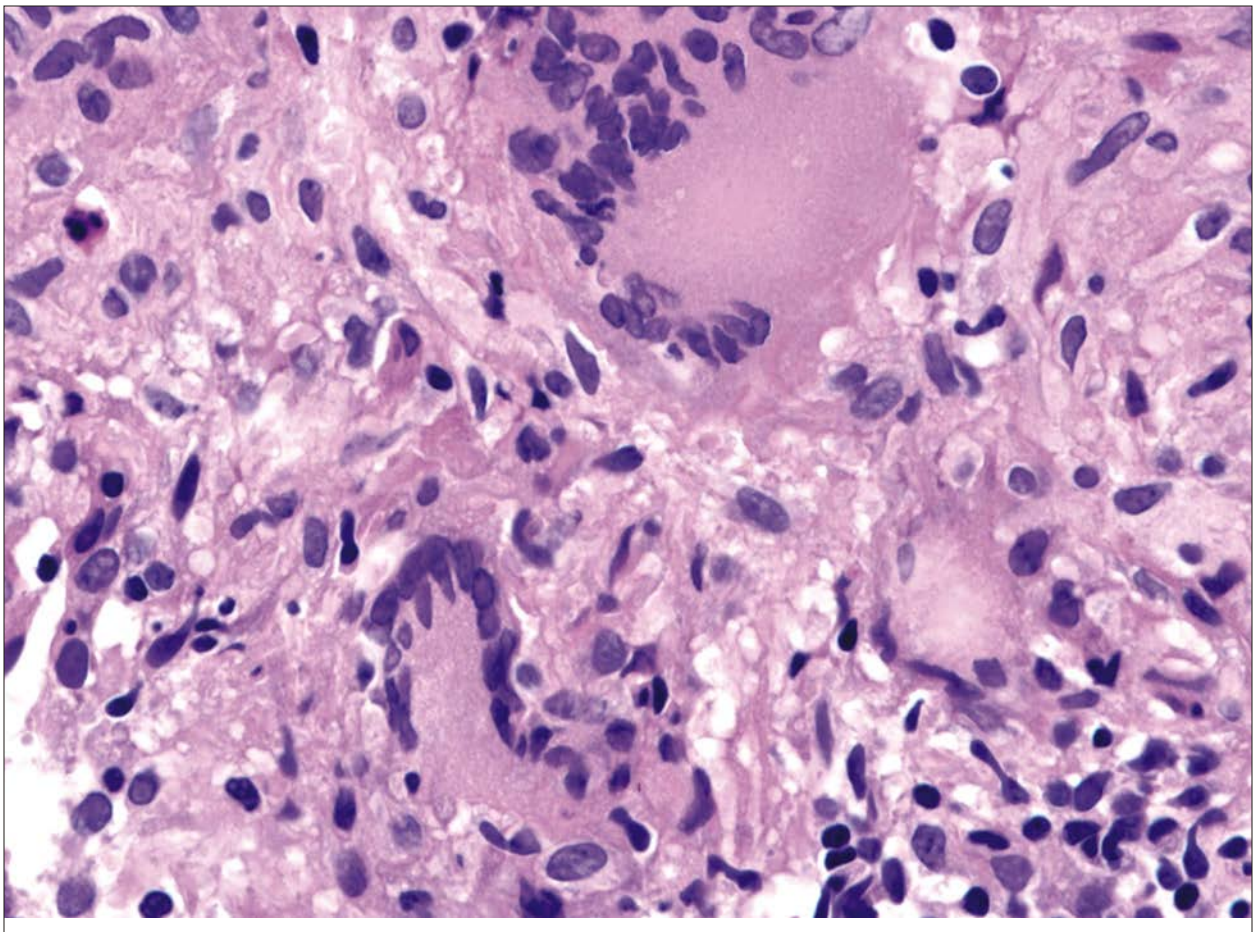

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Evaluation of Immunocytochemistry on Pleural Fluid for the Diagnosis of Pleural Tuberculosis

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ABSTRACT

The aim of this study was to evaluate the diagnostic potential of immunocytochemistry against the Bacille Calmette-Guérin (BCG) antigen on pleural fluid for the diagnosis of pleural tuberculosis. Immunocytochemistry refers to the process of localizing proteins in cells and exploiting the principle of antigens binding to their respective antibodies. Visualization is enabled by tagging the antibody with color producing tags. Consecutive pleural fluid specimens were cytospun and stained for the BCG antigen. Specimens were cultured on Lowenstein Jensen media. After incubation, culture slopes were washed with distilled water and washings used to perform real-time Polymerase Chain Reaction (PCR) assay for mycobacteria. Immunocytochemistry detected mycobacteria in 10/102 (9.8%) specimens compared to 22/102 (21.6%) by culture and 26/102 (25.5%) by real-time PCR. This gave a sensitivity of 27% [95%CI: 16, 34] and specificity of 96% [95%CI: 92, 99] ($p = 0.002$). Immunocytochemistry detecting the BCG antigen was not useful for the diagnosis of pleural tuberculosis.

Key words: Antigen, rapid, BCG, PCR, Mycobacterium tuberculosis

INTRODUCTION

A steady increase has been observed in the number of new extrapulmonary tuberculosis (EPTB) cases which accounted for close to 16% of notified new cases of tuberculosis (WHO, 2009). In HIV patients it is the second most frequent extrapulmonary presentation of the disease (Valdes *et al*., 2003). Tuberculosis pleural effusion is induced when mycobacteria release antigenic proteins into the pleural cavity, thus triggering an imperfectly understood delayed hypersensitivity reaction and the accumulation of fluid in the cavity (Valdes *et al*., 2003).

The definitive diagnosis of tuberculosis pleural effusion involves demonstration of *Mycobacterium tuberculosis* by microbiological, cytopathological or histopathological methods (Sharma and Mohan, 2004). Securing tissue or body fluids for analysis requires the use of invasive methods such as an incisional biopsy or needle aspiration biopsy (Sharma and Mohan, 2004).

Acid-Fast Bacilli (AFB) smears of pleural fluid are seldom positive with a detection rate of <5% of cases due to its paucibacillary nature (Trajman *et al*., 2008). The rate of culture detecting *M. tuberculosis* in pleural fluid ranges from 23 to 56% (Trajman *et al*., 2008). Histology of pleural biopsy tissue/fluid samples coupled with mycobacterial culture is the most sensitive method currently available but could miss upto 20% of cases (Trajman *et al*., 2008).

Pleural fluid adenosine deaminase (ADA) activity has been shown to be a valuable biochemical marker that has a high sensitivity and specificity for tuberculosis diagnosis (Chen *et al.*, 2003), but its diagnostic usefulness depends on the local prevalence of tuberculosis, laboratory methodology and population ethnicity (Chen *et al.*, 2003). A recent meta-analysis by Liang *et al.* (2008) included sixty-three studies to evaluate the diagnostic accuracy of ADA for pleural tuberculosis, showing an overall sensitivity of 92% and specificity of 90%. They concluded that ADA is a useful diagnostic marker for pleural tuberculosis but should be interpreted in parallel with clinical findings and other conventional diagnostic procedures (Liang *et al.*, 2008).

Another diagnostic approach making use of biopsy tissue from the pleural space is histologic examination (Baba *et al.*, 2008). An imflammatory response to *M. tuberculosis* is strongly suggested by the presence of caseous granulomas (Perez-Rodriguez *et al.*, 2003). Studies with immunohistochemistry (IHC) using antibodies directed against mycobacterium antigens have demonstrated higher sensitivities than for the AFB staining and culture method, as the technique does not require an intact cell wall for detection of mycobacterial antigens (Mustafa *et al.*, 2006). Its role is to establish mycobacterial etiology of caseating granulomas of lung, lymph nodes and tissue specimens with TB (Goel and Budhwar, 2007).The major drawback using IHC is that it requires invasive methods to obtain and in cases where a blind biopsy is taken representative tissue may not be secured (Sharma and Mohan, 2004).

In this study, we evaluated an immunocytochemical (ICC) method for the rapid and less invasive diagnosis of pleural TB. Immunocytochemistry refers to the process of localizing proteins in cells and exploiting the principle of antigens binding to their respective antibodies. Visualization is enabled by tagging the antibody with color producing tags. This technique is novel and displays great promise due to its simplicity, practicality and quick turnaround times.

MATERIALS AND METHODS

Consecutive pleural fluid samples sent for adenosine deaminase (ADA) analysis, from patients suspected of having pleural tuberculosis, to the Chemical Pathology Laboratory, National Health Laboratory Services (NHLS), Dr George Mukhari Hospital Complex, Pretoria were collected over a two month period in 2007. The specimens were centrifuged at 10,000 rpm for 10 min, the sediment divided into 1 mL aliquots and stored at -20°C. Specimens were cultured on Lowenstein Jensen (LJ) slopes by inoculating 1 mL of the centrifuged sediment and incubated at 37°C in an aerobic environment until growth was visualized or noted as negative after eight weeks. Ziehl-Neelsen staining was performed on all culture positives to confirm the presence of acid-fast bacilli (AFB).

All the LJ slants were rinsed with 300 μL dH_2O and a 100 μL collected in 1.5 mL polypropylene screw-cap tubes. The Amplicor Respiratory Specimen Preparation Kit (Roche Diagnostics, Germany) was used for DNA extraction. The real-time Polymerase Chain Reaction (PCR) was carried out using the LightCycler® prototype TB Kit (Roche Applied Sciences, South Africa) and the reaction carried out using the LightCycler® version 1.5 (Roche Diagnostics, Germany). The reactions were prepared and the amplification cycles carried out according to the manufacturer's instructions. Melting curve analyses were done on all products where a temperature range between 50-53°C indicated *M. avium*, 55-57°C *M. tuberculosis,* 59-62°C *M. kansasii* and 67°C for the internal control. Analysis of the results was done using the LightCycler® Software v.4.05 (Roche Diagnostics, Germany).

A volume of approximately 100 µL of each sediment was cytocentrifuged, at 1500 rpm for 3 min, onto a slide using the Cytospin 3 (Shandon, UK). The slides were then fixed using Cytological fixative (Pharmingen, San Diego, USA), left to air dry and then further fixed by immersing in absolute methanol overnight at room temperature. The slides were then wrapped in aluminum foil and stored at -20°C until immunostaining.

Immunocytochemical staining was performed by the unlabelled peroxidase-antiperoxidase method (Sternberger *et al.*, 1970). In brief, the smears were carried to a series of incubations in a humidifying chamber at room temperature in the following sequence: 5% hydrogen-peroxide and methanol (1:10) for 10 min, rinsed with distilled H_2O for 30 sec and washed in phosphate buffered saline (PBS) buffer pH 7.4 for 5 min. The slides were then incubated for 30 min with an optimised dilution of 1:5000 polyclonal rabbit anti-*Mycobacterium bovis* to *Mycobacterium* species (Dako, Denmark). The slides were washed for 5 min with PBS buffer pH 7.4 and then incubated in EnVision™ solution (Dako, Denmark). The slides were then washed thoroughly with PBS buffer pH 7.4 for 5 min. Thereafter substrate was freshly prepared by dissolving 20 µL of diaminobezidine tetrachloride (DAB) + chromogen (Dako, Denmark) in 1 mL of substrate buffer (pH 7.4) (Dako, Denmark). Samples were then incubated with substrate for 5-6 min at room temperature.

The smears were then counterstained with haematoxylin for 5 min at room temperature, thoroughly washed with dH_2O and mounted with Faramount Aqueous Mounting Medium (Dako, Denmark). The stained smears were examined using a light microscope at 40x magnification to visualize the presence of a brownish purple colour reaction within the cytoplasm of lymphocytic cells.

Ethical approval was obtained from the Research, Ethics and Publications committee, University of Limpopo, South Africa. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using an extended gold standard of culture and real-time PCR at a 95% confidence interval. Statistical significance was calculated using the Fisher exact test.

RESULTS

A total of 102 pleural fluid specimens submitted to the Chemical Pathology Laboratory at the Dr George Mukhari Hospital complex were collected over a two month period.

After a maximum of eight weeks incubation a total of 22 (21.6%) specimens showed visible growth on the culture media. The presence of acid-fast bacilli from cultures was confirmed by Ziehl-Neelsen staining.

Real-time PCR for mycobateria performed on the washing of culture slopes were positive in 26 (25.5%) cases. These included the 22 culture positives and an additional four on which no visible growth was observed on the slopes. Melting curve analysis using the LightCycler® Software v.4.05 (Roche Diagnostics, Indianapolis, USA) identified 25/26 of the PCR positive products to be *M. tuberculosis* and 1/26 as a mixed culture of *M. tuberculosis* and *M. avium*.

Immunocytochemistry using the anti-BCG antibody was positive for 10 (9.8%) specimens. Of the 10 positive by immunocytochemistry six were both culture and real-time PCR positive and one positive by real-time PCR only.

DISCUSSION

A diagnosis of tuberculosis using immunocytochemistry directed at the BCG antigen could only be made in 10 of the 102 (9.8%) specimens. Six of the positives were both culture and real-time PCR

positive and one by real-time PCR alone. Thus, immunocytochemistry showed a sensitivity of 26.9% [95%CI: 16, 32], specificity of 96.1% [95%CI: 92, 99] along with a PPV of 60% [95%CI: 42, 89] and a NPV of 79.3% [95%CI: 76, 81] (p=0.002). The test had a very low sensitivity in detecting the BCG antigen when compared to the extended gold standard of culture and real-time PCR. The sensitivity is comparable with that of PCR performed directly on pleural fluid which ranged between 20-31% as described by Lima *et al.* (2003). In comparison other immunodiagnostic methods directed against a variety of antigens and using a variety of techniques, reported sensitivities ranging form 12-87% (Gopi *et al.*, 2007).

CONCLUSION

This study has shown that immunocytochemistry using the BCG antibody although rapid was not useful for the diagnosis of tuberculous pleural effusions. Further studies to increase the sensitivity of immunocytochemistry using different antibodies or a panel of antibodies targeting a diverse range of antigens are required to evaluate this technique.

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REFERENCES

Baba, K., A.M. Dyrhol-Riise, L. Sviland, N. Langeland, A.A. Hoosen, H.G. Wiker and T. Mustafa, 2008. Rapid and specific diagnosis of tuberculous pleuritis with immunohistochemistry by detecting *Mycobacterium tuberculosis* complex specific antigen MPT64 in patients from a HIV endemic area. Appl. Immunohistochem. Mol. Morphol., 16: 554-561.

Chen, M.L., W.C. Yu, C.W. Lam, K.M. Au, F.Y. Kong and A.Y. Chan, 2003. Diagnostic value of pleural fluid adenosine deaminase activity in tuberculous pleurisy. Clin. Chim. Acta, 341: 101-107.

Goel, M.M. and P. Budhwar, 2007. Immunohistochemical localization of *Mycobacterium tuberculosis* complex antigen with antibody to 38kDa antigen versus Ziehl-Neelson staining in tissue granulomas of extra-pulmonary tuberculosis. Indian J. Tubec., 54: 24-29.

Gopi, A., S.M. Madhavan, S.K. Sharma and S.A. Sahn, 2007. Diagnosis and treatment of tuberculous pleural effusion in 2006. Chest, 131: 880-889.

Liang, Q.L., H.Z. Shi, K. Wang, S.M. Qin and X.J. Qin, 2008. Diagnostic accuracy of adenosine deaminase in tuberculous pleurisy: A meta-analysis. Respiratory Med., 102: 744-754.

Lima, D.M., J.K. Colares and B.A. da Fonseca, 2003. Combined use of the polymerase chain reaction and detection of adenosine deaminase activity on pleural fluid improves the rate of diagnosis of pleural tuberculosis. Chest, 124: 909-914.

Mustafa, T., H.G. Wiker, S.G. Mfinanga, O. Mørkve and L. Sviland, 2006. Immunohistochemistry using a *Mycobacterium tuberculosis* complex specific antibody for improved diagnosis of tuberculous lymphadenitis. Mod. Pathol., 19: 1606-1614.

Perez-Rodriguez, E., D.J. Castro and R.W. Light, 2003. Effusions from Tuberculosis. In: Textbook of Pleural Diseases, Light, R.W and Y.C.G. Lee (Eds.). Arnold, London, pp: 329-344.

Sharma, S.K. and A. Mohan, 2004. Extrapulmonary tuberculosis. Indian J. Med. Res., 120: 316-353.

Sternberger, L.A., P.H. Hardy Jr., J.J. Cuculis and H.G. Meyer, 1970. The unlabeled antibody enzyme method of immunohistochemistry: Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem., 18: 315-333.

Trajman, A., M. Pai, K. Dheda, A. van Zyl Smit and A.A. Zwerling *et al.*, 2008. Novel tests for diagnosis tuberculosis pleural effusion: What works and what does not? Eur. Respiratory J., 31: 1098-1106.

Valdes, L., P. Antonio, J.E. San and J.M.M. Vazquez, 2003. Tuberculous pleural effusions. Eur. J. Int. Med., 14: 77-88.

WHO, 2009. Global tuberculosis control-epidemiology, strategy, financing. World Health Organization, Report 2009. http://www.who.int/tb/publications/global_report/2009/en/index.html.