

Evaluation of two novel Ziehl-Neelsen methods for tuberculosis diagnosis

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Summary

Background: Currently, the diagnosis of tuberculosis (TB) in Ghana relies on direct sputum smear, Ziehl-Neelsen (ZN) staining method. This method has low sensitivity and poses some health risks. The study was to compare the, direct sputum smear, (ZN) staining method against two newer ZN methods; 1% Sodium hypochlorite (NaOCL)-xylene floatation and 1% NaOCL sedimentation methods, to determine the most sensitive and the safest.

Study design: A prospective descriptive study involving 150 adult patients attending Komfo Anokye Teaching Hospital, Kumasi, Ghana suspected of pulmonary tuberculosis, using the three ZN microscopy methods: direct sputum smear, 1% NaOCL sedimentation, and 1% NaOCL-xylene floatation, for the detection of acid fast bacilli (AFB). Sputum culture on Lowenstein-Jensen (LJ) slopes was used as the gold standard for determining the sensitivity and specificity rates.

Results: The sensitivity rates of NaOCL sedimentation, NaOCL-xylene floatation and direct smear methods were 77.2%, 71.8% and 66.3% respectively. The specificity rate was 95.9% for all three methods. Whereas the difference between the NaOCL sedimentation and the direct smear methods was statistically significant ($P=0.0446$), that between the NaOCL-xylene floatation and direct smear was not ($P=0.1788$).

Conclusion: In spite of the cost of chemicals, the hypochlorite sedimentation method was found to be the most accurate and the safest.

Key-words: Tuberculosis, Laboratory infection, Sputum microscopy.

Résumé

Introduction: Actuellement, le diagnostic de la tuberculose (TB) au Ghana compte sur crachat barbouille, méthode de barbouiller de Ziehl-Neelsen (ZN). La sensibilité de cette méthode est en baisse et elle menace quelque risques pour la santé.

L'objet de cette étude est de comparer le crachat barbouille direct, (ZN) méthode de barbouiller contre deux plus nouvelles; 1% méthode de ZN hypochlorure de sodium (NaOCL) - flottation xylene et 1% NaOCL méthode de sédimentation afin de décider le plus délicat et sans danger.

Plan d'étude: Une étude en perspective et descriptive impliquant 150 patients adultes soignés dans le centre hospitalier universitaire de Komfo Anokye, Kumasi, Ghana, présumé de la tuberculose pulmonaire à travers l'utilisation de trois méthodes de la microscopie ZN: Crachat barbouille direct, 1% NaOCL sédimentation, et 1% NaOCL-flottation xylene, pour la détection d'acide rapide bacilli (ARB). Culture

Crachat sur la pente de Lowenstein-Jensen (LJ) a été utilisé comme étalon-or pour décider le caractère délicat et taux de la spécificité.

Résultats: Taux de caractère délicat de la sédimentation de NaOCL, NaOCL-xylene flottation et méthode directe de barbouiller étaient 77,2%, 71,8% et 66,3% respectivement. Taux de la spécificité était 95,9% pour toute les trois méthodes. Tandis que la différence entre la sédimentation de NaOCL et les méthodes de barbouiller direct était statistiquement important ($P=0,446$), celui entre la flottation xylene NaOCL et barbouiller direct n'était pas ($P=0,1788$).

Conclusion: En dépit du frais du produit chimique, la méthode de la sédimentation d'hypochlorure était notée d'être la plus correcte et sans danger.

Introduction

Tuberculosis (TB) remains a deadly disease worldwide. In Ghana, it is estimated at 30,000 new cases and 15,000 deaths each year.^{1,2} TB in man is caused by the *Mycobacterium tuberculosis* complex, made up of *M. tuberculosis* (Mtb), *M. bovis*, *M. africanum* and *M. microti*.³

For the laboratory diagnosis of TB, based on the detection of the TB bacilli in clinical specimens, methods available are, microscopy, culture techniques or nucleic acid amplification tests, or serological methods.³⁻⁵

The increasing health threat of the emergence of multi-drug resistance TB (MDRTB), and the devastating effect of the combination with HIV/AIDS, call for rapid diagnosis, hastening the search for simpler, rapid, cheaper, but safer methods for diagnosis of TB.⁴⁻⁷

Though sputum culture on Lowenstein-Jensen (LJ) medium to isolate *Mycobacterium* species is the "gold standard" in the diagnosis of TB, growth may take up to 8 weeks.³⁻⁷

Microscopy, using either Rhodamine-auramine or ZN stain, serology and nucleic acid amplification tests remain the logical candidates. Serological methods, though rapid and probably safe, have been unpopular due to their low sensitivity and specificity,^{4,8} and the nucleic acid amplification tests such as polymerase chain reaction (PCR) and Gene probes though rapid and safe require higher training and are more expensive.^{5,8}

The WHO National Tuberculosis Control Programme has adopted Ziehl-Neelsen (ZN) staining sputum smear microscopy, to make TB diagnosis accessible to all patients at all levels of health institutions.^{2,4} For, apart from the use in primary TB diagnosis, ZN microscopy is also used in the monitoring of reduction of bacilli by effective treatment.^{5,9} ZN, therefore, remains the cornerstone of TB diagnosis in many low-income countries.^{6,7}

The ZN method, though simple, rapid, inexpensive and

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specific, however, has low sensitivity.^{3-5,9,10} The microscopic detection of acid-fast bacilli (AFB) require nearly 10⁴ of bacilli per millilitre of sputum.^{4,11}

Again, the handling of sputum is also dangerous to laboratory workers.^{7,12-14} In the ZN technique, a fixed sputum smear is covered with strong carbol fuchsin and heated. The dye is retained, despite decolorisation with sulphuric acid, showing acid-fast bacilli (AFB).³⁻⁵ The heating stage of ZN creates aerosol and is dangerous if it is not done in a safety cabinet.¹²⁻¹⁶ Unfortunately, many laboratory workers do not use safety cabinets as reported by Hass.¹⁵

It has been observed that the increasing awareness of the health threat of the emergence of multi drug resistance TB (MDRTB), the devastating association with HIV/AIDS, has hastened the search for simpler, rapid, cheaper, but safer methods for diagnosis of TB.^{7,12-16}

Concentration of sputum, either by centrifugation or sedimentation, prior to smear microscopy has been shown to be superior to direct smear microscopy.^{9,17} Concentrating the sputum alone does not make the sputum safer, but could rather make it dangerous.

Soltys et al¹⁸⁻²⁰ have shown that prior treatment of sputum with 1% hypochlorite (NaOCL) before ZN staining, kills the *Mycobacteria* rendering the procedure safer than the older direct smear method. Two methods, using sedimentation and floatation, after prior treatment of sputum with hypochlorite (NaOCL) before ZN staining are available.^{19,20} We are not aware of any studies evaluating the three methods in terms of accuracy, sensitivity and safety.

Our objective was to evaluate the three methods, direct sputum smear; sodium hypochlorite (NaOCL)-xylene floatation and sodium hypochlorite (NaOCL) sedimentation, ZN staining methods, to determine the most sensitive and the safest for TB Diagnosis.

Subjects and methods

The study was undertaken at the Chest clinic and the Microbiology laboratory of Komfo Anokye Teaching Hospital, Kumasi, Ghana (KATH), for 6 months in 2003. It was approved by the SMS Ethical Committee, and informed consent was granted by the subjects.

Study area

KATH, an 800-bed hospital, situated in Kumasi, the capital of Ashanti Region of Ghana, population (approx. 700,000), is a referral hospital for northern Ghana. Referrals

are also seen from all the regions and other neighbouring countries.

Subjects

Sputum specimen of 150 patients suspected of pulmonary tuberculosis, aged 12 to 65 years, who had not received prior TB treatment were screened using three ZN staining methods: direct smear, 1% NaOCL-xylene floatation smear and 1% NaOCL sedimentation smear ZN microscopy methods. The methods have already been described previously as reported:

- (i) Direct smear microscopy:³⁻⁵
- (ii) 1% NaOCL-xylene floatation smear microscopy:¹⁹
- (iii) 1% NaOCL sedimentation smear microscopy:²⁰

Culture³⁻⁵

All specimens were cultured on glycerol Lowenstein-Jensen (LJ) slopes for the isolation of *M. tuberculosis*, and identified using standard biochemical tests. The isolation rate of *Mycobacteria tuberculosis* was used as the gold standard for determining the sensitivity and specificity rates for the other methods under review.

Hypothesis testing

Test of difference of population proportions was used to evaluate the differences between smears made directly from sputum and those made from hypochlorite sedimentation and hypochlorite-xylene floatation processing. Statistical significance was defined at a confidence interval of 95% (P < 0.05).

Results

Of the 150 samples processed 48 (32.0%), 42 (28%) and 35 (23.3%) were positive for AFBs by the 1% NaOCL-xylene floatation, NaOCL sedimentation and direct smear methods respectively. Using the results of sputum culture as gold standard, the sensitivity rates of 1% NaOCL sedimentation, xylene floatation and direct smear methods were determined as 77.2%, 71.8% and 66.3% respectively. (Table 1). The specificity rate was 95.9 % for all three methods.

Whereas the difference between the NaOCL sedimentation and the direct smear methods was statistically significant (P= 0.0446), that between the NaOCL-xylene and direct smear was not (P=0.1788). Hence the NaOCL sedimentation method is the most sensitive, and the safest.

Of the 150 specimen cultured, 19 were excluded from the culture results, because bacterial and fungal contamination made identification of growth unreliable.

Table 1 Results of Ziehl-Neelsen staining of sputum smears prepared by 3 methods.

	Microscopy results by indicated method		
	NaOCL sedimentation	NaOCL-xylene floatation	Direct smear
Total no of positive cases	48	42	35
Total no of negative cases	102	108	115
Total no of specimens examined	150	150	150

Table 2 Comparison of culture with the 3ZN staining methods

	Culture (LJ slope)	Microscopy results by indicated method		
		NaOCl sedimentation	NaOCl-xylene floatation	Direct smear
Total no of positive cases	61	43	37	30
Total no of negative cases	70	88	94	101
False positive	0	3	3	3
False negative	0	18	24	31
Sensitivity (%)	100	77.2	71.8	66.3
Specificity (%)	100	95.9	95.9	95.9
Total no of specimens evaluated	131	131	131	131

NB Numbers of the 3 methods have been adjusted from 150 to 131 for comparison with the 131 culture positive samples.

Table 3 Correlation of sensitivity to smear preparation and AFB concentration

Score ^a	No of smears obtained by		
	NaOCl sedimentation	NaOCl-xylene floatation	Direct smear
3+	28	24	24
2+	6	8	6
1+	8	7	3
Scanty	6	3	2

Semi-quantitative grading based on grading of results from smear examination (taken from "Tuberculosis Microscopy, A laboratory manual for Ghana")

Negative	No AFB found in at least 100 fields
Scanty (exact number):	1 - 9 AFB found in 100 fields
(1 +)	10 - 99 AFB found in 100 fields
(2+)	1- 10 AFB found per field in at least 50 fields
(3+)	More than 10 AFB per field in at least 20 fields

Three slides were found positive by all three microscopy methods, but negative by culture.

Table 2 shows the comparison of the three methods to culture in the detection of AFBs.

Table 3 shows the sensitivity of AFB detection in sputa containing unequal concentration of bacilli. Sputa with a high concentration of AFBs, (i.e. 3+ and 2+), sensitivity was similar for all 3 methods. Of the 48 AFB positive smears (3+ and 2+), 34, 32 and 30 were by NaOCl-sedimentation, NaOCl-xylene floatation method and direct smear microscopy respectively.

For sputa with a low concentration of bacilli (1+), the sedimentation method was superior to the other two. NaOCl-sedimentation detected 14 as against 5 for direct microscopy. (P=0.0559). The xylene floatation detected 10 as against 5 by the direct method (P=0.1469).

Discussion

Microscopic examination of sputum for acid-fast bacilli (AFB) plays a key role in the initial diagnosis of pulmonary tuberculosis (PTB), monitoring of treatment and the determination for the eligibility for release from isolation.^{4,5,9}

The study found the sensitivity rates of 1% NaOCl sedimentation, 1% NaOCl-xylene floatation and direct smear methods to be 77.2%, 71.8% and 66.3% respectively. The specificity rate was 95.9% for all three methods. Whereas the difference between the NaOCl sedimentation and the direct smear methods was statistically significant (P= 0.0446), that between the NaOCl-xylene floatation and direct smear methods was not (P=0.1788).

The study confirms as shown⁹ that treatment of sputum with NaOCl followed by overnight sedimentation increases the sensitivity of sputum microscopy significantly. This is closely followed by the NaOCl -xylene floatation method, and then the direct smear microscopy.

Work related infections in personnel working in mycobacteriology laboratory is significant because aerosols produced during smear preparation, staining and processing for culture are sources of infection.¹²⁻¹⁴

Of the 150 specimens 48 (32.0%) smear-positive cases were detected by the NaOCl-sedimentation method as against 35 (23.3%) by direct smear microscopy. This means that 13 AFB positive patients were missed by the direct method. (Table 2)

Study showed that of the 13 missed positive smears, 9 were seen in sputa with low concentration of bacilli (1+ and scanty). The low sensitivity of direct smear has been noted¹⁰ Meticulous preparation and multiple examination of smears could increase the sensitivity of smear microscopy to 55%^{7,10} but, experience shows that it is often very difficult to maintain a high level of performance in overburdened control programmes^{7,13}

Major disadvantages of the sedimentation method are the laborious sample preparation and the delay in reporting. However, since current diagnosis by smear microscopy requires three specimens collected over two days, the increased sensitivity would compensate for any delay. The avoidance of a centrifuge is an added advantage as noted elsewhere.²⁰

The 42 smears in 150 detected as positive by the NaOCl-xylene floatation method was slightly lower than that obtained by sedimentation 48 in 150 (p=0.2843). The sensitivity of the method is 71.8% as against the 66.3% of direct smear; this is not significant statistically (p=0.1469). Though the method is not as sensitive as the NaOCl sedimentation method, it is as safe as the sedimentation method but has the advantage of being quicker, with results being available within 24 hours.

Again, the xylene floatation method detected more positives from specimens with low number of bacilli than the other two. Indeed, 7 cases reported negative by direct smear microscopy, 5 were positive but had low number of bacilli (1+ and scanty) (Table 3).

A major disadvantage of xylene is its inflammability, making it a fire hazard in the laboratory. Xylene, with a specific gravity of 0.86 at 20°C/4°C (compare 1.00 for water) is lighter and immiscible with water. The debris remain in the water layer, but the AFBs concentrates in the xylene, attributable to the unique hydrophobic, high lipid content of the cell walls of acid-fast bacilli (AFBs), which makes them buoyant²¹ making them more discernible for diagnosis.

Three slides found positive by all the three microscopy methods were found to be negative on culture. This might be due to organisms probably killed during decontamination with NaOCl or they could be species like *Mycobacterium bovis*, which do not grow on glycerol, since only glycerol LJ slopes were used.

In spite of the cost of chemicals, the hypochlorite sedimentation method was found to be the most accurate and the safest

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