Original Article

The efficacy of diagnostic battery in Pott's disease: A prospective study

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Abstract

Background: The diagnosis of Pott's disease is mostly based on clinicoradiological observations substantiated by the bacterial culture, staining and histopathology. Since, no single technique is enough to conclude Pott's disease in diagnosis, the present study was undertaken to correlate the clinicoradiological, microbiological, histopathological and molecular method to evaluate the effectiveness in diagnosis of Pott's disease.

Materials and Methods: 62 clinicoradiologically suspected cases of Pott's disease were included in this study. The specimens for diagnostic work up were collected either during surgery or by computed tomography guided fine needle aspiration. All these specimens were tested for tuberculosis (TB) through Ziehl-Neelsen (ZN) microscopy, BACTEC culture, histopathology and polymerase chain reaction (PCR). The final diagnosis was established by the results of performed tests and clinicoradiological improvement of cases at the end of 6 months on anti tubercular treatment.

Results: Out of 62 cases, 7 were excluded from this study as these were turned out to be neoplastic lesions on histopathology. Amongst remaining 55 cases, the TB was diagnosed in 39 (71%) on histopathology, 37 (67.5%) on PCR, 27 (49%) on BACTEC culture and 20 (36.3%) on ZN microscopy. Ultimately 45 cases were tested as positive and 10 were detected as negative for TB in combination of ZN microscopy, BACTEC culture and histopathology. PCR was positive in 37 of 45 cases and 10/55 cases remained negative. On clinical analysis of these 10 cases, it was noted that these were cases of relapse/poor compliance. The combination of PCR and histopathology was also shown positive for TB in 45 cases. Hence, the PCR showed a fair positive agreement (K^c = 0.63) against the combined results of all performed traditional methods.

Conclusions: The combination of PCR and histopathology is a rapid and efficient tool for diagnosis of Pott's disease.

Key words: Pott's disease, tubercular spine, BACTEC culture, histopathological and molecular method, Ziehl-Neelsen microscopy

Introduction

Pott's disease accounts for 50% of the cases of skeletal tuberculosis (TB), 15% of the cases of extrapulmonary tuberculosis (EPTB) and 2% of all cases of TB.[1] The diagnosis of Pott's disease is mostly based on clinicoradiological observations substantiated by staining and culture methods to detect the causative organism. The typical tubercular lesions of the spine can be diagnosed by radiological methods as their sensitivity is increased by the advent of newer imaging techniques like computed tomography and magnetic resonance imaging (CT and MRI). These techniques give sufficient information about lesions in the bone and tissues; although the definite diagnosis is based on tissue/pus examination.[2] The conventional bacteriological examination includes Ziehl-Neelsen (ZN) microscopy and culture for acid fast bacilli (AFB). ZN microscopy method is a popular technique routinely used in the clinical laboratories worldwide, due to its simplicity, cost effectiveness and rapidity, but it suffers with low sensitivity and requires 10^4 to 10^5 bacilli/ml in the clinical specimens to be positive.[3,4] The culture of *Mycobacterium tuberculosis (M. tuberculosis)* is a gold standard method for diagnosis but it also has limitations like that required 6 to 8 weeks due to the slow growth of *M. tuberculosis* and is often negative, it still needs 10^1-10^2 bacilli/ml (live bacilli) in clinical specimens for culture recovery and also stringent test conditions that is difficult to implement at primary or secondary clinical laboratories.[5,6]

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Moreover, histopathological examination plays a valuable role in the diagnosis of Pott's disease but sometime it may be inconclusive and in addition need high expertise and the final reporting also takes more than 1 week.[6] Recently, the molecular biology technique, polymerase chain reaction (PCR) represents a major advance in the diagnosis of TB.[6] With the use of amplification systems, nucleic acid sequences unique to *M. tuberculosis* can be detected directly in clinical specimens, offering better accuracy than ZN microscopy and greater speed than culture. The PCR has shown very promising results for early and rapid diagnosis of the disease due to its detection limit of one to 10 bacilli in various clinical specimens.[6] The present study was undertaken to evaluate the efficiency and effectiveness of different laboratory diagnostic modalities along with the role of PCR in the diagnosis of clinicoradiological suspected cases of Pott's disease.

Materials and Methods

62 clinicoradiological suspected cases of Pott's disease with neurological complications [Table 1] were prospectively enrolled in this study, from 2008 to 2011 in the Department of Neurosurgery, at a tertiary care hospital, India. The specimens of these patients such as pus and tissue were obtained either during surgery or under CT guidance.

We included clinicoradiological suspected cases of Pott's spine who underwent either open biopsy or CT guided aspiration at our institute. We excluded those subjects who did not give consent for biopsy or CT guided aspiration or biopsy showed neoplastic pathology.

Laboratory examinations

The specimens were examined by following methods:

a. Histopathology - The tissues stained with Hematoxylin and Eosin and ZN stain were analyzed under the microscope for epithelioid cell granulomas with or without the presence of langerhans giant cell and AFB.[7]
b. Periodic acid-Schiff (PAS) stain - Fungal examination was performed by PAS stain according to standard laboratory procedure.[7]
c. ZN microscopy - Smears were stained using the ZN method and examination for AFB were done under light microscopy.[8]
d. BACTEC 12B culture - BACTEC vials were incubated and interpreted as per Becton Dickinson (BD, Sparks, MD, USA) manual instructions.[9]
e. *P*-Nitro-α-acetylamino-β-hydroxy propiophenone (NAP) Test - The NAP was done for identification and differentiation of *Mycobacterium tuberculosis* complex (MTBC) and nontubercular *Mycobacterium* from culture isolates.[9]
f. Molecular diagnosis - PCR for TB was done using a MTBC specific sequence *IS6110* (123 base pairs [bp]) primer.

Table 1: Clinicoradiological evidence of suspected Pott's disease

Sign	Symptoms	Radiological features
Tenderness	Pain	At least two continuous vertebral involvement
Gibbous	Evening rise temperature/fever	Paradiscal vertebral changes in two continuous vertebrae
Psoas abscess	Loss of appetite	Soft tissue involvement
Restriction of movement	Loss of weight ≥10% over 1 month	Abscess/psoas abscess
Sinus tract	Cough/hemoptysis >3 months	Gibbus/bony involvement

Only five signs/symptoms/radiological features have been taken with the help of multiple published series. Patients who were having at least two signs and two symptoms and three radiological features (first is mandatory) which were labeled as suspected Pott's disease

Genomic DNA was extracted from pus specimens according to Van Soolingen *et al*.[10] technique. DNA extractions from tissues were done with Hipura genomic DNA extraction kit according to the manufacturer protocol[3]. Hipura™ MB505 bacterial and yeast genomic DNA miniprep purification spin kit, Hi-Media laboratories Private limited Mumbai, India.

The amplification reaction was performed in a 20 µl final volume of each tube. The reaction mixture contained 10 µl Pyrostart Fast PCR Master Mix 2X (dNTP, Taq polymerase with $MgCl_2$, Fermentas, India), 1 µl (10 pmole) of each primer, 3 µl water (nuclease free) and 5 µl of extracted DNA. The oligonucleotide primers[11] were used forward and reverse sequence: $^{5'}$CCT GCG AGC GTA GGC GTC GG$^{3'}$ and $^{5'}$CTC GTC CAG CGC CGC TTC GG$^{3'}$ respectively (SBS Gene tech Co. Ltd.). These primers amplified a target fragment of 123 bp from the insertion like sequence element *IS6110* of MTBC.

The amplification reactions were subjected to 40 cycles. It was performed in a programmable thermal cycler (MJ Research, PTC-100, GMI, Inc. USA). Each cycle comprised denaturation at 94°C for 2 min, annealing at 68°C for 2 min and primer extension at 72°C for 1 min. After the 40 cycles, the additional extension for 10 min at 72°C was carried out.

The amplified products were separated on 2% agarose gel, visualized on an ultraviolet-transilluminator (Bangalore Genei, Bangalore, India). The presence of 123 bp fragment indicated as a positive test for MTBC [Figure 1]. The positive controls included the DNA of H_{37Rv} strain and negative control included PCR grade water.

A definite treatment either by surgery (46 cases) or by only anti tubercular treatment (ATT) (9 cases) was offered. We followed the following major indications for surgical intervention in our setup: (1) Advanced cases

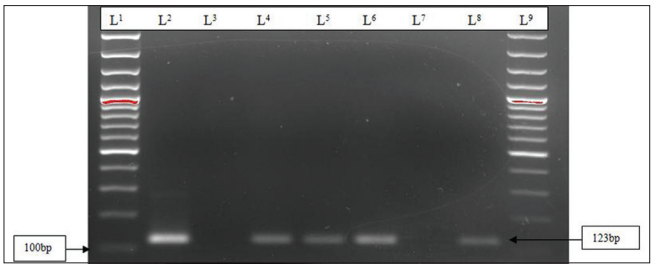

Figure 1: Polymerase chain reaction *IS6110* gene for detection of Mycobacterium tuberculosis complex at 2% agarose gel. Lane 1 (L1) and L9 ladder 100 base pairs, L2 positive and L3 negative control, L4, 5, 6, 8 showed positive and L7 showed negative specimens

of neurological involvement such as marked sensory or sphincter disturbances, flaccid paralysis or severe flexor spasms. (2) Prevertebral cervical abscess with difficulty in respiration and deglutition, seemingly difficult resolution on chemotherapy during conservative treatment. (3) Worsening of already present neurological complications during the treatment. Recurrence of neurological complications. (4) New neurological deficits developing during the course of treatment. (5) No improvement in neurological complications/morbidity even after 4-6 weeks of ATT.

A four drug regimen chemotherapy consisting of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) were given to all patients in their appropriate doses according to the weight of patients as per departmental protocol, following or along with surgery. Four-drug regimen for at least 4-6 weeks to gauge the response was given (8-12 mg/kg/day Rifampicin, 4-6 mg/kg/day Isoniazid and 15-20 mg/kg/day Ethambutol in a single daily dose; and Pyrazinamide 15-20 mg/kg/day in two divided doses), if not contraindicated the drug therapy was continued for 18 months[12]. In cases of no improvements or hepatotoxicity, the patients were switched to modified ATT with the addition of ofloxacin and streptomycin and modification in doses of isoniazid and rifampicin or discontinuation of them. The therapeutic response was observed by clinical improvement in the form of healing sign that is an improvement in motor power/spasticity, sensory loss of limbs, autonomic dysfunctions and constitutional symptoms [Table 2]. The liver function test was done as per protocol to rule out the subclinical hepatotoxcity. The radiological assessment was done by using followup MRI, the first at the end of 6 months of the commencement of the ATT and was compared with pretreatment images by the same senior radiologist of our institute.

This study was approved by 41st Institutional Ethics Committee (A-04 PGI/IMP/EC/41/28/2/2008).

Statistical analysis

The final diagnosis was established by using the collective results of ZN microscopy, BACTEC culture, histopathological findings and response to ATT. The efficiency of test tools was calculated as (total positive cases/total number of cases) ×100. The positive concordance between traditional tools and PCR were assessed using the Kappa coefficient (K^c > 0.75, excellent agreement; <0.4, poor agreement; ≥0.4 and ≤0.75, good to a fair agreement)[13] and significance difference was analyzed by the Chi square (χ^2) test with the help of the SPSS 15.1 version. The cases were labeled as definite TB if at least one performed test (ZN microscopy/BACTEC culture/or histopathology) was found positive.

Table 2: Criteria for clinical improvement: (First (pain) is mandatory and any other two improved symptoms/sign)

Sign/Symptoms	
Pain	Decrease significantly or absent without any pain killer
Weight	Improved/gain
Well being	Improved
Evening fever	Decrease significantly or absent
Cough	Decrease significantly or absent
Movement restriction	Decrease significantly or completely
Autonomic dysfunctions	Improved/or not

Results

The study group consisted of 62 specimens from suspected Pott's spine individuals. On the basis of histopathological examination, 7 cases (5 specimens from open biopsy and 2 from fine needle aspiration [FNA]) were excluded from the study as they were diagnosed to be neoplastic. Out of rest 55 cases, 46 specimens were obtained through open biopsy during surgery and 9 were CT guided FNA. Further, in all (46) open biopsy specimens, 7 were positive for epithelioid cell granulomas with AFB, 27 revealed granulomatous lesions suggestive of TB with or without the presence of the langerhans giant cell. However, the histological diagnosis could not be reached in 12 specimens because of either suspicious granulomatous lesions or on account of inconclusive histology. Five of nine CT guided FNA specimens were diagnosed as tubercular and 4 specimens showed no granulomatous/no malignant lesions. The overall tubercular positive/suggestive rate was 71% (39/55) on histopathology. The staining for fungal infection was throughout negative in these specimens.

Specimens characteristic for microbiological and PCR *IS6110* methods; their positive correlation

The received specimens included 31 (56.4%) pus and 15 (27.3%) tissues through open biopsy along with 6 (11%) pus and 3 (0.54%) tissues were obtained through CT guided FNA. The overall collected specimens were consisted 37 pus and 18 tissue specimens (either open

biopsy or by CT FNA) were analyzed. The ZN microscopy was positive in total 20 (36.4%) of 55 cases (18/37 cases in pus and 2/18 cases in tissue specimens). The BACTEC culture was positive 27 (49%) of 55 cases (22/37 in pus and 5/18 in tissue specimens). All 27 grown isolates were MTBC strains confirmed by NAP test. The desired amplification of PCR *IS6110* was positive in 37 (67.5%) of 55 cases (30/37 cases in pus and 7/18 in tissue specimens) [Table 3]. The Kappa coefficient of PCR with ZN microscopy ($K^c = 0.37$, [$P = 0.001$]) showed poor agreement and with the BACTEC culture ($K^c = 0.57$, [$P < 0.001$]) it showed a good agreement [Figure 2].

Comparison and correlation of histopathological results with molecular method (PCR *IS6110*) findings

On histopathological results; 39 (71%) cases were positive and 16 (29%) were negative for TB. Out of 39 positive cases, 31 (56.4%) were positive on PCR also. Although amongst 16 negative cases 6 were positive on PCR method. Thus, the false negative and positive of PCR was found to be 8 (14.4%) and 6 (11%) respectively. Therefore, the Kappa coefficient of PCR with histopathology was ($K^c = 0.4$ [$P = 0.004$]) slightly good [Figure 2].

Efficiency of PCR *IS6110* against final diagnosis and correlation with combined results of all performed traditional tests

In 55 cases, it was possible to reach a final diagnosis through the combined result of all performed tests (without consideration of PCR results) and with ATT response. Hence, among all 55 cases, 45 (82%) cases were TB positive test and 10 (18%) were negative by all tests carried out (ZN microscopy, BACTEC culture and histopathology). Although PCR showed 67.3% (37/55) sensitivity at the final diagnosis, while PCR showed 37/45 positive and no any false positive out of 10 negative cases by all traditional tests. Therefore, the Kappa coefficient of PCR with the combined results of all traditional tests ($K^c = 0.63$ [$P < 0.001$]) ultimately reached to a fair positive agreement [Figure 2].

Possible diagnostic battery

The present study, the combination of (a) ZN microscopy with BACTEC, the total 31 positive cases due to only 20 cases positive on ZN microscopy and an additional 11 cases were positive on BACTEC culture. (b) ZN microscopy with histopathology, the total positivity showed 43 cases due to 23 cases more positive on histopathology against ZN microscopy. (c) BACTEC culture with histopathology, the total 45 cases to be positive due to 39 cases positive on histopathology and an additional 6 cases were positive on BACTEC culture, where the histopathology was negative. (d) The total 45 cases were positive in combination of ZN microscopy + BACTEC culture + histopathology. (e) ZN microscopy with PCR, the total positivity was 38 cases, in which 18 more cases were positive on PCR method. (f) BACTEC culture with PCR, the total 38 positive cases due to only 27 cases positive on BACTEC culture and additional 11 cases were positive on PCR. (g) Histopathology with PCR, the total 45 cases positive on this possible battery due to 39 cases positive

Table 3: Result of laboratory findings

Types of specimen	ZN microscopy		BACTEC culture		PCR IS6110	
	Positive	Negative	Positive	Negative	Positive	Negative
Pus (31)	15	16	18	13	25	6
Tissues (15)	1	14	4	11	6	9
FNA pus (6)	3	3	4	2	5	1
FNA tissues (3)	1	2	1	2	1	2
Total	20	35	27	28	37	18

PCR=Polymerase chain reaction, FNA=Fine needle aspirate, ZN=Ziehl-Neelsen

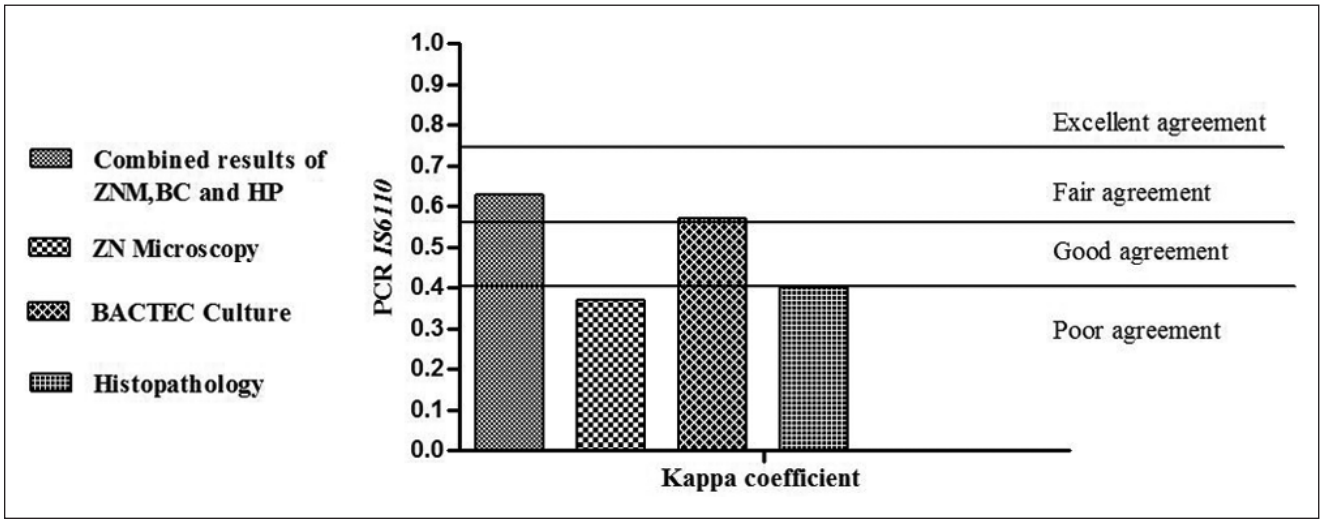

Figure 2: Bar diagram showing kappa coefficient concordance between PCR and traditional tools. (ZNM=ZN microscopy, BC=BACTEC culture and HP = Histopathology)

Table 4: Possible diagnostic battery

Battery	Combination of diagnostic different tools	Positive (%)	Negative (%)
A	ZN microscopy+BACTEC culture	31 (56.4)	24 (43.6)
B	ZN microscopy+histopathology	43 (78.2)	12 (21.8)
C	BACTEC culture+histopathology	45 (82)	10 (18)
D	ZN microscopy+BACTEC culture+histopathology	45 (82)	10 (18)
E	ZN microscopy+PCR	38 (69)	17 (31)
F	BACTEC culture+PCR	38 (69)	17 (31)
G	Histopathology+PCR	45 (82)	10 (18)
H	ZN microscopy+BACTEC culture+histopathology+PCR	45 (82)	10 (18)

ZN=Ziehl-Neelsen, PCR=Polymerase chain reaction

on histopathology and an additional 6 cases were positive on PCR. (h) The battery of ZN microscopy + BACTEC culture + histopathology + PCR showed ultimately 45 cases were positive because PCR could not give any more positive against the mentioned battery D and G [Table 4].

Discussion

Pott's disease, accounts for 2% of all TB infections.[1] The diagnosis of Pott's disease is principally based on classical clinical manifestations of spinal infection supplemented by modern imaging like CT and MRI.[14,15] The CT and MRI in particular can detect the subtle changes in the spine, from initial changes in the intensity pattern of the vertebrae to the extreme changes like deformity of the spine including soft tissue shadow, granulation tissue and pus etc.[16] However, the various pathologies including neoplastic and other inflammatory conditions simulate with Pott's diseases on imaging. The current study excluded 7 of 62 cases; they were diagnosed to be neoplastic by the histopathological examination. It signifies that even strong clinicoradiological diagnosis based on classical or nonclassical radiological and clinical findings can be wrong on histopathological evaluation.

The remaining 55 cases, 20 (36.3%) were tested positive with ZN microscopy and 27 (49%) with BACTEC culture. An earlier report by Jain *et al*.[17] found 14.8% positivity on ZN microscopy and 11.11% positivity on culture method in cases of spinal TB. Rasit *et al*.[18] found 6.25% positivity on culture in spinal TB. In our study the ZN microscopy and BACTEC culture reports revealed higher positivity in relation to the earlier published series. Our higher detection rate was because of the fact that we used the liquid culture medium BACTEC 12B medium. It is the rapid, sensitive and efficient method for the isolation of *Mycobacterium* in a clinical laboratory as compared to solid medium Lowenstein–Jensen.[19] Moreover, it is established today that all the traditional tests need to have a minimum concentration of bacilli for yielding positive results as mentioned in the introduction. Hence, if the specimens, which are naturally having more concentration of bacilli, like sputum and pus it may become easy to detect *M. tuberculosis*.[20] Similarly, in our study the pus specimens had higher recognition of tubercular bacilli on ZN microscopy and BACTEC culture methods [Table 3].

The histopathological findings are much more reliable in the diagnosis of EPTB. The study quoted the detection rate of EPTB including Pott's spine varying from 53% to 81%.[18,21,22] The histopathological diagnosis could be reached in 71% (39 out of 55) of the cases which were either confirmed by the presence of AFB in 12.7% or strongly suggestive of tubercular pathology. In our series, we found 16 cases were negative on histology. Among these negative cases, 6 cases were identified as tubercular on microbiological and PCR examination. 10 out of 16 cases had been under anti tubercular therapy for more than 6 months and another reason of histology negative might be a possible sampling error during the collection of the representative tissues for processing. The positivity of FNA specimens seems to higher than surgical specimens, however, we require a long sample size for definite conclusion.

Molecular diagnostic tool, such as PCR has been shown to have higher efficiency in the diagnosis of pulmonary TB as well as EPTB.[4] It has been studied on clinical specimens of sputum, cerebrospinal fluid, EDTA-blood, pleural fluid, fluid from fistulae and pus from wounds.[23,24]

In an attempt to increase diagnostic precision, we performed PCR *IS6110,* which was detected in 37/55 (67.3%) cases. Various studies documented the increased positivity by PCR targeting *IS6110* in specimens of EPTB i.e., Sekar *et al*.[25] found 63% positivity, Negi *et al*.[26] 73% and Tiwari *et al*.[27] 62% positivity among clinical specimens of EPTB. Our PCR result is fairly similar to earlier published series. The overall positive agreement shows a fair agreement (K^c = 0.63) between PCR and definite TB. The false negative results were observed in 14.5% cases out of 45 cases diagnosed by all performed traditional tests. No any false positive observed against our combined result of all performed tests. The reasons of false negative results of PCR may be on account of insufficient specimens, irregular distribution of *Mycobacterium* bacilli in specimens, the presence of extensive necrosis and presence of inhibitors.[28]

The limitation of our study are a low positive correlation (K^c = 0.4) of PCR with histopathological findings. This can be explained up to some extent by the specimen variation as that is different specimens (such as pus, tissue) from the same subjects. Surgeons prefer to send tissue specimens for histology and pus for ZN microscopy,

BACTEC culture and PCR. In this study histology was done on tissue specimens (all 55 cases), while ZN microscopy, BACTEC culture and PCR were done in 37 pus and 18 tissue specimens. In our diagnostic battery, all performed traditional tests were diagnosed 45/55 cases and PCR was alone detected 37/55 cases. Thus, positive results of PCR with the clinicoradiological assumption it would be possible to make a decision in suspected Pott's disease to start early treatment to prevent irreversible complications.

In the present study, it is observed that no single diagnostic modality is having a good positivity rate. Hence, a combination of diagnostic battery is required for rapid diagnosis and better results. The combination of BACTEC culture and histopathology has detected a maximum of 45 cases. While the combination of PCR and histopathology has also detected same number of cases [Table 4]. As culture takes long incubation time for results, the combination of PCR and histopathology can be applied for rapid detection of Pott's disease.

To conclude, for diagnosis of Pott's disease, the combined results of ZN microscopy, BACTEC culture and histopathology are most appropriate. However, for rapid diagnosis the combination of PCR amplification and histopathology offer a better prospect.

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