Diagnosis of Extrapulmonary Tuberculosis by Smear, Culture, and PCR Using Universal Sample Processing Technology

Soumitesh Chakravorty,¹ Manas Kamal Sen,² and Jaya Sivaswami Tyagi^{1*}

Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029,¹ and
Department of Respiratory Medicine, Safdarjung Hospital, New Delhi 110029,² India

Received 21 November 2004/Returned for modification 2 May 2005/Accepted 30 May 2005

Definitive and rapid diagnosis of extrapulmonary tuberculosis is challenging since conventional techniques have limitations. We have developed a universal sample processing (USP) technology for detecting mycobacteria in clinical specimens. In this study, this technology was evaluated blindly on 99 extrapulmonary specimens collected from 87 patients. USP-processed specimens were submitted to smear microscopy for detection of acid-fast bacilli (AFB), culture, and two PCR tests targeting *devR* (*Rv3133c*) and *IS6110* gene sequences. On the basis of clinical characteristics, histology and cytology, conventional microbiology results, and response to antitubercular therapy, 68 patients were diagnosed with tuberculosis. Although USP smear and culture were significantly superior to conventional microbiology, which was not optimized ($P < 0.0001$), these approaches fell short of PCR tests ($P < 0.0001$). The low yields by smear and culture are attributed to the paucibacillary load in the specimens. The highest sensitivity in PCR was achieved when *devR* and *IS6110* test results were combined; the sensitivity and specificity values were 83 and 93.8%, 87.5 and 100%, and 66.7 and 75%, respectively, in pleural fluid, needle-biopsied pleural tissue, and lymph node specimens. In conclusion, the application of USP technology, together with clinicopathological characteristics, promises to improve the accuracy and confidence of extrapulmonary tuberculosis diagnosis.

Significant mortality and morbidity is caused by tuberculosis in developing countries, including India (31). Tuberculous pleural effusion is the most common exudative pleural effusion prevalent in India in contrast to the west, where malignant effusions are more frequent (27). However, the disease most often remains undiagnosed and, even worse, untreated. The chief difficulty with extrapulmonary specimens is that they yield very few bacilli and consequently are associated with low sensitivity of acid-fast bacillus (AFB) smear and culture. Acid-fast staining was positive in fewer than 10% of patients in most reports, whereas pleural fluid cultures for *M. tuberculosis* were positive in up to 12 to 70% of cases and pleural biopsies revealed granulomas in 50 to 97% of patients with tuberculous pleural effusion (1). The role of PCR in the diagnosis of tubercular pleural effusion has been evaluated extensively as an alternative diagnostic tool and has yielded variable results, with sensitivities ranging between 42 and 100% and specificities ranging between 85 and 100% using various PCR targets such as *IS6110*, 65kDa, TRC4, GCRS, etc. (9, 10, 22, 23, 26, 29, 30). The most common form of extrapulmonary tuberculosis is tuberculous lymphadenopathy (2, 17, 20), and its diagnosis remains a challenge since granulomatous lymphadenopathy has an extensive differential diagnosis. Several conditions, including sarcoidosis, fungal infections, and other inflammatory conditions, can present the same cytology and/or histopathology as tuberculous lymphadenopathy. A diagnosis of tuberculosis is then confirmed by the presence of AFB and/or isolation of *M. tuberculosis* on culture. However, owing to the paucibacillary nature of the specimens, the sensitivity of AFB smear

and culture are low; cultures grow mycobacteria in 39 to 80% of cases (25). Thus, there remain samples that are both AFB and culture negative, and increasingly, conventional methods and cytological investigations are being used in conjunction with PCR techniques to further help in the detection and characterization of pathogenic mycobacteria associated with human lymphadenitis (18).

Many reports, including those from our laboratory, have demonstrated the value of PCR in the diagnosis of extrapulmonary tuberculosis, including pleural effusion and lymphadenitis (3, 12, 14, 24, 28, 29). However, the presence of PCR inhibitors in clinical samples hampers the use of amplification techniques with full confidence and ease (4, 7, 16, 24, 28), and there is a pressing need for a robust, reproducible, and uniform method of inhibitor removal from clinical specimens (sputum, fluids, and tissues). The universal sample processing (USP) technology we recently described (5, 6) efficiently processes all types of specimens for use in smear microscopy, culture, and inhibition-free PCR, thus allowing diagnosis by conventional bacteriology and molecular methods on the same specimen aliquot. It has shown impressive sensitivity and specificity on sputum specimens (6).

The extraordinary sensitivity of USP smear microscopy (300 to 500 AFB/ml of specimen) (5) is attributed to the efficient removal of the counterstaining background without any deleterious effect on *M. tuberculosis*, enabling the smearing and visualization of 5 to 10 times more processed material than the conventional methods on a microscopic slide (5, 6). It was reasoned that such a sensitive methodology should be very well suited for the diagnosis of extrapulmonary TB by smear microscopy, where the specimens are primarily paucibacillary in nature. The present study was undertaken to evaluate the utility and performance of USP technology in association with

* Corresponding author. Mailing address: Department of Biotechnology, All India Institute of Medical Sciences, New Delhi, India. Phone: 91-11-26588491. Fax: 91-11-26588663. E-mail: jstyagi@aiims.ac.in.

TABLE 1. Diagnostic categories of patients and specimens

Diagnosis	No. of patients	No. of pleural fluid specimens	No. of pleural tissue specimens ^a	No. of lymph node specimens
Tuberculosis	67	53	8	15
Malignancy	11	11	2	0
Amebiasis	4	4	1	0
Congestive heart failure	1	1	0	0
Sarcoidosis	1	0	0	1
Reactive/nonspecific inflammation	3	0	0	3
Total	87	69	11	19

^a Pleural fluid was also analyzed from all of these patients.

histo/cytopathological examination and a comprehensive clinical follow-up of patients in the diagnosis of tuberculous pleural effusion and lymphadenopathy. We also sought (i) to compare USP smear and culture versus conventional smear and culture; (ii) to compare the *devR* PCR method to assay with the commonly used target *IS6110*; and finally (iii) to assess which sample, pleural effusion or biopsy tissue, was better in terms of test performance in patients from whom both sample types were obtained and analyzed. We concluded that consideration of PCR and microbiology results in the context of clinicopathological characteristics improves the diagnostic accuracy of extrapulmonary tuberculosis.

MATERIALS AND METHODS

Patients, clinical information, and clinical specimens. All patients were drawn from those attending the Out-Patients' Department of Respiratory Medicine, Vardhaman Mahavir Medical College and Safdarjung Hospital, New Delhi, India. Informed medical consent was obtained from all subjects. A detailed clinical history, physical examination, baseline laboratory investigations (hemogram, tuberculin test, chest skiagram, blood levels of glucose, urea, creatinine, bilirubin, transaminases, and urinalysis), histology/cytology, and microbiological

and PCR tests were conducted for each subject. The study was done prospectively in a blinded manner in which the clinical diagnosis was not available to the laboratory personnel. Ninety-nine samples from eighty-seven patients were included in the study and included pleural fluids ($n = 69$), pleural tissues ($n = 11$), and lymph node biopsies ($n = 19$). Both pleural fluid and pleural tissue specimens were collected from 11 patients (Tables 1 and 2). The inclusion criteria adopted in the selection of patients were (i) untreated patients with peripheral (superficial) tuberculous lymphadenitis of cervical and axillary regions and (ii) patients with features of fever, cough, pleuritic pain, malaise, anorexia, and extensive exudative pleural effusion on diagnostic pleural tap, a with clinical working diagnosis of tuberculous pleural effusion. Patients with histories of tuberculosis in the preceding 2 years, those already receiving antituberculosis therapy, and those with other coexisting medical and surgical illness were excluded from the study.

Pleural fluid cytology was taken to be suggestive of tuberculosis only in the presence of an exudative fluid (protein level, >3 g/dl) with predominantly lymphocytic cytology. Tissue histology was taken to be suggestive of tuberculosis only if evidenced by presence of granulomatous inflammation with epithelioid cells, Langerhans type giant cells, and caseation necrosis. Fine-needle aspiration (FNA) cytology of tissue was taken as suggestive of tuberculosis only if evidenced by the presence of necrosis, hypocellularity, and epithelioid cells with or without giant cells and with or without AFB.

Pleural fluid. Ten milliliters of pleural fluid was tapped under aseptic precautions. Half of the aliquot was analyzed by conventional microbiology, including smear microscopy by Ziehl-Neelsen (ZN) staining and culture as described below and cytological (ZN and Giemsa staining) and biochemical (protein and glucose) examinations. An equal volume was stored at -20°C for processing by the USP method and subsequent ZN staining, culture for *M. tuberculosis*, and PCR.

Lymph node and pleural tissue biopsies. Lymph node biopsy was done under local anesthesia using an automatic 18-gauge core biopsy system echogenic needle (15-cm length, 17-mm channel cut specimen notch; Microvasive; Boston Scientific Corp.) using an antigravity approach. Pleural biopsy was also performed under local anesthesia using a Cope's pleural biopsy needle. The biopsy was divided into three equal parts. One part was fixed in 10% formalin, embedded in paraffin wax, and subjected to histopathological examination and ZN staining. The second portion was processed for conventional culture as described below. The third portion was suspended in normal saline and stored at -20°C for processing by USP method and subsequent ZN staining, culture for *M. tuberculosis*, and PCR.

All pleural fluid and pleural tissue specimens were subjected to cytology and histology, respectively. Among the lymph node specimens ($n = 19$), 12 were

TABLE 2. Results of culture, AFB smear, histology, cytology, and PCR analyses for 99 specimens from 87 subjects

Method of diagnosis	No. of positive specimens/no. of specimens tested ^a					
	Pleural fluid ($n = 69$)		Pleural tissue ^b ($n = 11$)		Lymph node ^c ($n = 19$)	
	TB ($n = 53$)	NTB ($n = 16$)	TB ($n = 8$)	NTB ($n = 3$)	TB ($n = 15$)	NTB ($n = 4$)
Conventional culture	0/53	0/16	0/8	0/3	0/15	0/4
USP culture	5/53	0/16	0/8	0/3	1/15	0/4
Conventional smear	1/53	0/16	1/8	0/3	1/15	0/4
USP smear	14/53	0/16	1/8	0/3	1/15	0/4
Conventional smear and culture (combined)	1/53*	0/16	1/8	0/3	1/15	0/4
USP smear and culture (combined)	19/53*, **	0/16	1/8	0/3	2/15	0/4
Tissue histology	ND	ND	7/8	0/3	9/9†	0/3†
FNA cytology	53/53	0/16	ND	ND	12/12‡	0/3‡
Tissue histology and FNA cytology (combined)	53/53	0/16	7/8	0/3	15/15	0/4
<i>devR</i> PCR	40/53	1/16	5/8	0/3	7/15	1/3 ^d
<i>IS6110</i> PCR	40/53	1/16	6/8	0/3	9/15	1/4 ^e
<i>devR</i> and <i>IS6110</i> PCR (combined)	44/53**	1/16 ^e	7/8	0/3	10/15	1/4
USP smear, culture, and PCR (combined)	46/53	1/16	7/8	0/3	10/15	1/4

^a TB, tuberculosis subjects; NTB, nontuberculosis subjects; ND, not done. *, $P < 0.0001$ (comparison between combination of conventional smear and culture and combination of USP smear and culture); **, $P < 0.0001$ (comparison between combination of USP smear and culture and combination of *devR* and *IS6110* PCR); †, seven specimens were not analyzed by histology; ‡, four specimens were not analyzed by cytology.

^b These subjects were also assessed by examination of pleural fluid.

^c One subject was assessed by examination of pleural fluid, pleural tissue, and lymph node.

^d One specimen was not analyzed by *devR* PCR.

^e The PCR false-positive result in the lymph node group was from a patient with sarcoidosis, and that in the pleural fluid group was from a malignant effusion.

analyzed by histology, 15 were analyzed by cytology, and 8 were analyzed by both histology and cytology.

All patients were satisfactorily monitored during and after the course of treatment. One patient with tuberculosis lymphadenopathy had a relapse within 6 months of completion of antitubercular therapy and had to undergo retreatment. Two patients have since died (one from coronary artery disease and one from acute respiratory distress syndrome).

Specimen processing by USP methodology. Specimens were either processed fresh or no more than 72 h after storage at -20°C by USP methodology as described previously (5). Briefly, pleural fluid specimens were centrifuged at $\sim 20,000 \times g$, and the sediments were washed once or twice with USP solution (4 to 6 M guanidinium hydrochloride, 50 mM Tris-Cl [pH 7.5], 25 mM EDTA, 0.5% Sarkosyl, and 0.1 to 0.2 M β -mercaptoethanol). Tissue biopsies were incubated in USP solution, minced finely, and homogenized in the presence of 1-mm glass beads in a mini bead beater (Biospec Products) for 30 to 90 s. The vial was centrifuged at 200 to $400 \times g$, and the homogenate was recentrifuged at $25,000 \times g$ for 10 min at 25°C . The sediment obtained was washed once each with USP solution and sterile water, resuspended in 0.05% Tween 80, and subjected to different tests as indicated: ZN staining (10%), culture on Lowenstein-Jensen slopes (50%) and PCR (40%) as described previously (5, 6).

Specimen processing by conventional methodology for culture. The pleural fluid specimens were centrifuged at $3,000 \times g$, and a portion of the sediment was directly inoculated onto LJ slopes; the other portion was used for preparation of direct smear. The tissue biopsy specimens were minced and homogenized in a sterile homogenizer. A portion of the homogenate was directly inoculated onto LJ slopes.

DNA isolation and PCR. Reagent and sample preparation, PCR amplification, and product detection were performed in separate areas/rooms using dedicated equipment, aerosol-resistant filter guard pipette tips, and a unidirectional work flow scheme to minimize the possibility of any false-positive result due to carryover amplicon contamination. Positive and negative controls for both sample preparation and PCR assays were utilized in every experiment. The processed sediment earmarked for PCR was resuspended in 10% Chelex-100 resin, 0.3% Tween 20, and 0.03% Triton X-100, heated at 94°C for 40 min, and the supernatant was used in PCR. The IS6110 assay was performed as described previously (11) except that the reactions were annealed at 60°C . The *devR* assay used 0.5 μM concentrations of the primers *devRf3* (5'-ATCTGTGTCCCGCATGCC-3') and *devRr3* (5'-GTCCAGCGCCACATCTTT-3'). The reactions were subjected to 10 min at 94°C , followed by 45 cycles each of 1 min at 94°C , 1 min at 52°C , and 30 s at 72°C , with a final extension of 10 min at 72°C . The amplified DNA products were visualized under UV light after agarose gel electrophoresis by ethidium bromide staining.

All centrifugations were performed in fixed-angle rotors for efficient recovery of the sediment.

Statistical analysis. The final diagnosis was established by using the collective results of smear, culture, histology/cytology, clinical diagnosis, and response to antitubercular therapy during follow-up. Because of low culture yields from samples of extrapulmonary tuberculosis, histology, cytology, and microbiology results, together with the response to antitubercular therapy, were considered as the gold standard in the present study. The results of individual tests, i.e., microbiology, histology/cytology, and PCR, were compared to the final diagnosis of tuberculosis made with individual specimens as the unit of analysis (Table 2). Significance was determined by the χ^2 test with Yates' correction. The PCR results were classified as true positives (T_p), true negatives (T_n), false positives (F_p), and false negatives (F_n). Sensitivity was calculated as $[T_p/(T_p + F_n)] \times 100$; specificity was calculated as $[T_n/(T_n + F_p)] \times 100$; positive predictive value was calculated as T_p obtained/total number of positives; negative predictive value was calculated as T_n obtained/total number of negatives; and efficiency was calculated as $[(T_p + T_n)/N] \times 100$, where $N = 69, 11$, and 19 for pleural fluid, pleural tissue, and lymph node specimens, respectively.

RESULTS

Clinical specimens. Between October 2002 and January 2003, 87 patients were enrolled who were finally classified into tuberculosis ($n = 67$) and nontuberculosis ($n = 20$) categories. The maximum number of samples was from patients in the age group of 20 to 39 years ($n = 50$), and the median age of the subjects was 35 years, two-thirds of whom were men. Only one patient in the tuberculosis-lymphadenopathy group, and no patients in the other groups had household contact with tuber-

culosis. The predominant site of lesion was right-handed ($n = 46$) in the tuberculosis-pleural effusion group and cervical ($n = 17$) in the tuberculosis-lymphadenopathy group. The major presenting clinical features included fever, malaise, chest pain, and cough. Of the 87 patients, 58 were tuberculin positive. The predominant associated characteristic among the tuberculosis patients was tobacco smoking ($n = 16$), and among nontuberculosis patients it was also tobacco smoking ($n = 5$). All 99 clinical specimens were subjected to routine biochemical analysis, cytology and/histology, microbiological tests, and PCR. One patient had tuberculous lymphadenopathy with pleural effusion. Details of the diagnostic categories of patients and samples are provided in Table 1.

Since demonstration of AFB in histopathological specimens and recovery by culture are both quite difficult, the diagnosis of extrapulmonary tuberculosis (including lymph node and pleural involvement) is often based on the presence of granulomatous inflammation with or without caseation. FNA cytology is being increasingly used as the main diagnostic procedure for tuberculous lymphadenitis. Factors favoring cytologic diagnosis of tuberculosis in such specimens include hypocellularity in smears, evidence of necrosis, and the presence of epithelioid cells with or without giant cells (8). Clinical, microbiological, and cyto/histopathological diagnosis, along with response to treatment, served as the gold standard in the present study against which all other diagnostic parameters were compared. Among the USP tests that included smear microscopy, culture of *M. tuberculosis*, and PCR, the latter was found to correlate maximally with the gold standard (see below and Table 2).

Smear microscopy and culture. USP smear microscopy detected 16 specimens (14 pleural fluid and 1 each of lymph node and pleural tissue biopsy) as positive versus 3 detected by direct-smear microscopy (Table 2). The overall sensitivity of USP smear microscopy was 21.1% (16 of 76), whereas that of the conventional method was only 3.9% (3 of 76). Six specimens were also determined to be positive by USP culture, showing an overall sensitivity of 7.9%, whereas conventional culture failed to detect *M. tuberculosis* in any specimen (Table 2). All cultures were positive for *M. tuberculosis*.

PCR. The detailed performance of the *devR* and IS6110 PCR assays in pleural fluid, pleural tissue, and lymph node specimens is represented in Tables 2 and 3. The sensitivity ranged between 66.7 and 83%, and the specificity ranged between 75% and 100% in all groups of specimens. The combined results of both assays showed the best sensitivity characteristics compared to the individual IS6110 and *devR* assays.

The performance of PCR was also compared in 11 patients, where both pleural tissue and pleural fluid specimens were collected (Table 2). Concordance was noted in 9 of 11 patients with respect to *devR* and IS6110 PCR results, and the sensitivity of the combined PCR results (both targets) was identical (7 of 8 [87.5%]). Nine specimens (four pleural fluid, two pleural tissue, and three lymph node) were determined to be positive by IS6110 but negative by *devR* PCR. This could be due to the higher sensitivity of the IS6110 assay, which targets multiple sequences of *M. tuberculosis*. On the other hand, six specimens (four pleural fluid, one pleural tissue, and one lymph node) were *devR* positive but IS6110 negative, suggesting that these clinical *M. tuberculosis* strains could be lacking IS6110 sequences. Thus, the combined results of both PCR

TABLE 3. Performance of PCR in diagnosis of extrapulmonary tuberculosis

Parameter ^a	Pleural fluid (<i>n</i> = 69)			Pleural tissue (<i>n</i> = 11)			Lymph node (<i>n</i> = 19)		
	<i>devR</i>	IS6110	<i>devR</i> and IS6110 combined	<i>devR</i>	IS6110	<i>devR</i> and IS6110 combined	<i>devR</i>	IS6110	<i>devR</i> and IS6110 combined
Sensitivity (%)	75.5	75.5	83	62.5	75	87.5	46.7	60	66.7
Specificity (%)	93.8	93.8	93.8	100	100	100	66.7	75	75
PPV (%)	97.6	97.6	97.8	100	100	100	87.5	90	90.9
NPV (%)	53.6	53.6	62.5	50	60	75	25	33.3	37.5
Efficiency (%)	79.7	79.7	85.5	72.7	81.8	90.9	47.4	63.2	68.4

^a PPV, positive predictive value; NPV, negative predictive value.

assays were superior to those of the individual assays, although the differences were not statistically significant. In terms of efficiency, analysis of pleural tissue and pleural fluid scored higher than analysis of lymph node specimens; the PCR efficiencies were 90.9, 85.5, and 68.4% in pleural tissue, pleural fluid, and lymph node, respectively.

DISCUSSION

The diagnosis of extrapulmonary tuberculosis is challenging for a number of reasons: the lack of adequate sample amounts or volumes; the apportioning of the sample for various diagnostic tests (histology/cytology, biochemical analysis, microbiology, and PCR), resulting in nonuniform distribution of microorganisms; the paucibacillary nature of the specimens; the presence of inhibitors that undermine the performance of nucleic acid amplification-based techniques; and the lack of an efficient sample processing technique universally applicable on all types of extrapulmonary samples. The poor performance of conventional microbiological techniques in extrapulmonary specimens has stimulated the increased use of PCR tests in the laboratory diagnosis of tuberculosis. The exact diagnostic role of PCR assay for *M. tuberculosis* in high-prevalence areas for tuberculosis has to be assessed in appropriate control groups, particularly in the case of extrapulmonary tuberculosis. In contemporary practice, clinicians neither start nor stop treatment for this condition based solely on PCR results. Taking this into account, the present blind study was conducted to prospectively evaluate the role of the multipurpose USP technology in the diagnosis of tuberculous pleural effusion and lymphadenopathy. Keeping in mind the relatively low yield of organisms by AFB smear and culture in extrapulmonary specimens, we evaluated the test results against histology, cytology, and microbiology and comprehensive clinical follow-up.

We show here that PCR would be particularly useful for the diagnosis of tuberculous pleural effusion and lymphadenopathy in patients where conventional diagnosis fails and where the provisional diagnosis of tuberculosis is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB. USP methodology was found to be highly suitable for processing fluid and tissue samples and completely compatible with AFB detection, culture, and PCR. The strengths of this technology included (i) the ability to apply smear microscopy, culture, and PCR tests on the same sample aliquot and (ii) its efficiency and robustness given the scanty amounts of tissue and the proteinaceous (coagulum-laden) nature of pleural fluid that were often obtained. It is now well

recognized that optimal sample preparation contributes in no small measure to the success and reliability of PCR-based assays (16, 19, 28). There are numerous examples in the literature of amplification-based test performances being marred by inhibitory substances present in clinical specimens, notably biopsies and proteinaceous pleural effusions which could include blood, host proteins, and even eukaryotic DNA that can inhibit amplification when present in a high concentration (7, 16, 19, 21, 24, 28). The USP methodology addresses precisely this lacuna and proved to be highly efficient in inhibitor removal from biopsies and proteinaceous pleural effusions.

The sensitivities of USP smear and culture were far superior to those of the conventional methods ($P < 0.0001$) but fell short of PCR. Fourteen of the sixteen specimens detected as positive by smear microscopy were pleural fluid, suggesting that positivity could be perhaps further enhanced by using a larger volume of body fluid. The low diagnostic yield by culture confirmed the findings of earlier studies on extrapulmonary disease diagnosis. However, it should be noted that culture was not optimized since culture in liquid medium was not attempted. Of 53 tuberculous pleural fluid samples, only 5 were determined to be positive by USP culture (9.4%) versus none by conventional culture. None of the pleural tissue specimens were determined to be positive by culture, and only 1 lymph node biopsy each was determined to be positive by USP smear and culture. The relatively better sensitivity of USP culture is likely due to a combination of factors that include more efficient recovery of bacilli by centrifugation at higher speed ($20,000 \times g$ versus $3,000 \times g$, respectively, in the USP versus conventional methods), use of relatively more specimen for analysis, and removal of contaminants prior to culture (5). The pleural fluid specimens were concentrated for conventional and USP methods, albeit at different g values (see above) and, despite USP treatment, smear and culture by the USP method performed better than the conventional methods. We have established previously that the acid-fast properties of bacteria are not compromised by exposure to USP solution (5), so we do not believe the low yields by smear microscopy to be a consequence of USP treatment. On the other hand, in histology sections three possible technical reasons could have contributed to the relatively poor yields by biopsy culture and smear using conventional methods: the cellular background, which makes it relatively more difficult to spot bacilli, compounded by the reported reduction in sensitivity of acid-fast microscopy in formalin-fixed paraffin-embedded tissue (13) and, lastly, the examination of only a small fraction of the biopsy. However, these handicaps were bypassed in the USP

smear and culture technique. In principle, toxicity of USP in case of USP culture could have reduced the yield, although we demonstrated that USP is not any more toxic than the conventional NALC-NaOH method (5). Therefore, we believe the low yields of culture by both conventional and USP methods was likely due to the paucibacillary load in the specimens, which was further compounded by use of a small sample volume or biopsy material. Thus, the performance of the smear, culture, and PCR tests could perhaps be improved further by processing larger volumes/amounts of specimen.

Two of the AFB smear-positive but culture-negative specimens were determined to be negative by PCR; we have no explanation for this except for the nonuniform distribution of bacilli in the aliquots apportioned for the diagnostic tests. The absence of PCR inhibitors was confirmed in the DNA preparation of both the specimens using suitable internal DNA amplification controls. In instances in which one PCR method was positive (either *IS6110* or *devR*) but the other method was negative, it is conceivable that amplicon carryover caused one of the two methods to yield a false-positive result. This possibility cannot be rigorously excluded despite the use of a unidirectional work flow scheme. Over and above these discrepancies, among the USP tests, PCR had the highest diagnostic utility. The ideal specimen was pleural tissue biopsy and pleural fluid. Both the PCR assays were equally useful to diagnose tuberculous pleural effusion and lymphadenopathy. The diagnostic accuracy increased further when the results of both the PCR assays were combined and boosted the confidence of the treating physician on PCR. Of 99 samples, 2 were false positive by PCR (by both assays). One of them was from a patient diagnosed with sarcoidosis after he showed poor response to a complete course of antitubercular therapy. Sarcoidosis is a multisystemic granulomatous disease of uncertain etiology. Many attempts to detect mycobacterial DNA in clinical samples from patients with sarcoidosis by PCR have been reported, and yet the possible role of mycobacterial infection in the pathogenesis of sarcoidosis has been widely debated and remains unresolved (15). The second false-positive specimen was from a patient with malignant pleural effusion in whom there was no clinical evidence of tuberculosis. In India where the prevalence of tuberculosis is very high, the immunosuppressive nature of a malignant lesion is capable of reactivating a latent tubercular infection; hence, malignancy and tuberculosis may coexist. The occurrence of false negatives in the present study was a matter of greater concern. There were nine (17%) false-negative samples among the pleural effusions and five (33%) among the lymph node biopsies. There are a number of possible reasons for false negativity: the paucibacillary nature of the disease, a possible hypersensitivity mechanism, or the availability of only a small amount or volume of sample after it was distributed for various pathological and biochemical investigations. Excision biopsy of the entire lymph node under local anesthesia and direct vision would have sampled larger tissue specimens and could have improved the yield of organisms. In support of the observation is the fact that the only lymph node biopsy specimen determined to be positive by AFB smear was of the latter type.

In summary, the USP method of specimen processing allowed us to carry out three diagnostic tests, namely, smear microscopy, culture, and PCR, conveniently on a single spec-

imen without dividing it into portions for the individual tests, which might have resulted in nonuniform distribution of the tubercle bacilli or cross contamination during tube-to-tube transfers. The use of a single specimen-processing platform enabled us to analyze with confidence the results of different tests leading to a more definitive diagnosis. Because of their excellent specificity the predictive value of positive amplification tests was very high in pleural effusion. However, because of lower sensitivity, the predictive value of a negative PCR was somewhat less, especially in lymphadenopathy. A clinicopathological correlation with microbiological and molecular tests results was observed to be an ideal approach to diagnose these forms of extrapulmonary tuberculosis. The application of this technology could seemingly attain particular relevance in developing countries such as India, which carry a heavy burden of extrapulmonary disease.

ACKNOWLEDGMENTS

S.C. received a Senior Research Fellowship from the ICMR. M.K.S. received a short-term fellowship from the World Health Organization to work in the Department of Biotechnology, All India Institute of Medical Sciences, at the time this study was conducted. J.S.T. acknowledges the Department of Biotechnology, Government of India, and the All India Institute of Medical Sciences for financial assistance.

We thank S. Kailash for assistance with the statistical analysis. We also thank Sanjay Kumar for technical assistance.

REFERENCES

- Aggarwal, A. N., D. Gupta, and S. K. Jindal. 1999. Diagnosis of tubercular pleural effusion. *Indian J. Chest Dis. Allied Sci.* **41**:89–100.
- Appling, D., and R. H. Miller. 1981. Mycobacterial cervical lymphadenopathy: 1981 update. *Laryngoscope* **91**:1259–1266.
- Baek, C. H., S. I. Kim, Y. H. Ko, and K. C. Chu. 2000. Polymerase chain reaction detection of *Mycobacterium* from FNA for diagnosis of cervical tuberculosis. *Laryngoscope* **110**:30–34.
- Burkhardt, H. J. 2000. Standardization and quality control of PCR analyses. *Clin. Chem. Lab. Med.* **38**:87–91.
- Chakravorty, S., and J. S. Tyagi. 2005. Novel multipurpose methodology for detection of mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture, and PCR. *J. Clin. Microbiol.* **43**:2697–2702.
- Chakravorty, S., M. Dubeja, M. Hanif, and J. S. Tyagi. 2005. Utility of universal sample processing methodology, combining smear microscopy, culture, and PCR, for diagnosis of pulmonary tuberculosis. *J. Clin. Microbiol.* **43**:2703–2708.
- Claridge, J. E., III, R. M. Shawar, T. M. Shinnick, and B. B. Plikaytis. 1993. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J. Clin. Microbiol.* **31**:2049–2056.
- Dasgupta, A., R. N. Ghosh, A. K. Poddar, C. Mukerjee, P. K. Mitra, G. Gupta, and U. Ganguly. 1994. Fine needle aspiration cytology of cervical lymphadenopathy with special reference to tuberculosis. *J. Indian Med. Assoc.* **92**:44–46.
- de Lasseigne, A., D. Lecossier, C. Pierre, J. Cadranet, M. Stern, and A. J. Hance. 1992. Detection of mycobacterial DNA in pleural fluid by means of the polymerase chain reaction: comparison of two protocols. *Thorax* **47**:265–269.
- de Wit, D., G. Maartens, and L. Stein. 1992. A comparative study of the polymerase chain reaction and conventional procedures for the diagnosis of tuberculous pleural effusion. *Tubercle Lung Dis.* **73**:262–267.
- Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford. 1990. Polymerase chain reaction amplification of repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J. Infect. Dis.* **161**:977–981.
- Ersoz, C., A. Polat, M. S. Serin, L. Soyulu, and O. Demircan. 1998. Fine needle aspiration (FNA) cytology in tuberculous lymphadenitis. *Cytopathology* **9**:201–207.
- Fukunaga, H., T. Murakami, T. Gondo, K. Sugi, and T. Ishihara. 2002. Sensitivity of acid-fast staining for *Mycobacterium tuberculosis* in formalin-fixed tissue. *Am. J. Respir. Crit. Care Med.* **166**:994–997.
- Goel, M. M., V. Ranjan, T. N. Dhole, A. N. Srivastava, A. Mehrotra, M. R. Khushwaha, and A. Jain. 2001. Polymerase chain reaction versus conventional diagnosis in aspirates of tuberculous lymph nodes. *Acta Cytol.* **45**:333–340.
- Hance, A. J. 1998. The role of mycobacteria in the pathogenesis of sarcoidosis. *Semin. Respir. Infect.* **13**:197–205.

16. Honore-Bouakline, S., J. P. Vincensini, V. Giacuzzo, P. H. Lagrange, and J. L. Herrmann. 2003. Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. *J. Clin. Microbiol.* **41**:2323–2329.
17. Hooper, A. A. 1972. Tuberculous peripheral lymphadenitis. *Br. J. Surg.* **59**: 353–359.
18. Kidane, D., J. O. Olobo, A. Habte, Y. Negesse, A. Aseffa, G. Abate, M. A. Yassin, K. Bereda, and M. Harboe. 2002. Identification of the causative organism of tuberculous lymphadenitis in Ethiopia by PCR. *J. Clin. Microbiol.* **40**:4230–4234.
19. Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. C. Bange, and E. C. Bottger. 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J. Clin. Microbiol.* **31**:2882–2889.
20. Krishnaswami, H., G. Koshi, K. G. Kulkarni, and C. K. Job. 1972. Tuberculous lymphadenitis in south India—a histopathological and bacteriological study. *Tubercle Lung Dis.* **53**:215–220.
21. Mangiapan, G., M. Vokurka, L. Schouls, J. Cadranet, D. Lecossier, J. van Embden, and A. J. Hance. 1996. Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *J. Clin. Microbiol.* **34**:1209–1215.
22. Nagesh, B. S., S. Sehgal, S. K. Jindal, and S. K. Arora. 2001. Evaluation of polymerase chain reaction for the detection of *Mycobacterium tuberculosis* in pleural fluid. *Chest* **119**:1737–1741.
23. Parandaman, V., S. Narayanan, and P. R. Narayanan. 2000. Utility of polymerase chain reaction using two probes for rapid diagnosis of tubercular pleuritis in comparison to conventional methods. *Indian J. Med. Res.* **112**: 47–51.
24. Pfyffer, G. E., P. Kissling, E. M. Jahn, H. M. Welscher, M. Salfinger, and R. Weber. 1996. Diagnostic performance of amplified *Mycobacterium tuberculosis* direct test with cerebrospinal fluid, other nonrespiratory, and respiratory specimens. *J. Clin. Microbiol.* **34**:834–841.
25. Prakash, U. B., and H. M. Reiman. 1985. Comparison of needle biopsy with cytologic analysis for the evaluation of pleural effusion: analysis of 414 cases. *Mayo Clin. Proc.* **60**:158–164.
26. Querol, J., J. Minguez, E. Garcia-Sanchez, M. A. Farga, C. Gimeno, and J. Garcia-de-Lomas. 1995. Rapid diagnosis of pleural tuberculosis by polymerase chain reaction. *Am. J. Respir. Crit. Care Med.* **52**:1977–1981.
27. Shah, A. 1992. Tuberculous pleural effusion: a diagnostic problem. *Indian J. Chest Dis. Allied Sci.* **34**:115–116.
28. Singh, K. K., M. Muralidhar, A. Kumar, T. K. Chattopadhyay, K. Kapila, M. K. Singh, S. K. Sharma, N. K. Jain, and J. S. Tyagi. 2000. Comparison of in-house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. *J. Clin. Pathol.* **53**:355–361.
29. Verma, A., N. Dasgupta, A. N. Agarwal, J. N. Pande, and J. S. Tyagi. 1995. Utility of a *Mycobacterium tuberculosis* GC-rich repetitive sequence in the diagnosis of tuberculous pleural effusion by PCR. *Indian J. Biochem. Biophys.* **32**:429–436.
30. Villena, M., J. Robollo, J. M. Aguada, A. Galan, L. Encuentra, and E. Pulenque. 1998. Polymerase chain reaction for the diagnosis of pleural tuberculosis in immunocompromised and immunocompetent patients. *Clin. Infect. Dis.* **26**:212–214.
31. World Health Organization. Global tuberculosis control: surveillance, planning, financing. World Health Organization, Geneva, Switzerland. [Online.] W.H.O./CDS/TB/2002.295. <http://www.who.int/gtb/publications/globrep02/index.html>.