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Prospective evaluation of BDProbeTec strand displacement amplification (SDA) system for diagnosis of tuberculosis in non-respiratory and respiratory samples

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Nucleic acid amplification techniques (NAATs) have been demonstrated to make significant improvements in the diagnosis of tuberculosis (TB), particularly in the time to diagnosis and the diagnosis of smear-negative TB. The BD ProbeTec strand displacement amplification (SDA) system for the diagnosis of pulmonary and non-pulmonary tuberculosis was evaluated. A total of 689 samples were analysed from patients with clinically suspected TB. Compared with culture, the sensitivity and specificity for pulmonary samples were 98 and 89 %, and against final clinical diagnosis 93 and 92 %, respectively. This assay has undergone limited evaluation for non-respiratory samples and so 331 non-respiratory samples were tested, identifying those specimens that were likely to yield a useful result. These were CSF ($n = 104$), fine needle aspirates ($n = 64$) and pus ($n = 41$). Pleural fluid ($n = 47$) was identified as a poor specimen. A concern in using the SDA assay was that low-positive samples were difficult to interpret; 7·8 % of specimens fell into this category. Indeed, 64 % of the discrepant results, when compared to final clinical diagnosis, could be assigned as low-positive samples. Specimen type did not predict likelihood of a sample being in the low-positive zone. Although the manufacturers do not describe the concept of a low-positive zone, we have found that it aids clinical diagnosis.

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INTRODUCTION

Nucleic acid amplification techniques (NAATs) have been established in the diagnosis of tuberculosis, making significant improvements in the time to diagnosis with benefits both for the individual patient and in the provision of services. It has been shown that the addition of molecular techniques to our mycobacterial diagnostic service reduced the time to confirmed diagnosis from a mean of 37·5 to 22 days (Davies *et al.*, 1999). Rapid diagnosis reduces the costs of management of multidrug-resistant-tuberculosis, with estimated savings of between £50 000 and £150 000 per annum being achieved following early detection (Drobniewski *et al.*, 2000). Many NAATs have been employed successfully for the detection of *Mycobacterium tuberculosis* (Huggett *et al.*, 2003). Several commercial systems exist and are in routine use, for example AMPLICOR (Roche Diagnostics; Beavis *et al.*, 1995), Gen-Probe (Gene-Probe; Gamboa *et al.*, 1998), ligase chain reaction (Abbot; Lindbrathen *et al.*, 1997), RealArt *M. tuberculosis* PCR kit (Artus) and the BDProbeTec SDA system (Becton Dickinson; Down *et al.*, 1996) adopted in this study. The BDProbeTec is a semi-automated real-time system which allows simultaneous amplification and detection of *M. tuberculosis* target DNA IS*6110* using amplification primers and fluorescently labelled probe.

Commercial systems provide benefits of quality assurance for reagents, user-friendly formats and automated handling of large numbers. However, they are often only fully evaluated for specific clinical specimens and indications, and amplification methods for *M. tuberculosis* are commonly only validated on smear-positive respiratory samples. Non-pulmonary tuberculosis represents a tougher diagnostic challenge. We therefore performed a prospective evaluation of the BDProbeTec system for diagnosis of both non-pulmonary and pulmonary samples. The BDProbeTec system is marketed as a qualitative system, providing a positive or negative result. However, numerical data are produced

Abbreviations: FNA, fine needle aspirates; NAAT, nucleic acid amplification techniques; SDA, strand displacement amplification; TB, tuberculosis.

and a positive result has a value of >3400 MOTA (Metric Other Than Acceleration). During the course of this evaluation we noted that the samples with positive SDA results <40 000 MOTA were responsible for the majority of diagnostic anomalies and so we extended the analysis to investigate the results in this 'low-positive zone'.

METHODS

Specimens. Specimens were obtained prospectively from those submitted routinely to the Molecular Diagnostic Service of the Royal Free Hospital between and including January 2001 and December 2002. Molecular analysis was performed on all those patients in whom the clinician managing the case suspected tuberculosis and requested a *M. tuberculosis* NAAT.

Microbiological methods. Specimens were stored at 4 °C prior to decontamination. Respiratory specimens were treated with an equal volume of *N*-acetyl-L-cysteine (NALC)-NaOH (final concentration 2 %) for 15 min at room temperature and were neutralized with sterile phosphate buffer (0·067 M, pH 6·8). After centrifugation at 3000 ***g*** for 30 min, the pellet was resuspended in 10 ml sterile distilled water and further centrifuged at 3000 ***g*** for 30 min. Half the deposit was inoculated into the culture medium and the remaining half subjected to SDA investigation.

Liquid non-respiratory specimens were centrifuged at 3000 ***g*** for 30 min. Half the resultant pellet was inoculated into culture medium and half submitted to SDA. Similarly, following homogenization in a sterile Griffiths tube homogenizer, tissues were divided into two equal portions. Pellets were resuspended in 2·0 ml phosphate buffer and subjected to both culture and SDA analysis.

All specimens were screened microscopically after concentration using the Auramine stain with positive results confirmed using Ziehl–Neelsen staining. Culture was performed using the MBAlert 3D (bioMérieux) liquid culture system following the manufacturer's instructions.

Molecular methods. Specimens were treated according to the manufacturer's instructions. In brief, a volume of 100–500 µl decontaminated specimen was washed with 1 ml wash buffer (BD-SDA buffer 1) prior to centrifugation in a microfuge at 12 200 ***g*** for 3 min. The supernatant was decanted and mycobacteria killed by heating the pellet to 105 °C for 30 min. DNA was released from cells in the deposit by resuspension in 100 µl lysis buffer (BD-SDA buffer 2), followed by sonication in a soft polymer tube at 65 °C for 45 min. Samples were then neutralized by addition of 600 µl BD-SDA neutralization buffer.

A 150 µl aliquot of the DNA extract was added to a priming well containing dehydrated primers and probes, in the microtitre plate supplied. To ensure complete rehydration of reagents the plate was incubated at room temperature for 20 min. This priming mix was then incubated at 72·5 °C for 10 min. In a separate microtitre plate, amplification wells containing enzymes, dNTPs and buffer were activated by heating to 54 °C for 10 min. Amplification was activated by the addition of 100 µl of the priming mix to the corresponding amplification well and mixing. Plates were then transferred to the ProbeTec analyser.

Each assay run includes positive and negative controls and each test well contains an internal control. Samples that gave a fluorescence reading of >3400 MOTA were regarded as positive.

Clinical data. Specimens were only entered into the study if a minimal clinical dataset was available (patient identifiers and specimen date, sample type and anatomical site, SDA assay, smear and culture results, symptomatic and radiological evidence of tuberculosis). A final clinical diagnosis of tuberculosis was confirmed by cross-reference to the Statutory Infectious Diseases Notification records.

Statistical analysis. Analysis was performed on the basis of each specimen or sample and not on the basis of patient. Sensitivity and specificity of the assay was calculated for each specimen type using the formula described previously by Motulsky (1995).

RESULTS

Over the course of this study a total of 358 respiratory and 331 non-respiratory specimens from 307 patients were investigated. Multiple specimens (2–5/patient) were received from 132 patients, these included sampling of the same site on separate occasions as well as sampling of multiple sites on the same occasion. Each specimen was treated separately in this analysis, as a specimen represents an independent diagnostic event. In assigning a final diagnosis all specimens were considered together with microbiological and clinical data. The respiratory specimens consisted of sputum (169) and bronchial washings/lavages (189). Non-respiratory specimens included cerebrospinal fluid (CSF; 104), fine needle aspirates (FNA; 64), ascitic fluid (18), pus (41), pleural fluid (47), fluids (17) and other specimens (40).

The results of culture, SDA, acid-fast bacilli (AFB) smear and final clinical diagnosis are summarized in Tables 1 and 2. Ninety-five patients had a clinical diagnosis of pulmonary tuberculosis and 69 had a diagnosis of extra-pulmonary tuberculosis. On culture, 83/358 respiratory samples were positive for *M. tuberculosis* (Table 1) a further 14 samples grew non-tuberculosis mycobacteria (NTM), these were

Table 1. SDA, culture, smear and clinical diagnosis (CD) of 358 respiratory samples (189 bronchial washings, 169 sputum)

Mtb SDA result	Culture-positive				Culture-negative			
	Smear-positive		Smear-negative		Smear-positive		Smear-negative	
	CD: not TB	CD: TB	CD: not TB	CD: TB	CD: not TB	CD: TB	CD: not TB	CD: TB
Positive	0	48	0	33	0	2	19	9
Negative	0	0	0	2	0	0	240	5

Table 2. SDA, culture, smear and clinical diagnosis (CD) of 331 non-respiratory samples

Specimen type	Mtb SDA result	Culture-positive				Culture-negative			
		Smear-positive		Smear-negative		Smear-positive		Smear-negative	
		CD: not TB	CD: TB	CD: not TB	CD: TB	CD: not TB	CD: TB	CD: not TB	CD: TB
Ascitic fluid ($n = 18$)	Positive	0	0	0	2	0	0	0	0
	Negative	0	0	0	2	0	0	12	2
CSF ($n = 104$)	Positive	0	1	0	1	0	0	3	2
	Negative	0	0	0	0	0	0	91	6
Fluids ($n = 17$)	Positive	0	2	0	2	0	0	0	0
	Negative	0	0	0	2	0	0	10	1
FNA ($n = 64$)	Positive	0	3	0	5	0	1	4	0
	Negative	0	0	0	3	0	1	46	1
Pleural fluid ($n = 47$)	Positive	0	0	1	2	0	0	3	0
	Negative	0	0	3	4	0	0	29	5
Pus ($n = 41$)	Positive	1	1	0	10	1	1	2	2
	Negative	2	1	1	1	1	0	16	1
Others ($n = 40$)	Positive	0	1	0	1	0	0	4	4
	Negative	0	0	1	1	1	0	24	3

Mycobacterium avium (1), *Mycobacterium kansasii* (3), *Mycobacterium fortuitum* (5), *Mycobacterium xenopi* (3) and two unidentified environmental mycobacteria. Of the non-respiratory samples 54/331 were culture-positive for *M. tuberculosis* (Table 2) and one specimen grew *M. avium*. For respiratory samples 111/358 (31 %) were positive by SDA and for non-respiratory samples 60/331 (18 %) were positive by SDA.

Using the manufacturer's cut-off of 3400 MOTA, the sensitivity and specificity for respiratory samples was 98 and 89 %, respectively, compared to culture (Table 3). When compared to clinical diagnosis the sensitivity was reduced but the specificity increased (Table 3).

An analysis of assay performance was undertaken for each non-respiratory specimen type where there were adequate sample numbers for valid interpretation (Table 3). When compared to culture, CSF samples gave good sensitivity and specificity (100 and 95 %). The assay characteristics for FNA and pus specimens were adequate to provide useful data, however, pleural fluid samples had very poor sensitivity (30 %).

All other non-respiratory sites were evaluated on the basis of potential clinical relevance and individual case histories.

Review of the false-positive results identified 42 of which 27 (64 %) fell between <3400 MOTA and <40 000 MOTA, designated the low-positive zone. The remaining 15 had values above 40 000 MOTA. Forty-six per cent of the low-positive zone specimens (27/58) were discrepant and of these 15 were respiratory specimens. The remaining 12 non-respiratory specimens came from a range of sites with no single type predominating (pleural fluid, 3; fluid, 2; FNA, 2; ascitic fluid, 1; CSF, 3; pus, 1).

Table 3. Sensitivity (sens.), specificity (spec.), positive predictive value (PPV) and negative predictive value (NPV) of Mtb SDA for various specimen types compared to smear, culture and final diagnosis for each sample

Sample	Smear				Culture				Final diagnosis			
	Sens. (%)	Spec. (%)	PPV	NPV	Sens. (%)	Spec. (%)	PPV	NPV	Sens. (%)	Spec. (%)	PPV	NPV
Respiratory specimens	100	80	45	100	98	89	73	99	93	92	81	97
CSF*	100	94	14	100	100	95	29	100	40	97	57	94
FNA	80	85	31	98	73	91	62	94	64	92	69	90
Pleural fluid	100	87	0	100	30	92	50	83	18	89	33	78
Pus	100	58	22	83	71	75	66	78	78	83	78	87

*Note of caution: of 104 CSF samples tested only two were positive.

DISCUSSION

The purpose of NAAT in the diagnosis of tuberculosis is to identify patients requiring treatment rapidly. Using the methods in this way means that applying them to specimens that are smear-positive provides the best diagnostic yield. However, such a policy is less rational than it first appears, as often the clinical presentation, examination and radiology mean that a positive smear is adequate to make a presumptive diagnosis. This is especially true when infections with non-tuberculosis mycobacteria such as *M. avium intracellulare* are less likely with patients on highly active antiretroviral therapy (HAART). We have previously shown that results from smear-negative patients have the biggest impact on treatment decisions (Conaty *et al.*, 2004). Thus, the application of NAAT to specimens that are smear-negative or from a non-pulmonary source is important.

The BDProbeTec SDA system is now well established in the diagnosis of respiratory tuberculosis and the data presented here reflect both the manufacturer's data and that of other groups (Barrett *et al.* 2002; Mazzarelli *et al.*, 2003). For respiratory samples, SDA improved sensitivity (Table 3) over smear alone, which has a sensitivity of 58 % against culture and 53 % against final clinical diagnosis. In this study, the specificity of the SDA was notably low (89 %), when compared to other NAATs, although it is comparable to the test characteristics published by the manufacturers (91 %). We have previously reported that there is cross-reactivity between the target (IS*6110*) and mycobacteria other than tuberculosis (McHugh *et al.*, 1997), such cross-reactivity may contribute to the decreased specificity of the test. This view is supported by the observation that specificity against culture is good in those samples that are likely to have fewer competing bacteria (CSF, FNA and pleural fluid) as compared to pus and respiratory specimens. Of course it should be noted that 'final diagnosis' includes an element of bias, as the SDA result often contributes to the final diagnosis.

In this study we have identified samples that are low-positive ($>$3400–$<$ 40 000 MOTA), which formed 7·8 % of all specimens. Of the discrepant results, when compared to final clinical diagnosis, 64 % could be assigned to the low-positive zone. There were 54 samples in this low-positive zone, of which 46 % were discrepant. Specimen type did not predict the likelihood of a sample being in the low-positive zone, 15/27 discrepant results were respiratory samples, the remaining 12 samples were from a wide range of non-respiratory sites with no single site being over-represented ($P > 0{\cdot}05$). Although the manufacturers do not describe the concept of a low-positive zone, we have found that its introduction has been an aid to interpretation for clinical diagnosis. These samples represent a small proportion of the total, but our experience is that they demand a disproportionate effort to achieve a satisfactory final diagnosis. The implications for a patient of being treated inappropriately for tuberculosis are substantial. Thus, we propose that any sample with results in the low-positive zone is automatically repeated and a further specimen sought. Only if all three results are concordant should a result be reported. The biological significance of the false-positive results in the low-positive zone remains unclear, the presence of low levels of *M. tuberculosis* DNA as a result of environmental exposure or non-viable infection are both possibilities. The critical point is that these patients did not go on to develop clinical disease which emphasizes the need to view the molecular test result within the context of the whole clinical picture.

In this 2-year review of our practice we have identified those non-respiratory samples for which an SDA result may be interpreted with some confidence. CSF is a critical specimen with good sensitivity and specificity as compared to smear and culture (Table 3). It should be noted that this analysis is based on 2/104 positive samples. The manufacturers report a sensitivity of 125 c.f.u. ml^{-1} for the SDA assay, this is tenfold greater than the likely number of organisms in CSF, thus the test is being used at the limit of its analytical sensitivity in this context, and this may contribute to the poor sensitivity in comparison to final diagnosis (40 %). This reflects the importance of non-microbiological markers, such as CSF pleocytosis and biochemistry in the diagnosis of TB meningitis and identifies a role for the SDA in rapid diagnosis. Recently, Johansen *et al.* (2004) described a modified extraction protocol and subsequent re-evaluation of the 3400 MOTA cut-off for CSF samples; this is a promising development that may increase the sensitivity for this important specimen. The low sensitivity but high specificity of the SDA for FNA samples reflects low organism numbers often found in these specimens, however, the good specificity makes the test very useful clinically. Conversely, applying the SDA to pus samples showed good sensitivity but poor specificity, thus, although a negative result may be helpful, the poor specificity suggests that SDA should be applied to pus samples with caution. Pleural fluid is known to be a poor sample for smear and culture testing due to sampling and dilution effects (Valdes *et al.*, 2003). This is also the case for SDA testing, sensitivity of this test for pleural fluids is so low that the test is of little value.

This is the largest prospective review of NAAT for the diagnosis of non-respiratory samples. As a result we are able to focus our resources on those specimens that are most likely to yield clinically relevant results. We confirm the value of NAAT techniques in diagnosis from respiratory samples, and particularly enhanced sensitivity from smear-negative specimens (Conaty *et al.*, 2004). Also, we have demonstrated that certain non-respiratory samples are appropriate for NAAT diagnosis but there is a significant risk of biological false-positive results and so these tests must be interpreted with caution.

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