Table 2. The Performance of the BDProbeTec ET Assay for Detection of *M. tuberculosis* Complex in Prospectively and Retrospectively Collected FFPE Tissues Compared to Results of Final Clinical Diagnosis

BDProbeTec ET assay	Final clinical diagnosis		
	Notified with TB	NTM*	Others†
Prospective (<i>n</i> = 47)			
Positive	18	0	0
Negative	2	6	21
Retrospective (<i>n</i> = 19)			
Positive	6	0	0
Negative	9‡	2	2

*, Nontuberculous mycobacteria; †, non-mycobacterial infection or autoimmune diseases (sarcoidosis, Wegeners granulomatous); ‡, two had high MOTA values below the cutoff value.

Discussion

Rapid diagnosis of TB has a major impact on the management of patients. The detection of MTC in clinical samples leads to initiation of correct treatment thereby avoiding or diminishing severe complications as well as spread of the disease. Currently, several amplification methods for direct detection of MTC in clinical specimens have become commercially available and application of these methods may offer significant improvements in the management of patients. Despite the existence of numerous evaluations of amplification methods their feasibility remains to be elucidated in certain sample categories. Previous evaluations of commercially available methods have focused on fresh clinical specimens and evalua-

tions on FFPE specimens have been performed by using in-house PCR or nested PCR in selected specimens.

In this study, we demonstrated the feasibility and reliability of strand displacement amplification method in prospectively, consecutively collected FFPE tissue samples in two low incidence countries. The majority of the specimens were from patients in whom TB was not initially suspected and specimens were thus solely sent to histopathological examination. Of all 47 FFPE samples, 20 were from patients who commenced treatment for TB on receipt of the histopathologic results. The assay detected MTC DNA in 18 (90%) of these samples, providing the TB diagnosis in 1 working day where no fresh material was left or available for mycobacterial culture. The two false-negative results may be due to paucibacillarity in this type of specimens or loss of DNA during the paraffin extraction and sample preparation for amplification. Further optimization of these procedural steps should thus be considered. We observed no false-positive results. By using a faster, kit-based, standardized strand displacement amplification method, our results extend the findings reported by Salian and co-workers, who demonstrated the feasibility of in-house PCR using IS6110 target DNA on 60 FFPE tissue samples prospectively collected in various geographical areas yielding a 73.6% sensitivity and 100% specificity compared to final TB diagnosis.¹⁵

The sensitivity of the amplification method of FFPE specimens depends on several factors such as the duration of the fixation, the fixative, the amplicon size, and the age of the paraffin block. All prospectively and retrospectively collected specimens in our study were fixed in 10% buffered formalin for 16 to 18 hours. The assay showed excellent performance for the detection of *M. tuberculosis* bacteria in prospectively collected FFPE samples. However, the sensitivity was considerably lower in the 3- to 5-year-old archival samples, which might be explained by DNA damage due to long storage. It is well known that old archival FFPE samples are not as suitable as fresh samples as the time-dependent physical degradation of DNA in FFPE tissue affects the success rate of the amplification. Goelz et al²⁶ reported that successful amplification of human DNA from various tissue samples stored 4 to 6 years often necessitated smaller size of the amplified fragments than DNA from samples stored less than 2 years. In the present study, the size of the amplicon was 95 bp. An epidemiological study performed by Qian and co-workers²⁷ on 85 FFPE lung biopsies from Chinese patients with pulmonary TB collected from 1950 through 1990 revealed significant time-related differences in the detection rate of DNA. The results indicated that the old DNA templates might have become damaged and fragmented due to years of storage. This problem should be taken into account, when the assay is used in archival samples stored more than 2 years. The sensitivity might be improved through optimization of the paraffin and DNA extraction procedures. Heller et al²⁸ described an easy and fast method for the extraction of DNA from FFPE tissue by using sonic bath and glass beads. After paraffin extraction with xylene, we applied the BDProbeTec ET procedure to detection of MTC DNA. DNA extraction was carried out by heating in an oven,

followed by sonication. Although sonication with glass beads thus might be comparable to proteinase K digestion, which is considered the best known extraction method requiring extensive hands-on work, it may not be suitable in all situations. Extraction of long DNA fragments is less efficient and adequate copies of target DNA may not be liberated. Since long-term storage of samples tend to degrade DNA, the extraction step might need modification if the assay is used in such samples. According to the manufacturer, the limit of detection for the assay as determined by serial dilution is nine CFU per reaction or 125 CFU/ml. The lower sensitivity in archival specimens in the present study could be due to sampling error as the bacteria are unevenly distributed and the number of bacteria thus varies in each tissue section. In the present as well as in a previous study, we found that the assay yielded low inhibition rate.²¹ To increase the sensitivity particularly in archival specimens, it could therefore be considered to include more tissue sections (>3) in each run.

There are reports of higher sensitivity (58% to 100%) than observed in this study. However, in these studies the FFPE specimens were collected retrospectively and selected from high-risk patients or from patients where TB diagnosis had already been confirmed by positive culture.¹⁶⁻¹⁹ Ruiz-Manzano and co-workers²⁹ retrospectively evaluated two commercially available kit-based assays: Amplified *Mycobacterium* TB Direct Test and the LCx *Mycobacterium tuberculosis* Assay. In 74 FFPE pleural biopsy samples they showed a 52.6% and a 63.2% sensitivity, respectively, and a 100% specificity for both assays. Proteinase K digestion method was used in all of these studies.

Disease caused by NTM has increased due to the HIV epidemic and various immunosuppressive treatments.³⁰ The clinical approach to NTM infection differs significantly from that to infections with MTC.³¹ The histopathological findings in infections caused by NTM are the same as for TB and a variety of other infectious and autoimmune diseases and would thus not contribute to the NTM diagnosis. In accordance with previous studies, we observed no cross-reaction between MTC and NTM species.

We conclude that the BDProbeTec ET Direct Detection Assay provides rapid reliable results when applied to prospectively collected FFPE tissue samples with necrotizing granulomatous inflammation. Thus, the assay provides an additional opportunity to identify MTC where TB is not initially suspected and no fresh specimen is available for routine microbiological TB diagnostics. Although the assay showed good performance, it cannot replace culture, which is essential for drug susceptibility testing, NTM species identification, and surveillance of active transmission by DNA fingerprinting analysis.

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