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ZIEHL-NEELSEN MICROSCOPY IN THE DIAGNOSIS OF TUBERCULOSIS IN SETTINGS OF HIGH HUMAN IMMUNODEFICIENCY VIRUS PREVALENCE

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ABSTRACT

Objective: To determine the accuracy of Ziehl-Neelsen microscopy in the diagnosis of TB in setings of high HIV prevalence.
Design: Cross-sectional descriptive study.
Setting: Hospitals serving areas of high human immunodeficiency virus prevalence in western Kenya. The study was conducted between September 2007 and September 2009.
Results: In total, 341/872 (39.1%) of the TB suspects were positive in ZN, 53.1% (181/341) of them culture positive. Only 3.8% (20/531) of the ZN smear negatives were culture positive. Of the 695 suspects evaluated for both *Mycobacterium* and HIV infection, 255 (36.7%) were ZN smear positive, 42.7% of them HIV positive. Out of the 440 ZN smear negatives, 37% were HIV positive. Similarly, 168 suspects were culture positive, 46.4% of them HIV positive. The HIV infection did not significantly reduce ZN smear positivity rate (P = 0.42) and culture sensitivity (P = 0.09). The ZN sensitivity and specificity were 88.1% and 79.7%, respectively. The predictive values were 58.0 (PPV), and 95.5% (NPV), respectively. However, the area under the ROC curve was 0.84, with 95% CI between 0.80-0.87 and P< 0.001). The ZN smear microscopy had a lesser ability to distinguish between TB and non-TB cases compared to culture.
Conclusion: ZN microscopy causes a significant over-diagnosis of TB in settings of high HIV/AIDS prevalence. There is need for further studies on this subject taking into consideration the various confounding factors.

INTRODUCTION

Human immunodeficiency virus (HIV) and tuberculosis (TB) co-infection interact patho-physiologically, clinically and epidemiologically (1). Many differences between TB and TB-HIV patients have been described, yielding important diagnostic implications. In immuno-competent patients, pulmonary TB accounts for about 80% of the cases (2). In early stage of HIV infection, the frequency and presentation of TB is similar to that observed in immuno-competent and HIV negative patients, while at a later stage of HIV infection it varies with the severity of immunosuppression (1,3). As a consequence, while extra-pulmonary TB accounts for only 20% of the TB cases in HIV negative patients, it accounts for 53-62% of the HIV positive patients (4,5). Tuberculosis is also more likely to be disseminated in HIV positive than in HIV negative cases and is more difficult to diagnose by conventional diagnostic procedures because of immunosuppression due to HIV/AIDS progression (1).

The Ziehl-Neelsen (ZN) smear microscopy to detect Acid Fast Bacilli (AFB) in clinical specimens is still the corner-stone and the only cost-effective tool for diagnosing pulmonary TB and to monitor anti-TB chemotherapy in most resource-poor settings (6,7). This is not a favourable situation as culture is the

gold standard for diagnosis of TB and to determine treatment success (8). Moreover, the diagnosis of TB in HIV infected patients is difficult because of absence of conventional symptoms, negative sputum smears, atypical chest radiography, higher prevalence of extra-pulmonary TB, and resemblance to other opportunistic pulmonary infections, posing a diagnostic challenge (9). The HIV infected smear positive patients tend to excrete significantly fewer TB bacilli in the sputum than HIV negative patients, which may lead to the AFBs being missed on microscopic examination. Therefore, HIV/AIDS infection has been associated with increased sputum smear negative TB cases which may be culture positive (10,11).

Tuberculin negative test (12) and smoking (13) have also been associated with lower smear microscopy and sputum culture positivity rates. However, although data on factors associated with an increased risk of smear negative in combination with culture positive results are still scarce (14), it is known that a substantial number of TB patients in high HIV prevalent and TB high-burden countries will be smear-negative (10,14). The growing number of individuals with TB-HIV co-infection (50-60%) in Kenya (15,16) poses several challenges in early diagnosis, prevention, prophylaxis and treatment of TB (17). There is also the emergence of non-tuberculous mycobacteria (NTM) as opportunistic pathogens among HIV/AIDS patients, and ZN smear microscopy does not differentiate between TB and NTM disease. The current study was designed to determine the accuracy of ZN smear microscopy and demonstrate its tendency to diagnose NTM disease as TB in Western Kenya in the current era of high HIV prevalence.

MATERIALS AND METHODS

Study site and population: The study was done at chest and paediatric clinics at one provincial, one level 5, and eight district hospitals in western Kenya. These were Busia, Bungoma, Kisumu, Migori, Kisii (Level 5), Narok, Kericho, Uasin Gishu and Lodwar district hospitals, and Nakuru Provincial General Hospital. Western Kenya includes the expansive former Rift Valley, Nyanza and Western Provinces, with a cumulative population of about 19.8 million people, constituting about 52.1% of the Kenyan population, according to the Kenya Census of 2009 (18). Western Kenya region is served by many health facilities including health centres, sub-district hospitals, district hospitals and three provincial hospitals, and one teaching and referral hospital. There was no data available on TB and HIV in the individual areas where the selected hospitals for the present study are located. However, data available in the 2007-2009 annual reports of the Division of Leprosy, Tuberculosis and Lung Disease (DLTLD)

(15,19,20) indicate up to 60% TB cases to be HIV co-infected.

Sampling frame and patient characteristics: Purposeful sampling was employed in recruiting TB suspects into the study as they sought healthcare at chest and paediatric clinics. Suspects between 9 months and 80 years old were enrolled into the study. Patients were suspected of having TB if they had a cough of more than two weeks not responding to antibiotic treatment (21).

Inclusion and exclusion criteria: Those suspected of having TB and resident in western Kenya for at least six months, not on anti-TB chemotherapy and consented to participate in the study were recruited. Suspects who had not lived in Western Kenya for the last 6 months, those already on anti-TB chemotherapy, and those unwilling to participate in the study and not meeting the above inclusion criteria were excluded. Children under 18 years were enrolled into the study after receiving consent from legal guardians.

Collection of demographic data: Counseling and collection of demographic data were done by clinicians / nurses running the chest and paediatric clinics. Interview questionnaire was used to obtain the demographic data. The data collected included age, gender, previous anti-TB treatment, and HIV status.

Collection of sputum and blood samples: Three sputum specimens (spot, early morning, spot) were collected from 872 TB suspects under the supervision of trained and competent medical staff. The patients were requested to cough so that expectoration would come from deep down the chest as possible, and spit into a sterile 50 ml blue cap tubes. For children less than five years of age and those less than ten years of age unable to expectorate sputum had sputum induction performed at the Nakuru Provincial and Kisii level five hospitals. The sputum induction involving six children was done by paediatricians using hypertonic (3%) saline (22). The samples were refrigerated at 4°C awaiting transportation in cool boxes to the Mycobacteria Reference Laboratory, Moi University School of Medicine (MRL, MUSOM) weekly for analysis (ZN smear microscopy, culture, identification of isolates and drug susceptibility testing). Samples were processed within seven days of collection in order to minimise loss of viability of the mycobacteria. Consenting 695 participants also underwent phlebotomy for HIV testing. The blood was delivered into Vacutainer Brand STERILE interior EDTA (K3) tubes and stored at –20°C awaiting processing. The samples were transported in cool boxes to MRL, MUSOM, Eldoret, and processed within two weeks. The safety for research assistants and healthcare workers during collection and

handling of sputum specimens was ensured by observing the WHO guidelines (23).

HIV testing: Testing for HIV infection was done by screening serum with the Trinity Biotech Uni-GoldTM test 24 and confirmed with the enzyme linked immunosorbent assay (25).

Microscopic examination of specimens: Sputum smears were examined for acid-fast bacilli (AFB) after staining following ZN method (26). The degree of ZN smear positivity was quantified as 1+ for 10 -100 AFBs per 100 fields, 2+ for 1-10 AFBs per field (50 fields), and 3+ for > 10 AFBs per field (20 fields). For less than 10 AFBs per 100 fields, the exact number of AFBs was indicated. A suspect was considered to be ZN smear positive if at least one specimen was positive.

Isolation of mycobacteria and identification of mycobacteria: Sputum specimens were processed for isolation of mycobacteria following standard protocols (27). A participant with at least one positive culture (MGIT and / or LJ) was considered as a TB case, while those with three negative culture results were regarded as not having TB. The mycobacterial isolates were identified as M. tuberculosis complex or species of non-tuberculous mycobacteria (NTM) using Hain's GenoType® Mycobacterium CM and GenoType® Mycobacterium AS Molecular Genetic Assays, following manufacturer's instructions (28). The suspects with ZN smear positive but culture negative sputa were treated as smear negative pulmonary TB cases.

Quality control for ZN microscopy and culture: Standard Operating Procedures (SOPs) used at Mycobacteria Reference Laboratory of Moi University School of Medicine were followed for microscopy and culture. M. tuberculosis (H37Rv) was used as a positive control, and Escherichia coli as a negative control and all new batches of microscopy reagents. Similarly, M. tuberculosis (H37Rv) positive control and a negative E. coli were inoculated once a day together with the first batch of LJ slants and MGIT tubes for that day. An experienced microscopist read an arbitrary 10% positive and 10% negative slides randomly selected, with concordance of 99% and 97%, respectively. The smears with discordant results were confirmed as positives or negatives using MGIT cultures.

Data analysis: Data were entered in MS Excel 8.0 and analysed using Epi Info version 3.5.1. Descriptive statistics were used to summarise data into proportions. Logistic regression was used to calculate odds ratio (OR), 95% confidence intervals (CI) and P-values to assess how HIV co-infection infection related to ZN smear microscopy and culture. The sensitivity, specificity, predictive (NPV and PPV) values were computed. The k coefficient and ROC curve were calculated using ZN smear results as nominal data.

Ethical issues: The proposal for this study was approved by ITROMID / KEMRI's Scientific Steering Committee (SSC) and Ethics Review Committee (ERC) [SSC No. 837] and by Moi University School of Medicine (MU-SOM) / Moi Teaching and Referral Hospital (MTRH) Institutional Research and Ethics Committee (IREC) [FAN No.00092]. The study was conducted in accordance with the Declaration of Helsinki 29. Results on TB, NTM disease and HIV infection were availed to respective healthcare givers for appropriate patient care. The HIV positive cases were referred for post-test counselling and enrolment to HIV / AIDS Programme.

RESULTS

A total of 872 eligible TB suspects were enrolled into the study and screened for TB. However, 177 suspects declined HIV test without explanation.

ZN smear microscopy and culture: Specimens from 39.1% (341 / 872) participants were ZN smear positive, of which 53.1% (181 / 341) were culture positive and 46.9% (160 / 341) were culture negative. The distribution of culture negative sputa among the various degrees of ZN smear positive samples were 13.1% (21 / 160) for 3+, 23.1% (37 / 160) for 2+, 30.6% for 1+ and 33.1% (53 / 160) the sputa with less than 10 AFBs per 100 fields. There was no correlation between ZN smear positivity and culture negativity. Only 3.8% (20 / 531) of the ZN smear negative were culture positive. Hence, 41.4% (361 / 872) suspects were infected with mycobacteria, of which 44.3% (160 / 361) were culture negative and 55.7% (201 / 361) were culture positive. Of the culture positives, 92.5% were *M. tuberculosis* complex and 7.5% were NTM. The five isolates from the NTM infection cases were identified as *M. intracellulare* (three isolates), and one each as *M. fortuitum* and *M. peregrinum*. The remaining ten NTM isolates could not be identified to species level. The 46.9% (160 / 341) ZN smear positive, culture negative cases were also treated as TB cases. None of the cultures yielded combined tuberculous and non-tuberculous mycobacteria co-infection.

Smear microscopy, culture and HIV infection: Among the 695 TB suspects tested for HIV infection, 255 were ZN smear positive (109 of the HIV infected) and 168 suspects were culture positive (78 of them HIV infected) (Table 1).

Table 1

ZN microscopy, TB culture and HIV infection

N = 695	ZN smear microscopy		TB culture	
	ZN smear positive	ZN smear negative	Culture positive	Culture negative
HIV positive	109	163	78	194
HIV negative	146	277	90	333
Totals	255	440	168	527

Socio-demographic variables among TB and TB-HIV patients: Among the 695 TB suspects tested for HIV infection, 152 suspects (103 males and 49 females) had TB alone and 112 suspects (63 males and 49 females) were co-infected with HIV. Majority of the TB and TB-HIV cases were in the 15-44 age bracket, with males more affected (Table 2).

Table 2

Socio-demographic variables among TB and TB-HIV patients

Age-group	TB patients (N = 152)		TB-HIV patients (N = 112)	
	Males	Females	Males	Females
0-14	3(2.9)	2(4.1)	2(3.2)	2(4.1)
15-24	20(19.4)	11(22.4)	13(20.6)	8(16.3)
25-34	37(35.9)	22(44.9)	26(41.3)	21(42.9)
35-44	24(23.3)	6(12.2)	15(23.8)	13(26.5)
45-54	11(10.7)	5(10.2)	4(6.3)	3(6.1)
55-64	4(3.9)	2(4.1)	2(3.2)	1(2.0)
> 64	4(3.9)	1(2.0)	1(1.6)	1(2.0)
Total	103 (100)	49 (100)	63(100)	49(100)

ZN smear and culture results among TB and TB-HIV patients: A total of 264 (166 males and 98 females) were among the TB cases who accepted HIV test, and 42.4% (112/264) were co-infected. Males constituted 56 % (63/112) and females 44% (49/112) of TB-HIV cases. Among the 152 non-HIV TB cases, 140 were ZN smear positive while 82 cases were culture positive. Among the 112 TB-HIV cases, 106 cases were ZN smear positive, while 72 cases were culture positive (Table 3).

Table 3

ZN smear and culture results and logistic regression output for TB and TB-HIV patients

Variable	TB patients N = 152		TB-HIV patients N = 112		OR	95%CI	P-value
	+ve	-ve	+ve	-ve			
ZN smear	140 (92.1%)	12 (7.9%)	106 (94.6%)	6 (5.4%)	1.5143	0.5505-4.1653	0.4215
Culture	82 (53.9%)	70 (46.1%)	72 (64.3%)	40 (35.7%)	1.5366	0.9309-2.5362	0.0929

Sensitivity, specificity, predictive values and receiver-operating characteristic (ROC) curve: The sensitivity and specificity of ZN smear microscopy were 88.1% and 79.7%, respectively. The positive and negative predictive values were 58% and 95.5%, respectively (Table 4). The value of the area under the ROC curve was 0.839, meaning that a randomly selected TB suspect from culture positive cases would have a test value larger than that for a randomly chosen suspect from culture negative cases in 83.9% of the time 30. The 95% confidence interval (95%CI) was 0.80-0.87. Since the P-value was low (P < 0.001), then the area under the ROC curve was significantly different from 0.5 (null hypothesis: Area = 0.5). There was evidence that ZN smear microscopy had a lesser ability to distinguish between TB and non-TB cases compared to culture (Figure 1).

Figure 1
ROC Test result ZN smear microscopy:
Area under curve = 0.839; Standard error = 0.02; P < 0.001; 95CI, 0.80-0.87
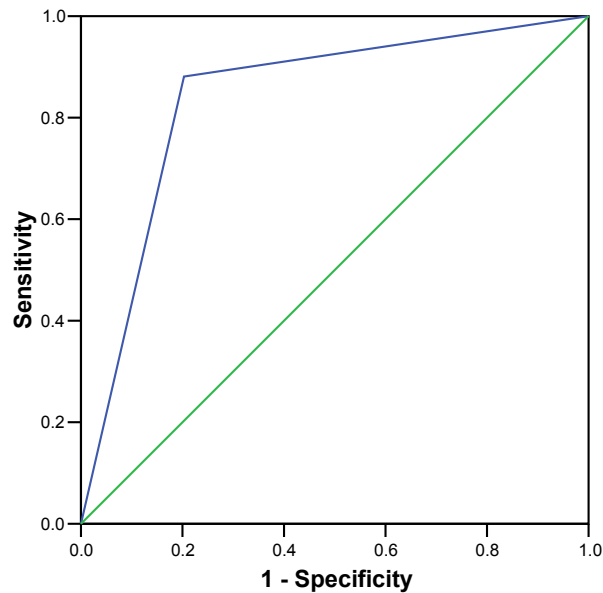
Agreement and kappa statistic: Culture classified 24.2% of all the 695 TB suspects tested for HIV as TB cases, and 75.5% of the suspects as non-TB cases. If culture were to classify the TB suspects independent of classification by ZN smear microscopy, then 24.2% (61.71) of the 255 suspects classified as TB cases by ZN smear microscopy would be classified as TB cases. Similarly, 24.2% (106.29) of the 440 suspects classified as non-TB cases by ZN smear microscopy would be classified as non-TB cases by culture (Tables 4). Computations of percentage observed agreement and the percentage agreement expected by chance alone give 87.48% and 56.895%, respectively. The value of kappa (k) in this case comes to 0.71 (Table 4). Since the k value lies between 0.40 and 0.75 31, the two TB diagnostic tests are intermediate to good in agreement.

Table 4
Sensitivity, specificity and negative and positive predictive values, percentage
observed agreement, percentage agreement expected by chance alone, and k value

N = 695	Culture		
	positive	negative	Total
ZN smear	148	107	255(36.7%)
	20	420	440(63.3%)
	168 (24.2%)	527 (75.8%)	695(100%)

% sensitivity = 88.1; % specificity = 79.7; % NPV = 95.5; % PPV = 58.0

Percentage observed agreement = 87.48%

Percentage agreement expected by chance alone = 56.895%

Kappa (k) = 0.71.

Antiretroviral therapy: During the period of the present study, 16.9% (46/272) of the HIV cases (14 TB-HIV and 2 NTM-HIV) were put on antiretroviral therapy (ART), 65.2% females and 34.8% males, respectively.

DISCUSSION

Many previous studies have reported HIV infection to lower the rates of ZN smear microscopy sensitivity (7) and M. tuberculosis culture positivity (32), with smear positivity rates among TB cases as low as 31% (33, 34). Studies in India even reported smear negativity rates as high as 82% (6). The sputum smear negativity tends to be associated with advanced HIV disease and progression in immuno-suppression (6). In the present study, HIV co-infection did not significantly affect ZN smear [P = 0.42] and culture [0.09], which differs from previous studies (7). Plausible explanation for the apparent non-influence of HIV co-infection on ZN smear and culture sensitivity is that most the HIV positive TB cases may have been in the early HIV infection stage during which the frequency and presentation of TB is similar to that observed in immuno-competent and HIV negative cases (5,6). This explanation may be further supported by the fact that only 16.9% (46/272) of the cases were on anti-retroviral therapy (ART). However, the present study did not establish HIV cases needing ART, as neither the CD4 cell count nor viral loads were determined. The current study also did not examine the correlation between the clinical presentation of individual patients and the ZN smear microscopy and culture outcomes. Nationally, however, of the 1.4 million Kenyans infected with HIV, about 42.6% (243,000/570,000) of those needing ART were on therapy (35). This means that Universal Access to ART in Kenya still remains a big challenge.

Moreover, from a significant proportion of the microscopically smear positive sputa, no mycobacteria could be cultured on Lowenstein-Jensen medium, nor on the more sensitive liquid MGIT media. Only in 51% of the TB cases diagnosed by ZN smear microscopy could M. tuberculosis be grown from sputum. This implies that ZN microscopy as a diagnostic tool for TB among HIV/AIDS cases has become imprecise. However, the high number of ZN smear positive but culture negative cases observed in the present study may also be attributed either loss of viability since most sputum samples were cultured on the 7th day after collection, or the presence of fastidious NTM, or the presence of acid-fast non-mycobacteria organisms. Not all acid fast bacilli represent the M. tuberculosis complex. Non-tuberculous mycobacteria (39) and some other bacterial species including Nocardia species which are widespread (40) yield positive results in ZN smear detection of acid-fast bacilli (AFB). Similarly, a significant proportion of patients especially those HIV positive may give negative ZN smear results, although they are culture positive (41). It is therefore evident that TB diagnosis and treatment in Western Kenya is not completely evidence-based, and cases of non-tuberculous mycobacterioses are often put on anti-TB chemotherapy. Cases of non-tuberculous mycobacterioses are often put on anti-TB chemotherapy, even though the treatment of NTM disease is generally not directly analogous to TB treatment. Multi-drug regimes are used for NTM TB-like disease treatment, the cornerstone agents being a newer macrolide (azithromycin, clarithromycin) (42), ethambutol, and rifamycin, and require prolonged durations of therapy aimed at facilitating clearance of the mycobacteria and minimising the emergence of drug resistance (41,42). Consequently, yet to be found out is which ZN smear positive microorganisms cause this high degree of false-positivity and whether they are emerging infections.

Previous studies have also indicated that positive blood cultures for TB (uncommon in HIV negative TB patients) may be a reflection of disseminated TB in patients with advanced HIV/AIDS. Due to alteration of the normal host immune response to *M. tuberculosis* in HIV co-infected patients, cavitation and transfer of tubercle bacilli into respiratory secretions is markedly reduced, thereby hampering TB diagnosis by microscopy and culture (8,32). The positivity of TB culture also varies with the nature of the specimen, ranging from 26 to 42% (36). However, due to practical reasons the ZN smear microscopy so far remains the most feasible microbiological method for the diagnosis of PTB in developing countries due to its rapidity, low cost, and high positive predictive value for *tubercle bacilli* (37). Cultures are not performed for routine diagnosis and monitoring of TB treatment due to high costs and biosafety infrastructure requirements (37). However, Dowdy *et al*. (38) reported that the TB culture is a potentially sensitive and cost-effective tool for HIV positive TB patients in resource-constrained settings, but integration with existing clinical systems and strengthening of post-analytical processes are required to maximise their impact.

With the high prevalence of HIV/AIDS in the developing countries, there is the possibility that a substantial number of TB patients will be smear-negative; or some HIV/AIDS patients may be smear-positive due to NTM and acid-fast non-mycobacteria organisms. Considering the relatively low sensitivity of ZN smear microscopy it is possible that a substantial number of HIV co-infected TB patients may already be missed to be initiated on treatment, a worrying situation since untreated TB from also smear negatives contributes to ongoing transmission (43). The increased prevalence of extra-pulmonary forms of TB in HIV infected patients is a further challenge to the management of TB in resource-poor settings, where access to histopathology and advanced imaging tests are limited or unavailable (35). As TB rates continue

to increase in HIV endemic regions, there is merit for evaluation of the performance of diagnostic techniques as one of the TB control strategies. There also remains a need to develop more user friendly TB diagnostic techniques, which can be efficiently adapted to use in the high-burden and low-income countries (1). There is also a need for large studies to identify selected clinical and laboratory parameters which could be used to identify TB in HIV positive individuals more accurately (31).

Tremendous progress has been made in the diagnosis of TB in developed countries including replacing cultures with new molecular methods for TB diagnosis and drug susceptibility testing (DST). However, TB diagnosis and DST methods have remained relatively unchanged (ZN smear microscopy and DST using LJ slants) in resource-poor settings. The level of sophistication and cost associated with the new and more sensitive techniques have made their general applicability unfeasible in developing countries (44), where the basis for TB diagnosis has continued to be ZN smear microscopy to visualise acid-fast bacilli (AFB), which may capture 50-60% of the cases when done by experienced personnel. However, in most low-income countries, much lower rates of case detection are achieved due to poor quality microscopes, heavy workload and shortage of appropriately trained technical staff. The latter could have also contributed to low accuracy of ZN smear microscopy due to moderate experience of most of the technical staff involved in the current study. Moreover, ZN smear microscopy is usually negative in advanced disease among HIV/AIDS co-infected patients and extra-pulmonary TB cases. The proportion of cases detected by microscopy in low-income countries is often as low as 20-30% of all cases (44, 45).

In the developed world, molecular detection and speciation of mycobacteria are currently the corner stone of the laboratory diagnosis of TB and NTM disease. Commercial DNA probes (AMPLICOR nucleic acid amplification test – Accuprobe) and the Gene – Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD) have been available in the developed world for sometime. These tests are based on species-specific DNA probes that hybridise with ribosomal RNA (rRNA) released from the mycobacteria (44). The other methods (INNO-LiPA Mycobacteria and the Hain's GenoType Mycobacterium CM/AS) are based on reverse hybridisation, in which the mycobacteria 16S-23S internal transcribed spacer region or the 23S gene region are amplified by polymerase chain reaction (PCR), and the amplicons are subsequently hybridized with probes for several mycobacterial species on membrane strips (44).

This study had a limitation. The number of HIV cases needing ART could not be established. This is because the clinical characteristics (HIV immunosuppression phase / CD_4 cell counts, duration of disease, chest radiological features) that could also interfere with ZN smear and culture positivity rates were not determined.

In conclusion, the ZN microscopy as a diagnostic tool for TB appears to be imprecise and causes a significant over-diagnosis of TB among HIV/AIDS patients in western Kenya where there is high prevalence of HIV. There is need for further studies in this subject taking into consideration the various confounding factors. It is also unclear which micro-organisms other than mycobacteria cause positivity in ZN smear microscopy especially among HIV/AIDS patients and this needs to be examined further.

ACKNOWLEDGEMENTS

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