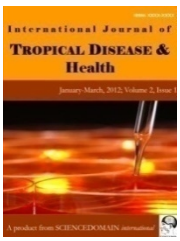

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Validity of Acid-Fast Smear of Gastric Aspirates for the Diagnosis of Childhood Pulmonary Tuberculosis among Human Immunodeficiency Virus-Infected Children

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Authors' contributions

This work was carried out in collaboration between all authors. Author EIK designed the study, wrote the protocol, saw about specimen collection, and wrote the first draft of the manuscript. Authors CKO and VUN managed literature searches, specimen analysis and performed the statistical analysis. All authors read and approved the final manuscript.

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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 expressed about the continued relevance of this test among the HIV-infected 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 validity 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: Diagnostic study. Suspected tuberculosis patients registered in the paediatrics department of University of Benin Teaching Hospital

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were prospectively recruited from January 2010 to March, 2011.
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 gastric aspirate (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 Mycobacterium tuberculosis complex (MTBC). MTBC Culture was taken as the "Reference test" for calculations of sensitivity and specificity.
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, the sensitivity was 38.3% [95%CI:24.4 – 52.2] while the specificity was 95.7% [95% CI:90.9 – 100.0]. Among the HIV-uninfected patients, the sensitivity was 22.0%[95%CI:10.5 – 33.5] while the specificity was 99.0%[95% CI:96.9 -100.0]. The sensitivity of the acid-fast smear in the HIV-infected group of patients was significantly higher than that of the HIV-uninfected group (p = 0.0401).The difference in specificity between the two groups was not statistically significant (p=0.496). There was also no significant difference in their respective positive-predictive values (85.7 vs 91.7)(p = 0.60).
Conclusion: The sensitivity of GA acid-fast smear was significantly higher among the HIV infected group.

Keywords: Gastric aspirates; validity; sensitivity; specificity; acid-fast smear; Human immunodeficiency virus; tuberculosis; diagnostic accuracy.

1. 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 (TB) 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 Mycobacterium tuberculosis complex (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 gastric aspirate (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 sensitivity and specificity of acid-fast smears for the diagnosis of pulmonary TB in children, with a view to ascertaining its continued relevance.

2. METHODOLOGY

2.1 Study Location, Population, Design and Duration

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. The prevalence of HIV in this region was 4.1% in 2010; while the estimated incidence and prevalence of tuberculosis has respectively reduced from 311/100,000 population per year and 616/100,000 population in 2006 to 133/100000 population per year and 199/100,000 population, currently [15-17]. The hospital is a referral (tertiary health care) centre for Edo, Ondo, Delta and other states of Nigeria.

Recruitments for this diagnostic study took place between January 2010 and March, 2011. Study population was patients who were registered in either the outpatient and inpatient units of the paediatric department of UBTH and who had features suggestive of pulmonary tuberculosis.

A total of 263 patients were prospectively recruited. Any paediatric patient who had one or more of the following features was included: illness duration of more than two (2) weeks, despite broad-spectrum antibiotics; chronic cough (cough of > two-week duration) 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) ≥ 10mm; erythrocyte sedimentation rate (ESR) ≥ 100/hour; and typical chest radiographic findings. Patients who were already on anti-TB treatment, anti-retroviral therapy and those who did not consent to the rigors of the research protocols were excluded.

2.2 Data Collection

Data collection was based on a semi-structured researcher-administered study questionnaire, clinical observation and laboratory investigations. Voluntary HIV counseling and serial testing algorithm was adopted for all the patients in line with routine guidelines prescribed by the Nigerian Federal Ministry of Health [18]. The PEPFAR clinic and laboratory diagnosed and classified the cases.

A total of two or three consecutive daily specimens were collected from each child. Children whose second specimens did not yield acid-fast smears underwent a third gastric aspirate collection procedure. Each gastric aspirate specimen was tested for acid fast smear positivity and MTBC culture positivity.

2.2.1 Specimen handling 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^{o}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^{o}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.

Grading followed CDC standards [19]:

>9 bacilli / Oil immersion field = 4+; 1 – 9 bacilli /Oil immersion field = 3+; 1 – 9 bacilli / 10 Oil immersion fields = 2+; 1 – 9 bacilli / 100 Oil immersion fields = 1+; 1-3 bacilli/300 Oil immersion fields = doubtful, repeat ; 0 bacilli / 300 Oil immersion fields = No AFB seen.

Grades of smear-positive findings, 1+ to 4+, from at least one of the three patient's samples were taken as positive results. A smear – positive finding of "doubtful" grade required at least another smear-positive sample from the same patient for an impression of positive result to be made. All results were consensus opinions of the three smear microscopists (who have been working in TB laboratory for more than 5 years); and they were blinded to the clinical/HIV-status of the patients from whom the samples were collected. They were also blinded to the culture results.

For GA culture, the decontaminated, centrifuged and homogenized sediment was used to inoculate acidified Ogawa medium which was incubated at 37ºC in CO2 enriched ambient air for the first week and then in ambient air for the rest of 4 - 8 weeks. Culture bottles were placed horizontally for the first 24 hours to enable absorption of innocula; after which they were placed upright.

Sterile Ogawa media were used as negative controls. There were no positive culture controls, as use of positive controls is currently being discouraged in order to prevent contamination [19].

Cultures were examined every other day for the 1st 7 days within which rapidly growing mycobacteria and other contaminants were detected. Culture contamination rate was 3%. Assessment of contamination was based on observation of growth before two (2) weeks, observation of growth in association with media deterioration, Gram stain results, and Z-N staining results.

Subsequently, cultures were examined weekly for the remaining 7 weeks till growth were detected. Cultures were reported as negative and discarded after 8 weeks of incubation.

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

HIV status was assessed by examining the respondents' plasma by using two different kits: Determine® HIV1/2 kit, a visually read immunoassay (Inverness Medical Innovations South Africa Ltd, South Africa); and HIV 1 & 2 STATPAK Assay kit, which is an immunochromatographic test for the qualitative detection of HIV-1 and HIV-2 antibodies (CHEMBIO Diagnostic system, INC, New York, USA). Manufacturers' instructions were strictly adhered to in carrying out the tests. The tests had built-in quality controls.

2.3 Data Analysis

SPSS version 16 software was used in the analysis. Data was analyzed with a view to calculating the sensitivity and specificity of acid-fast smear of gastric aspirates in the HIV-infected sub-population and comparing with values among the HIV-uninfected sub-population. Confidence intervals for the sensitivities and specificities were also calculated.

Sensitivity was defined as the proportion of smear-positive cases among culture-proven cases; while specificity was defined as the proportion of smear-negative cases among culture-negative cases. Positive-predicitive values were defined as number of true positives divided by the total number of smear positives. Negative-predictive values were defined as the number of true negatives divided by the total number of smear-negatives.

Tests of statistical significance of the differences in sensitivity and specificity between the HIV-infected and the HIV-uninfected groups were done. Chi^2 was used to compare smear-positivity between the groups; while Z-statistic of difference between proportions was used to

assess significance of the difference between their sensitivities and specificities. Level of significance was 0.05.

3. RESULTS

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

Table 1. Characteristics of Participants by HIV status

	HIV-infected	**HIV-uninfected**			
Characteristics	**Frequency (%) (N = 116)**	**Frequency (%) (N = 147)**	**Total (%) (N = 263)**	**Chi^2**	**P-VALUE**
Sex					
Male	50(43.1)	62(42.2)	112(43.0)	**0.02**	-----
Female	66 (56.9)	85 (57.8)	151(57.0)		
Age					
≤ 2.0	77(66.4)	75 (51.0)	152(57.8)	**4.60**	**0.21**
2.1 – 4.0	23 (19.8)	34 (23.1)	57(21.7)		
4.1 – 6.0	10 (8.6)	20 (13.6)	30(11.4)		
6.1 – 8.0	1(0.9)	5(3.5)	6(2.3)		
8.1 – 10.0	5(4.3)	13(8.8)	18(6.8)		
Illness duration > 2 Weeks, despite antibiotics					
Yes	47(40.5)	22(15.0)	69 (26.0)	**21.87**	**0.001**
No	69(59.5)	125 (85.0)	194 (74.0)		
Chronic cough(> 2 weeks), with WEIGHT loss or lack of weight gain					
Yes	98 (84.4)	115(78.2)	213(80.6)	**1.65**	**0.20**
No	18 (15.6)	32(21.8)	50 (19.4)		
Positive history of contact					
Yes	17(14.7)	11(7.5)	28 (10.6)	**3.51**	**0.07**
No	99 (85.3)	136(92.5)	235 (89.4)		
Tuberculin sensitivity test (TST)					
≥ 10mm	56 (48.3)	102 (69.4)	158 (60.0)	**12.04**	**0.001**
<10mm	60 (51.7)	45 (30.6)	105 (40.0)		
Erythrocyte sedimentation rate (ESR)					
≥ 100/hour	11(9.5)	2 (1.4)	13 (5.8)	**9.10**	**0.007**
<100/hour	105 (90.5)	145 (98.6)	250 (94.2)		
Typical chest radiologic findings.					
Present	115(99.1)	109 (74.1)	224(84.6)	**32.05**	**0.001**
absent	1(0.9)	38 (25.9)	39 (15.4)		

One hundred and sixteen (44%) of the patients were HIV-infected while 147(56.0%) of them were HIV-uninfected. All the HIV-infected were in-patients and had either stage III or stage IV disease. None was on antiretroviral therapy.

There was no significant sex difference between the two groups. Mean age of HIV-infected and HIV uninfected are 1.86±1.56 years and 2.63±2.15 years. Despite the younger average age of the HIV-infected group, the difference between the ages of the groups was not significant (Table 1).

The most frequent bases for participant inclusion were “Chronic cough, in association with wasting/lack of weight gain” and “typical chest radiologic findings”.

There was no significant difference in the proportion of HIV-infected participants included on the basis of “Chronic cough in association with wasting/lack of weight gain” relative to that of the HIV-uninfected (84.4 vs 78.2); whereas “typical chest radiologic findings” was significantly more prevalent among the HIV-infected relative to the HIV-uninfected(99.1% vs 74.1%).

Other inclusion criteria that were significantly more prevalent among the HIV-infected were “illness duration of > 2weeks despite broad spectrum antibiotics” and “Erythrocyte sedimentation rate (ESR) > 100mm” Contrarily, “Tuberculin Sensitivity Test > 10mm” was significantly more prevalent among the HIV-uninfected. (Table 1)

The proportion of smear-positive cases was significantly higher among the HIV-infected than among the HIV-uninfected (18.1% vs 8.9%, respectively). $Chi^2 = 5.8$; $p = 0.01$. (Table 2)

Table 2. Acid-fast smear results by HIV status

HIV status	Acid-fast smear-results		Total (%)
	Positive (%)	Negative (%)	
HIV-infected	21(18.1)	95(81.9)	116(44.1)
HIV-uninfected	12 (8.9)	135(91.1)	147 (55.9)
Total (%)	33 (12.5)	230(87.5)	263(100.0)

Among HIV-infected patients, the culture-positive proportion was 40.5%. Eighteen (18) of the 47 culture–positive cases were positive by AFB smear; which gave a sensitivity of 38.3% [95% CI: 24.4 – 52.2] In the same group of patients, the specificity was 95.7% [95% CI: 90.9 – 100]; as 66 of the 69 culture-negatives were also AFB-negative. Eighteen (18) of the 21 smear-positives were also culture-positive (truly positive); while three (3) smear-positives were culture-negative (False-positives). Smear positivity in these three (3) was due to Non-tuberculous mycobacteria (NTM); and all occurred in the “under-2” age group. Positive predictive value (PPV) was 85.7% [95%CI=71.2-100.0]. Sixty-six (66) of the 95 smear-negatives were also culture-negative (true-negatives); while 29 of the smear-negatives were culture-positive (false-negatives). Thus Negative predictive value was 69.5%[95% CI=64.8 – 74.3] (Table 3).

Among the HIV-uninfected patients, the culture-positive proportion was 34.0%; while the smear-positive proportion was 8.2%. Eleven (11) of the 50 culture-positive results were also smear-positive; giving a sensitivity of 22.0% [95% CI: 10.5 – 33.5]. In the same group of patients, 96 of the 97 culture-negatives were also AFB smear-negative; yielding a specificity of 98.9% [95% CI: 96.9 – 100.0]. Eleven (11) of the 12 smear-positives were also culture-positive (Truly-positive). Positive-predictive value was, therefore, 91.7%[95% CI = 76.4-100.0]. Only one smear-positive was culture-negative (False-positive), but the culture was contaminated and the patient was aged 3.5 years. Ninety-six (96) of the 135 smear-

negatives were also culture-negative, yielding a negative predictive value of 71.1%[95% CI = 64.4-78.8] (Table 3).

Table 3. Sensitivity and specificity of acid-fast smear among the HIV-infected and THE HIV-uninfected groups

HIV statuses	ACID-fast smear results	MTBC culture results		
		Positive	**Negative**	**Total**
HIV –infected				
	positive	18	3	21
	negative	29	66	95
	Total	47	69	116
HIV-unifected	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 (38.3 vs 22.0)[p = 0.0401].

The difference in specificity between the HIV-infected group and the HIV-uninfected group (95.7 vs 99.0) was not statistically significant (p=0.49). Similarly, the difference in the positive-predictive values (85.7 vs 91.7) was not statistically significant. (p = 0.60).

4. DISCUSSION

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 cavitory pulmonary lesions [2,20]. 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 [21].

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 [22,23].

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 cavitory pulmonary changes among the HIV-infected childhood TB cases relative to their HIV-uninfected counterparts was reported from South Africa [24]. Since these cavities are associated with a high mycobacterial load [24], it is expected that the sensitivity of bacteriologic tests should increase among the HIV infected. In keeping with this principle, the culture-positive proportion and smear-positive proportion 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]. Furthermore, in our experience with paediatric gastric aspirates, it is unusual to see more than Grade I AFB count on smear microscopy, but it is noteworthy that the only specimen that yielded up to Grade II was found to be from a Stage IV HIV patient.

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. Higher incidence of severe disease may well explain this result. It was observed that all the HIV-infected patients had clinically severe diseases, a condition that is known to activate TH2 cytokine response which is associated with progression of pulmonary cavities, rather than TH1 cytokine response which is protective.[20,25-31] Significantly greater proportion of HIV-infected participants than the HIV-uninfected, were included on the bases of “unresolved prolonged illness despite antibiotic therapy”; “ESR > 100mm”; and “typical radiological signs”, all of which suggest severe disease in children. However, a limitation of this study is the fact that it was not designed to quantitatively control for the effect of disease severity.

The finding that there was no significant difference between the specificity and PPV findings among the HIV-infected, relative to the HIV-uninfected, is in agreement with previous studies that have shown smear microscopy specificity differences between the two groups to be insignificant [22,23,32]. Since all the strictly false-positive smear-results occurred among under-2 HIV-infected participants, smear-positives from this age group should be interpreted with caution, especially if they are HIV-infected.

Over the past six decades, the question of validity of gastric aspirate (GA) smear microscopy (acid-fast smear) has been recurrent [33]. 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 the Nigerian 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. This is a small-scale hospital-based study. There is need for large-scale and community-based studies to ascertain the effect of HIV-infection on all the TB diagnostic criteria, with a view to revision of the national guidelines. Almost all the HIV-infected participants had typical radiologic findings; and significantly more HIV-infected than HIV-uninfected, had “TST>10mm” and “ESR>100mm”. These are useful parameters for assessing a TB case (new and old cases). It seems that the

diagnostic criteria for TB management ought to be re-evaluated for programmatic application at national level.

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 [24]. 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.

5. 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

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

ETHICAL APPROVAL

Approval was sought and obtained from the ethics and research committee of this hospital for this research work.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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