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Diagnostic performance of isothermal strand displacement amplification of *Mycobacterium tuberculosis* IS 6110 in tissue samples

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ABSTRACT

Background: Visualized histopathological findings in tissue samples are not specific for tuberculosis while mycobacterial cultures from such specimens have low yields and long turn around times. A rapid, sensitive method is therefore needed for detection of *Mycobacterium tuberculosis* in paucibacillary tissue samples.

Methodology: In this paper, a total of 158 tissue specimens, including 42 culture-positives, were tested for the presence of *Mycobacterium tuberculosis* by strand displacement amplification of DNA targeting the region of the insertion element IS 6110 and detected by a chemiluminescence based commercial platform (BDProbeTec™ ET System). The amplification results were correlated to histopathology, microscopy and microbiological culture.

Results: The strand displacement amplification based assay showed low overall sensitivity (31.5%) but high specificity (97.5%) which varied across various tissue types. Only 35.7% of culture-positive biopsies were positive by the molecular assay. Some discrepancy were attributed to suboptimal performance of the traditional methods.

Conclusions: The assay is useful to rule in the disease in common tissue specimens (lung, pleura and lymph node); but less so in other tissue types. The poor sensitivity in tissue specimens necessitates careful interpretation of data generated by the assay in conjunction with a clinical suspicion of tuberculosis for making decision regarding empirical treatment. The complexity of the disease pathology along with the low bacillary load and clumping tendency require selection of more sensitive methods or gene targets.

Introduction

Diagnosis of tuberculosis (TB) in tissues represents a diagnostic challenge since the disease tends to be paucibacillary with a subsequent low yield of acid-fast microscopy [1,2]. While bacteriological confirmation is helpful and essential for speciation and susceptibility testing, it is time-consuming owing to the long generation time of the organism, as well as the technical difficulties in growing mycobacteria from tissue samples. For these reasons, a rapid histopathological diagnosis of TB is valuable to initiate treatment promptly and apply appropriate infection control measures. In additions, rapid diagnosis of TB has a considerable cost impact on healthcare institutions [3].

Mycobacterium tuberculosis bacilli are visualized in tissue sections using specialized stains, including the traditional Ziehl-Neelsen stain. Diagnosis of TB from representative tissue biopsy samples is supported by the presence of caseating

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granuloma, the classic histopathological picture of TB. A toxic glycolipid component of the mycobacterial cell wall is thought to induce caseation [4]. While the presence of caseous necrosis is highly suggestive of TB, this finding can be apparently confused with invasive mycoses, such as cryptococcosis and histoplasmosis. Necrotizing non-granulomatous lesions can also be attributed to tissue TB [5], and thus can be confused with other necrotizing pathological lesions. Furthermore, the histopathological features of TB may show considerable variation and are largely dependent on the underlying immune response [6]. The anergic histopathological presentation of TB is being increasingly recognized with the advent of the era of immunosuppressed patients, making the distinction between TB and other chronic conditions particularly difficult on morphological grounds alone. This shows the critical need for rapid, accurate tissue diagnostics, such as the nucleic acid amplification tests for TB (NAATs-TB). The chief role of those assays is a rapid mean for presumptive diagnosis of TB, which justifies the initiation of therapy in cases where clinical judgment alone does not favor doing so. Genetic targets that have been used for detecting *M. tuberculosis* include IS 6110, 65KD heat shock protein, MPB 64, 38KD protein and ribosomal RNA [7]. Various molecular approaches have been successfully applied for detecting mycobacterial DNA by commercial platforms with the principle based on either conventional PCR techniques followed by sequencing of the amplified product, solid phase hybridization, or real-time amplification methods like the strand displacement amplification (SDA). The advantages of such systems are better quality control of reagents, user-friendly format and potential of automation [8]. However, these commercial systems have been inadequately validated in non-respiratory specimens, and the accuracy of NAATs-TB in this context remains unclear with previous reporting of inadequate diagnostic accuracy and, in particular, false positives in various clinical settings [9]. Of significance is the poor sensitivity in detecting mycobacterial DNA in clinical specimens compared with cultures [10].

The SDA, an isothermal amplification assay, is based on the ability of the Klenow fragment of *Escherichia coli* DNA polymerase to start at the site of a single stranded nick in double stranded DNA. This is followed by extending one strand from the 3^- end and displacement of the downstream strand of the DNA [11]. The replicated DNA and the displaced strands are then substrates for further annealing, nicking and strand displacement. This results in geometric amplification ($\sim 10^8$ folds amplification reaction in 2 h). Thus, the SDA process has a low detection limit of around 10–50 copies, so when the target is present in several copies in the genome of the organism (e.g. IS 6110 in *M. tuberculosis*), only a few number of the bacterium is required for detection [12]. The BDProbeTec system (Becton Dickinson), adopted in this study, provides a mycobacterial-specific platform for SDA coupled with a chemiluminescence detection system. This allows the whole sensitive assay to be performed in 4 h following the processing and decontamination of specimens if required, so the assay is a convenient alternative for real-time PCR [13]. Since its initial description and evolvement, the ProbeTec has been evaluated mainly in respiratory specimens, but also in non-respiratory fluid samples [14,15]. Limited data, however, exist regarding its performance in tissue biopsies. A study was conducted to evaluate the diagnostic performance of the BDProbeTec system in various tissue samples and its concordance with histopathological examination (HPE) and bacteriological culture.

Materials and methods

Study settings and specimens

This comparative study was conducted between and including January 2011 and April 2012 in the Diagnostic Laboratories of a tertiary care center in Saudi Arabia. One hundred and fifty-eight tissue samples were processed for both SDA and HPE. Tissue samples for SDA were sent in normal saline and for histopathology in 10% formalin. All the samples were kept at 4 °C before processing.

Bacteriological methods

All the tissue biopsies were dissected, manually macerated and homogenized using a sterile tissue grinder then subjected to decontamination by a standard *N*-acetyl-L-cysteine (NALC) digestion method. Briefly, specimens were treated with an equal volume of NALC–NaOH (final concentration 2%) for 15 min at room temperature, neutralized with sterile phosphate buffer (pH 6.8), and centrifuged at 3000*g* for 30 min. The sediment pellet was then re-suspended in 2 ml phosphate buffer and subjected to both culture and SDA analysis using the BDProbeTec ET system following manufacturer's instructions. Culture was performed using two Löwenstein-Jensen slopes (with and without pyruvate), and the MGIT (Becton Dickinson) liquid culture system according to the manufacturer's instructions. All specimens were screened microscopically after concentration using the Auramine stain with positive results confirmed using Ziehl-Neelsen staining.

Molecular methods

Following the manufacturer's instructions, 500 μl post-decontamination specimens were washed with 1 ml wash buffer 1, and then centrifuged at 12,200*g* for 3 min. The supernatant was discarded and mycobacteria were subjected to killing by heat (105 °C) for 30 min. DNA was released from the organisms by re-suspending the deposit in 100 μl lysis buffer 2, followed by sonication at 65 °C for 45 min. Samples were then neutralized by adding 600 μl neutralization buffer. A volume equivalent to 150 μl DNA extract was added to the priming well containing dehydrated primers and probes in the microtiter plate. The plate was incubated at room temperature for 20 min to allow complete rehydration of reagents. The priming mix was incubated at 72.5 °C for 10 min. Enzymes, dNTPs and buffer were activated in a separate amplification microtiter plate by heating to 54 °C for 10 min. Hundred microliter of the priming mix was added to each corresponding well and mixed to initiate amplification. Plates were then transferred to the BDProbeTec analyzer. Each assay run was done in duplicate and included positive and negative controls, along with the assay supplied internal control to avoid false-nega-

tive results caused by amplification inhibitors in the tissue specimens. Samples that gave a MOTA (method other than acceleration) fluorescence score of ⩾3400 were regarded as positive, and those with a MOTA score of ⩽3400 were considered negative for mycobacterial DNA.

Histopathological examination

Tissue specimens received for HPE were fixed in 10% formalin, embedded in paraffin, cut to 3-μm thick sections, and stained with hematoxylin–eosin (H&E) before microscopic examination. Presence of any or a combination of the following criteria – well-defined granulomatous inflammation, caseous necrosis, typical caseating granulomas or Langhans giant cells – indicated possible tubercular involvement of the tissue regardless of the Ziehl-Neelsen staining status and the result was compared with the parallel bacteriological culture and nucleic acid amplification assay. Poorly defined or non-specific inflammatory findings were categorized as histopathologically negative for typical tubercular lesions.

Discrepant findings

If there was any discrepancy among culture, BDProbeTec SDA or HPE results, a second aliquot of the frozen specimen that had been processed for amplification was retested by the BDProbeTec. When discrepancy among the laboratory assays persisted, cases were traced for the possibility of TB as a final diagnosis.

Data analysis

Analysis was performed using Graphpad Prism 5.0 on a specimen-basis, not on a patient-basis, to allow assessment of the parallel laboratory assays.

Results

Out of 42 culture-positive cases, only 15 (35.7%) were detected by nucleic acid amplification using the SDA-based BDProbeTec ET system (Table 1). Histopathology was positive in 44 cases; of which 3 were culture-negative. The two cases where TB was a final diagnosis (lymph node in a known TB patient and peritoneal tissue specimen from a dialysis patient who improved dramatically on empirical anti-TB treatment), SDA was helpful as an early marker of TB in these cases as well as in one lung tissue specimen where histopathology was non-specific which was smear-negative, but SDA-positive and later culture-positive. An alternative final diagnosis was considered in one case of granulomatous joint reaction where neither culture nor SDA was positive. Table 2 illustrates the performance of the different laboratory assays in various types of tissue specimens included in the study. Only one case of tissue TB (intestinal biopsy) showed positive reactions in all bacteriological, molecular and histopathological examination. The sensitivity of SDA ranged between 0% and 50% compared with culture, and between 12.5% and 50% in relation to histopathology. Overall specificity ranged between 96.6% and 92.8%. The PPV for SDA was variable among various tissue types (pleura, lymph node and intestinal tissue). Sensitivity, specificity, and positive and negative predictive values of the SDA are shown in Table 3, assuming that either histopathology or bacteriological cultures are the standard reference assays in either column.

Discussion

Nucleic acid amplification tests (NAATs) have the potential to shorten clinical detection of *M. tuberculosis* from weeks to a few hours. The semi-automated real-time BDProbeTec SDA system allows simultaneous amplification and detection of the *M. tuberculosis* DNA-specific target IS 6110 using the amplification primers and fluorescently labeled probe [14]. The isothermal nature of SDA eliminates the need for cyclers and makes it ideally suited to *in situ*-based applications in which the morphology of the tissue sections is preserved [16]. Additional practical advantages of the system include: the presence of internal amplification control (thus spiking is not required), the semi-quantitative nature of the assay, multiplexing and batching potential for testing a large number of samples, and the autoclave-based lysis of samples. These features favor the adoption of the simplified protocol in routine TB laboratory settings [13]. However, data regarding its performance in tissue specimens are limited.

The relatively low temperature at which the SDA-based assays is carried out (52.5 °C) is expected to result in non-stringent amplification and the occurrence of non-specific binding, especially when the target is absent or present in low copies compared with the background DNA. This drawback is reflected as poor specificity with a negative diagnostic performance of the testing platform. In this study, however, when the BDProbeTec was utilized to supplement histopathology in detecting TB in characteristic tissue lesions, the high positive predictive value (PPV) in common specimens

Table 1 – Results of strand displacement amplification (SDA), culture, microscopy and histopathological examination (HPE) for the 158 tissue specimens.

SDA	HPE positive (44)				HPE negative (114)			
	Culture-positive (41)		Culture-negative (3)		Culture-positive (1)		Culture-negative (113)	
	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative
Positive (15)	1 (intestine)	11	2 (Peritoneum and lymph node)	0	0	1 (Lung)	0	0
Negative (143)	0	29	0	1 (Joint)	0	0	0	113

Table 2 – Performance of the strand displacement amplification (SDA) against microbiology and histopathological examination (HPE) per specimen type.

Type of tissue (number of samples)	SDA	HPE positive (44)				HPE negative (114)			
		Culture-positive (41)		Culture-negative (3)		Culture-positive (1)		Culture-negative (113)	
		Microscopy positive (1)	Microscopy negative(40)	Microscopy positive (2)	Microscopy negative (1)	Microscopy positive (0)	Microscopy negative (1)	Microscopy positive (0)	Microscopy negative (113)
Lung (51)	Positive	0	1	0	0	0	1	0	0
	Negative	0	7	0	0	0	0	0	42
Pleura (45)	Positive	0	2	0	0	0	0	0	0
	Negative	0	4	0	0	0	0	0	39
Lymph nodes (38)	Positive	0	8	1	0	0	0	0	0
	Negative	0	13	0	0	0	0	0	15
Intestine (11)	Positive	1	0	0	0	0	0	0	0
	Negative	0	1	0	0	0	0	0	9
Others (13)	Positive	0	0	1 (Peritoneum)	0	0	0	0	0
	Negative	0	4 (bone, muscle)	0	1 (joint)		0	0	7
Total (158)	Positive	1	11	2	0	0	1	0	0
	Negative	0	29	0	1	0	0	0	113

demonstrates that a positive NAAT-TB by BDProbeTec is unlikely to be a false one. This was applicable to tissue specimens originating from pleura, lymph node, intestine and lung. Although the table shows a PPV of 66.78% in lung tissue, this was interpreted by the presence of a case of non-specific chronic inflammatory findings in the lung where TB was the final diagnosis based on culture. Thus, the PPV and specificity were restored when the performance of NAAT was compared with the culture (Table 3). For other less common types of tissues including orthopedic specimens, specificity was moderate and the assay performance was less accurate in ruling out or ruling in the diseases. This may reflect differences in tissue matrix digestibility and efficiency of DNA extraction from such tissues, e.g. calcified bone or the inhibitory activity owing to some tissue components [17]. Since there is no gold standard laboratory test for extra-pulmonary TB, this marginally low specificity may represent lack of concordance with the reference methods rather than poor performance of the assay. This is thought to be the case since the clinical data supported the diagnosis of TB in 2 samples that were positive by SDA but not culture which has low yield in tissues. The peritoneal tissue sample with a positive NAAT-TB but negative culture and HPE (Table 2) was clinically diagnosed as TB and responded to anti-tuberculosis treatment. However, it is difficult to infer the performance of the assay in this type of specimen owing to the limited number of specimens tested. The other case with a positive HPE but negative culture was a lymph node obtained from a patient who was diagnosed as a case of TB by culture from a non-tissue specimen who failed treatment and presented with disseminated TB. For such cases, the NAAT is useful as a clue to TB, with and without suggestive histopathological findings, awaiting culture results although the clinical decision needs to consider the lack of technical experience and data about the system performance in tissues [18]. The inaccuracy of the reference methods negatively impacted the apparent specificity and PPV of the assay in comparison with the culture (Table 2). The overall good specificity findings can be attributed to the technical improvements on the assay to optimize specificity, including the thermophilic adjustment, and the use of the recently available thermostable polymerases and organic solvents. In addition, the specificity is largely influenced by the target genes and primer design.

Sensitivity of the SDA assay was a main concern in this study. Overall sensitivity was poor (Table 3) as SDA was able to detect only one third of the TB cases as suggested by HPE and/or bacteriological cultures. The MOTA score was reviewed for false negative cases by SDA and found some scores were close to the cut-off value, but could not identify an alternative threshold to raise the sensitivity without compromising the specificity in tissue samples (data not shown). The reported sensitivity is inadequate to recommend using the assay for routine confirming or ruling out TB in suspected tissue specimens with granulomas in diagnostic settings. A report of SDA-BDProbeTec was published with higher sensitivity than observed in this study, although the assay was performed on formalin-fixed, paraffin-embedded tissues [19]. The findings, however, were fit into the published data about other non-SDA NAATs for detection of TB in tissues [20–22] in which the sensitivity of NAATs for detection of TB in the tissues was

Table 3 – Sensitivity, specificity and predictive values of the BDProbeTec ET system in comparison with the standard laboratory assays for various specimen type (a) histopathological examination is used as a reference, (b) culture is the reference.

Type of tissue (number of samples)	HPE				Culture			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Lung (51)	12.5	97.7	66.78	75.12	22	100	99.99	77.61
Pleura (45)	33.3	100	99.99	80.21	33.3	100	99.99	80.21
Lymph nodes (38)	39	100	99.99	81.59	38	93.8	69.39	80.36
Intestine (11)	50 (only 2)	100	99.99	84.39	50 (only 2)	100	99.99	84.39
Others (13)	20	87.5	25.60	72.63	0	77.8	0	67.78
Total (158)	32	98.2	87.25	78.95	30.9	96.6	77.07	79.08

found to be variable between as low as 24% to very high (>90%). The poor sensitivity of a molecular NAAT could be related to the limit of detection of the assay and the strain genotype. It should be noted that biopsy specimens are usually obtained from sites with macroscopic pathology. Such sites are expected to have a vigorous immunological reaction and very few live bacilli [23]. Also, the abundance of copies of the target gene sequence is an important determinant of NAAT sensitivity. The commercial molecular assays are based upon kits innovated in the developed world so they represent certain lineages of strains. There have been reports about Mycobacterial isolates from some geographical areas like the Indian subcontinent lacking or having only a few copies of IS 6110 [24], compared with (8–15) copies usually found in strains from the Western Countries where most molecular assays are developed. A recent study by Al-Hajoj et al. found that the Asian mycobacterial lineages represent a significant proportion of the circulating strains in Saudi Arabia [25]. However, the amplification scores of most false-negative specimens were close to the cut-off value, suggesting that it is an assay limitation rather than a strain-related finding. In addition, the diagnostic performance of molecular assays for *M. tuberculosis* relies much on the bacterial load in tissues, quality of the representative specimen, laboratory infrastructure and efficiency of DNA extraction. Increasing the sample input volume improves the performance of molecular assays [26], and a brief incubation of biopsy tissues on Löwenstein-Jensen slopes was found to improve mycobacterial DNA extraction from tissues and to raise the sensitivity of NAAT by around 50% without compromising the specificity [17]. For all types of tissues, the negative predictive value (NPV) was found to be less than 90%, so the BDProbeTec system was not suitable to rule out TB in tissues with granulomas. This is of great clinical significance as NAATs will be clinically useful when they reliably rule out TB in tissues with negative acid-fast staining. This was not the case in the presented data raising the questions about the reliability and cost-effectiveness of their routine use in tissue specimens.

The BDProbeTec system is marketed as a qualitative system as the original paper describing the method showed that the numerical data produced (MOTA) was not a quantitative measurement that can be used to infer the level of target DNA [27]. The scores are thus considered purely qualitative since they are influenced by several parameters other than the amount of the target in the clinical specimens like the sample matrix, kinetics associated with the molecular interactions and slight variations in the reagent, hardware, environment or workflow. McHugh et al. noted that diagnostic inaccuracies were encountered in the low-positive zone with lower scores [14]. It was found that while the sensitivity was poor in lung tissues, the amplification score was excessively high in both positive lung tissues (>100,000 MOTA) compared with those found in other tissues. This suggests a relatively higher bacillaryl load which could be incidental, since only two cases of lung tissues were positive by the BDProbeTec system, or it could be owing to the different pathological events in pulmonary TB. The microscopy yield was unsurprisingly low (7%) in the study. Alternative staining techniques have been proposed to improve microscopic yield in tissues, such as Triff stain which demonstrates both the organisms and the background tissue pathology allowing better correlation with the pathological process [28], the Silver impregnation Dieterle or Warthin Starry methods [29], and the fluorescence Auramine–Rhodamine dye, which is only suitable for cytological examination [30]. The immunohistochemical detection method has also been developed, but is not widely used [31].

A drawback of this study design is that molecular analysis was performed on specimens sent to the TB laboratory, and it does not include tissues sent to pathology without a corresponding specimen in the TB laboratory. This might have contributed to the high specificity results found. When all histopathological tissues are included, it is expected that there will be some TB cases as suggested by histopathology findings. Although this study contained a small subset of TB cases with only a small number of certain tissue types (intestine, peritoneum, orthopedic specimens), it showed an inadequate diagnostic performance of a commercially available NAAT system on tissue specimens and the need for a more reliable testing system for testing non-respiratory specimens. Such a system needs to be user friendly and internally-quality controlled. Global efforts are currently focused on developing diagnostic systems useful for certain parts of the world with high disease burden and low economy. While investing in these diagnostics is valuable to meet the Millennium TB Targets, simple commercial diagnostic platforms with optimal diagnostic performance are required for countries with a good economy, modest burden of the disease, high proportion of extra-pulmonary TB cases, and low expertise in the laboratory settings. The in-house developed and quality-controlled amplification assays are unsuitable options for such countries with a rapid turnover of staff and low technical experience. The evaluation of the newly launched GeneXpert and the

Genotype Hain Assays in tissue samples will highlight the effectiveness of those assays for routine use in tissue samples with possible TB involvement. This will not only aid in rapid identification of TB, but will also screen for drug-resistant TB.

Conclusions

In summary, the data suggest that the BDProbeTec Test is a specific technique used for detection of *M. tuberculosis* complex directly in common tissue biopsies. The number of positive cases of TB in this study is small; therefore, further studies are required to confirm the findings. The sensitivity of the assay is inadequate, so it is not recommended for routine diagnostic use in such extra-pulmonary samples. The major problem with the assay in this evaluation was false-negative results, which could be related to inherent or technical grounds. While NAATs are expected to revolutionize the diagnosis of mycobacterial diseases owing to their speed, accuracy and sensitivity, their application for a reliable diagnosis of extra-pulmonary TB is lagging behind. The search for a commercially available, quality-controlled system for TB in tissue specimens remains a continuing challenge.

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