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BRIEF METHOD

Molecular and Standard Approaches to the Diagnosis of Mycobacterial Granulomatous Lymphadenitis in Paraffin-Embedded Tissue

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The diagnosis of tuberculosis is mainly based on acid fast staining and culture of processed sputum or other biologic material (Shinnick and Good, 1995). In the recent upsurge of tuberculosis cases associated with the HIV pandemics, extrapulmonary diseases have become a common finding; among them, lymphadenopathy remains the most frequent presentation (Raviglioni et al, 1992). Sometimes a diagnosis may be first considered in paraffin-embedded biopsy material, without any material having been sent for culture before. Acid fast staining is the most rapid method for the detection of mycobacterial infection in tissue sections but frequently presents negative results, sometimes because of the fact that antimycobacterial therapy changes capsule integrity, preventing acid fast staining, or because of a small quantity of bacilli (Wiley et al, 1990). Immunohistochemical staining for *Mycobacterium* has shown higher sensitivity than acid fast staining, probably because of the detection of fragmented bacilli (Carabias et al, 1998). Recently, the rapid diagnosis of tuberculosis by nucleic acid amplification using the PCR technique has become feasible in fresh material and has been also used for formalin-fixed paraffin-embedded tissue (Marchetti et al, 1998). Another new molecular technique, the ligase chain reaction (LCR), available for the detection of *Mycobacterium tuberculosis* in fresh respiratory specimens (Jouveshomme et al, 1998) and also for extrapulmonary material (Gamboa et al, 1998), has also been applied to formalin-fixed paraffin-embedded tissue (Ruiz-Manzano et al, 2000). The LCx *M. tuberculosis* assay is the first commercial semiautomated nucleic acid amplification test using the LCR. Its target sequence, which encodes protein antigen b, is specific for the *M. tuberculosis* complex. In paraffin-embedded tissue, despite lower sensitivity (63.2%) as compared with fresh respiratory specimens (78–92.9%) and extrapulmonary material (78.5%), this test shows higher sensitivity when compared with another amplification system using PCR (52.6%) and 100% specificity.

In the present study we evaluated this new molecular diagnostic mycobacterial test in paraffin-embedded tissue and compared it with acid fast bacilli (AFB) and immunohistochemistry using anti-bacillus Calmette-Guérin (BCG) staining in granulomatous lymphadenitis. We retrospectively studied 155 biopsy samples from adults with a diagnosis of granulomatous lymphadenitis, who were seen at the University Hospital, FMUSP, from 1985 to1998, excluding those with determined nonmycobacterial etiology. The biopsy samples were examined with AFB and BCG staining, with qualitative and semiquantitative scoring. Nucleic acids were extracted from paraffin-embedded lymph node tissue blocks and submitted to this commercial technique for specific detection of the *M. tuberculosis* complex by the LCR (LCx *M. tuberculosis*; Abbott Laboratories, Abbott Park, Illinois). Ten 5-μm sections from each paraffin block were carefully placed in one 1.5-ml sterile Eppendorf tube. Sections from one positive and one negative paraffin sample were also examined for each reaction group. The microtome blade was changed after each sample was sectioned to avoid cross-contamination. Sections were deparaffinized by twice adding 1 ml of xylene, vortexing for 2 to 5 seconds, and centrifuging at 13,000 rpm for 5 minutes at room temperature. The residual xylene was removed by twice adding 1 ml of absolute ethanol, vortexing for 2 for 5 seconds, and centrifuging at 13,000 rpm for 5 minutes at room temperature. The preparations were vortexed with 350 μl of Milli-Q (Millipore, Bedford, Massachusetts) for 2 to 5 seconds and processed according to the assay protocol (LCx *M. tuberculosis*). The results were qualitatively expressed as positive or negative as deter-

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mined with an automated optical detector and quantitatively expressed using the arbitrary units supplied by the manufacturer.

Qualitative data were compared by the χ^2 test, and quantitative data were analyzed using Pearson correlation. All differences were considered significant when the probability of equality was less than 0.05 ($p < 0.05$), with a type II error of 0.10. All tests were performed using the Statistic 4.0 software (Statsoft, Inc., Tulsa, Oklahoma).

Detection of bacilli by combined AFB and BCG staining showed high agreement when compared with qualitative LCR (Table 1) and allowed an increased rate of diagnosis of mycobacterial infection in most samples. Analysis of quantitative LCR according to the semiquantitative score obtained by AFB and BCG staining, as illustrated in Figure 1, showed some intermediate values (gray zone) for LCR, which were considered to be negative in qualitative analysis. There was a positive correlation between frequency of stained bacilli by standard staining and LCR.

Our analysis shows that anti-BCG immunohistochemistry had higher positivity (59.35%) compared with AFB staining (47.09%) and that LCR for *M. tuberculosis* could be very useful when using paraffin-embedded tissues, increasing agent detection by 14.85% compared with AFB stains and by 2.59% compared with anti-BCG immunohistochemistry. The LCR for *M. tuberculosis* was positively correlated with AFB and BCG staining, with 55.48% agreement, but some positive biopsy samples for both standard methods gave no sign of LCx *M. tuberculosis*. This fact could be ascribed both to nontuberculous mycobacterial infection and to false-negative results caused by prolonged fixation before embedding. When quantitative data were correlated, several "gray zone" cases (~10%) were found, suggesting that new

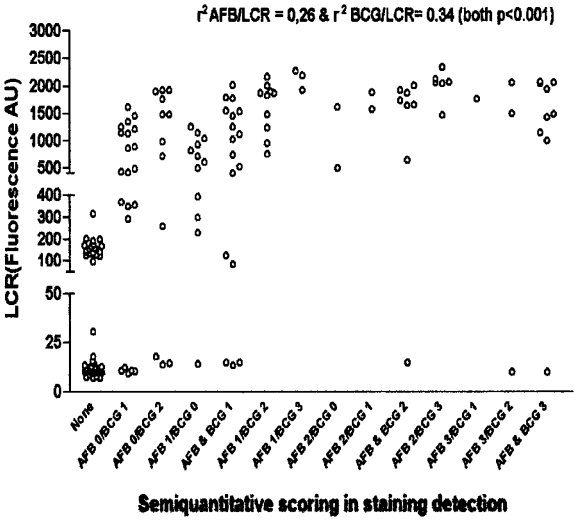

Figure 1.
Quantitative data obtained by ligase chain reaction (*LCR*) for *M. tuberculosis* according to the score results obtained by acid fast bacilli (*AFB*) and bacillus Calmette-Guérin (*BCG*) staining. The numbers in the legends represent the semiquantitative scoring for each stain. The correlation between isolated stains and LCR is also presented.

Table 1. Distribution of Qualitative LCR in Granulomatous Lymphadenitis Paraffin-Embedded Tissues According to AFB and BCG Staining

	LCR		
BCG and AFB	Negative	Positive	Totals
BCG and AFB negative	35	14	49
Row percent	71.43%	28.57%	59.76%
Total percent	22.58%	9.03%	31.61%
BCG negative and AFB positive	3	11	14
Row percent	21.43%	78.57%	19.18%
Total percent	1.94%	7.10%	9.03%
BCG negative	38	25	63
Row percent	60.32%	39.68%	
Total percent	24.52%	16.13%	40.65%
BCG positive and AFB negative	13	20	33
Row percent	39.39%	60.61%	40.24%
Total percent	8.39%	12.90%	21.29%
BCG and AFB positive	8	51	59
Row percent	13.56%	86.44%	80.82%
Total percent	5.16%	32.90%	38.06%
BCG positive	21	71	92
Row percent	22.83%	77.17%	
Total percent	13.55%	45.81%	59.35%
Total	59	96	155
Total percent	38.06%	61.94%	

LCR, ligase chain reaction; BCG, bacillus Calmette-Guérin; AFB, acid fast bacilli.

Analysis of quantitative LCR according to the semiquantitative score obtained by AFB and BCG staining, as illustrated in Figure 1, showed some intermediate values (gray zone) for LCR, which were considered to be negative in qualitative analysis. There was a positive correlation between frequency of stained bacilli by standard staining and LCR.

adequate thresholds for paraffin-embedded tissues could result in even better sensitivity.

In conclusion, the use of LCR for *M. tuberculosis* together with the AFB and BCG staining methods is very effective for mycobacterial diagnosis in paraffin-embedded tissue, with 61.94% total positivity. This is a higher positivity compared with standard methods used in surgical pathology laboratory routine and is especially important because it permits mycobacterial species identification.

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