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ARTICLE

HIV-related incremental yield of bleach sputum concentration and fluorescence technique for the microscopic detection of tuberculosis

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Abstract Bleach sputum concentration and fluorescence microscopy (FM) are reportedly more sensitive than direct Ziehl-Neelsen (ZN) sputum smears for tuberculosis detection, and might be particularly valuable for human immunodeficiency virus (HIV)-positive patients excreting fewer bacilli. This study, implemented in Yaoundé, Cameroon, determined the yield from both direct and bleach-concentrated FM and ZN duplicate smears against culture on Löwenstein-Jensen medium, with HIV testing from the sputa. From 418 HIV-positive and 518 HIV-negative tuberculosis suspects, 185 (44.3%) and 243 (46.9%) cultures, respectively, grew *Mycobacterium tuberculosis*. Direct ZN was positive for, respectively, 87 (47.0%) and 202 (83.1%) of the culture-positive cases. Proportional incremental yield over direct ZN from ZN and FM bleach smears was 14.9% ($P<10^{-3}$) and 17.2% ($P<10^{-4}$) for HIV-positive versus 4.9% ($P<10^{-2}$) and 2.0% (non-significant) for HIV-negative cases. There was no gain from direct FM. Bleach FM showed 2% excess false positives. The bleach concentration, therefore, increases the yield of ZN and FM, particularly from HIV-positive patients, but with a higher risk for false positives with bleach FM. With excellent baseline direct ZN, the gain remains modest. Field studies under real-life conditions are needed to determine whether it is worth the risks and operational challenges in HIV high-prevalence populations. FM was not more sensitive than ZN in this study, probably because of sub-optimal objective power and background staining. Culture on solid media with sparing laurylsulfate decontamination was clearly superior for HIV-positives, but it remains to be seen if culture also leads to more cases started on treatment routinely.

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Introduction

With close to two billion people infected worldwide, an incidence estimated at 8.8 million cases and 1.8–2 million deaths annually, tuberculosis (TB) remains a major public health problem, particularly in low- and middle-income countries [1]. The human immunodeficiency virus (HIV) epidemic has contributed considerably to the increased global incidence of TB, and countries with high HIV prevalence often also show the highest rates of TB worldwide [2]. Due to its speed, simplicity and low cost, in high endemic countries, sputum smear microscopy remains the primary and, often, still the only tool for the diagnosis of pulmonary tuberculosis, where it is also highly specific for

Mycobacterium tuberculosis [3]. The Ziehl-Neelsen (ZN) technique is universally applicable, even under field conditions, but it constitutes a high workload. A lack of ZN sensitivity has been emphasised, particularly with respect to HIV co-infected persons [4, 5], while its tediousness and safety have also recently received more attention [6].

For these reasons, ZN has been largely replaced by fluorescence microscopy (FM) in industrialised countries. Although it could also be rewarding in low- and middle-income settings [7], FM is still rarely used there because of numerous constraints.

In recent years, sputum concentration techniques have been reported to result in increased microscopy sensitivity with preserved specificity. Mainly, sodium hypochlorite (household bleach) has received considerable attention; however, contradictory reports on bleach effectiveness exist, and concerns regarding operational feasibility and preserved specificity with programmatic mass application have been raised [8–11]. Although the technique might be especially rewarding in HIV-positive TB cases, because of the higher frequency of paucibacillary sputa [12, 13], this has rarely been investigated [14].

We determined the incremental yield of bleach-concentrated smears for acid-fast bacilli (AFB) microscopy by the HIV serological status of TB suspects with culture on Löwenstein-Jensen (LJ) as the gold standard. Both direct and concentrated ZN and FM smears performed at a reference laboratory and direct ZN at a busy routine diagnostic laboratory were included in the comparison.

Methods

Subjects and specimens

The study subjects were consecutive, new TB suspects recruited at the Jamot Referral Hospital laboratory in Yaoundé, Cameroon, which attracts a high proportion of bacteriologic-positive cases from all over the country. One morning sputum was selected per suspect, irrespective of aspect or volume, and transported the same day to the National Tuberculosis Reference Laboratory (NTRL). During four days per week, the first ten suspects registered at the Jamot laboratory were enrolled. A routine smear was performed at the Jamot laboratory, but all study tests were performed at the NTRL.

Sample processing and testing

Sputum samples were screened for HIV using the OraQuick® HIV-1/2 assay (OraSure Technologies, Bethlehem, PA) according to the manufacturer's instructions, including the use of appropriate controls. HIV-positive samples were confirmed by enzyme-linked immunosorbent assay (ELISA) testing (Genscreen HIV-1/2, Bio-Rad Laboratories, Hercules, CA). After making two new direct smears, the sputum specimens were divided into two equal parts. An equal volume of household bleach was added to one of these, using locally purchased 12° free chlorine, diluted by half with distilled water. After mixing and occasional shaking during 15 min, the tubes were topped up with sterile distilled water and centrifuged at low speed (400g) for 5 min. Two fairly thick smears were made using two drops of sediment. After coding for blinding, one each of direct and bleach sediment smears were stained using the standard 0.3% basic fuchsin hot ZN technique [15, 16]. The two remaining duplicate smears were stained for FM using 0.1% auramine O stain and 0.1% thiazine red counterstain [17]. The smears were read by two technicians, who were blinded to the results of the Jamot laboratory readings, to those of the other smears from the same sputum and to the culture results. Due to the different aspect of the smears, blinding to the sample preparation and staining technique were not possible. The technicians read fairly equal numbers of the various types of smears over the study period. One length was read for all types of smears. AFB quantification followed the World Health Organization (WHO) scale, converting the FM results from the 500× magnification used [16].

The second part of the sputum was decontaminated by the sodium laurylsulfate method [18] and inoculated on two LJ slants, with incubation at 37° for eight weeks or until growth was detected. The presence of mycobacteria was confirmed by ZN smear from positive cultures, and strains were further identified using biochemical methods [19].

Quality assurance of bacteriologic tests

Series of smears with positive/negative discordant results at the NTRL were re-examined by the principal investigator. Moreover, all of the available direct study ZN smears read at the NTRL were restained and blindly reread at a Bangladesh Tuberculosis Programme known for its high-quality AFB microscopy [20].

Data recording and analysis

The data were processed in Epi-Info 6.04d, using double data entry and validation, with statistics calculation in STATA 8.0 (Stata Corporation, College Station, TX). Student's *t*-test, chi-square test or Fisher's exact test was used as appropriate. McNemar's chi-square test was used to compare the results of duplicate smears. The relative risk (RR) with a 95% confidence interval (CI) was estimated between HIV-positive and HIV-negative patients. All statistical analyses were conducted with a significance level of 5%.

Table 1 Demographic data and human immunodeficiency virus (HIV) status of tuberculosis (TB) suspects

		HIV-positive (%)	HIV-negative (%)	Total (%)	RR (CI)
		n=418 (44.6)	*n*=518 (55.3)	*n*=936	*P*
Sex					
	Female	233 (54.1)	198 (45.9)	431 (46.0)	1.46 (1.27–1.68, $P<10^{-6}$)
Age (years)					
Age groups (% of HIV sub-group)					$P=<10^{-5}$
	<15	7 (1.7)	13 (2.5)	20 (2.1)	
	15–24	28 (6.7)	127 (24.5)	155 (16.6)	
	25–34	156 (37.3)	124 (23.9)	280 (29.9)	
	35–44	135 (32.3)	83 (16.0)	218 (23.3)	
	45–54	63 (15.1)	67 (12.9)	130 (13.9)	
	55–64	20 (4.8)	49 (9.5)	69 (7.4)	
	≥65	9 (2.1)	55 (10.6)	64 (6.8)	

RR=relative risk
CI=confidence interval

All tests at the NTRL were carried out anonymously. Permission for the study was granted by the Cameroon Ethical Committee.

Results

Of 1,006 TB suspects enrolled, 936 had complete and valid results. Of the 70 other subjects, 62 were excluded due to the absence of an HIV serology result and the remaining for missing bacteriology results.

The demographic data of the 936 subjects analysed are summarised in Table 1 by HIV status. Forty-five percent were HIV-positive. The patients were mainly young adults, with a median age of 35 years (range 4–88), and there was little difference (non-significant, NS) between HIV-positive and HIV-negative subjects. There were 505 (54.1%) males, but females were more often affected by HIV (54.0% versus 36.6% sero-positives, RR 1.46, $P<10^{-6}$). Subjects between 25 and 44 years of age constituted 70% of HIV-positive but only 40% of HIV-negative cases ($P\leq10^{-5}$).

Table 2 shows the quantified culture results by HIV status. Cultures grew *Mycobacterium tuberculosis* (any number of colonies) for 185 (44.3%) of HIV-positive and 243 (46.9%) of HIV-negative suspects (RR 0.94, NS). The quantifications differed significantly with HIV status, with less abundant growth from HIV-positive suspects (RR 1.80, $P<10^{-5}$). While 30.3% of their cultures grew low numbers of colonies (1–19), this was the case for 11.1% of cultures from HIV-negative patients. Mainly colony counts below five, without positive smear, were very unequally distributed, with 38 of 49 (78%) belonging to HIV-positive cases.

Three hundred and thirty-nine specimens were positive on any smear, 323 (75.5%) from culture-positives and 16 (3.1%) from culture-negatives (details not shown). Table 3 shows the results of smear microscopy by HIV status and culture result, and Table 4 shows the incremental yields for the same, considering only culture-positive specimens. From two to 14 smear-positives were reported from culture-negatives. While the difference was not significant between ZN and direct FM smears, smear-positive/culture-negative results were significantly more numerous for bleach FM (14 or 2.8%, $P<10^{-2}$). All smears were significantly less sensitive among HIV-positive suspects (RR varying from 0.39 for ZN carried out at Jamot laboratory to 0.65 for FM after bleach concentration at the NTRL, P always$<10^{-6}$). Using direct ZN at the NTRL as the baseline, incremental yields proportional to the baseline

Table 2 Culture results by HIV status

Culture result	HIV-positive (%)	HIV-negative (%)
	n=418	*n*=518
Negative	233 (55.7)	275 (53.1)
Positive	185 (44.3)	243 (46.9)
Quantified results (% of all positives)		
1–4 colonies, smear-negative	38 (20.6)	11 (4.5)
1–4 colonies, smear-positive	1 (0.5)	4 (1.7)
5–19 colonies	17 (9.2)	12 (4.9)
1+	33 (17.8)	15 (6.2)
2+	36 (19.5)	42 (17.3)
3+	60 (32.4)	159 (65.4)
All low positives (1–19 colonies)	56 (30.3)	27 (11.1)
All 1+ to 3+	129 (69.7)	216 (88.9)

1+: 20–99 colonies
2+: 100–199 colonies
3+: 200 colonies or more

Table 3 Microscopy positives by HIV serology and culture result

Microscopy positives from	HIV-positive, culture			HIV-negative, culture			Total, culture			RR HIV-positive (CI)	P
	Pos. n=185	Neg. n=233	Total n=418	Pos. n=243	Neg. n=275	Total n=518	Pos. n=428	Neg. n=508	Total n=936		
ZN direct Jamot (% of n)	75 (41.0)	0	75 (17.9)	198 (81.5)	2 (0.7)	200 (38.6)	273 (63.8)	2 (0.4)	275 (29.4)	0.39 (0.31–0.48)	$<10^{-6}$
ZN direct NTRL (% of n)	87 (47.0)	0	87 (20.8)	202 (83.1)	4 (1.4)	206 (39.8)	289 (67.5)	4 (0.8)	293 (31.3)	0.57 (0.48–0.67)	$<10^{-6}$
ZN bleach NTRL (% of n)	100 (54.0)	1 (0.4)	101 (24.2)	212 (87.2)	4 (1.4)	216 (41.7)	312 (72.9)	5 (1.0)	317 (33.9)	0.44 (0.36–0.53)	$<10^{-6}$
FM direct NTRL (% of n)	87 (47.0)	0	87 (20.8)	203 (83.5)	2 (0.7)	205 (39.6)	290 (67.8)	2 (0.4)	292 (31.2)	0.56 (0.48–0.66)	$<10^{-6}$
FM bleach NTRL (% of n)	102 (55.1)	6 (2.6)	108 (25.8)	206 (84.8)	8 (2.9)	214 (41.3)	308 (72.0)	14 (2.8)	322 (34.4)	0.65 (0.57–0.75)	$<10^{-6}$

HIV=human immunodeficiency virus
RR=relative risk
CI=confidence interval
ZN=Ziehl-Neelsen
FM=fluorescence microscopy
NTRL=the National Tuberculosis Reference Laboratory
bleach=bleach sputum concentration technique

ranged from −5.5% to +8.0% (−13.8% to +17.2% among HIV-positive cases). Bleach ZN showed the largest proportional increment overall (8.0%, CI 5.2–11.9), but among HIV-positive cases, bleach FM showed the largest proportional increment overall (17.2%, CI 10.3–27.2). Overall and for HIV-positive suspects, bleach ZN or FM increments over direct smear were significant ($P<10^{-5}$, $P<10^{-2}$, respectively), but there was no significant difference between them (not shown) and neither between direct ZN and FM. For HIV-negative suspects, only the bleach ZN increment was significant ($P<10^{-2}$).

Table 5 shows the positive microscopy results for the various methods used. The proportion of low positive (rare AFB and 1+) smears was low for Jamot ZN smears, independent of HIV status, but at the NTRL, it was significantly higher among HIV-positive subjects (from 20% to 44% versus from 7% to 30%, RR 1.45 to 3.21; P= 0.025 to $P<10^{-4}$). The bleach concentration significantly reduced this proportion, for ZN (from 33% to 20%, P= 0.04, and from 14% to 7%, P=0.02), as well as for FM (from 44% to 26%, P=0.01, and from 30% to 8%, $P<10^{-6}$), for HIV-positive and HIV-negative suspects, respectively. Microscopy from samples bringing about less than 20 colonies on culture was positive for only 4/27 (15%) and 4/56 (7%) among HIV-negative HIV-positive patients, respectively, adding up all variations (data not shown in Table 5). With so few colonies in culture, there was one positive direct ZN and one rare AFB direct FM result (both HIV-negative), while bleach ZN and bleach FM each brought about two positives and two rare AFBs from HIV-negative, against, respectively, one and four rare AFBs from HIV-positive patients.

The rechecking of 52 series with discordant results at the NTRL showed two false negatives for each of the microscopy variations, except bleach FM, and one high false positive for direct ZN and bleach FM (data not shown). There was also one low false positive for each of the ZN variations, but three and eight for direct and bleach FM, respectively (P=0.01 for bleach FM). The same total number of positive results was found in Bangladesh as at the NTRL (231 versus 230, details not shown) from the rechecking of 794 of the direct ZN smears.

Discussion

With the current emphasis on detection for TB control, bleach concentration and FM microscopy have received considerable attention recently [14, 21]. Both are generally considered as more sensitive than direct ZN, with unusually large incremental yields reported by some authors [22–25].

Bleach would also make microscopy safer and easier to perform [14]. In contrast with sodium hydroxide-concentrated

Table 4 Incremental yield from microscopy variations by HIV status, culture-positives only

Increment compared to baseline direct ZN at the NTRL	HIV-positive	*P*	HIV-negative	*P*	Total	*P*
ZN direct NTRL positives, baseline number	87		202		289	
ZN direct Jamot, % increase and CI	−13.8; −7.6 to −23.2	$<10^{-2}$	−2.0; −0.6 to −5.3	NS	−5.5; −3.3 to −9.0	$<10^{-2}$
ZN bleach NTRL, % increase and CI	14.9; 8.5–24.6	$<10^{-3}$	4.9; 2.5–9.2	$<10^{-2}$	8.0; 5.2–11.9	$<10^{-5}$
FM direct NTRL, % increase and CI	0; 0–5.3	NS	0.5; 0.03–3.2	NS	0.3; 0.02–2.2	NS
FM bleach NTRL, % increase and CI	17.2; 10.3–27.2	10^{-4}	2.0; 0.6–5.3	NS	6.6; 4.1–10.2	$<10^{-2}$

HIV=human immunodeficiency virus
NTRL=the National Tuberculosis Reference Laboratory
CI=confidence interval
ZN=Ziehl-Neelsen
FM=fluorescence microscopy
bleach=bleach sputum concentration technique
NS=non-significant

smears [26], bleach concentration was shown not to require high-power centrifugation [23], with greatly increased yield, even after sedimentation [27], making it suitable even for low-resource settings. However, the technique is still not widely implemented for various reasons, i.e. the extremely variable yield of bleach ZN, with its increment proportional to direct ZN, ranging from 2% to 233% [14], warnings concerning false positives and false negatives from bleach, and associated logistical challenges [8].

Similar logistical challenges exist also for FM, since the classical equipment has been found to be inappropriate for low-income countries for a variety of reasons. The new type of LED lamp FM seems far more appropriate, and preliminary trials have shown that this might be the future for low-income countries [28].

This study was undertaken in a TB and HIV high-prevalence population, where these techniques could be most rewarding. In our reference laboratory, the sensitivity of a single direct ZN smear was 67%, but was far lower among the HIV-positive than HIV-negative suspects (47% versus 83%, respectively). Using bleach, the sensitivity of ZN increased to 73% (54% and 87% for HIV-positive and HIV-negative suspects, respectively), or an 8% (15% and 5%, respectively) proportional increase from the baseline. Our results are, therefore, less encouraging than the 28% and 7% proportional increment in HIV-positive versus HIV-negative cases reported by Bruchfeld et al. [29], the only publication so far on the differential yield of bleach by HIV status. This might be explained by the excellent quality of direct ZN at the NTRL, as testified by its 12% proportional incremental yield over ZN performed at the Jamot routine laboratory. This shows that, apart from using bleach or FM, the quality improvement of direct ZN can also add considerably to its yield. The rechecking of discordant series showed no effect of bleach on ZN specificity, while the proportion not confirmed by culture was also the same for direct and bleach ZN.

By using bleach concentration combined with FM, the gain seemed slightly larger for HIV-positive cases (17%

Table 5 Low positive and total positive microscopy results by HIV status (culture-positive specimens only)

Microscopy variation	HIV-positive		HIV-negative		RR (CI)	*P*
	Rare AFB and 1+ (%)	Total positive	Rare AFB and 1+ (%)	Total positive		
ZN direct Jamot	10 (13.3)	75	20 (10.1)	198	1.32 (0.65–2.69)	NS
ZN direct NTRL	29 (33.3)	87	29 (14.4)	202	2.32 (1.48–3.64)	$<10^{-3}$
ZN bleach NTRL	20 (20.0)	100	15 (7.1)	212	2.83 (1.51–5.29)	$<10^{-3}$
FM direct NTRL	38 (43.7)	87	61 (30.1)	203	1.45 (1.06–2.00)	0.025
FM bleach NTRL	27 (26.5)	102	17 (8.2)	206	3.21 (1.83–5.61)	$<10^{-4}$

HIV=human immunodeficiency virus
RR=relative risk
CI=confidence interval
ZN=Ziehl-Neelsen
FM=fluorescence microscopy
bleach=bleach sputum concentration technique
NTRL=the National Tuberculosis Reference Laboratory
Rare AFB: 1–9 AFBs per 2-cm smear length in ZN; 1–19 AFBs per 2-cm smear length in FM
1+: 10–99 AFBs per 2-cm smear length in ZN; 20–199 AFBs per 2-cm smear length in FM

increment over baseline), but the confidence limits overlapped with ZN bleach, and it was not more sensitive than bleach ZN when considering HIV-negative or all cases together. The rechecking of discordant series suggested more false positives for bleach FM, in line with the higher proportion of bleach FM positives that remained culture-negative. Surprisingly, direct FM brought the same yield as direct ZN, without a suggestion of false positives.

Of 339 specimens positive from any smear, 16 (4.7%) remained culture-negative, testifying to the high quality of culture at the NTRL. Even using only LJ medium, fast transport together with prompt processing and a soft decontamination method (laurylsulfate) resulted in a very high yield of culture (428 positives versus 339 from any smear). While the most rewarding smear variation detected close to 90% of HIV-negative cases, this was only 55% among HIV-positives, whereby it should be stressed that no sputa had been excluded because of quality or quantity. At the NTRL, a significantly higher proportion of low positive smears (rare AFB and 1+) was consistently found among HIV-positive suspects, as has been reported before. Even with low-speed centrifugation, the concentration effect of bleach was clear from a reduced proportion of low positives in ZN and FM. Cultures from HIV-positive subjects yielded significantly fewer colonies as well, 30% against 11% were low positives. A few of those were detected as rare AFBs, but only after bleach concentration.

ZN quality at the NTRL was very good, as shown by blinded rechecking and internal quality control. This may explain the absence of extremely high incremental yields from bleach concentration, as sometimes reported. Since poor-quality sputa were not excluded, our results could, thus, be considered as representative of a routine setting with excellent ZN and moderately high HIV prevalence.

Our study has some limitations

A slightly reduced specificity has been reported for our HIV rapid test from sputum [30, 31]. Nevertheless, its accuracy can be considered as adequate for the purpose because of the very high HIV prevalence and resulting excellent positive predictive value, and since we confirmed positive results by ELISA.

The quality of FM at the NTRL may not have been optimal, considering the absence of an increment over ZN. This was probably due to the sub-optimal magnification (500×, with a smaller field than for the often recommended 200× magnification), besides a too bright (thiazine red) background with a very strong light source (mercury vapour lamp).

One sputum only was examined per suspect. A recent study in Zimbabwe has reported the value of repeated FM smears from sodium hydroxide (NaOH)-concentrated sputa, approaching culture sensitivity, even among HIV-positive patients. However, since 4% NaOH was used for decontamination, their culture sensitivity may have been lower [32].

Finally, our results were obtained in a carefully controlled reference laboratory setting. Because of all of the logistical challenges involved, the results should not be extrapolated to mass application in a field setting without further operational trials.

In conclusion, our results suggest that, in HIV low-prevalence and low-income countries, bleach incremental yield over optimally performed ZN may not justify the additional work, equipment and supplies required, or the risk of false results due to the instable reagent or other causes [8, 9]. However, bleach-concentrated ZN smears do appear to be promising in HIV high-prevalence populations. This should now be confirmed by its impact on case detection and treatment via field studies, using series of two to three good sputum samples processed in a network of routine health centres, with attention also to feasibility and acceptability.

Contrary to most reports, FM was not better than ZN in our study, with or without bleach concentration, possibly due to technical details such as the magnification and background staining used. However, FM has also proven to be very difficult to introduce and sustain for routine work in low-income settings. Its value for the increased detection of TB in HIV high-prevalence settings should be further studied, preferably with more appropriate, user-friendly FM systems, and particularly in over-loaded urban laboratories.

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