Evaluation of the BD ProbeTec ET system for the direct detection of *Mycobacterium tuberculosis* from clinical samples

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ABSTRACT

Early detection of disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC) is essential in controlling transmission of tuberculosis. Clinical evaluation of the BD ProbeTec ET system for the direct detection of MTBC organisms in clinical specimens is carried out by comparing results obtained with conventional liquid culture and reference laboratory identification. In 186 specimens (159 respiratory, 27 non-respiratory), the BD ProbeTec ET assay detected eight out of 11 MTBC culture-positive specimens, with overall sensitivity, specificity, positive predictive value and negative predictive value of 85%, 100%, 100% and 99%, respectively. The data show that the BD ProbeTec ET assay is a highly sensitive and specific technique, and gives an accurate result from a clinical specimen within three to four hours.

KEY WORDS: Amplification tests.
Mycobacterium tuberculosis.
Tuberculosis.

Introduction

The World Health Organization (WHO) estimates that one-third of the world's population currently has tuberculosis (TB), with approximately two million deaths per year. Despite the availability of effective anti-tubercular drugs, the incidence of the disease has been rising steadily, and TB remains the leading infectious cause of death in adults worldwide.[1] Although the major burden of TB occurs in developing countries, the number of cases in the UK has increased by 25% over the past 10 years.[2] A high proportion of these cases occur among certain groups, such as those living in major cities who have recently arrived from countries with a high disease prevalence.[3] The increasing number of cases of human immunodeficiency virus (HIV) infection and use of immunosuppressive therapy also makes an important contribution to the increase of TB.[4] Over 7000 cases of TB are reported in the UK each year.[5]

Tuberculosis is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), which comprises the closely related organisms *M. tuberculosis* (MTB), *M. bovis, M. africanum, M. microti* and *M. canettii*. The vast majority of human cases of TB, which may be pulmonary or extra-pulmonary, are caused by MTB. Pulmonary disease is spread by droplet inhalation, and is a particular problem in poor, overcrowded or unhygienic conditions. An untreated patient may infect 10–15 other people in a year.[6] Early detection is effective in stopping the chain of transmission, with considerable benefits for public health,[7] as a rapid diagnosis leads to speedier initiation of treatment. Appropriate treatment will cause infectious patients to become non-infectious within two weeks.[8] Extra-pulmonary TB is not considered to be contagious.[1]

The number of infections caused by non-tubercular mycobacteria (NTM) is also increasing, especially among the immunocompromised.[9] These organisms are opportunist, environmental mycobacteria that cause a range of diseases, from granulomatous lesions of the skin to pulmonary disease or even disseminated infection. Generally, NTM are not considered to be transmissible from human to human,[10] but it is important to diagnose all mycobacterial infections and to differentiate between NTM and MTB. A rapid diagnosis of NTM avoids unnecessary contact tracing and administration of anti-tubercular drugs, with significant cost benefits.[11]

Of particular concern is the emergence of MTB strains that are resistant to isoniazid and rifampicin – two of the most important first-line drugs. Outbreaks of multidrug-resistant TB (MDR-TB) are difficult and expensive to treat and contribute to the increase in mortality from TB.[12] A single case of MDR-TB may cost £50,000–£70,000;[13] however, rapid detection of MDR-TB greatly reduces overall treatment costs by the appropriate isolation of cases in negative-pressure rooms and the administration of more effective drugs.[14]

As a truly effective vaccine is not yet available,[15] rapid treatment of active infection, whether resistant or susceptible to first-line drugs, remains the most important method of infection control.[11]

A clinical diagnosis of pulmonary TB may prove difficult, as symptoms such as persistent cough, night sweats and weight loss accompany many other systemic diseases.[8] Chest X-ray and tuberculin skin tests may also prove inconclusive, especially in immunocompromised patients.[8] Thus, in many cases, diagnosis relies on laboratory findings. Clinical diagnosis of extra-pulmonary TB may prove even more

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difficult, due to the very wide spectrum of disease and its non-specific clinical manifestations.[16]

Conventional methods for the detection and identification of MTB are based on microscopy for the presence of acid-fast bacilli, and on culture and biochemical tests. Microscopy of stained smears of sputum and other samples is a simple, cost-effective screening method,[16] but it is relatively insensitive as at least 5 x 10^3 acid-fast bacilli/mL are required for detection.[9] This method also has low specificity[17] because it is not possible to distinguish reliably MTB from NTM. Culture, on the other hand, has a much higher sensitivity (only 10 viable MTB/mL are required[18]), and thus it is considered to be the gold-standard method. However, the slow-growing mycobacteria may take two to four weeks to reach detectable numbers. The isolate must then be sent to a regional TB reference laboratory for identification, which may delay the result a further two to three days. Delays in diagnosis delay appropriate treatment, which, in turn, may lead to increased transmission.[7]

The ideal diagnostic test for TB should, therefore, be highly sensitive and specific, and give reliable results from a clinical specimen within a matter of a few hours. The test should also differentiate MTB from other mycobacteria. A number of nucleic acid amplification tests (NAATs) have been developed that fulfil these criteria, and one such test is the BDProbeTec ET direct TB assay (Becton Dickinson, Oxford). The aim of this study is to evaluate the use of this assay in a busy diagnostic microbiology laboratory at Northampton General Hospital (NGH) and to assess cost-effectiveness for routine TB detection.

Materials and methods

The incidence of TB in Northamptonshire for 2005 was close to the national rate of 12.8/100,000 population.[19] A total of 1800 samples from 820 patients suspected of having TB were received in the microbiology laboratory at NGH during 2005, and 86 new notifications were made.[19] For this study, 186 samples submitted to NGH for routine acid-fast microscopy and TB culture between November 2005 and January 2006 were assayed on the BD ProbeTec ET system. All positive cultures were sent to the Regional TB Reference Laboratory at Heartlands Hospital, Birmingham, for identification and antibiotic sensitivity testing.

The BD ProbeTec ET system is a semi-automated assay based on the simultaneous amplification and detection of a 95 bp region of the target DNA sequence IS*6110* by strand displacement amplification (SDA). IS*6110* is an insertion sequence (IS) present in the genome of MTBC organisms, with up to 25 copies/cell, and is used as a target in many alternative molecular TB detection assays (e.g., polymerase chain reaction [PCR]-based techniques).[16,20] The BD ProbeTec ET system employs an isothermal process that utilises the restriction enzyme *Bso*B1. The restriction enzyme recognition sequence is nicked by *Bso*B1, but not completely cut, due to the presence of a thiolated nucleotide, dC_5TP. *Bst* polymerase displaces the existing strand of DNA and synthesises a new one. The displaced strand acts as a template for further amplification. Amplified product is detected by an increase in fluorescence.

All samples were from patients suspected of having a mycobacterial infection, having been sent to NGH for conventional diagnostic testing. A total of 159 respiratory specimens (128 sputa, 31 bronchial aspirates or bronchoalveolar lavages) and 27 non-respiratory specimens (19 pleural fluids, four tissue samples, four other samples) were processed on receipt, stained and examined by fluorescence microscopy for acid-fast bacilli. Samples were then decontaminated and inoculated in a liquid culture medium. A sample of each decontaminated specimen was stored at –20°C to be processed in batches using the BD ProbeTec ET assay.

Preparation of samples

Sputum samples and centrifuged pellets from bronchial aspirates were liquefied by the addition of an equal quantity of 1% dithiothreitol and this was vortex-mixed intermittently over 20 min. Then, the mixture was centrifuged at 4000 rpm for 10 min, and the supernatant discarded. A smear was made from the deposit, stained by the auramine-phenol method and examined for acid-fast bacilli by fluorescence microscopy. The deposit was decontaminated with 4% (w/v) NaOH for 25 min, with vortex-mixing at 5-min intervals. The suspension was centrifuged again and the deposit neutralised with 0.5 mol/L H_2SO_4.

Pleural fluids were centrifuged at 4000 rpm for 10 minutes. A smear was made from the deposit, which was stained with auramine-phenol and examined by fluorescence microscopy. The deposit was decontaminated with 0.25 mol/L H_2SO_4 for 25 min, with vortex-mixing at 5-min intervals. The suspension was neutralised with 4% (w/v) NaOH.

Tissue samples were homogenised in a small amount of distilled water, then decontaminated using the procedure adopted for pleural fluids.

The decontaminated and neutralised samples were centrifuged at 4000 rpm for 10 min. The deposits were resuspended in 1.5 mL phosphate-buffered saline (PBS, pH 7.2).

Liquid culture

Liquid culture medium, supplied by the manufacturer, was inoculated with 0.5 mL decontaminated sample and incubated on an MB/BacT system (bioMérieux UK, Basingstoke), which continually monitors the specimen for production of CO_2 as an indication of bacterial growth. The remainder of the sample was stored at –20°C.

BD ProbeTec ET assay

The BD ProbeTec ET assay was performed according to the manufacturer's instructions. The frozen, decontaminated samples were thawed at room temperature and vortex-mixed. Then, 500 µL was added to 1 mL of wash buffer. The mixture was vortex-mixed and centrifuged at 12,200 x*g* for 3 min.

The supernatant was discarded and the pellet was heated for 30 min at 105°C in the BD ProbeTec ET oven to render the bacteria non-viable. All tubes were pulse centrifuged to eliminate aerosols that may occur due to condensation on the inside of the lid. The pellet was then resuspended in 100 µL of sample lysis buffer. The mixture was vortex-mixed and sonicated in a BD ProbeTec ET sonicator for 45 min at 65°C. The sample was then pulse centrifuged and 600 µL of sample neutralisation buffer was added.

For priming and amplification, 150 µL of each prepared sample was dispensed into a priming microwell containing dehydrated amplification primers and fluorescence-labelled detector oligonucleotide probes, and incubated for 20 min at room temperature. The priming plate was transferred to a BD ProbeTec ET priming and warming heater for 10 min at 72.5°C. Then, 100 µL from each priming well was pipetted into amplification wells prewarmed to 54°C. These wells contained dehydrated restriction enzyme and DNA polymerase. The microwell plate was sealed with a plate sealer and placed in the BD ProbeTec ET instrument, which incubated the plates for 1 h at 52.5°C. Positive and negative controls were included in each run.

For interpretation, the BD ProbeTec ET system provided a MOTA (metric other than acceleration) value on completion of the test. This represented the area under the relative fluorescence unit curve, which formed as the amplification product concentration increased and a fluorescein-labelled probe hybridised to the product. According to the manufacturer, samples with MOTA values >3400 are positive for MTB complex DNA. Each test well contained an internal amplification control (IAC). The result was regarded as negative if the MOTA was <3400 and the IAC MOTA was >5000. A MOTA value <3400 and IAC <5000 indicated sample amplification inhibition, and thus a void result.

Table 1. Repeated results from discrepant samples.

Sample No	MOTA 1	MOTA 2	IAC 1	IAC 2
9	2	126	149	39
58	58	8	1716	256
101	128	149	44,303	54,354
107	8	0	70,395	46,374
158	7	2390	35,719	45,933

Table 2. Culture and BD ProbeTec ET results of the smear-positive samples.

BD ProbeTec ET	Culture-positive	Culture-negative
Positive	7	0
Negative	2*	0

*one *M. xenopi*, one *M. avium* complex

Table 3. Culture and BD ProbeTec ET results of 176 smear-negative samples.

BD ProbeTec ET	Culture-positive	Culture-negative
Positive	1	9*
Negative	10†	154
Void	2‡	0

*Previous TB, treated.

†one *M. tuberculosis*, two *M. tuberculosis* plus *M. chelonae*, four *M. avium* complex, two *M. kansasii*, one *M. gordonae*.

‡two *M. avium* complex.

Statistical analysis

Analysis was performed on the basis of reference laboratory identification of positive cultures. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using the formulae described by Bland.[21] Samples with BD ProbeTec ET results that were discordant with culture and reference laboratory results were repeated (Table 1).

Results

The results of this study are summarised in Tables 2 and 3.

Smear-positive, culture-positive samples

Eight samples were positive by all three tests, and the reference laboratory subsequently identified seven of these as MTB. The remaining sample failed to grow at the reference laboratory. Two samples were smear- and culture-positive, but negative by BD ProbeTec ET. These were identified by the reference laboratory as *M. xenopi* and *M. avium* complex.

Smear-negative, culture-positive samples

Thirteen smear-negative samples were culture-positive and, of these, one was positive by BD ProbeTec ET, which was identified by the reference laboratory as MTB. The amplification reaction was inhibited so the BD ProbeTec ET results were void in two samples. Freezing and re-thawing of the prepared sample may remove inhibition but, in these two samples, a repeat test after freezing resulted in further void results. Of the remaining 10 smear-negative, culture-positive, BD ProbeTec ET-negative samples, four were identified as MAC, two as *M. kansasii* and one as *M. gordonae*. There were three discrepant results – reference laboratory results identified one culture as MTB, and two cultures (both from the same patient) as MTB and *M. chelonae*. The three samples were negative on retesting by BD ProbeTec ET.

Smear-negative, culture-negative samples

Of the 163 smear-negative, culture-negative samples, nine were positive by BD ProbeTec ET. All others were negative.

For smear-positive samples, the calculated values for both sensitivity and specificity were 100%. For smear-negative samples, sensitivity and specificity were 25% and 98%, respectively. Overall sensitivity and specificity were 85% and 100%, respectively, while PPV and NPV were 100% and 99%, respectively.

Discussion

Early detection of TB infection is crucial to controlling transmission of the disease. Acid-fast staining and subsequent microscopy has been the mainstay of TB diagnosis for over 100 years and is generally considered to be an indication of infectivity, although it has low sensitivity and specificity.

However, clinical presentation and radiological findings may sometimes mean that a positive smear result is all that is needed to make a presumptive diagnosis and initiate treatment.[20] In these cases, the value of the smear is irrefutable, but there are many instances of false-negative and false-positive results.

The type and quality of the specimen, the thickness of the smear, and the expertise of the microscopist all influence the result. In addition, mycobacteria tend to grow in clumps, giving an uneven distribution throughout the sample, and this also may contribute to the incidence of false-negative results.

In contrast, the presence of acid-fast bacilli in a smear may not necessarily be a positive result. For example, if the patient has been treated, non-viable bacteria may be seen, or the bacteria may be NTM.

In many patients with a high clinical suspicion of TB, a positive smear test may lead to their isolation and the use of costly empirical treatment. This may be unnecessary if the culture is later identified as NTM,[11] which is on the increase in the UK due to the rise in the number of susceptible people such as the elderly or those infected with HIV.[16]

Patients with HIV are also at increased risk of reactivation of latent TB;[22] thus, differentiation of the infecting *Mycobacterium* species is very important. *M. avium* complex is most commonly found in immunocompromised patients, whereas *M. kansasii, M. malomoense* and *M. xenopi* are often isolated from elderly people with other pulmonary diseases.[23]

There are further difficulties in the identification of extra-pulmonary TB. Specimens from non-pulmonary sites tend to have very low numbers of mycobacteria per mL, and often give a false-negative result.[16] The number of patients with extra-pulmonary TB has increased at a faster rate than those with pulmonary TB over the past decade. Notifications of extra-pulmonary TB rose from 1897 in 1994 to 2800 in 2004. In comparison, reported cases of pulmonary TB rose from 3694 to 3923 over the same period.[5]

Culture of the organism, although much more sensitive than identification in a smear, is too slow and an infected person may transmit the disease to others before the result is known, even if they are smear-negative.[24] The consequences of delay in diagnosis are not only an increase in the number of cases of TB, but also a requirement for wider tracing and screening of contacts. This, in turn, has major cost implications, which could be reduced considerably by rapid, accurate diagnosis.

Increasing rates of drug resistance also highlight the importance of a rapid diagnosis to prevent transmission. In the UK, approximately 6% of TB strains isolated are resistant to at least one of the first-line drugs, and 1% are resistant to multiple drugs.[2] Patients with MDR-TB remain infectious longer and require up to two years' treatment with the more toxic second-line drugs.[25]

During the past decade, several NAATs have been developed for the detection of MTB complex in clinical specimens. These include the PCR-based Amplicor (Roche, Lewes, East Sussex, UK), transcription-mediated amplification-based AMTD (Gen-Probe, San Diego, California), ligase chain reaction-based LCx (Abbott, Maidenhead, Berkshire, UK), and strand displacement amplification (BD ProbeTec ET). The advantage of the last system over other NAATs is the use of an internal amplification control in each microwell, which detects the presence of inhibiting substances.

Simultaneous amplification of the IS*6110* target and the internal control is detected by an increase in a fluorescence signal. The target probe is labelled with rhodamine/dabcyl and the control probe is labelled with fluorescein/dabcyl. Dabcyl quenches fluorescence energy from rhodamine or fluorescein when the two molecules are in close proximity. As the target is extended by polymerase and then displaced, the two molecules are able to move apart and thus fluorescence can be detected.

As the two fluorescence signals are emitted at different wavelengths, the BD ProbeTec ET system can detect both simultaneously. Continuous monitoring of the increase in fluorescence means that results are available in real-time.

A further advantage to this system is that the amplification microwells are sealed prior to amplification and are not re-opened. Therefore, contamination with amplified product is unlikely to occur, which means that sample processing can be carried out in the same room in which amplification takes place. This is not the case for other NAATs, which require a separate room for the amplification step.

In the present study, 186 specimens were assayed on the BD ProbeTec ET system and the results were compared to culture and reference laboratory identification. There were 23 anomalous results.

Of the eight samples positive by smear, culture and BD ProbeTec ET, only one failed to be identified at the reference laboratory and was excluded from the statistical analysis,. A review of this sample showed that very scanty acid-fast bacilli were seen on the original smear and on a smear taken from the culture, indicating decreased viability. Consequently, the Birmingham reference laboratory was unable to culture the organism, as the confirmatory assay it uses relies on a good growth of mycobacteria to provide a high DNA yield.

The assay used is the Hain Genotype Mycobacterium CM (Hain Lifescience, Nehren, Germany). The manufacturer states that poor quality or low quantity of isolated DNA may not allow efficient amplification and subsequent hybridisation to the probes. The BD ProbeTec ET assay utilises much longer heat lysis and sonication steps, which could account for the better isolation of small quantities of DNA.

The two smear-positive, culture-positive and BD ProbeTec ET-negative, and the seven smear-negative, culture-positive and BD ProbeTec ET-negative samples were all identified as NTM. The IS*6110* target sequence is highly specific for MTB complex organisms,[26] although McHugh *et al*.[27] have reported its presence in other mycobacteria. This, however, has not been confirmed by any other group.

In the present study, all cultures identified as NTM, except for two that showed irreversible inhibition, were negative by BD ProbeTec ET. These two samples were excluded from the statistical analysis. No cross-reactions were found between MTB and NTM in this study, despite the fact that two NTM samples were smear-positive, indicating a high mycobacterial load.

The smear-negative, culture-positive, BD ProbeTec ET-negative sample identified as MTB was a swab from a neck abscess. The MOTA scores for this sample were 7 and, on repeat, 2390. The manufacturer's cut-off for positives is 3400. The repeated result could be considered a low positive, although this concept is not recognised by the manufacturer. The BD ProbeTec ET direct detection assay is recommended for respiratory samples only, due to the low bacterial load present in many non-respiratory samples.

Few studies have been carried out using the BD ProbeTec ET system for non-respiratory samples.[12,28] Rüsch-Gerdes

and Richter[12] reported a sensitivity of 83.3%, but a positivity rate of only 6%. Johansen *et al*.[28] reported a sensitivity of 60.7% and a positivity rate of 51%. Statistical analysis was not possible in the present study because the neck abscess swab was the only non-respiratory sample that produced a positive MTB culture. Clearly, further investigation is required to assess the assay's performance for non-respiratory samples.

The two samples identified as MTB plus *M. chelonae* may have been variants that lacked the IS*6110* sequence, so the primers were unable to bind. These variants have been reported in several studies, being identified as MTB by restriction fragment length polymorphism (RFLP).[29] The MOTA scores for these two samples were 128 and 8, and, on repeat, 149 and 0. The IAC scores were 44,303 and 70,395, and 54,354 and 46374 for the repeat test. These IAC scores, being >5000, indicate that there was no amplification inhibition.

Another possibility is that the presence of *M. chelonae* in the samples may have interfered with hybridisation to the probe. This phenomenon could be the subject for further study. The manufacturer has reported no cross-reactions with this organism, although it states that the effect of co-infected samples has not been determined.

The nine smear-negative, culture-negative, BD ProbeTec ET-positive samples were all from treated TB patients. The BD ProbeTec ET assay does not distinguish between the presence of viable and non-viable mycobacteria, and DNA can persist in a post-treatment sample for more than 12 months.[30] Specificity is 100% if these samples are removed from the analysis. This compares well with the manufacturer's stated value of 97.8%, and with that of other studies. Several groups have reported specificities between 96% and 99.8%.[12,28,31,32]

Overall sensitivity of 85% in the present study is close to the 91% claimed by the manufacturer. Bergmann *et al*.,[31] Barrett *et al*.,[32] Johansen *et al*.[28] and Rüsch-Gerdes and Richter[12] report sensitivities of 93.8%, 97.1%, 82.7% and 90.3%, respectively.

Rapid detection methods should be highly sensitive and specific for MTB but also must be sufficiently adaptable to fit in with the routine laboratory workflow. The simplest way to use the ProbeTec ET assay would be to store a portion of each decontaminated sample, after smear and culture had been performed, and to run the assay in batches. Although a practical approach for the present study, it may not be feasible for routine work, where the object of using a rapid test is to obtain an accurate result within hours. Clearly, batching would delay the results and thus limit any advantage over routine liquid culture.

National Institute for Health and Clinical Excellence (NICE) guidelines[25] recommend that NAATs for MTB complex be used only for direct detection from clinical specimens if patient care would be altered by rapid identification, or if a large amount of contact tracing must be carried out. At NGH, only one or two samples a week would fall into this category and warrant BD ProbeTec assay detection.

Performed as a single assay, each test would cost £30. Reagents are supplied in packs costing approximately £200 and are sufficient for 100 tests. Once opened, they must be used within a month, resulting in much wastage. The cost of the equipment would also be prohibitive in such a situation.

In conclusion, the BD ProbeTec ET direct assay is a rapid and accurate test, showing high sensitivity and specificity, but the low number of positive samples received at NGH could not justify routine use. Its use in some settings (e.g., major cities with a large population of high-risk groups, and in patients with risk factors for MDR-TB) may be cost-effective, but Northamptonshire is a county with a low incidence of TB, which saw no cases of MDR-TB in 2005.[19]

Nucleic acid amplification tests are invaluable for rapid detection and differentiation of mycobacteria, but they are unlikely to be used in routine diagnostic microbiology laboratories in areas of low incidence, due to a lack of resources. Thus, it is likely that their use will remain restricted to reference laboratories and to large city hospitals, at least for the foreseeable future.

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