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The utility of immunohistochemistry for detecting mycobacterial infections in bronchoalveolar lavage & bronchial washings

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***Background & objectives*: Tuberculosis, most commonly caused by *Mycobacterium tuberculosis* (MTB), is an infectious bacterial disease, with a major impact on global health. In this study, immunohistochemistry (IHC), acid-fast bacilli (AFB) culture and Ziehl-Neelsen (ZN) staining, techniques were compared on bronchoalveolar lavage (BAL) and bronchial washings (BW) with respect to sensitivity and specificity for detecting mycobacteria, taking culture as the gold standard.**

***Methods*: Consecutive BAL and BW specimens were included in the study, over a period of one year for which AFB cultures were available. Samples with diagnosis other than inflammatory pathology such as malignancies or inadequate samples were excluded. A total of 203 BAL and BW specimens from patients with age ranging from 14 to 86 yr were analyzed for the presence of mycobacteria. The utility and efficacy of ZN stain and IHC in detecting mycobacteria was tested using AFB culture as a gold standard.**

***Results*: Out of 203 cases, 10.3 per cent (n=21) were positive on AFB culture. Of these, 5.9 per cent (n=12) smears were positive for ZN stain, whereas IHC positivity was seen in 8.4 per cent (n=17) of the cases. ZN staining had a sensitivity of 57.1 per cent and a specificity of 100 per cent whereas, IHC had a sensitivity of 81 per cent and a specificity of 81.9 per cent.**

***Interpretation & conclusions*: Comparison with AFB culture (gold standard), IHC was found to be superior to ZN stain in terms of sensitivity, whereas ZN stain was found to be superior to IHC in terms of specificity. These findings therefore suggest that IHC may be a useful adjunct to ZN stain in the detection of mycobacteria in specimens from the respiratory tract.**

Key words Bronchoalveolar lavage - bronchoalveolar washing - culture - immunohistochemistry - mycobacteria

Tuberculosis is an infectious and contagious bacterial disease. It continues to be one of the most prevalent infectious diseases in India, with an incidence of 1.98 million cases per year[1]. It is estimated that one third of the world population is infected with *Mycobacterium tuberculosis* (MTB)[2] . As the incidence of multidrug resistant tuberculosis and HIV-associated tuberculosis, especially by atypical mycobacteria, is on the rise and majority of people with TB have latent infection, the development of new diagnostic and screening tools has become necessary in order to facilitate early diagnosis and control of the disease[3].

High specificity has been observed in the detection of mycobacteria in specimens such as

sputum, bronchoalveolar lavage (BAL) or induced sputum for the diagnosis of pulmonary tuberculosis[3,4]. In this study, three tests were compared, namely immunohistochemistry (IHC), culture of acid-fast bacilli (AFB) and Ziehl-Neelsen (ZN) staining, for the diagnosis of MTB, considering culture as the gold standard, to determine their efficacy, sensitivity as well as the ease of detection of mycobacteria.

Material & Methods

The study was carried in the department of Microbiology, Apollo Hospital, Hyderabad, Telangana, India. All consecutive BAL and bronchial washings (BW) specimen submitted to the department, over a period of one year (from May 2017 to April 2018) for which AFB cultures were available, were included in the study. Samples with a diagnosis other than inflammatory pathology like malignancies or inadequate samples were excluded. The study was approved by the Institutional Ethics Committee.

A total of 203 BAL and BW specimens were analysed from patients with age ranging from 14 to 86 yr. Giemsa, Papanicolaou and Ziehl-Neelsen (ZN) stains were performed on smears as per standard procedures[5]. ZN stain was done after destaining the Giemsa-stained smears in retrospective cases, while in prospective cases; it was done on fresh smears using the standard procedure. Hematoxylin and eosin (H and E) staining and IHC were done on cell blocks to determine the presence of mycobacteria.

Immunohistochemistry (IHC) was performed on cell blocks prepared from the respective BAL and BW using *M. tuberculosis* concentrated (catalogue no. CP 140 A; Biocare Medical, USA) and pre-diluted (1:200) rabbit polyclonal antibody (catalogue no. PP 140 AA; Biocare Medical) in an automated slide stainer, Ventana BenchMark® XT (Roche, USA) by following manufacturer's instructions. This antibody is reactive with mycobacteria species including *M. tuberculosis*, *M. avium*, *M. phlei* and *M. parafortuitum*. BD BACTEC MGIT (mycobacteria growth indicator tube) 960 automated mycobacterial detection system (Becton Dickinson, USA) was used for AFB culture. It was used as a gold standard for comparing the efficacy of ZN and IHC stains.

Results & Discussion

To the best of our knowledge, this is one of the few studies with such a large sample size comparing the efficacy of ZN stain and IHC for detecting mycobacteria

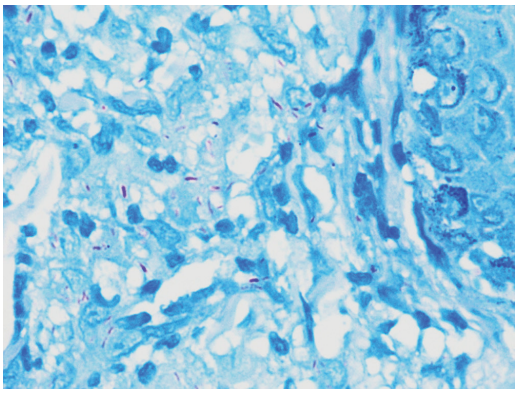

Fig. 1. Acid-fast bacilli (ZN stain, ×100).

on fluids from BAL and BW. Out of the 203 samples studied, 141 (69.50%) were male and 62 (30.50%) were female. Of these, 112 were BAL samples (55.17%) and 91 were BW (44.83%).

In the present study, 175 cases were reported as smears on cytology showing an inflammatory pathology and were negative for malignancy. These showed inflammation composed of neutrophils and lymphocytes admixed with benign squamous cells and ciliated columnar cells with alveolar macrophages. Ten of the 203 specimens were reported as smears showing inflammation with tuberculous etiology based on the presence of AFB on ZN stain. Thirteen were reported as negative for malignancy; three showed inflammation with fungal hyphae and two showed inflammation along with numerous macrophages.

Out of the 203 specimens, 21 were AFB culture positive, accounting for 10.3 per cent of the total number of cases. This included both typical and atypical mycobacteria. On ZN staining, the smears showing beaded, bright pink rods were reported as AFB positive (Fig. 1). Twelve out of 203 (5.91%) cases showed positivity on ZN staining. None of the samples showed false positivity (Table I).

The lowest ZN positivity of mycobacterial strains (0%) was reported by Radhakrishnan *et al*[6] and by Padmavathy *et al*[7], whereas the highest positivity of the same (50%) was reported by Purohit *et al*[8]. Typically, ZN staining is positive only when the bacillary count is more than 10,000 organisms/ml of the specimen[9]. Other reasons for false negativity could be technical problems leading to suboptimal staining. Antimycobacterial therapy can also alter capsule integrity to render organism non-acid-fast[10]; hence, partially treated smears may also be negative on ZN stain. As the AFB gets engulfed and phagocytosed by

Table I. Results of immunohistochemistry (IHC) and Ziehl-Neelsen (ZN) staining (n=203)

Results	IHC, n (%)	ZN, n (%)
True positive	17 (8.37)	12 (5.91)
False positive	33 (16.25)	0 (0)
True negative	149 (73.39)	182 (89.65)
False negative	4 (1.97)	9 (4.43)
Sensitivity of detecting mycobacteria (%)	81	57.10
Specificity of detecting mycobacteria (%)	81.90	100
Positive predictive value (%)	34	100
Negative predictive value (%)	97.40	95.30
Positive likelihood ratio	4.48 (95% CI: 3.08-6.48)	Infinity
Negative likelihood ratio	0.23 (95% CI: 0.10-0.56)	0.43 (95% CI: 0.26-0.70)

CI, confidence interval

the macrophages, only fragments of bacilli are left in the lesion which are not identified by ZN stain[11]. Due to intense phagocytic activity of the macrophages in tuberculous granulomas, the morphological characteristics of AFB often get distorted. This may account for the low detectability on ZN staining similar to the finding in the present study.

The IHC staining of bacilli showing different staining patterns is shown in Figure 2. IHC positivity was reported as intracellular as well as extracellular fine brown granularity or as slender rods (Fig. 2C). In the present study, 50 out of 203 (24.63%) showed positive staining on IHC, 17 (8.37%) were true positive *i.e*. these cases were culture positive. However, 33 cases were false positive (16.25%) *i.e*. these cases were IHC positive but culture negative (Figs 2G and H).

The bacilli were mostly seen in clusters (Figs 2C and D), however, a few were also seen as singly scattered (Fig. 2A). Two fluids which showed histiocytes and neutrophils on smears, particularly displayed intracellular positivity in histiocytes or within aggregates of histiocytes and neutrophils (Figs 2C and D). In these smears, AFB were seen on ZN staining. The contaminants and dark brown course granularity resulting in false positive interpretation are shown in Figures 2G and H.

The lowest IHC positivity of 64 per cent was reported by Mustafa *et al*[12], whereas the highest positivity of 100 per cent was reported by Barbolini *et al*[13] and Goel and Budhawar[14] on tissue sections. The possible reasons for high false positivity in this study could be contamination due to various reasons such as unsterile sampling technique, unsterile containers, long storage time of sample or contamination during processing. For example, the use of egg albumin stored for a long time for preparing cell blocks is a good medium for the growth of contaminants. The contaminants were recognized as short, stubby rods (Fig. 2B) rather than slender beaded rods or fine granularity. Usually, these were seen in groups or big clusters, often extracellular.

The other reason for false positivity could be the use of the polyclonal antibody, which might have resulted in the uptake of the stain by mycobacteria other than MTB. Morphologically, typical and atypical mycobacteria cannot be distinguished on immunohistochemical stain[15]. This indicates that the use of polyclonal antibody for IHC may increase the chances of false positivity. IHC stains the dead as well as fragmented bacilli. It has the capacity to stain antigenic dust as well, which is not stained by ZN stain[16]. This may be the cause of the higher sensitivity of IHC.

In this study, differentiating IHC stain from intracellular anthracotic pigment (Fig. 2E) and haemosiderin pigment (Fig. 2F) in the macrophages was a challenge. It was found that the anthracotic pigment appeared darker and blackish as compared to the positive IHCstain which is brown, and haemosiderin pigment showed coarse staining pattern only within macrophages.

Three cases were positive for AFB culture as well as ZN stain; however, these did not show IHC positivity. The reason for this false negativity cannot really be ascertained but could possibly be due to technical issues.

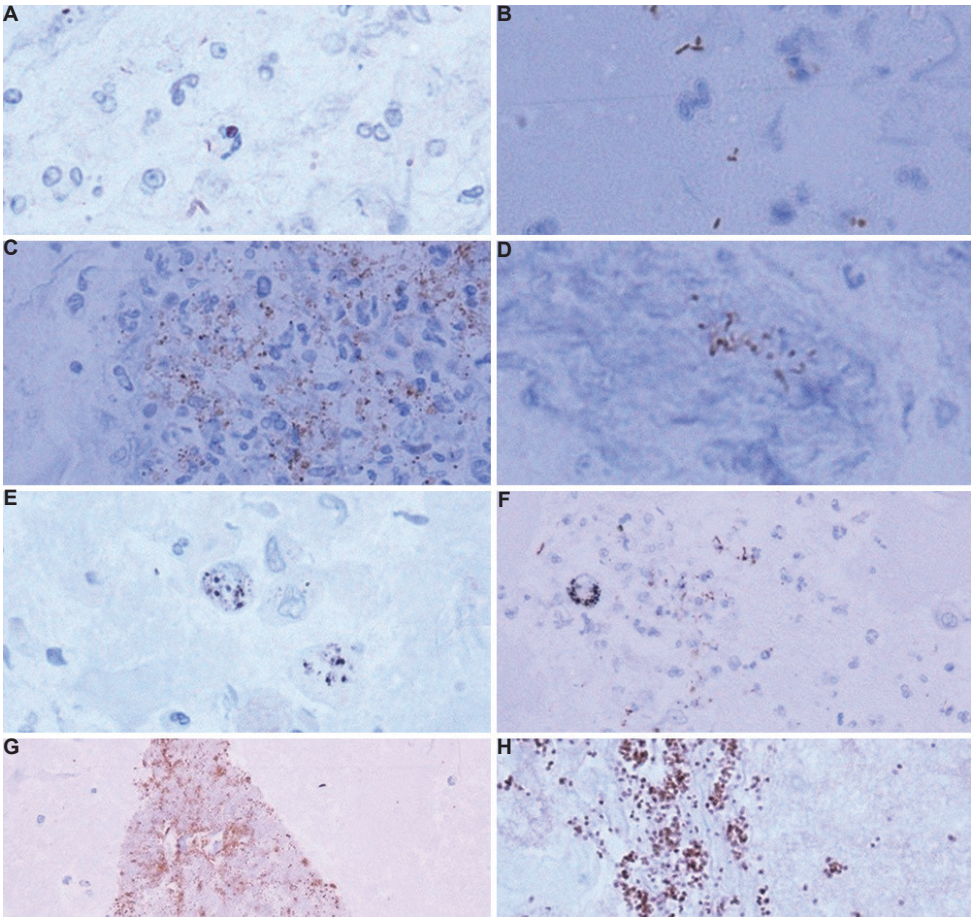

Fig. 2. (**A**) Weak IHC positivity showing bacilli in clusters (×100). (**B**) Extracellular, stubby, dark bacilli, possibly contaminant (×100). (**C**) IHC positive bacilli entrapped in cluster of histiocytes and neutrophils (×40). (**D**) Cluster of IHC positive bacilli (×100). (**E**) Macrophages showing intracellular black anthracotic pigment (IHC, ×100). (**F**) IHC-positive bacilli and central macrophage showing blackish intracellular anthracotic pigment (×40). (**G**) Contaminant, (IHC, x40) (**H**) Dark brown course granularity, false positive (IHC, ×40) IHC, immunohistochemistry.

The result of IHC staining (81% true positivity) in the current study is similar to the studies by Baba *et al*[17], and Purohit *et al*[8]. The results of studies using monoclonal antibodies however, appear to be superior, with 100 per cent IHC positivity[6,13]. As reported by Purohit *et al*[8], the overall sensitivity and specificity of IHC with monoclonal antibodies such as anti-MPT64 were 92 and 97 per cent, respectively, while the corresponding values for anti-BCG were 88 and 85 per cent. Comparison with various studies on IHC for MTB is shown in Table II.

The sensitivity and specificity of ZN stain was 57.1 and 100 per cent, respectively, whereas for IHC staining these were 81 and 81.9 per cent, respectively. The combined ZN stain and IHC results had a sensitivity of 69 per cent and specificity of 90.1 per cent. The likelihood ratio positive and likelihood ratio negative for IHC and ZN stains are included in Table I.

The sensitivity of IHC depends on various factors such as distribution of mycobacterial antigen within the granuloma, the clinical stage of disease, duration of anti-tubercular treatment received prior to sampling and specificity of the primary antibody[18]. The reason for 100 per cent positivity in few previous studies could be the use of monoclonal antibody.

Most of the studies published in literature have done studies on tissue sections, where PCR was used for tuberculosis as a standard for comparison, unlike the present study in which fluids were used as samples to compare ZN and IHC results with AFB culture, which is considered a gold standard in the diagnosis of tuberculosis. Culture with the new and robust BACTEC machine is not only considered reliable but also quick as compared to conventional culture methods. Studies comparing the utility of IHC and ZN stain in detecting mycobacteria in tissue sections are included in Table II.

Table II. Comparison of immunohistochemistry and Ziehl-Neelsen staining results in literature with our study

Study	Number of cases	Type of antibody used	ZN positivity (%)	IHC positivity (%)	ZN sensitivity (%)	ZN specificity (%)	IHC sensitivity (%)	IHC specificity (%)
Radhakrishnan *et al*[6], 1991	10	IgG anti-mycobacterial Ab	0	100	NA	NA	NA	NA
Mukherjee *et al*[19], 2002	50	Polyclonal anti-BCG	44	87	NA	NA	NA	NA
Mustafa *et al*[12], 2006	55	Polyclonal anti-BCG	0	64	NA	NA	90	83
Baba *et al*[17], 2008	25	Ani-MPT and anti-BCG	NA	80	NA	NA	81	100
Kundu *et al*[18], 2014	100	Polyclonal anti-BCG	23	72	31	96	95	35
Barbolini *et al*[13], 1989	23	Monoclonal antibodies, 60.15, 61.3	15	100	NA	NA	NA	NA
Luo[20], 1990	137	Polyclonal anti-BCG	34	69.3	NA	NA	NA	NA
Padmavathy *et al*[7], 2005	50	Polyclonal anti-BCG	0	68	NA	NA	NA	NA
Purohit *et al*[8], 2007	120	Ani-MPT and anti-BCG	50	80	13	NA	92	97
Humphrey and Weiner[21], 1987	59	Polyclonal anti-BCG	NA	77.7	NA	NA	NA	NA
Oliveira *et al*[22], 2004	3	NA	NA	100	NA	NA	NA	NA
Goel and Budhwar[14], 2007	36	Monoclonal	NA	100	NA	NA	NA	NA
Present study (on BAL and BW)	203	Rabbit polyclonal antibody	5.91	24.63	57	100	81	82

NA, not available; BAL, bronchoalveolar lavage; BW, bronchial washings; IHC, immunohistochemistry; ZN, Ziehl-Neelsen; Anti-BCG, anti-Bacillus Calmette-Guérin

According to literature, IHC staining gives superior results as compared to the ZN staining, with a positivity of 100 per cent using monoclonal antibody[13]. In our study also, the sensitivity of IHC (81%) was found to be better than ZN staining (57.10%). But as the morphology of mycobacteria on IHC stain is varied, distinguishing these from contaminants was a challenge and the main limitation of this study. The false-positive results were high, thus limiting the use of this method alone for the detection of mycobacteria in fluids.

Overall, based on the study findings, AFB culture as a gold standard, neither IHC nor ZN stain appears to be useful as independent methods for the detection of mycobacteria, but these can be used as adjuncts to each other along with other diagnostic tests. A combination of tests can give better and more accurate results for the detection of mycobacteria in bronchoscopic fluids and aid in timely institution of therapy. Further studies with a larger sample size are, however, needed to validate the findings of this study and also to validate the use of IHC as an independent diagnostic test in the diagnosis of tuberculosis.

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