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Two vs. three sputum samples for microscopic detection of tuberculosis in a high HIV prevalence population

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SUMMARY

SETTING: A busy urban hospital in Cameroon.

OBJECTIVES: To compare the yield in bacteriologically proven tuberculosis (TB) cases examining two morning vs. three spot-morning-spot sputum specimens (MM vs. SMS) by direct microscopy for acid-fast bacilli (AFB).

DESIGN: Repeated temporal cross-over between MM and SMS sampling for successive TB suspects, using culture as gold standard.

RESULTS: A total of 799 suspects were screened using the MM strategy, identifying 223 smear-positives, and 808 suspects with the SMS strategy, yielding 236 smear-positives. Of the MM, 256 were culture-positive, of whom 195 (76%) were smear-positive. For SMS, these figures were respectively 281 and 206 (73%), a non-significant difference. The MM and SMS strategies also detected respectively 28 and 30 smear-positive cases not confirmed by culture. No cases were lost to treatment with either strategy.

CONCLUSIONS: In this population with a high prevalence of human immunodeficiency virus (HIV) with late case presentation, smear microscopy of two morning specimens detected at least as many positive cases as the classical strategy, and no cases were lost before treatment. Two specimens for initial TB suspect screening can thus be recommended, also without excessive workload. Comparative studies in populations presenting with paucibacillary sputum are needed to determine the equivalent quality and yield of an alternative strategy with two spot specimens at consultation.

KEY WORDS: microscopy; Ziehl-Neelsen; tuberculosis; sputum collection strategy; yield

IN THE LAST DECADE, tuberculosis (TB) detection has been perceived as a greater problem than case holding and treatment outcome, particularly in low-income countries with a high prevalence of human immunodeficiency virus (HIV) infection.[1] While the search for superior detection techniques continues, microscopy remains the most appropriate method in most settings, and it has been argued that more can be gained from improving it than from introducing advanced and, in principle, far more sensitive techniques, such as liquid culture.[2]

The optimal sputum collection strategy, in terms of number and type of sample to be examined for indiscriminate screening of TB suspects, remains one of the most frequent questions in optimising microscopy. A recent meta-analysis has confirmed the low yield of the third specimen in the classical spot-morning-spot (SMS) strategy.[3] Across a large variety of studies and settings, the weighted incremental yield of smear-positive cases for the third specimen above that of the two other specimens remained very close to 2.3%. At the ideal 10% positive TB suspect frequency, this amounts to examining over 900 specimens to detect these last 2–3 cases, i.e., several months of work when the necessary care is taken. On the other hand, the number of suspects that needs to be examined has increased by three fold, four fold or even more in high HIV burden populations. This seriously jeopardises the quality of Ziehl-Neelsen (ZN) microscopy, further reducing its yield.[4] This danger, or at least inefficiency, has resulted in a recent World Health Organization (WHO) recommendation to allow TB control programmes to opt for a two-specimen collection strategy, and the concomitant criterion of one positive smear to notify a patient as smear-positive. However, this was made conditional to the presence of a well-functioning external quality assurance (EQA) system and an excessively high workload for the human resources available.[5]

Considering the crucial importance of the human factor (mainly alertness and motivation),[6] the tediousness of ZN microscopy and the deleterious effect of fatigue on its quality, the above recommendation remains very cautious. This may be explained by the

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fact that we do not really know the yield of two vs. three specimens. Virtually all studies reviewed by Mase et al. have extrapolated the yield of a two-sputum strategy from studies or settings actually using a three specimen strategy,[3] so that by definition two sputum samples could never yield more cases than three. It cannot, however, be excluded that if the workload could be reduced by a third, the quality of the tests would improve sufficiently to result in increased detection. As an exceptional real-life comparison did not use a gold standard for detection, the reported equivalence of numbers of smear-positive cases detected remains less convincing.[7]

The present study was designed to document the yield of culture-proven TB cases using the classical SMS strategy vs. examination of two morning specimens (MM). It was conducted in a peri-urban population from Cameroon, with about 50% HIV-positive TB cases and late presentation for diagnosis.

MATERIALS AND METHODS

Suspects consecutively presenting with smear-positive TB were enrolled at the routine laboratory of the Provincial Hospital in Limbe, South-West Province of Cameroon, alternating each quarter between an SMS and MM sputum collection strategy over an 18-month period (April 2006 to September 2007). SMS collection followed international guidelines.[8] During the quarters where MM collection was applied, patients were instructed to collect the first sputum sample on rising in each of two numbered containers on the two mornings following the consultation day, and to deliver them together to the laboratory on the second day. A spot specimen was also collected but kept in reserve, to be examined only if the suspect did not return with the morning specimens.

The Limbe Laboratory examined a direct smear from each of the specimens according to the Cameroon National TB Programme (NTP) instructions, including a hot ZN staining technique and a 1% fuchsin stain. Smears were declared negative if no acid-fast bacilli (AFB) were detected after screening 100 high-power fields (HPF). Grading of smears containing AFB followed the WHO/International Union Against Tuberculosis and Lung Disease (The Union) scale.[8] Patients notified as smear-positive were treated with WHO-recommended standard regimens following NTP guidelines. One positive smear was sufficient to register a case as smear-positive.

Fresh morning specimens delivered on the same day were kept refrigerated and transported within 72 h to the culture laboratory at the *Conseil des Eglises Baptiste et Evangélique de Cameroun* (CEBEC) (the Baptist Hospital), Douala. In the CEBEC provincial reference laboratory, a new smear was made after decontamination of the specimens with the laurylsulfate method and concentration by centrifugation. The smears were examined blind using the same ZN technique.[9] Two slopes of Löwenstein-Jensen medium were simultaneously inoculated per specimen, and incubated at 37°C, discarding negative slopes after 10 weeks. Identification of TB strains was based on growth rate and colony morphology; further identification using conventional biochemical methods was performed only if indicated by atypical TB features.[10] Any TB culture-positives were reported to the principal investigator, and field supervisors were instructed to assure start of treatment for the culture-positive, smear-negative cases. Start of treatment was checked for all smear- and/or culture-positives.

Double data entry with data cleaning and analysis was performed in Epi Info 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). Pearson's χ^2 was used for the comparison of smear-positive cases detected by collection strategy. Any number of AFB in at least one specimen was considered sufficient to declare a case smear-positive, and any number of TB colonies was considered as a positive culture. The proportion of smear-positives out of all culture-positives detected by routine screening in Limbe was the main outcome parameter analysed for the evaluation of the collection strategies.

We also compared the proportions of true AFB-positive suspects and of false-negative smear results by collection strategy. For this analysis, a positive was accepted as true if confirmed by a positive smear or culture (or both). However, culture results were not taken into account to determine true- and false-negatives among the SMS and MM smears, as the large difference in operating characteristics between smear and culture methods would have obscured quality differences between the laboratories far more than the concentrated reference laboratory smears used exclusively.

For each cohort (SMS or MM), a minimum of 750 suspects had to be enrolled to show significance of a difference reaching at least 7%, estimating 20% positive cultures, with 80% of those also positive on smear.

RESULTS

Over the entire study period, 1607 suspects were enrolled: 808 during the three quarters of SMS sampling, and 799 during the three alternating quarters of MM sampling (Table 1). AFB smear-positive suspects detected at the routine laboratory varied between 24% and 33% by quarter, with on average 29% positives using SMS sampling vs. 28% using MM.

In total, 281 (35%) suspects were culture-positive with SMS sampling vs. 256 (32%) with MM (Table 2). Of these, 206 vs. 195 (73.3%, 95% confidence interval [CI] 67.7–78.3 vs. 76.2%, 95%CI 70.4–81.2, non-significant) were also smear-positive at the routine laboratory. Another 27 and 19 smear-positives

Table 1 Numbers of suspects screened and smear-positive cases detected at the routine laboratory, by collection strategy and quarter

Quarter*	Sputum collection strategy	Suspects			Positives
		Negative *n*	Positive *n*	Total *n*	%
2006/II	SMS	208	92	300	31
2006/III	MM	202	79	281	28
2006/IV	SMS	153	74	227	33
2007/I	MM	211	68	279	24
2007/II	SMS	211	70	281	25
2007/III	MM	163	76	239	32
All	SMS	572	236	808	29
All	MM	576	223	799	28
All		1148	459	1607	29

* 2006/II to 2007/III = second quarter 2006 to third quarter 2007.
SMS = spot–morning–spot samples; MM = two morning samples.

Table 2 Yield of culture-positive cases by collection strategy

Sputum collection strategy	Total *n*	Smear-positive		
		n	%	95%CI
SMS				
Culture-positive	281	206	73.3	67.7–78.3
Culture-negative	510	27	5.3	3.6–7.7
Culture-contaminated	17	3	17.6	4.7–44.2
Total	808	236	29.2	26.1–32.4
MM				
Culture-positive	256	195	76.2	70.4–81.2
Culture-negative	524	19	3.6	2.3–5.7
Culture-contaminated	19	9	47.4	25.2–70.5
Total	799	223	27.9	24.9–31.2

SMS = spot–morning–spot samples; CI = confidence interval; MM = two morning samples.

had a negative culture with the SMS and MM strategies, while the culture was contaminated for respectively three and nine smear-positives.

Table 3 shows an estimate of AFB smear quality at the routine laboratory by collection strategy. The routine results have been re-classified as true- or false-positives/-negatives, based on the results of smear or culture at the CEBEC culture laboratory. The SMS strategy was thus found to yield 222 true and 14 (5.9%) false-positive vs. 36 false-negative results, resulting in 86% sensitivity relative to the culture laboratory. For the MM strategy, these figures were 214 true-positives, 9 (4.0%) false-positives and 22 false-negatives, giving a relative sensitivity of 91%. The difference in sensitivity was statistically non-significant ($P = 0.08$), with a borderline result for false-negative percentage (6.3 vs. 3.8%, $P = 0.056$).

All suspects enrolled submitted their sputum samples with either strategy, and all cases found to be smear-positive started treatment. Approximately 5% of the positive cases required tracing as they did not return for their results with either strategy, while no cases had to be traced due to non-return with the morning sputum samples and a positive reserve spot specimen (data not shown).

DISCUSSION

In spite of the numerous publications comparing the yield of two vs. three smears for the detection of smear-positive cases, many doubts remain, as reviewed by Mase et al.[3] This may explain the relatively restrictive 2007 WHO recommendation allowing a change to two specimens if pressed by inadequate human resources, with the requisite change in the definition of smear-positive from at least two to one positive samples requiring coverage by good EQA.

We believe that these restrictions are not justified. First, it has been clearly demonstrated that the technician is the main determining factor for quality of AFB microscopy;[6,11] second, in our experience a high microscopy workload automatically excludes high quality, which cannot be entirely corrected by good EQA. Improved quality with reduced numbers might thus actually result in increased detection, but no earlier studies have determined the true yield of a two-specimen screening strategy under real-life conditions. Our study is the first to do so, comparing a

Table 3 Routine smear quality by collection strategy using smear and culture at the reference laboratory as gold standard

Sputum collection strategy	Routine smear evaluation						
	True-positive *n*	False-positive *n*	True-negative *n*	False-negative *n*	False-positive %	False-negative %	Sensitivity %
SMS							
Culture-positive	206	0	47	28			
Culture-negative/-contaminated	16	14	489	8			
Total	222	14	536	36	5.9	6.3	86
MM							
Culture-positive	195	0	48	13			
Culture-negative/-contaminated	19	9	506	9			
Total	214	9	554	22	4.0	3.8	91

SMS = spot–morning–spot samples; MM = two morning samples.

two- vs. a three-specimen strategy applied during different periods under routine conditions, with culture as the gold standard to exclude bias from temporal changes. Although the positivity rate of smears was higher during the SMS periods, this difference may have been caused by seasonal confounding effects, as the proportion of culture-positives detected was higher with MM. As in many other countries, Cameroon patients are more likely to have less time and money to visit the health services during the harvest and school re-enrolment period (third quarters 2006 and 2007, MM strategy), with higher case detection afterwards (fourth quarter 2006, SMS). The higher yield of MM compared to culture was not significant, but considering the concentrated smears at the reference laboratory, there was suggestion of more false-negatives with SMS (borderline significant). No cases were lost to treatment with either strategy. Although rather exceptional in other settings, this may be explained by the fact that, in Cameroon, patients were more likely to present late, but were highly motivated to complete the diagnostic procedure and to start treatment.

The most important practical question, which is not addressed in Mase et al.'s review on yield, concerns the type of samples to be collected with a two-sputum screening strategy. Our study compares the classical SMS strategy with sputum samples collected on two successive mornings and delivered together on the second day. This choice was based on publications reporting a (much) higher yield for morning compared to spot sputum as well as on findings from our own, earlier studies. Urbanczik has reported up to double the number of positives from morning sputum.[12] The classic Andrews and Radhakrishna paper on which the SMS strategy is based did not see much difference between the two types of sputum in a highly centralised setting with late case detection, except in the group with early disease on chest X-ray, where morning sputum yielded proportionally 50% more positives than spot sputum.[13] We earlier reported incremental yields of 94% on the first and 99% on the first two of three morning specimens in Bangladesh. In this HIV-free population with fairly late case presentation, fresh morning sputum yielded 9%, and older, liquefied morning sputum up to 11% more positives (WHO/Union scale) than spot. Although 10% of suspects did not return with their three specimens on day 3, this represented only 1.5% of cases. As many of those were also not motivated to take treatment, this actually meant higher efficiency of a morning-sputum-only strategy. The study concluded that two morning specimens delivered together on day 2 were most efficient, but that a spot specimen should be examined immediately for clearly sick patients.[14] Furthermore, our experience from the field is that in reality only the first sputum may be examined (properly), whatever the strategy or guideline; opting for the best possible quality first specimen therefore seemed logical.

A few recent studies on the equivalence of same day spot-spot collection (SS) have led to the recommendation of a radically different, so-called 'front-loading', strategy. Extrapolated from a series of four specimens actually examined (SMS and an additional spot), the yield of SS was only a few per cent lower than SMS. Because of the lower cost to the service and the patients, as well as the presumed prevention of loss of cases during the diagnostic process, SS was recommended as the best strategy.[15,16] However, the difference between the bacillary load of spot and morning specimens in these settings (Abuja, Nigeria and Southern Ethiopia) may have been exceptionally low. Very few low-positives were encountered, and the smear positivity rate of more than 20% indicates late case detection. Moreover, the smears were examined up to three times, which would obscure the difference in yield caused by higher or lower bacillary load. The yields might thus be entirely different in settings with early detection, particularly in high HIV prevalence populations, as well as in a routine setting with often superficial examination of smears, a limitation pointed out by the authors. In addition, proper instructions for sputum production have been shown to be important for optimal yield.[17] This factor is likely to be more important for spot than for morning sputum, and might thus cause a bias in experimental studies that deviate too much from the reality in the field.

In our opinion, preventing loss of cases during the diagnostic process will require more than just a change in collection strategy. For various reasons, very few laboratories process sputum on the same day, and improved contact with patients may be at least as important as speeding up this process.[18] With the SMS strategy the rate of primary default could be reduced to around 1% by obligatory registration of all smear-positives detected, with a default outcome report in case of non-start, rather than continuing to ignore the problem (Md A H Salim, Damien Foundation Bangladesh, personal communication).

CONCLUSIONS

Two successive MM sputum specimens can give a higher yield than SMS by improving the quality of the tests. The 2007 WHO recommendation can thus be extended, omitting the conditions of high workload and lack of human resources. Although effective EQA is always useful as another method of improving quality, its absence is not a counter-indication for a two-specimen, one-positive strategy, which will by itself create conditions that permit better quality.

Whether the collection of two SS at presentation also has a comparable yield and results in reduced loss of cases before start of treatment needs to be evaluated by comparative studies under real-life, routine conditions, using culture as the gold standard,

and targeting populations with a lower average bacillary load at first presentation.

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RÉSUMÉ

CONTEXTE : Un hôpital urbain très actif au Cameroun.
OBJECTIFS : Comparer le rendement en cas de tuberculose bactériologiquement démontrée (PTB+) de deux échantillons de crachats du matin vs. trois échantillons de crachats « sur place-matin-sur place » (MM vs. SMS) lors de l'examen microscopique direct à la recherche de bacilles acido-résistants (BAAR).
SCHÉMA : Alternance répétée dans le temps entre les échantillonnages de MM et de SMS chez les patients successifs suspects de tuberculose avec utilisation de la culture comme gold standard.
RÉSULTATS : On a dépisté 799 suspects par la stratégie MM, avec dépistage de 223 patients PTB+, et d'autre part 808 suspects par la stratégie SMS dont 236 patients étaient PTB+. Parmi le groupe MM, la culture a été positive chez 256 et parmi ceux-ci, 195 (76%) étaient PTB+. Les valeurs correspondantes pour le groupe SMS ont été respectivement 281 et 206 (73%) ; cette différence n'est pas significative. Les stratégies MM et SMS ont détecté également 28 et 30 PTB+ non confirmés par la culture. Dans les deux stratégies, aucun cas n'a été perdu pour le traitement.
CONCLUSIONS : Dans cette population à prévalence élevée de VIH et se présentant tardivement pour le diagnostic, l'examen microscopique des frottis de deux échantillons du matin a détecté au moins autant de cas positifs que la stratégie classique, et aucun de ces cas n'a été perdu avant traitement. On peut donc toujours recommander deux échantillons pour le dépistage initial des suspects de tuberculose, ce qui évite une charge excessive de travail. Des études comparatives dans des populations à expectoration paucibacillaire s'imposent pour déterminer l'équivalence en matière de qualité et de rendement de stratégies alternatives comportant deux échantillons sur place lors des consultations.

RESUMEN

MARCO DE REFERENCIA : Un hospital urbano de alta ocupación en Camerún.

OBJETIVOS : Evaluar el rendimiento de la confirmación bacteriológica de casos de tuberculosis (TB), comparando la búsqueda de bacilos acidorresistentes por examen microscópico en dos muestras matinales (MM) y en tres muestras : instantánea, matinal e instantánea (SMS).

MÉTODOS : Se recogieron muestras de esputo repetidas de los pacientes consecutivos que acudieron con presunción de TB, alternando en el tiempo (trimestral), entre MM e SMS. El método de referencia fue el cultivo para micobacterias.

RESULTADOS : En 799 casos presuntos se practicó la estrategia MM y se encontraron 223 pacientes con TB y baciloscopia positiva ; con la estrategia SMS se estudiaron 808 casos y se encontraron 236 con baciloscopia positiva. En el grupo MM hubo 256 casos con cultivo positivo y de ellos 195 (76%) habían tenido baciloscopia positiva. En el grupo con la estrategia SMS hubo 281 casos con cultivo positivo, de los cuales 206 (73%) habían tenido baciloscopia positiva, una diferencia sin significación estadística. No hubo confirmación por cultivo en 28 casos con baciloscopia positiva del grupo MM y en 30 casos del grupo SMS. Con ninguna de las estrategias se perdieron casos para el tratamiento.

CONCLUSIONES : En esta población con alta prevalencia de infección por el virus de la inmunodeficiencia humana y presentación tardía de los casos de TB, la obtención de dos muestras matinales para baciloscopia condujo a la detección de por menos tantos casos positivos como la estrategia convencional y no se perdió ningún paciente para el tratamiento. Por lo tanto, siempre se pueden recomendar dos muestras matinales en la evaluación inicial de pacientes con presunción de TB, sin sobrecargar en exceso el laboratorio. Se precisan estudios comparativos en poblaciones con escasos bacilos en el esputo, a fin de determinar la equivalencia de la calidad y el rendimiento de esta estrategia opcional con dos muestras instantáneas en la consulta.