

## The bleach method improves the detection of pulmonary tuberculosis in Laos

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### SUMMARY

**SETTING:** Laos has a high prevalence of tuberculosis (TB) and a low prevalence of human immunodeficiency virus/acquired immune-deficiency syndrome (HIV/AIDS). *Mycobacterium tuberculosis* culture is not yet available. Case finding is based on the microscopic detection of acid-fast bacilli (AFB) in sputum. Liquefaction and concentration using sodium hypochlorite (bleach) could improve its yield.

**OBJECTIVE:** To assess the gain due to the bleach method and its feasibility in Laos, and to compare the classical method sputum processing (direct microscopy) and the bleach method for AFB detection over 3 consecutive months at a central site (Vientiane) and for 1 month at a peripheral site (Attapeu).

**RESULTS:** Of 1675 sputum samples collected from 612 patients, respectively 206 (12.3%) and 275 (16.4%) were AFB-positive by the direct and bleach methods ( $P = 0.0007$ ), i.e., an increase in smear positivity rate of 33.5% (95%CI 31.2–35.8). The superiority of the bleach method was confirmed, regardless of the site, the aspect of the sputum and delay before analysis. This method yielded 24 more TB patients.

**CONCLUSION:** The bleach method, which is very easy to use, could significantly increase the yield of sputum smear microscopy for the detection of pulmonary TB in Laos.

**KEY WORDS:** tuberculosis; case detection; microscopy; sputum; sodium hypochlorite

TUBERCULOSIS (TB) is a major public health concern in Laos, where it is the seventh leading cause of mortality.<sup>1,2</sup> The population, which is mainly rural (73%, according to the 2005 census) and poorly educated, has poor access to health care facilities. The strategy of the National Tuberculosis Programme (NTP) is to reduce the reservoir of infection by early detection of patients with pulmonary TB and the use of DOTS. *Mycobacterium tuberculosis* culture and drug susceptibility testing are not yet available in Laos. Case detection is based on direct sputum smear microscopy for acid-fast bacilli (AFB). In 2007, this strategy identified 44% of incident cases (67 new cases per 100 000 population); the incidence of all forms of TB was estimated at 151/100 000.<sup>3</sup> Direct microscopy is less sensitive in patients with human immunodeficiency virus (HIV) co-infection,<sup>4,5</sup> who represent 3.3% of TB incident cases in Laos.<sup>3</sup>

Several improvements have been suggested to increase the yield of microscopic detection: serial sputum specimen examination,<sup>6</sup> fluorescent microscopy with auramine or rhodamine stain, chemical fluidisation of sputum with concentration by sedimentation or centrifugation.<sup>7,8</sup> Sodium hypochlorite (NaOCl), or bleach, has been used for over a century in this appli-

cation. Concentrations of 2–5% NaOCl digest sputum products and inactive mycobacteria without altering their structure, so that even when killed they can still be stained and observed. This provides greater security for laboratory use.<sup>9</sup> Further centrifugation concentrates the AFB in the mixture and increases the rate of positivity. A literature review revealed a significant increase in the positivity rate related to bleach digestion of sputum in 15 of 19 studies.<sup>10</sup>

Whether this method provides a marked improvement for low-income countries such as Laos, both for case finding and for treatment monitoring, remains to be proved. The technique is still not recommended by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (The Union), mainly because of a lack of standardisation.<sup>11</sup> Further studies are requested to evaluate its feasibility at the peripheral level,<sup>7</sup> and its impact and cost-effectiveness.<sup>12</sup>

The sensitivity and specificity of the bleach method could not be evaluated due to the lack of mycobacterial culture. The present study aimed to quantify the gain in positivity of microscopic detection and to test its implementation in a peripheral laboratory participating in the Lao NTP.

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Article submitted 17 February 2009. Final version accepted 20 May 2009.

[A version in French of this article is available from the Editorial Office in Paris and from the Union website [www.theunion.org](http://www.theunion.org)]

## MATERIALS AND METHODS

### Study site

The study was conducted in two stages: 1) the method was first developed and implemented for 3 months in a central laboratory at Mahosot hospital (National Reference Hospital, Vientiane); 2) to assess its feasibility, it was then implemented for 1 month in a peripheral laboratory, in Attapeu hospital (South Province of Laos), where TB is the third leading reason for consultation and the fifth leading reason for hospitalisation.

### Patients

On the assumption that the bleach method increases the sensitivity of detection by 50%,<sup>10</sup> the number of samples was calculated for theoretical sensitivity rates of 44% (direct method) and 66% (bleach method). With an average of 12% AFB-positive sputum samples routinely detected by the direct method, a unilateral test, a risk  $\alpha$  of 0.05, and a level of significance of 0.05, the sample size to be examined was 2024. All patients undergoing sputum microscopy for AFB (case finding or treatment monitoring) were included in the study. Where serial sputum specimens were tested, each specimen was individually included in the comparative analysis.

### Sample processing

Sputum samples were first classified according to their macroscopic aspect in the laboratory. Salivary samples were immediately removed and a new sample was requested. The time between sputum collection and analysis was recorded. A 20–30 mm smear was made on a new slide with a wooden applicator. After heat fixation, hot Ziehl-Neelsen (ZN) staining was performed: carbol fuchsin 0.3%, slow heating until steaming, rinsing after 5 min, destaining with 25% sulfuric acid, rinsing, counterstaining with methylene blue 0.3% for 1 min, rinsing and drying.<sup>13</sup> The remainder of the specimen was concentrated following the bleach method, as described by Gebre et al.<sup>14</sup> A solution of 5% NaOCl was prepared weekly by dilution in distilled water of household bleach manufactured in Thailand (6% NaOCl) and added to an equal volume in the sputum container. The mixture was homogenised by shaking and then incubated for 15 min at room temperature. A volume of 2–15 ml was transferred to a disposable plastic conical tube with an equal volume of distilled water. After centrifugation at 2000 rpm for 15 min, a drop of the pellet was transferred onto a slide, dried, heat-fixed and stained as the first smear.

Microscopic examination was performed by two experienced technicians for 20 min per smear for 200 high-power microscopic fields (HPF). The results were expressed as per the quantitative scale of The Union (Table 1).<sup>13</sup>

**Table 1** Distribution of results obtained on each sample by the direct method and the bleach method according to the semi-quantitative scale of The Union<sup>13</sup>

	Direct method					Total <i>n</i>	<i>P</i> value
	Negative	±	+	++	+++		
Bleach method							
Negative*	1400	0	0	0	0	1400	
±	51	14	0	0	0	65	
+	14	16	9	0	0	39	
++	3	4	15	2	0	24	
+++	1	2	23	31	90	147	
Total	1469	36	47	33	90	1675	<0.0001

\*No AFB on at least 100 HPF; ± = 1–9 AFB/100 HPF; + = 10–99 AFB/100 HPF; ++ = 1–10 AFB/HPF on at least 50 HPF; +++ = >10 AFB/HPF on at least 20 HPF.

AFB = acid-fast bacilli; HPF = high-power microscopic fields.

### Quality control

The anonymous slides were coded with numbers carried in a notebook kept by the investigator. All the smears, regardless of their method of preparation, were read separately by the two microscopists. After unblinding and comparison, results with agreement were validated. Discordant results were read a third time by the two technicians to reach a consensus. The concentration of the NaOCl solution was checked to 4.75% by the iodometric method.

### Ethical clearance

The project was approved by the Lao National Ethics Committee for Health Research.

### Data analysis

Data were entered using Epi Data 3.1 (Centers for Disease Prevention and Control, Atlanta, GA, USA) and analysed using Stata 8.0 (StataCorp, College Station, TX, USA). Agreement between the two readings was measured by the Cohen's kappa ( $\kappa$ ) coefficient. Using each sample as its own control, the two matched series were compared by the MacNemar's  $\chi^2$  test for binary variables and the Wilcoxon rank-sum test for quantitative variables, with a significance level of 0.05.

## RESULTS

Of 612 patients enrolled, 560 in Vientiane and 52 in Attapeu provided respectively 1552 and 123 sputum samples, i.e., an average of 2.7 samples per patient. The male/female ratio was 0.57, the mean age was 57 years (range 5–92), and 98% of patients were sampled for TB case detection. Only one patient had known HIV infection (Table 2).

Dual reading resulted in 10 disagreements, one on smears prepared by the direct method ( $\kappa = 0.99$ ) and nine on smears prepared by the bleach method ( $\kappa = 0.98$ ). There were six major discrepancies (positive vs. negative) and four differences in assessment of bacillary density. All were resolved after rereading. The

**Table 2** Origin of sputum samples

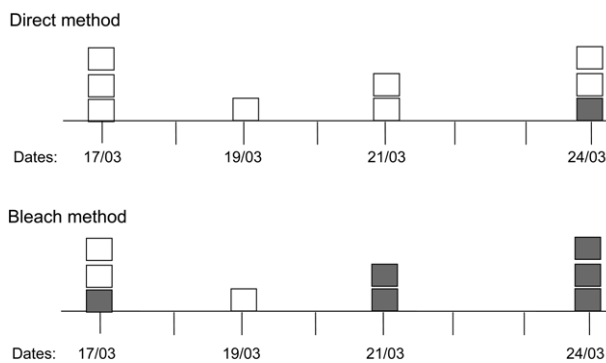
	Patients (N = 612)		Sputum samples (N = 1675)	
	n	%	n	%
Sex				
Male	351	57.4	715	42.7
Female	261	42.7	960	57.3
Age group, years				
<10	4	0.7	8	0.5
10–19	24	3.9	54	3.2
20–29	86	14.1	221	13.2
30–39	90	14.7	213	12.7
40–49	92	15.0	280	16.7
50–59	96	15.7	263	15.7
60–69	123	20.1	338	20.2
≥70	97	15.9	298	17.8
Study sites				
Vientiane	560	91.5	1552	92.7
Attapeu	52	8.5	123	7.3
Reasons for sampling				
Case finding	600	98.0	1625	97.0
Treatment monitoring	12	1.96	50	3.0
HIV-positive	1		9	

HIV = human immunodeficiency virus.

six major discrepancies were related to paucibacillary smears prepared by the bleach method at Mahosot hospital. All were reclassified as positive after the third reading.

Overall, AFB were detected on 206 smears prepared by the direct method (12.3%, 95%CI 10.7–13.9) and 275 smears prepared by the bleach method (16.4%, 95%CI 14.7–18.2), a statistically significant difference ( $P = 0.0007$ ), giving an increase in positivity of 33.5% (95%CI 31.2–35.8). The semi-quantitative results highlighted a significant gain in positivity ( $P < 0.001$ ) with the bleach method, especially for paucibacillary (1–9 AFB/100 HPF) and multibacillary (>10 AFB/HPF) smears (Table 1).

Positivity rates were higher using the bleach method, regardless of study site, the aspect of the sputum and



**Figure** Results of sputum microscopy on nine serial samples from a patient with HIV co-infection. □ = AFB-negative samples; ■ = AFB-positive samples. HIV = human immunodeficiency virus; AFB = acid-fast bacilli.

the delay between sampling and analysis. Gains in positivity were greater at the Attapeu site, on blood-containing specimens and when delays before analysis were <7 h (Table 3).

All TB patients detected by the direct method were also detected by the bleach method. Conversely, 20 TB patients (3.57%) in Vientiane and four TB patients (7.69%) in Attapeu were detected only by the bleach method ( $P = 0.14$ ).

Regarding the only HIV-positive patient enrolled in this study, nine serial sputum samples were tested over an 8-day period. Only one was AFB-positive by the direct method vs. six by the bleach method, providing a positive result 1 week earlier (Figure).

## DISCUSSION

The bleach method was applied for the first time in Laos for the case detection of pulmonary TB. It was compared to the direct method on a large number of sputum samples from two different sites, one central and one peripheral. Proving easy to use in Vientiane as well as in Attapeu, it can be introduced into routine

**Table 3** Qualitative results of sputum microscopy by the direct method and the bleach method by study site, reasons for sampling, the aspect of the sputum and the delay between sampling and analysis

	Sputum samples n	Direct method positive		Bleach method positive		Gain in positivity %	P value
		n	%	n	%		
Study sites							
Vientiane	1552	195	12.6	255	16.4	+30.8	<0.000
Attapeu	123	11	8.9	20	16.2	+81.8	0.002
Reasons for sampling							
Case finding	1625	205	12.6	268	16.5	+30.7	0.006
Treatment monitoring	50	0	0	1	2	—	0.3
Aspect of sputum							
Bloody	94	9	9.6	19	20.2	+111.1	0.001
Purulent	136	36	26.5	49	36.0	+36.1	<0.000
Mucopurulent	1445	161	11.1	207	14.3	+28.6	<0.000
Delay before analysis, h							
1–6	978	118	12.1	165	16.9	+39.8	<0.000
7–13	418	52	12.4	65	20.4	+25	<0.000
14–22	279	36	12.9	45	16.1	+25	0.002

laboratories after a brief training period for technicians responsible for AFB sputum smear microscopy.

The bleach method increases the effectiveness of TB case finding, detecting 24 patients who were not detected by the direct method. The highest gain in positivity (80.5%) was observed among paucibacillary samples (Table 1). It may also reduce delays in diagnosis and treatment; for two patients with serial sputum samples, including one with HIV co-infection, the bleach method provided positive results respectively 5 and 7 days before the direct method.

In the absence of a gold standard, i.e., mycobacterial culture, it was not possible to compare the sensitivity and the specificity of the two methods. Furthermore, the prevalence of HIV co-infection among the patients enrolled was too small to assess the benefits of the method in people living with HIV/AIDS.

The implementation of the bleach method yields an overall increase in positivity of 33.5% (95%CI 31.2–35.8). This result confirms those published in 10 of 13 studies without a gold standard (overall increments ranging from 7% to 253%).<sup>10</sup> Several methodological parameters may explain such a wide range: the target population; the numbers of patients enrolled and samples collected; whether the comparison was performed on patients or on individual samples; the exclusion of salivary samples; the source, preparation and conservation of the NaOCl solution; the incubation time; the power and duration of centrifugation (or duration of sedimentation); blinded reading; the minimum duration of smear reading; and the positivity criteria.<sup>7</sup>

This first application of the bleach method in Laos, performed on 612 patients and 1675 samples, provides answers to a number of objections in the literature against its use in routine microscopy for pulmonary TB.<sup>12</sup> Although the often-mentioned lack of standardisation and quality assurance are unacceptable defects in biological analysis, it is difficult to demand the same level of performance in a well-equipped central laboratory and a poorly equipped peripheral laboratory. Bleach digestion followed by centrifugation is a rustic method. If specific technical adjustments have been made in some centres, they are not likely to affect the results if the key parameters are met: 2–5% NaOCl concentration of the bleach solution, incubation at room temperature for 15 min and 15–30 min centrifugation. As the latter does not require high speed, it can be replaced by sedimentation for 12–18 h.<sup>10</sup> Centrifugation is often seen as a major obstacle because of the cost of the initial investment and consumables, the fact that power cuts are frequent and the risk of reusing disposable tubes,<sup>11</sup> which is why some centres prefer sedimentation.<sup>4,7</sup> Quality assurance of sputum microscopy is a mandatory component of any NTP.<sup>13</sup> Implementing the bleach method requires training in situ, which provides an excellent opportunity to strengthen the procedures in all laboratories participating in the NTP.<sup>15,16</sup>

Few studies have been conducted in peripheral laboratories. Reservations are expressed about the possibilities of transferring this new method successfully, given their isolation and lack of equipment. In our experience, the gain in positivity provided by the bleach method was higher in Attapeu (80.8%) than in Vientiane (30.8%). Although this difference may also reflect the better quality of examinations routinely performed in a central laboratory,<sup>5</sup> it strongly suggests the feasibility of its implementation in the provinces.

In Laos, the bleach method also addresses two concerns related to the distance of the target populations from the health centres: the risk of contamination by sample handling and the long delays between sputum collection and smear staining. The addition of 5% NaOCl to the container disinfects the sputum while making it liquid.<sup>17</sup> Although bleach also makes it impossible to isolate *M. tuberculosis*, as long as culture is not available the method will provide greater bio-safety for laboratory staff.<sup>18</sup> As regards the delays, of 597 samples analysed 7–22 h after sputum collection, the bleach method provided a 25% increase in positivity compared to the direct method.

Laos still has a low prevalence of HIV/AIDS (according to the WHO, the prevalence rate is between 0.2% and 0.4% among adults aged 15–49 years).<sup>19</sup> TB is the leading cause of death among patients living with HIV.<sup>19</sup> The risk of co-infection with *M. tuberculosis* therefore justifies systematic case finding in this population. The poor yield of sputum microscopy in HIV-positive patients undermines the TB control strategies.<sup>20</sup> The incremental yield of the bleach method has rarely been studied in high HIV/AIDS prevalence settings. However some studies show a higher increase in positivity among HIV-positive than among HIV-negative subjects.<sup>5,21</sup> Only one patient with HIV was included in our study. Although the bleach method proved very advantageous in this case, its efficacy for TB case finding in patients with HIV co-infection in Laos remains to be determined.

## CONCLUSION

Our study, conducted in a population with a low prevalence of HIV/AIDS, confirms the benefits of the bleach method for the microscopic case detection of pulmonary TB. This rustic, simple and inexpensive method could easily be integrated into the routine of a peripheral laboratory after a short training period and strengthening of quality assurance. Further multi-centre studies will ensure that when it is extended to all laboratories participating in the NTP, it will make the DOTS strategy more efficient in Laos.

## Acknowledgements

This study was funded by the Agence Universitaire de la Francophonie and the Chambre Syndicale Nationale de l'Eau de Javel, Neuilly-sur-Seine, France.

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## RÉSUMÉ

**CADRE :** Au Laos, pays de forte prévalence de la tuberculose (TB) et de faible prévalence d'infection par le virus de l'immunodéficience humaine/syndrome de l'immunodéficience acquise (VIH/SIDA), la culture de *Mycobacterium tuberculosis* n'est pas encore disponible. Le dépistage repose sur la détection microscopique des bacilles acido-alcool-résistants (BAAR) dans les crachats. La méthode de fluidification-concentration par l'eau de Javel pourrait en améliorer le rendement.

**OBJECTIF :** Évaluer l'apport de la méthode à l'eau de Javel et sa faisabilité au Laos.

**MÉTHODES :** Comparaison de la méthode directe et de la méthode à l'eau de Javel lors des recherches de BAAR effectuées pendant 3 mois consécutifs dans un site central

(Vientiane) et pendant un mois dans un site périphérique (Attapeu).

**RÉSULTATS :** Sur 1675 échantillons de crachats prélevés chez 612 patients, 206 (12,3%) ont été trouvés BAAR-positif par méthode directe et 275 (16,4%) par méthode à l'eau de Javel ( $P = 0,0007$ ), soit une augmentation du taux de positivité de 33,5% (IC95% 31,2–35,8), supériorité confirmée quels que soient le site, l'aspect des crachats et les délais avant analyse. Cette méthode a permis de dépister 24 patients (3,9%) supplémentaires.

**CONCLUSIONS :** Facile à appliquer, la méthode à l'eau de Javel peut accroître significativement le rendement du dépistage microscopique de la TB pulmonaire au Laos.

## RESUMEN

**MARCO DE REFERENCIA :** Laos presenta una alta prevalencia de tuberculosis (TB) y una prevalencia baja de infección por el virus de la inmunodeficiencia humana y sida (VIH/SIDA). Aún no se cuenta con el cultivo para *Mycobacterium tuberculosis*. La búsqueda de casos se

basa en la detección microscópica de bacilos acidorresistentes (BAAR) en el esputo. El método de liquefacción y concentración de las muestras con hipoclorito de sodio (lejía) podría mejorar el rendimiento de la prueba.

**OBJETIVO :** Evaluar las ventajas y la factibilidad del método de la lejía en Laos.

**MÉTODO :** Comparar el procesamiento convencional de las muestras de esputo (método directo) con el método de la lejía, en la detección de BAAR durante 3 meses consecutivos en un laboratorio central (Vientiane) y durante un mes en un laboratorio periférico (Attapeu).

**RESULTADOS :** De las 1675 muestras de esputo recogidas de 612 pacientes, 206 (12,3%) tuvieron un resultado positivo para BAAR con el método directo y 275

(16,4%) con el método de la lejía ( $P = 0,0007$ ), es decir, un aumento de 33,5% del índice de positividad (IC95% 31,2–35,8). La superioridad del método de la lejía fue independiente del laboratorio, el aspecto del esputo y del lapso transcurrido hasta el análisis y permitió detectar 24 pacientes suplementarios con TB.

**CONCLUSIÓN :** El método de la lejía es sencillo de realizar y podría aumentar en forma significativa el rendimiento de la baciloscopia en la detección de la TB pulmonar en Laos.

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