

VALIDITY OF ACID-FAST SMEAR OF GASTRIC ASPIRATES FOR THE DIAGNOSIS OF CHILDHOOD PULMONARY TUBERCULOSIS AMONG HUMAN IMMUNODEFICIENCY VIRUS-INFECTED CHILDREN

ABSTRACT

Background/Aims: Gastric aspirate specimen is accepted as an alternative specimen of choice to sputum in the diagnosis of childhood pulmonary tuberculosis (TB); and specimen microscopy is usually the only available bacteriologic confirmatory test for streaming cases into the National TB Control Programme treatment protocol. Doubts have been expressed about the continued relevance of this test among the HIV-infected. These doubts are based on observations that the bacteriologic yield of acid-fast smears of gastric aspirate specimens from these patients is markedly reduced. This study is aimed at determining the sensitivity and specificity of acid-fast smear of gastric aspirates among the HIV-infected and compare with those of the HIV-uninfected.

Design, Place and Duration of Study. Cross-sectional study design was adopted. . Suspected tuberculosis patients registered in the paediatrics department of University of Benin Teaching Hospital were prospectively and consecutively recruited from January 2010 to January 2012.

Methodology: A total of 263 children were recruited. Voluntary counseling and testing for HIV was done for each child. Two to three gastric aspirate specimens were also collected from each child using standard GA collection protocols. On each specimen, Zeihl-Neelsen (ZN) staining and culture on Ogawa medium were done. After two or more weeks of incubation, typical acid-fast bacilli isolates on Ogawa medium, which failed to grow on para-nitrobenzoic acid-Ogawa (PNB-Ogawa) media were taken as positive culture for MTBC. MTBC Culture was taken as the "Reference test" for calculation of sensitivity and specificity proportions.

Results: Of the 263 children surveyed 116(44.0%) were HIV-infected while 147 (56.0%) of them were HIV-uninfected. Among HIV-infected patients, 18 of the 47 culture – positive cases were positive by AFB smear; giving a sensitivity of 38.3%. In the same group of patients, 66 of the 69 culture-negatives were also AFB-negative, yielding a specificity of 95.7%. Among the HIV-uninfected patients, 11 of the 50 culture-positive results were also smear-positive, giving a sensitivity of 22.0%. In the same group of patients, 96 of the 97 culture-negatives were

also AFB smear-negative; yielding a specificity of 99.0%. The sensitivity value of the acid-fast smear in the HIV-infected group of patients was significantly higher than the value in the HIV-uninfected group (38.5 vs 22.0)[p = 0.0401]. The difference in specificity proportions between the HIV-infected group and the HIV-uninfected group (95.7 vs 99.0) was not statistically significant (p=0.496).

Conclusion: While there was no significant difference between the specificity scores of acid-fast smear between the HIV-infected and the HIV-uninfected, the sensitivity of the test was significantly higher among the HIV infected group.

KEY WORDS: Gastric aspirates, validity, sensitivity, specificity, acid-fast smear, Human Immunodeficiency Virus, tuberculosis, *Mycobacterium tuberculosis* complex

INTRODUCTION

Sub-Saharan Africa has been reported to bear about thirty-percent (30%) of the worldwide TB burden [1, 2]. In the same region, also resides the vast majority of people living with Human Immunodeficiency Virus (HIV)/Acquired immune deficiency syndrome (AIDS) (PLWHA) [3]. Consequently, about 80% of worldwide burden of HIV-TB co-morbidity is borne by this sub-region [4]. Tuberculosis is the leading cause of death among these HIV-infected [4, 5]. The disaster resulting from convergence of HIV and TB, along with multi-drug resistant TB, was the basis of the declaration of TB as a global emergency by WHO in 1993 [6]. The disease was later declared an emergency in Africa in 2005 and a national emergency by the Federal Government of Nigeria in 2006 [6, 7]. The response to the TB emergency, though satisfactory among adults, has not been satisfactory in children as a result of case detection challenges, and this is partly due to the peculiar low bacteriologic yield of paediatric specimens [8, 9]. There are several reports to the effect that the bacteriologic yield is further reduced by HIV co-infection [10, 11]. The reduced sensitivity has been attributed to the reduced capability of the immunocompromised to effect the characteristic chronic granulomatous inflammatory responses to MTBC infection [10].

In this region where there is high prevalence of both environmental and colonizing non-tuberculous mycobacteria, there is a high incidence of diseases caused by non-tuberculous mycobacteria (NTM) among the immunocompromised [11, 12]. Moreso, the frequency of NTM disease in stage III-IV HIV can be as high as TB, if not more [13]. Thus, questions of specificity of GA acid-fast smears also increase in HIV-TB co-morbidity because of the enhanced possibility of false-positive results due to increased risk of NTM disease in this sub-population [14].

Revalidating known diagnostic approaches to childhood tuberculosis is an important aspect of the WHO research agenda [9]. This study is aimed at assessing the effect of HIV-infection on the validity of acid-fast smears for the diagnosis of pulmonary TB in children, with a view to ascertaining its continued relevance.

METHODOLOGY

The study area was the University of Benin Teaching Hospital (UBTH), Benin. Benin is the capital city of Edo state and lies in the mid-western zone of Nigeria.

Cross-sectional study design was adopted. Study period was between January 2010 and January, 2012. Study population was patients who were registered in the paediatric department of UBTH and who had features suggestive of pulmonary tuberculosis.

Approval was sought and obtained from the ethics and research committee of this hospital for this research work. Informed written consent was obtained from the parents on behalf of their children. Only consenting patients were investigated. A total of 263 patients were prospectively and consecutively recruited. Inclusion criteria included illness duration of more than two (2) weeks; chronic cough in association with weight loss or lack of weight gain; positive history of contact with a known TB case; previous TB diagnosis; positive tuberculin sensitivity test (TST) $\geq 10\text{mm}$; erythrocyte sedimentation rate (ESR) $\geq 100/\text{hour}$; and typical chest radiographic findings. Patients who were already on anti-TB treatment were excluded.

Data collection was based on a semi-structured researcher-administered study questionnaire, clinical observation and laboratory investigations.

Voluntary HIV counseling and testing was done for all the patients. Gastric aspirate specimens were

tested for acid fast smear positivity and MTBC culture positivity.

Procedures: For Gastric aspirate (GA) specimen collection, each mother was instructed not to feed the child again after supper and, also, not to allow the child out of bed until the specimen was collected. By 6.00am, each child was immobilized on a firm surface, the distance between the nose and the stomach was measured. After wiping the nostrils with dry swab, a naso-gastric tube was passed. As the tube reached the throat, a puff on the face of the child would stimulate swallowing reflex that enabled extension of the tube into the stomach. At least 5-10 mls of aspirate was collected each time in each case. Any reactive vomitus was also collected. Three consecutive specimens were collected from each patient every morning into properly labeled universal bottles.

The specimens were tightly covered and promptly delivered to the laboratory, neutralized with sodium bicarbonate. pH was first assessed before neutralization, which was effected by gradual addition of sodium bicarbonate and monitoring pH change with the pH meter. The neutralized specimens were promptly refrigerated and temperature maintained at about 4-6°C till processing was done. Processing (microscopy and culture) of the neutralized specimens was done as early as possible but was never up to 24 hours from time of collection. Specimen manipulations were done in a biosafety cabinet type 2; and care was taken to avoid exposure to direct sunlight and heat.

A set of smears were made with sediments following decontamination with 4% NaOH and centrifugation at relative centrifugal force (RCF) of 3000 X gravity(g) for 15minutes. The smears of the sediments were prepared by placing 1-2 drops of homogenized sediment on a slide and letting it air-dry.

Positive control stained slides were prepared from skimmed milk suspension of known *M. tuberculosis* isolates. Negative control slides were made from Z-N staining of a smear of egg albumin. For each new batch of stains, new control Z-N staining slides were prepared. Fixing the smear with heat was achieved by placing the air-dried smear on a hot plate set at 70°C for 1 hour. Standard procedure and precautions were followed in the Z-N staining.

The stained slide was viewed with X100 (oil-immersion) objective of a compound microscope. The acid-fast bacilli were seen as straight or bent pink-red rods which could be banded due to differential staining of different sections of the individual bacilli. They were seen also in clusters.

148 Grading followed CDC standards [15]:
 149 >9 bacilli / Oil immersion field = 4+; 1 – 9 bacilli / Oil immersion field = 3+; 1 – 9 bacilli / 10 Oil
 150 immersion fields = 2+; 1 – 9 bacilli / 100 Oil immersion fields = 1+; 1-3 bacilli/300 Oil immersion fields = doubtful,
 151 repeat ; 0 bacilli / 300 Oil immersion fields = No AFB seen.

152 Grades of smear-positive findings, 1+ to 4+, from at least one of the three patient's samples were taken as
 153 positive results. A smear – positive finding of “doubtful” grade required at least another smear-positive sample
 154 from the same patient for an impression of positive result to be made.

155 For GA culture, the decontaminated, centrifuged and homogenized sediment was used to inoculate
 156 acidified Ogawa medium which was incubated at 37°C in CO₂ enriched ambient air for the first week
 157 and then in ambient air for the rest of 4 - 8 weeks. Culture bottles were placed horizontally for the first
 158 24 hours to enable absorption of inocula; after which they were placed upright.
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 162 Sterile Ogawa media were used as negative controls. There were no positive culture controls, as use of positive
 163 controls is currently being discouraged in order to prevent contamination [15].

164 Cultures were examined every other day for the 1st 7 days within which rapidly growing mycobacteria
 165 and other contaminants were detected. Culture contamination rate was 3%. Assessment of contamination
 166 was based on observation of growth before two (2) weeks, observation of growth in association with
 167 media deterioration, Gram stain results, and Z-N staining results.
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 171 Subsequently, cultures were examined weekly for the remaining 7 weeks till growth were detected.
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 173 Cultures were reported as negative and discarded after 8 weeks of incubation.

174 Isolates appearing after 3 or more weeks of inoculation and showing rough, non-pigmented colonies,
 175 and Z-N stain positivity were presumed to represent pathogenic species of Mycobacterium
 176 tuberculosis complex (MTBC). To further affirm that the isolate is MTBC and not non-tuberculous
 177 mycobacteria (NTM), a suspension of the isolates in water was used to inoculate an Ogawa medium
 178 containing 500µg/ml para-nitrobenzoic acid (PNBA). Absence of growth in this medium was in
 179 keeping with MTBC. Only positive cultures were sub-cultured in PNBA-Ogawa media.

185 HIV status was assessed by examining the respondents' plasma by using two different kits: Determine®
 186 HIV1/2 kit, a visually read immunoassay (Inverness Medical Innovations South Africa Ltd, South
 187
 188

189 Africa); and HIV 1 & 2 STATPAK Assay kit, which is an immunochromatographic test for the
 190 qualitative detection of HIV-1 and HIV-2 antibodies (CHEMBIO Diagnostic system, INC, New York,
 191 USA). Manufacturers' instructions were strictly adhered to in carrying out the tests. The tests had built-
 192 in quality controls.

196 Data Analysis

197 SPSS version 16 software was used in the analysis. Data was analyzed with a view to calculating the
 198 sensitivity and specificity of acid-fast smear of gastric aspirates in the HIV-infected sub-population and
 199 compare with values among the HIV-uninfected sub-population.

202 Sensitivity was defined as the proportion of smear-positive cases among culture-proven cases; while
 203 specificity was defined as the proportion of smear-negative cases among culture-negative cases.

205 Tests of statistical significance of the differences in sensitivity and specificity proportions between the
 206 HIV-infected and the HIV-uninfected groups were done. Level of significance was 0.05.

208 RESULTS AND DISCUSSIONS

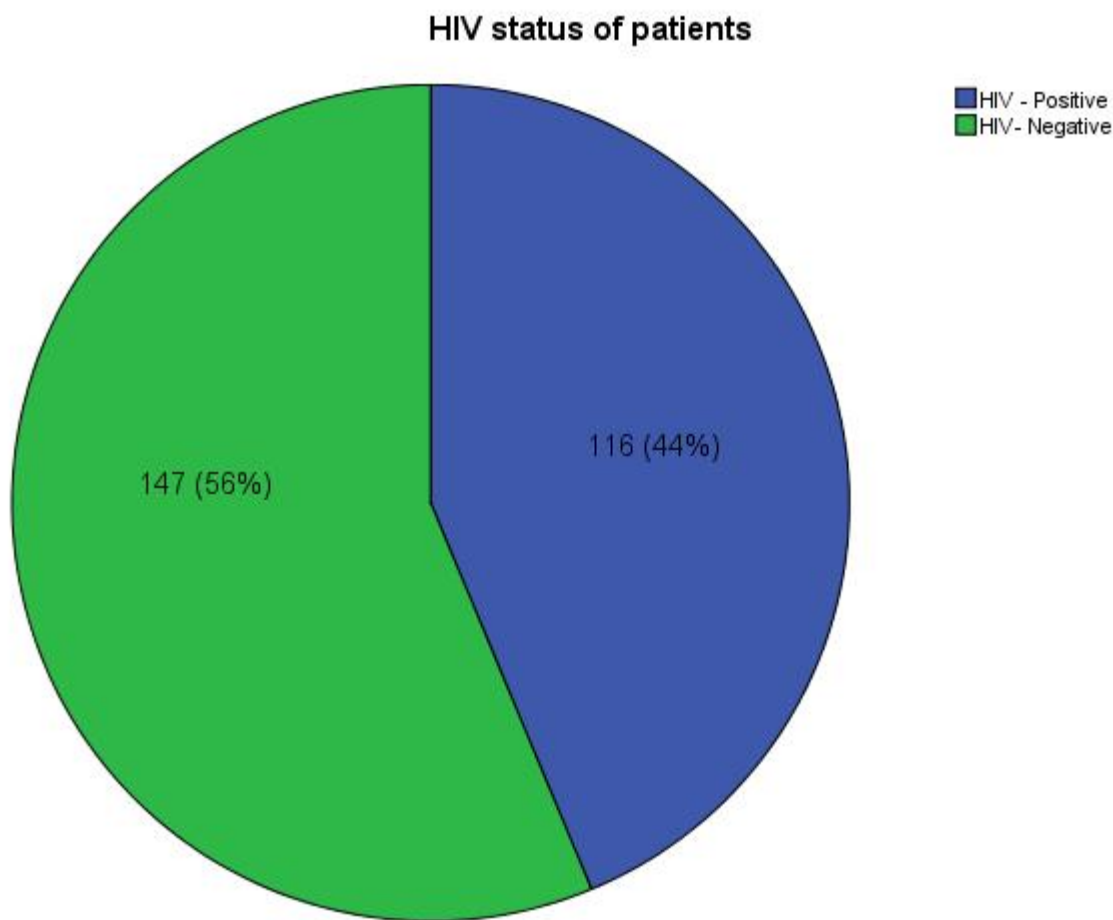
210 Gastric aspirate samples were collected from a total of 263 symptomatic paediatric patients who had features
 211 suggestive of pulmonary tuberculosis. One hundred and thirteen (113) [43.0%] of the patients were males while
 212 150 [57%] were females. (TABLE 1) The individual ages ranged from 3months to 10 years; while their mean age
 213 was 2.8±2.6 years.

214 **TABLE 1: SEX AND AGE-GROUP DISTRIBUTION OF THE PATIENTS**

AGE(YEARS)	NUMBER OF ALL PATIENTS (%)	NUMBER OF MALES (%)	NUMBER OF FEMALES (%)
≤ 2.0	152(57.8%)	65	87
2.1 – 4.0	57(21.7%)	23	34
4.1 – 6.0	30(11.4%)	10	20
6.1 – 8.0	6(2.3%)	1	5
8.1 – 10.0	18(6.8%)	11	7
TOTAL	263(100%)	113(43%)	150(57%)

216 As shown in FIGURE 1, Forty-four percent (44.0%) of the patients were HIV-infected while 56.0% of
 217 them were HIV-uninfected.
 218

219 **FIGURE 1: HIV STATUS OF PATIENTS**



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221 Among HIV-infected patients, the culture-positive rate was 40.5%; while the smear- positive rate was
 222 18.1%. Eighteen (18) of the 47 culture – positive cases were positive by AFB smear; which
 223 gave a sensitivity of 38.3%. (TABLE 2). In the same group of patients, the specificity was 95.7%; as 66
 224 of the 69 culture-negatives were also AFB-negative. (TABLE 2).
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 227

228 **TABLE 2: ASSESMENT OF THE VALIDITY OF ACID-FAST SMEAR AMONG THE HIV-INFECTED GROUP**

ACID-FAST SMEAR RESULTS	MTBC CULTURE RESULTS		TOTAL
	POSITIVE	NEGATIVE	
POSITIVE	18	3	21
NEGATIVE	29	66	95
TOTAL	47	69	116

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Among the HIV-uninfected patients, the culture-positive rate was 34.0%; while the smear-positive rate was 8.2%. Eleven (11) of the 50 culture-positive results were also smear-positive; giving a sensitivity of 22.0%.(TABLE 3) In the same group of patients, 96 of the 97 culture-negatives were also AFB smear-negative; yielding a specificity of 99.0%.(TABLE 3)

TABLE 3: ASSESMENT OF THE VALIDITY OF ACID-FAST SMEAR AMONG THE HIV-UNINFECTED GROUP

ACID-FAST SMEAR RESULTS	MTBC CULTURE RESULTS		TOTAL
	POSITIVE	NEGATIVE	
POSITIVE	11	1	12
NEGATIVE	39	96	135
TOTAL	50	97	147

The sensitivity value of the acid-fast smear in the HIV-infected group of patients was significantly higher than the value in the HIV-uninfected group (38.5 vs 22.0)[p = 0.0401]. (TABLE 4)

The difference in specificity proportions between the HIV-infected group and the HIV-uninfected group (95.7 vs 99.0) was not statistically significant (p=0.496). (TABLE 4)

TABLE 4: COMPARISM OF THE VALIDITY PARAMETERS BETWEEN THE HIV-INFECTED AND THE HIV-UNINFECTED GROUPS (SUMMARY)

		ACID-FAST SMEAR VALIDITY PARAMETERS	
		SENSITIVITY PROPORTIONS	SPECIFICITY PROPORTIONS
HIV-STATUSES OF PATIENTS	HIV-INFECTED	18 / 47	66 / 69
	HIV- UNINFECTED	11 / 50	96 / 106

Among the HIV-infected patients in this study, the sensitivity of gastric aspirate (GA) acid-fast smear (GA smear microscopy) was found to be 38.3%. This sensitivity value was significantly higher than the 22.0% found among the HIV-uninfected group. This finding is contrary to the report of a study on cohorts of HIV-infected and HIV-uninfected childhood TB patients from Ethiopia, in which there was reduced bacteriologic yield of GA specimens from the HIV-infected group relative to their HIV-uninfected counterparts [10]. The finding of higher sensitivity among the HIV-infected group in this study is not in keeping with established ideas that the paucibacillary status of paediatric respiratory specimens is further worsened by HIV-TB co-morbidity. HIV infection has been known to be associated with a reduced capacity of the patient to effect chronic granulomatous inflammatory responses, which defect is expected to reduce the tendency to form cavitary pulmonary lesions [2, 16]. However, contrary to this

known trend, and in keeping with our findings, a study in India reported an increased sensitivity of smear microscopy among the HIV-infected, although the patients were not strictly paediatric patients and the finding of increased bacteriologic yield was only in association with fluorescence microscopic techniques [17].

One un-proven explanation for the increased sensitivity among the HIV-infected is that there may be a possibly more effective concentration (by centrifugation) of specimens from HIV-infected patients; but this idea, which was muted by Mendelson [2], requires specific evaluation since previous reports show that the concentration-induced improvement in sensitivity occurs more with samples from HIV-uninfected TB patients irrespective the concentration method [18, 19].

Moreover, the enhanced bacteriologic yield of GA from HIV-TB co-morbid patients could also be a peculiarity of the sub-Sahara African and/or endemic regions where there are other possible coincident endemic and/or HIV-potentiated childhood pulmonary illnesses that may enhance cavity-formation among these patients [2]. Such coincident pulmonary illnesses include bronchiectasis and staphylococcal pneumonia [2]. An observation of increased cavitary pulmonary changes among the HIV-infected childhood TB cases relative to their HIV-uninfected counterparts was reported from South Africa [20]. Since these cavities are associated with a high mycobacterial load [20], it is expected that the sensitivity of bacteriologic tests should increase among the HIV infected. In keeping with this principle, the culture-positive rates and smear-positive rates in this study were consistently higher among the HIV-infected group than among the HIV-uninfected group [(40.5% vs. 34.0%) and (18.1% vs. 8.1%), respectively].

The increase in sensitivity of the test among HIV-infected patients, in this study, also suggests that HIV-TB co-infection may directly enhance cavity formation, rather than reduce it. It is known that the formation of caseous granuloma (caseation); the progression and liquefaction of the granuloma (liquefaction); and erosion of the liquefied granuloma into the airways (excavation), are the underlying processes in the formation of the typical cavitary lesions of TB [16]. Tumour necrosis factor-alpha (TNF- α) (released by activated macrophages) has been shown to play a central role in the formation and conditioning of the pulmonary TB parenchymal necroses (caseous granulomata) [21]. It has also been shown that TNF- α could play both protective and destructive roles [21]. Whatever type of role TNF- α will play has also been shown to be conditioned by the cytokine environment in which it is secreted [21]. Two, cytokine environments interplay in TB pathogenesis: TH1, in which the relevant

cytokines include interferon- γ (IFN- γ) and Interleukin-2 (IL-2); and TH2, which cytokine milieu include interleukin-4 (IL-4), IL-10, and prostaglandin E-2 (PGE-2). TH1 cytokine response to MTBC enables TNF- α to play a protective role and thus leads to granulomatous inflammatory response, immune containment of infection and enhanced delayed type hypersensitivity response (DTH) which is evidenced by positive tuberculin skin test [22]. TH2 cytokine response, on the other hand, enables TNF- α to play destructive roles and thus leads to severe parenchymal necrosis (progression and liquefaction of granulomata) [16].

TH2 cytokine response has been shown to occur in some specific situations. About 52 weeks after initial MTBC infection and the following TH1-mediated immune containment, there is a natural waning of TH1 response and supervening of TH2 response, in a large proportion of immunocompetent cases [21, 22]. From this perspective TH2 response should be more associated with late diagnosis and late onset of effective treatment. In keeping with this pathogenetic viewpoint, Biedermann has demonstrated that there is a high IL-4 release at late stages of TB and that IL-4, a TH2 cytokine, is responsible for down-regulating TH1 response [23]. It has also been elegantly demonstrated that there is a significant correlation between IL-4 expression and extent of cavitation [24].

Moreover, HIV- infected patients have also been known to express more TH2 than TH1 cytokines [26]. Furthermore, irrespective of HIV-status, increased TH2 response has been shown to be associated with high inoculum MTBC infections, advanced disease, high NTM colonization, residence near the equator [21, 25, 26]. All these factors, as well as late case presentations, are very relevant to the sub-Saharan African environment [20, 27].

With respect to the comparative specificity, there was no significant difference between the specificity finding among the HIV-infected, relative to the HIV-uninfected. This is in agreement with previous studies have shown smear microscopy specificity differences between the two groups to be insignificant [18, 19, 28].

Over the past six decades, the question of validity of gastric aspirate (GA) smear microscopy (acid-fast smear) has been recurrent [29]. The sensitivity of GA acid-fast smear is a validity parameter of great importance; since the proportion of acid-fast smear-positive results tends to determine the proportion of childhood pulmonary TB cases that will be integrated into national TB control programmes, in line with the DOTS/ Stop TB strategies [8]. The known and characteristic low bacteriologic yield of GA

acid-fast smears led to several consequences in endemic/developing countries: insignificant impact of TB control programmes in children; loss of interest of physicians in the test; exclusion of the test from many National TB Control programmes; and the near-total dependence on clinical diagnostic criteria for TB detection in the National TB control programme, as published by the Nigerian Federal Ministry of Health [5, 7]. Apathy towards this test might have been increased by the diagnostic complications imposed by HIV infection. The results of this study indicate that the test should actually be more relevant in the management TB in HIV-endemic settings, since both infections tend to coincide.

Our findings also indicate that HIV-TB co-morbid patients could pose greater TB transmission risks compared to their HIV-uninfected counterparts. This observation was corroborated by Schaaf [20]. It follows therefore that the stake of considerations for transmission prevention should be raised in childhood HIV-TB co-morbid patients, as the disease may not be pauci-bacillary.

CONCLUSION

The sensitivity of gastric aspirate smear microscopy was significantly higher among the HIV-infected group of patients than among the HIV-uninfected group. There was no significant difference in the specificity of the test between the HIV-infected and the HIV-uninfected groups.

CONSENT

All the authors have declared that written consents were obtained from the parents on behalf of the children for the publication of this work.

COMPETING INTERESTS

The authors declare that no competing interests exist.

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