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Papanicolaou induced fluorescence, Ziehl-Neelsen and Auramine O stains on lymph node fine needle aspiration biopsy specimens from children: A comparative study

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Background: The rapid diagnosis of extrapulmonary tuberculosis in children remains challenging. The presence of enlarged lymph nodes provides an opportunity to obtain diagnostic material through fine needle aspiration biopsy (FNAB). Mycobacterial culture, traditionally the reference standard, has a slow turnaround time and PCR-based methods are not widely available in developing countries. Direct visualization of mycobacteria on microscopy can be a rapid method to confirm the diagnosis. This study compared three staining methods to visualize mycobacteria.

Methods: Hundred FNAB specimens from persistently enlarged lymph nodes in children, clinically suspicious for tuberculosis, were evaluated for the presence of mycobacteria by three staining methods: Papanicolaou induced fluorescence (PIF) and Auramine O staining using fluorescence microscopy and Ziehl-Neelsen (ZN) staining using conventional light microscopy. These methods were evaluated against mycobacterial culture.

Results: PIF positivity was 30%, with 38% and 48% for Auramine O and ZN respectively. The combined ZN/PIF positivity was 56%. The highest diagnostic accuracy (73%) was demonstrated by ZN alone and in combination with PIF, with PIF alone showing the lowest (49%) accuracy. Although the combined test showed the highest sensitivity, it had the lowest specificity, while ZN was significantly more sensitive than both other staining modalities. No statistical difference in specificity was seen among the tests.

Conclusion: This study suggests that Auramine O staining on previously ZN stained slides does not significantly improve diagnostic accuracy. While currently widely available methods of direct visualization of mycobacteria suffer from low sensitivity, the ZN stain remains a useful diagnostic test, particularly in resource-constrained countries.

KEYWORDS
children, fine needle aspiration biopsy, lymphadenopathy, tuberculosis, Ziehl-Neelsen

1 | INTRODUCTION

In resource-constrained countries, pediatric tuberculosis (TB) is a diagnostic challenge due in part to the problems attributed to its clinical presentation exacerbated by the parallel human immunodeficiency virus (HIV) pandemic and difficulties with microbiological confirmation.[1]

Unlike adults, where definitive diagnosis of *Mycobacterium tuberculosis* in sputum depended on identification of the organism by various staining methods, including Ziehl-Neelsen (ZN) and Auramine O, poor

sputum production in children, difficulty in obtaining gastric aspirate specimens and the paucibacillary nature of disease have resulted in poor diagnostic yield.[2] The ZN stain is specific but has low sensitivity.[3] Mycobacterial culture has for long been the reference standard but its main limitation is a slow turnaround time of 2 to 6 weeks.[4] Xpert MTB/RIF (Cepheid, Sunnyvale, California), a real-time polymerase chain reaction assay, has shown remarkable sensitivity and specificity and was recommended by the World Health Organization (WHO) in 2013 as the first line diagnostic procedure for TB lymphadenopathy (LAD).[5] Persistent peripheral LAD is the most common sign of extrapulmonary TB in children.[6] These enlarged lymph nodes are usually easily accessible for fine needle aspiration biopsy (FNAB) and these specimens provide a better bacteriological yield than sputum and gastric aspirates.[7] However, the Xpert MTB/RIF assay is not freely available in resource-constrained countries.

This study compares three staining methods for mycobacteria in LAD specimens: Papanicolaou induced fluorescence (PIF) and Auramine O staining both using light emitting diode (LED) fluorescent light and ZN staining using conventional microscopy.

2 | MATERIAL AND METHODS

Archived cytology slides at the National Health Laboratory Services, Tygerberg Hospital for the period 2003 to 2008 were used for this study. The FNABs were from children below the age of fifteen years with persistently enlarged lymph nodes and clinically suspected to have tuberculosis. Informed consent for all study subjects was obtained from the legal guardian prior to FNAB.

At the time of collection, the standard operating procedure for FNAB was used by the aspirator in the clinic following administration of light sedation by and at the discretion of the referring clinician. FNAB was performed using a 10 mL syringe and 22 or 23-gauge needle. Four slides (conventional smears) were made before the needle was rinsed in a liquid mycobacterial culture medium (MGIT 960; Becton Dickinson, Franklin Lakes, New Jersey). Two slides were air-dried for Giemsa and ZN staining and two were spray fixed for Papanicolaou staining. The best slides in terms of quantity and quality of cytological material were selected for further evaluation by methods of direct mycobacteria visualization. For a previous study,[8] the Papanicolaou stained slides were subjected to screening under a royal blue LED light source (peak wave length 450 nm), to visualize any bacilli fluorescing with the typical morphology of mycobacteria (Figure 1).

ZN stains were performed (Figure 2) and screened as part of routine laboratory practice shortly after the specimens were collected.

For the current study, the ZN slides were de-stained and stained with Auramine O and viewed under the same LED light source that was originally used for PIF.

The raw data were entered on a spreadsheet and performance calculations, including test sensitivity, specificity, and predictive values, were undertaken using Statistica version 8 to compare the diagnostic performance of the three modalities to the reference standard of
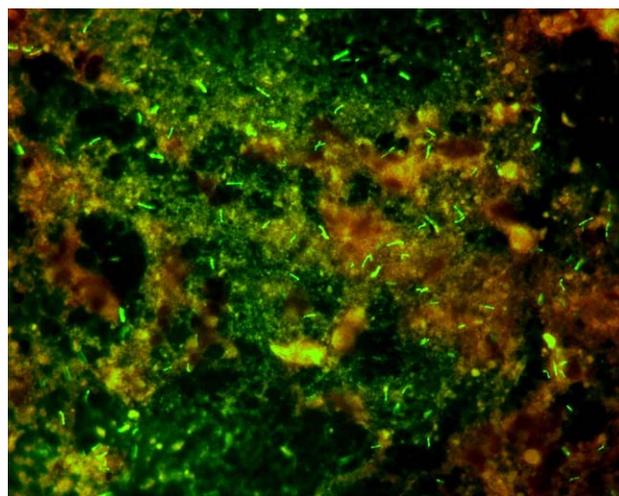

FIGURE 1 Papanicolaou induced fluorescence of mycobacteria in a lymph node aspirate (Papanicolaou, original magnification 1000× with a wide-band blue excitation filter) [Color figure can be viewed at wileyonlinelibrary.com]

culture. The study was approved by the Human Research Ethics Committee of Stellenbosch University.

3 | RESULTS

A total of 100 of 121 archival FNAB specimens were selected from a previous study[4] in approximately the same ratio of 1:3 culture negative: positive. The age ranged from 2.6 months to 15 years with a mean of 3.6 years (standard deviation 3.5) and an equal gender distribution. Only 52% had known HIV status, of these, 21 tested positive and 31 were HIV negative. The most frequently involved nodes were in the head and neck region (60%) while axillary nodes were accounted for in 24%. Of the 100 specimens evaluated for microbiology, culture was positive in 69% of cases of which 71% were confirmed as *M. tuberculosis*, 27.5% *M. bovis BCG* and 1.5% as nontuberculous mycobacteria. All positive MGIT samples underwent the MTBDR*plus* assay v1.0 (Hain
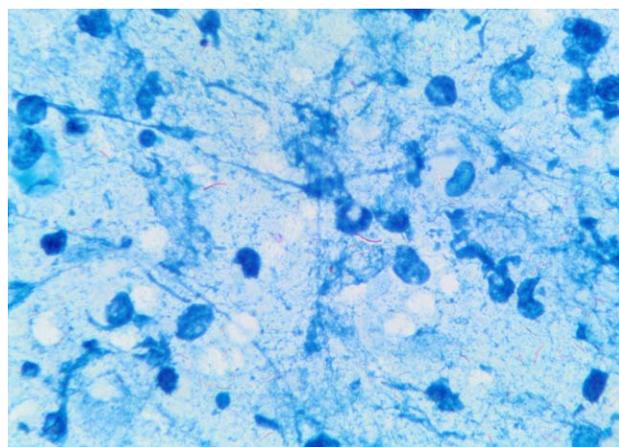
FIGURE 2 Ziehl-Neelsen stain demonstrating the typical appearance of acid-fast bacilli (original magnification 1000×) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The accuracy, sensitivity, and specificity for each of the tests (ZN, PIF and Auramine O)

Comparison with FNAB culture	Accuracy	Sensitivity	Specificity
ZN	73%	65%	90%
PIF	49%	35%	81%
Auramine O	61%	49%	87%
Combined (ZN and PIF)	73%	71%	77%
Testing differences with:	Accuracy P-value (95% CI)	Sensitivity P-value (95% CI)	Specificity P-value (95% CI)
ZN vs PIF	0.001[a]	0.000[a]	0.1081
ZN vs Auramine O	0.098	0.032[a]	0.6576
PIF vs Auramine O	0.118	0.063	0.3348

[a]Statistically significant.

CI, confidence interval; FNAB, fine needle aspiration biopsy; PIF, Papanicolaou induced fluorescence; ZN, Ziehl-Neelsen stain.

Lifesciences, Hehren, Germany) for identification of *M. tuberculosis* complex and first-line drug susceptibility testing (isoniazid and rifampicin). The mycobacterial isolates were further identified as *M. tuberculosis*, *M. bovis* BCG or nontuberculous mycobacteria (NTM) in a reference laboratory using the GenoType® MTBC and GenoType® Mycobacterium CM and, where necessary, the GenoType® Mycobacterium AS (Hain Lifesciences, Hehren, Germany) line probe assays, respectively.

All three tests (ZN, Auramine O and PIF), as well as the combined ZN/PIF were evaluated against mycobacterial culture. Of the 100 FNAB specimens, PIF positivity was 30%, with 38% and 48% for Auramine O and ZN respectively. The combined ZN/PIF positivity was 56%.

Table 1 demonstrates the accuracy, sensitivity and specificity of the three tests and those of the combined PIF/ZN in comparison with one culture.

The highest accuracy (73%) was demonstrated by ZN alone and in combination with PIF, with PIF alone showing the lowest (49%) accuracy. Although the combined test showed the highest sensitivity, it had the lowest specificity, while ZN was significantly more sensitive than both other staining modalities. No statistical difference in specificity was seen among the tests.

4 | DISCUSSION

This study compared three staining methods for mycobacteria in FNAB material obtained from children: PIF and Auramine O using LED fluorescent light and ZN using conventional light microscopy. In sputum, the Auramine O stain, widely used by microbiologists, has previously been reported to be 10% more sensitive than ZN in detecting mycobacteria.[9] PIF has previously been reported to have a similar diagnostic performance in FNAB material obtained from children compared to ZN.[10]

The results of this study suggest that ZN staining has superior diagnostic performance compared to Auramine O and PIF in lymph node FNAB in children. Potential limitations of this study include its retrospective nature, the uncertain effect that staining and destaining of the slides had on the eventual performance of the Auramine O stain and the fact that slides filed for up to 12 years had to be used for Auramine O staining. The reason why the same slides were used for the Ziehl-Neelsen and Auramine O stains was to compare the different methods on material of similar quality. Our material was limited to conventional smears (no liquid based cytology available) and because the quality of the material obtained can vary markedly from one needle pass to another, we had to use slides made from the same pass for all three methods. This is in contrast to sputum samples or liquid based cytology where two or more slides of similar quality can be prepared from the same material. We hope this study would stimulate other researchers to do a side by side comparison of these methods on FNA material obtained by liquid based cytology.

In contrast to our findings, Thakur et al. reported auramine-rhodamine staining to be more sensitive than ZN in lymph node aspirates using conventional smears but found ZN to be more specific.[11] Factors that can pose problems in the detection of *Mycobacteria* using fluorescent methods include fluorescence that can be displayed by some nonmycobacterial microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Nocardia* and yeasts, the presence of small naturally fluorescent particles in smears or in poor quality coverslips and even air-drying artifacts in Papanicolaou stained smears.[8,11] In cytology laboratories Auramine O is not used routinely, and use of this stain would add cost and complexity. Other negative factors are the potential carcinogenic effect of Auramine O in humans and the need for slides to be screened within a short period of preparation as the fluorescence diminishes over time. In addition, Auramine O permanently destroys cell morphology making further morphological review impossible. PIF and Auramine O fluorescence also require the use of a fluorescent microscope and a dark room for screening.

The Xpert MTB/RIF assay would be the ideal diagnostic modality in a high risk setting, providing a much higher sensitivity, but the technology is not yet a reality in many resource-constrained countries. The WHO 2013 policy update on the use of this technology in extrapulmonary TB[5] reported a pooled sensitivity across studies in adults of 84.9%

(95% CI, 72.1–92.4%) and a pooled specificity of 92.5% (95% CI, 80.3–97.4%). In children the pooled sensitivity of the Xpert MTB/RIF assay compared to culture as the reference standard was 86% (95% CI, 65–96%) and the pooled specificity was 81%.

The ZN stain with a sensitivity of 65% and a specificity of 90%, although inferior to the Xpert MTB/RIF, is easy to perform, stable, allows for identification of morphology, requires no additional equipment and continues to be effective in the reliable identification of mycobacterial infection in FNAB specimens in children.

In our opinion, to improve the diagnostic yield from FNAB in suspected extrapulmonary TB in cytology laboratories, investment in PCR-based methods such as the Xpert MTB/RIF assay would be a better option than investing in fluorescent microscopes to perform additional Auramine O staining or to replace ZN staining with Auramine O staining.

CONFLICT OF INTERESTS

None declared.

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REFERENCES

[1] Marais BJ, Graham SM, Cotton MF, Beyers N. Diagnostic and management challenges for childhood tuberculosis in the era of HIV. *J Infect Dis.* 2007;196:S76–S85.

[2] Eamranond P, Jaramillo E. Tuberculosis in children: reassessing the need for improved diagnosis in global control strategies. *Int J Tuberc Lung Dis.* 2001;5:594–603.

[3] Wright CA, Warren RM, Marais BJ. Fine needle aspiration biopsy: An undervalued diagnostic modality in paediatric mycobacterial disease. *Int J Tuberc Lung Dis.* 2009;13:1467–1475.

[4] Coetzee L, Nicol MP, Jacobson R, et al. Rapid diagnosis of pediatric mycobacterial lymphadenitis using fine needle aspiration biopsy. *Pediatr Infect Dis J.* 2014;33:893–896.

[5] WHO. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children: Policy update. *World Heal Organ.* 2013:1–79.

[6] Marais BJ, Gie RP, Schaaf HS, Hesseling a. C, Enarson D. a, Beyers N. The spectrum of disease in children treated for tuberculosis in a highly endemic area. *Int J Tuberc Lung Dis.* 2006;10:732–738.

[7] Wright CA, Hesseling AC, Bamford C, Burgess SM, Warren R, Marais BJ. Fine-needle aspiration biopsy: A first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy? *Int J Tuberc Lung Dis.* 2009;13:1373–1379.

[8] Van Wyk AC, Marais BJ, Warren RM, Van Wyk SS, Wright CA. The use of light-emitting diode fluorescence to diagnose mycobacterial lymphadenitis in fine-needle aspirates from children. *Int J Tuberc Lung Dis.* 2011;15:56–60.

[9] Steingart KR, Henry M, Ng V, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet.* 2006;6(September):570–581.

[10] Wright CA, van der Burg M, Geiger D, Noordzij JG, Burgess SM, Marais BJ. Diagnosing mycobacterial lymphadenitis in children using fine needle aspiration biopsy: cytomorphology, ZN staining and autofluorescence - making more of less. *Diagn Cytopathol.* 2008;36:245–251.

[11] Thakur B, Mehrotra R, Nigam JS. Correlation of various techniques in diagnosis of tuberculous lymphadenitis on fine needle aspiration cytology. *Pathol Res Int.* 2013;2013:1.