MAJOR ARTICLE

Clinical Evaluation of a 16S Ribosomal RNA Polymerase Chain Reaction Test for the Diagnosis of Lymph Node Tuberculosis

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Reports on the sensitivity of polymerase chain reaction (PCR) for the diagnosis of lymph node tuberculosis (TB) show divergent results. We evaluated the accuracy of the Roche Amplicor *Mycobacterium tuberculosis* PCR test with lymph node aspirate and biopsy samples.

Methods. The study was conducted at a public reference hospital in Lima, Peru. From the period of January 2003 to January 2004, we included patients who had lymphadenopathy and in whom the attending physician suspected TB. Aspirate and biopsy samples were submitted for culturing in Löwenstein-Jensen medium, for histopathologic testing, and for PCR. The sensitivity and specificity of PCR were calculated against a reference standard based on histopathologic findings and culture.

Results. Our study included 154 patients. Median age was 29 years (interquartile range, 21–40 years); 97 patients (62.9%) were men. Twenty-nine patients (18.8%) had acid fast bacilli–positive histopathologic findings, and 44 (28.6%) had a positive culture result. Using the combination of histopathologic findings and culture as reference standard, 55 patients (35.7%) had a diagnosis of tuberculous lymphadenitis. The sensitivity of the PCR test was 58.2%, and the specificity was 93.9%. For biopsy tissue only, the sensitivity of PCR was 52.7%, and the specificity was 97.0%. For aspirate samples only, the sensitivity of PCR was 47.3%, and the specificity was 96.0%.

Conclusion. The Amplicor PCR test revealed low sensitivity and high specificity for the diagnosis of lymph node TB. The sensitivity was higher in cases in which the bacillary load was high—in acid fast bacilli–positive samples and among HIV-infected patients. Considering the results of microbiological and PCR tests together, there was still a patient group in whom no final diagnosis could be established.

Tuberculosis (TB) is an important cause of human mortality: ~6% of all deaths in the world are due to this disease [1]. The diagnostic approach to TB has changed little since the time of Robert Koch (1882); the laboratory diagnosis basically relies on the staining of acid-fast bacilli (AFB) and on culturing in solid or liquid media. The World Health Organization estimates that only one-half of new cases of active TB per year are diagnosed and notified [2].

Extrapulmonary TB is particularly difficult to diagnose and accounts for 20% of TB cases among HIV-negative people [3]. Immune suppression due to advanced age and chronic diseases—in particular HIV infection—increases the risk of extrapulmonary presentations of TB [4].

Lymph nodes are the most frequent site of extrapulmonary TB, both among HIV-positive and HIV-negative people [5, 6]. In regions with high TB incidence rates, TB is a common cause of lymphadenopathy [7]. However, especially in tropical areas, several other possible diagnoses should be considered, some of which may result in histopathological findings that are similar to those of TB, such as fungal diseases, leprosy, brucellosis, toxoplasmosis, cat scratch disease, syphilis, and infections with mycobacteria other than *Mycobacterium tuberculosis* [7].

Routine tests used for the microbiological confirmation of lymph node TB have important disadvan-

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tages: the sensitivity of AFB staining is <50%. On the other hand, culturing takes 2–8 weeks to produce a definite result [7–9]. In general, diagnostic tests that analyze the amplification of nucleic acids could be a better alternative because of their unsurpassed detection sensitivity and the rapid availability of results (within hours). In addition, this technology could offer the possibility of pathogen subspecies identification. These techniques could represent an important progress in the detection of *M. tuberculosis,* starting from clinical samples.

Several variants of the PCR technique have proven to be accurate for the diagnosis of pulmonary TB using sputum samples [10–12]. In smear-positive, respiratory specimens, the sensitivity of the Amplicor PCR test (Roche Diagnostic Systems) is 90%–100%, and the test's specificity is close to 100% [12, 13]. The test has been approved by the US Food and Drug Administration for the direct detection of *M. tuberculosis* in respiratory specimens with positive AFB smear results [14]. In smear-negative samples, the sensitivities of the Amplicor PCR test and of other PCR tests are lower, ranging from 50% to 96% [10, 11, 13].

In extrapulmonary specimens, divergent sensitivities (from 27% to 100%) have been reported for several PCR tests, whereas specificity was generally found to be high [13]. In the case of lymph node samples, reported PCR test sensitivities range from 33% to 96%, and the specificity is >86% [8, 9, 15–17]. Most of these studies included relatively few patients [8, 9, 15, 17]. Moreover, differences in patient selection, reference standards used to evaluate PCR results, sample collection and preparation, and DNA extraction and PCR techniques make it difficult to compare the results.

We undertook a prospective study to evaluate the accuracy of a well-established PCR test in the diagnosis of TB through lymph node aspirate and biopsy samples, compared with a reference standard based on histopathologic findings and culture.

MATERIALS AND METHODS

Patients. The study was conducted at the Hospital Nacional Cayetano Heredia, a public reference hospital in Lima, Peru, during the period from January 2003 through January 2004. We included inpatients and outpatients with superficial lymphadenopathy in whom the attending physician suspected TB and requested a lymph node biopsy as part of the diagnostic evaluation.

Procedures. After providing written, informed consent, each participant was interviewed and examined, and a fine needle aspirate and a biopsy sample of an enlarged lymph node was obtained by a surgeon. We divided the aspirate sample in 3 parts: the first part, for use in PCR, was dissolved in sterile physiological serum and cryopreserved at −70°C, the second aliquot was immediately sent for culture, and the third part was used to prepare 2 smears (one for Ziehl-Neelsen and the other for Papanicolaou staining). Immediately after the aspiration, a biopsy was acquired through lymph node tissue excision or incision. This lymph node tissue was also divided in 3 parts: the first part, for PCR analysis, was cryopreserved at −70°C; the second part was stored at 4°C for ⩽4 days and cultured in Löwenstein-Jensen medium; and the third part was fixed in 10% formalin solution and sent to the histopathology department for analysis. All samples were coded before they were sent to the laboratories. Those in charge of diagnostic testing did not have access to the results of other tests, and were unaware of the clinical condition of the patients.

For the diagnosis of diseases other than TB, such as toxoplasmosis and Epstein-Barr virus infection, we took into account the results of serological tests that were requested by the attending physicians of the case patients. HIV ELISA tests are routinely requested at our hospital for patients with lymphadenopathy that is suggestive of TB. Written, informed consent for HIV testing and pre- and posttest counseling are provided by the hospital office of the National Control Program for Sexually Transmitted Diseases and AIDS.

For histopathological examination, the sections were stained with hematoxylin-esosin and Ziehl-Neelsen. Periodic Acid Schiff and Grocott staining were only performed when requested by the attending physician. If AFB were observed in any sample, the patient was considered to have a definite diagnosis of lymph node TB.

PCR. We used the commercially available Amplicor *Mycobacterium tuberculosis* PCR test (Amplicor MTB; Roche Diagnostic Systems). In a pre-PCR phase, the lymph node aspirate and biopsy samples were digested and decontaminated following the technical instructions of the manufacturer. All biopsy tissue samples underwent additional decontamination using a QIAamp DNA extraction kit (QIAGEN), according to the specifications of the manufacturer. In the case of lymph node aspirate samples, this additional QIAamp decontamination procedure was only performed if there was PCR inhibition in a first test. In the PCR phase, the samples were thermocycled to denaturalize the double DNA helix and to expose the target sequences to the indicators. These biotinilated indicators are derived from the 16S rRNA gene region specific to the genus *Mycobacterium:* KY18 (5′-CACATGCAAGTCGAACGGAAAGG-3′) and KY75 (5′-GCCGTATCGCCCGCACGCTCACA-3′). The indicators form hybrids with the target DNA of the *M. tuberculosis* complex and with the internal control of the Amplicor kit. Results determined by the colored complex that were formed in the detection microplaques were considered positive at a cut-off point of 0.35 (measured at a wavelength of 450 nm), as proposed by the manufacturer. Results were reported as positive or negative.

Statistical analysis. The primary end point was the sen-

sitivity and specificity of PCR for the diagnosis of lymph node TB. We used EpiInfo, version 6 (Centers for Disease Control and Prevention), to calculate the sample size. The expected sensitivity of the PCR test was 80%, and the expected specificity was 90%. We considered a margin of error for sensitivity and specificity of 10% and a confidence interval of 95%. From these figures, we calculated a target number of 150 patients.

The combination of histocytopathology and culture was used as a reference standard: a patient received a definite diagnosis of lymph node TB if AFB were observed or cultured in $\geqslant 1$ sample. All other patients were considered not to have TB. The sensitivity of the PCR test was computed as the proportion of patients with positive PCR results to the total number of patients with a definite diagnosis of TB. The specificity of the PCR test was computed as the proportion of patients with negative PCR results to the total number of patients who did not have TB. PCR results for biopsy and aspirate samples were first considered together and then separately. We also calculated positive and negative predictive values. Data were stored and analyzed with EpiData, version 3.0 (The EpiData Association) and EpiInfo 2000 (Centers for Disease Control and Prevention).

Ethics approval. The study protocol was reviewed and approved by the Institutional Review Board of the Universidad Peruana Cayetano Heredia, and the study was conducted following the guidelines of this university with respect to human participant protection.

RESULTS

One hundred sixty-six patients with superficial lymphadenopathy were enrolled during the study period. We excluded 12 patients because, on histopathological examination, no tissue compatible with lymph node structures was found. The remaining 154 patients were included in the analysis (figure 1). The median age was 29 years (interquartile range, 21–40 years); 97 (62.9%) were men. Forty-five patients (29.2%) were HIV infected. The main site of peripheral lymphadenopathy was the cervical region in 127 subjects (82.5%); 19 patients (12.3%) had inguinal and 8 (5.2%) had axillary lymphadenopathy.

Twenty-nine patients (18.8%) had a definite diagnosis of TB on the basis of the presence of AFB in histopathological examination of aspirate or tissue samples (figure 1). AFB were observed in 29 (100%) of 29 biopsy samples and in 9 (31.0%) of 29 aspirate samples. Histopathological findings for the remaining patients were as follows: 26 patients (16.9%) had necrotizing granulomas with caseum, epitheloid cells, and giant multinucleated Langhans cells, but no AFB; 23 (14.9%) had purulent granulomas—necrotizing or not—with or without caseum and with or without giant multinucleated Langhans cells; and 76 (49.4%) had findings inconsistent with TB.

Forty-four patients (28.6%) received a definite diagnosis of TB on the basis of positive culture results (figure 1); *Mycobacterium* species were isolated from 15 biopsy and 38 aspirate samples. Thirty-eight patients (24.7%) had positive PCR results; *M. tuberculosis* complex was detected in 30 aspirate and 32 biopsy samples (table 1).

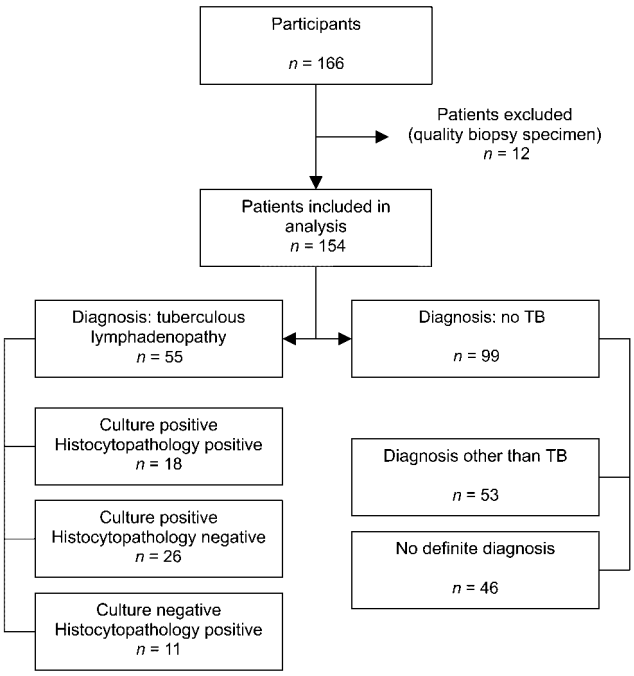

Figure 1. Flow chart of patients, according to diagnosis

Considering all test results together except for PCR, there were 55 patients (35.7%) who received a definite diagnosis of tuberculous lymphadenitis (figure 1), 1 of whom received a simultaneous diagnosis of lymphoma. Fifty-three patients (34.4%) had other diseases that could explain their lymphadenopathy: 16 patients had HIV adenitis, 9 had lymphoma, 1 had lymphoma and mycosis, 7 had cat scratch disease, 6 had mycosis (2 cryptococcosis, 2 paracoccidioidomycosis, and 2 histoplasmosis), 5 had tumor metastasis, 3 had Kaposi sarcoma, 2 had toxoplasmosis, 2 had lymphogranuloma venereum, and 2 had Epstein-Barr virus infection. In the remaining 46 subjects (29.9%), no definite diagnosis could be established (figure 1).

Using the combination of histopathologic findings and culture as a reference standard, 55 patients were classified as being in the TB group, and 99 patients were in the other group. The sensitivity of the PCR test was 58.2%, the specificity was 93.9%, the positive predictive value was 90.6%, and the negative predictive value was 78.7%. The sensitivity of PCR in biopsy tissue only versus the reference standard in biopsy or aspirate was 52.7%, and the specificity was 97.0%. For PCR in aspirate samples only, the sensitivity was 47.3%, and the specificity was 96.0% (table 1).

The PCR test detected 24 (83%) of the 29 cases of TB with an AFB-positive histocytopathologic test result. Among the 45

Table 1. Sensitivity and specificity of PCR in aspirate and/or tissue samples.

Sample substance tested	TB diagnosis (n = 55)	No TB diagnosis (n = 99)	Sensitivity, %	Specificity, %
Aspirate or tissue	32	6	58.2	93.9
Tissue only	29	3	52.7	97.0
Aspirate only	26	4	47.3	96.0

NOTE. Data are no. of samples with a positive PCR result, unless otherwise indicated. TB, tuberculosis.

patients with HIV, 17 (38%) received a diagnosis of tuberculous lymphadenitis. In this subgroup, the sensitivity of the PCR test was 94%, compared with histopathologic findings and culture.

DISCUSSION

The sensitivity of the Amplicor PCR test for the diagnosis of lymph node TB in this study was 58% against a reference standard of histopathologic findings and/or culture—lower than we had expected. In a study in Korea, the same PCR test on lymph node aspirate samples produced a sensitivity of 76% against a reference standard of histocytological findings and response to antituberculous treatment [17].

The PCR test failed to detect 23 of 55 patients with a diagnosis of TB that was confirmed through histopathologic findings and/or culture. The conditions of digestion and purification were, in our opinion, the major obstacle to the performance of the PCR test. Suboptimal target extraction and PCR inhibition can cause false-negative results [8, 16]. It is not yet clear whether inhibition occurs more frequently with extrapulmonary samples. In several studies, more PCR inhibition was observed with extrapulmonary than with pulmonary specimens [15, 18, 19], whereas the authors of other studies have reported the opposite [10, 20]. In this context, it is remarkable that the sensitivity of PCR for the diagnosis of tuberculous lymphadenitis was determined to be higher when PCR was performed on PBMCs than on lymph node tissue samples [21].

Mycobacteria tend to clump together; the irregular distribution of mycobacteria in a sample could contribute to the lack of sensitivity observed in some studies [22]. It has been shown that testing >1 sample per patient increases test sensitivity, particularly with smear-negative samples [10]. In this context, the sample volume used for PCR could also be important. There are reports of high sensitivities of in-house PCR assays that use a high sample volume compared with the sample volume used by commercially available PCR tests [8, 10, 15].

It is possible that, among the patients with AFB-positive histopathologic findings, there were infections with atypical mycobacteria. These infections could not have been detected by the 16S rRNA primer, which is specific to the *M. tuberculosis* complex. However, false-negative PCR results occurred in particular among HIV-negative people who have a low bacillary load (no AFB on histopathologic findings). Infection with atypical mycobacteria is unlikely among these patients [7].

The specificity of the PCR test was high (94%–98%) and fell within the range of previous reports [17, 21, 23]. Six patients had positive PCR results but negative culture results and had no AFB on histocytopathologic analysis; PCR results for these patients were considered to be false positive. However, 2 of these 6 patients had histopathologic results that were compatible with TB and were started on TB treatment. The remaining 4 patients received a diagnosis with non-Hodgkin lymphoma (1 patient), lymphadenopathy compatible with HIV infection (1 patient), and lymphadenopathy of unknown origin (2 patients). During follow-up, none of these 4 patients showed signs or symptoms of lymph node TB and, with the exception of the patient with lymphoma, the lymphadenopathy disappeared spontaneously. It is likely that, at least in those 4 patients, the PCR results were true false positives.

In this study, 1 aspirate and 1 biopsy sample were examined per patient. Culture yielded more positive results using aspirate samples, whereas AFB-staining yielded more positive results with tissue samples. Consistent with a former report, the sensitivity and specificity of PCR in aspirate samples, which are obtained through a minimally invasive procedure, were similar to that of tissue samples [15].

The PCR test performed best among patients with a high bacillary load; 83% of the patients with visible AFB on histopathologic findings had a positive PCR result, whereas in this subgroup, culture results were positive for only 62%. This observation is consistent with other reports of high PCR sensitivity in AFB-positive samples [13, 20, 24]. The sensitivity of PCR was also remarkably high for patients who were HIV positive (94%, compared with histopathologic findings and/or culture).

Where the PCR test is able to detect *Mycobacterium* species, it has advantages over the traditional diagnostic tests: the procedure is quick, and through additional PCR tests, it is possible to typify the strain of *Mycobacterium* that is isolated [16]. In addition, TB drug resistance could be determined at an early stage, thereby allowing the attending physician to indicate effective TB treatment from the beginning. In Peru, where the rate of multidrug resistance is increasing—especially among

HIV infected patients—this possibility could markedly improve the prognosis of TB [25].

In this study, we diagnosed several other infectious and neoplastic diseases in the patients suspected of having lymph node TB. Furthermore, in 30% of the study population, there was no definite diagnosis of any disease, despite a thorough and systematic evaluation. This illustrates how frequently an attending physician must make decisions only on the basis of clinical observation and indicates the need for better diagnostic tests.

We conclude that the 16S rRNA Amplicor PCR test did not improve the sensitivity for the diagnosis of lymph node TB compared with histopathologic findings and culture. The test performed better in HIV-infected patients, who tended to have a high bacillary load; in this specific patient population, PCR could be an interesting tool. Taking microscopic, microbiological, and PCR tests together, there was still a group of patients in whom no final diagnosis could be established.

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Potential conflicts of interest. All authors: no conflicts.

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