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# Recovery rates of *Mycobacterium tuberculosis* using five decontamination methods

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**Summary**

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains one of the leading infectious diseases in resource limited countries of the world, including Nigeria. For optimal care of patients with pulmonary TB, effective decontamination methods are required for isolation and identification of *M. tuberculosis* from other fast growing organisms found in sputum samples of infected patients. Five methods of sputum decontamination including the Petroff, oxalic, bleach, simplified concentration and Kudoh methods were assessed prior to mycobacterial culture. In all, thirty human sputum samples were processed and results analysed after eight weeks of incubation. Overall, there was a significant difference in the growth yield using the different methods (Friedman test statistic, $Q_K = 36.3$; $P<0.05$). Again, a significant difference (Friedman test statistic, $Q_K = 48.0$; $P<0.05$) was observed between the valuable and non-valuable yield of mycobacteria. Furthermore, the simplified concentration method had the best performance in terms of pure culture growth/minimal media contamination coupled with a cost benefit ratio of 0.10; the bleach method being the least.

Given these findings, coupled with laboratory challenges in developing countries as well as ease of use on the field/cost effectiveness; we propose the simplified concentration as an optimal decontamination method for use in resource limited settings where TB remains an endemic problem.

**Keywords**: *Decontamination, diagnosis, sputum, Mycobacterium tuberculosis*

**Résumé**

La Tuberculose (TB) causée par le *Mycobacterium tuberculosis* demeure l'une des maladies infectieuses principales dans les pays à ressources limitées tel que le Nigeria. Pour des soins optimaux des malades de la tuberculose pulmonaire, il faut des méthodes de décontamination efficace pour l'isolation et l'identification du *M. tuberculosis* des autres organismes à croissance rapide qui se trouvent dans les échantillons de crachat des patients infectés. Cinq méthodes de décontamination du crachat y compris les méthodes de Petroff, oxalique, de blanchiment, de concentration simplifiée et de Kudoh ont été évaluées avant la culture des mycobactéries. En tout, trente échantillons de crachat humains ont été traités et les résultats analysés après huit semaines d'incubation. De façon générale, il y avait une différence considérable dans les rendements de croissance en utilisant les différentes méthodes (test statistique de Friedman, $Q_K = 36,3$; $P<0,05$). Aussi, on a remarqué une différence importante (test statistique de Friedman, $Q_K = 48,0$; $P<0,05$) entre le rendement important et peu important de mycobactérie. De plus, la méthode de concentration simplifiée a eu une meilleure performance en termes de croissance de culture propre/ contamination minimale de media ajoutée à un ratio de profit de coût de 0,10; la méthode de blanchiment étant la dernière. Etant donné ces résultats, ajouté aux défis de laboratoire dans les pays en développement de même que la facilité de l'usage sur terrain/efficacité du coût ; nous proposons la concentration simplifiée comme méthode de décontamination optimale à l'usage des pays à ressources limitées où la TB demeure un problème endémique

## Introduction

Tuberculosis (TB) is still a leading health concern globally and the main cause of death by a single infectious agent, namely *Mycobacterium tuberculosis*. The disease spreads more easily in overcrowded settings and in the conditions of malnutrition and poverty; characteristics typical of developing countries [1] including Nigeria.

The diagnosis of TB infection is vital both clinically and epidemiologically [2] and the identification of infectious cases is a crucial step towards the control of TB globally. In the laboratory, diagnosis of TB relies exclusively on the detection of acid- fast bacilli (AFB) in sputum by smear microscopy, an approach that has been in existence since its introduction in the nineteenth century [3]. Though the diagnosis of pulmonary tuberculosis relies exclusively on the detection of AFB in the sputum [3]; the bulk of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by more rapidly growing normal flora organisms. These rapidly overgrow the entire surface

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of the medium and digest it before the slow growing tubercle bacilli begin to grow. Consequently, most specimens must therefore be subjected to a harsh digestion and decontamination process that liquefies the organic debris and eliminates the unwanted normal flora [4]. Thus, there is the need to identify methods to improve the sensitivity of microscopy. A number of methods have been developed for the decontamination of non-sterile specimens to enhance the recovery of *M. tuberculosis* [5]. Such sputum processing methods including centrifugation and sedimentation have been studied and found promising [6]. However, given the different economic implications of using some of these methods, there is a need to evaluate their cost effectiveness, ease of performance and level of mycobacterial yield especially in low resource countries like Nigeria where tuberculosis is endemic. Although each of these methods has been used at different times and places, however, limited study has compared their recovery rates for mycobacteria. This study was therefore carried out to evaluate the recovery rates of *M. tuberculosis* from smear positive sputum samples using five decontamination methods as well as to determine the cost benefits of using these different methods.

## Materials and methods

*Study design, study area, sample collection and sample number*

This analytical experimental study was carried out in Ibadan, Oyo State, Nigeria. Already processed and smear positive sputum specimens based on local algorithm were collected from three major Directly Observed Short-Course Therapy (DOTS) Centres namely Jericho Chest Hospital, TB and Leprosy Health centres at Molete and Moniya in Ibadan for this study. Ten specimens were collected during the first week and then every forth-night until the desired sample number was reached; with each batch transported to the laboratory in an ice-pack container for processing. The main inclusion criterion for selecting specimens was based on laboratory analysis (mainly smear microscopy) by the respective clinics. In all, 30 sputum specimens were collected.

*Laboratory analysis*

*Decontamination*

A total of 30 sputum samples from 30 different confirmed TB patients were subjected to five different decontamination methods at the TB laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan in order to compare their effectiveness in sputum decontamination prior to microscopy and culture. The methods used included: the Petroff, oxalic, bleach, simplified concentrated and Kudoh.

*Petroff's method*

This was carried out by adding 1ml of sputum sample to 2mls of NaOH; the tubes were capped tightly and gently shaken to digest the sample. The mixture was allowed to stand at room temperature for 15 minutes with occasional shaking and centrifuged at 3000x*g* for 15minutes. The supernatant was poured off and 15mls sterile saline was added and centrifuged again at 3000x*g* for 15minutes. Supernatants were decanted and smears made from the sediment.

*Oxalic method*

This technique was carried out as described by Yajko et al. [4]. Briefly, 1ml of sputum sample was added to 1ml of 5% oxalic acid in a sterile tube, and was mixed by vortexing for 15seconds. This was incubated at room temperature for 30 minutes with occasional shaking and the volume was brought to 5mls with sterile saline, mixed and centrifuged at 3000x*g* for 15minutes. The supernatant was discarded and the remaining material was brought to neutrality using 4% NaOH and pH paper as indicator. After neutralization, the solution was mixed and smears were made [5].

*Bleach method*

This method was carried out according to the description of Githui et al [7]. Briefly, equal volume of 5% NaOCl was added to the sputum sample and left at room temperature for 15minutes after which smears were made.

*Simplified concentration method:*

This method was carried out as described by Garay[6]. This method involved the preparation of solutions of 3% ammonium sulphate and 1% of NaOH which were added together. 2mls of the product was then mixed with 1ml of sputum sample, shaken by hand and left at room temperature for 12-15 hours after which smears were made.

*Kudoh method*

This was carried out using the method of Kudoh [8]. Briefly, a significant portion of the sputum sample was obtained with a sterile cotton swab, submerged in a tube containing 3mls of 4% NaOH solution and incubated at room temperature for 2 minutes; the applicator was removed and smear was made.

After the decontamination procedures above were carried out, smears from each of the samples were made on Middlebrook 7H11 agar slants prepared in duplicate with pyruvate or glycerol and later incubated at 37°C for at least eight weeks during which readings were made.

*Cost benefit ratio*

The cost benefit ratio was calculated in order to determine the cost effectiveness of the methods. Cost was determined by the cost of equipment, cost of reagents and cost of man-hour. However, cost of laboratory electrical consumption and bottles used were not considered while the benefit was determined by the number of tubes with harvestable mycobacterial yield.

using Chi square analysis. The data were statistically analysed using Friedman ANOVA for ranks.

Contamination was defined as complete overgrowth of the medium within the eight weeks of incubation and the lack of acid fast bacilli (AFB) by the time the medium was discarded. The contamination rate was calculated as (number of plates or tubes contaminated/total number of plates or tubes inoculated) x100.

## Results

The simplified concentration and Kudoh methods yielded the best pure culture growth (26.7% each), followed by Petroff's (16.7%) and Oxalic (16.7%) methods; the bleach method being the least (10%) (Table 1). Other findings such as the combination of culture growth and contaminant, and contaminant only are shown in Table 1.

**Table1:** Growth yields of the five decontamination methods

| | Simplified concentration n (%) | Kudoh n (%) | Petroff n (%) | Oxalic n (%) | Bleach n (%) |
|---|---|---|---|---|---|
| Growth | 8(26.7) | 8(26.7) | 5(16.7) | 5(16.7) | 3(10.0) |
| Growth/contaminants | 15(50.0) | 12(40.0) | 15(50.0) | 16(53.3) | 14(46.7) |
| Contaminants | 7(23.3) | 10(33.3) | 10(33.3) | 9(30.0) | 13(43.3) |

*n = Number of growth, growth/contaminants or contaminants*

**Table 2:** Valuable and non-valuable yields of the five decontamination methods

| | Petroff n (%) | Oxalic n (%) | Bleach n (%) | Simplified concentration n (%) | Kudoh n (%) |
|---|---|---|---|---|---|
| Valuable | 20(66.7) | 21(70.0) | 17(56.7) | 23(76.7) | 20(66.7) |
| Non Valuable | 10(33.3) | 9(30.0) | 13(43.3) | 7(23.3) | 10(33.3) |

*n = Number of valuable or non-valuable yield*

**Table 3:** Cost benefit ratio per sample

| | Petroff | Oxalic | Bleach | Simlplified | Kudoh |
|---|---|---|---|---|---|
| Cost (in $) | 3.18 | 2.12 | 3.03 | 2.37 | 2.21 |
| Benefit | 20 | 21 | 17 | 23 | 20 |
| Ratio | 0.16 | 0.15 | 0.18 | 0.10 | 0.11 |
| Time required(in hour) | 0.75 | 0.75 | 0.25 | 12 – 15 | 0.03 |

*Data analysis*

The number of culture tubes with pure growth of *M. tuberculosis;* growth with contaminants; and contaminants only were counted and statistical difference was based on 95% confidence interval

Evaluation of the five methods for their ability to prevent contamination of media by non-acid fast organisms was reported as either valuable (if there were colonies of AFB on the media) or non valuable (if no colony at all was present). By these

criteria, simplified concentration method had the highest valuable yields (76.7%) followed by oxalic acid (70%), Petroff's (66.7%), Kudoh (66.7%), and bleach (56.7%). However; considering the non valuable yield, the bleach method was the highest (43.3%) while the simplified concentration method was the least (23.3%) (Table 2). Furthermore, with respect to the cost effectiveness of the methods, the simplified concentration method was the most cost effective with cost benefit ratio of 0.10 followed by Kudoh (0.11), Oxalic (0.15), Petroff (0.16) and bleach (0.18) methods, respectively (Table 3). In the same vein, the simplified concentration method was the cheapest of all the five methods as $2.06 worth of reagents and chemicals were required to process one sample followed by oxalic method ($2.12). Kudoh method ($2.17), Petroff's method ($2.24) and the bleach method ($2.72), respectively (Table 3). Analysis of the growth, contaminants and both valuable and non-valuable yield are presented in Tables 4 and 5.

the best result. This method may therefore be suitable for use in low resource countries of Africa and on the field given the promising results and more so; this method was the cheapest of the five methods evaluated. In addition, the simplified concentration method is simple to perform and does not require additional equipment like centrifuge, but just an overnight incubation, which is the only limitation it has, as there is one day delay in getting the result. Meanwhile, the Kudoh method competed favorably with simplified concentration method in terms of pure growth yield, but its ability to reduce contaminants was lower (66.7%). This finding is in agreement with earlier report which showed high contaminant rate with the Kudoh method [8]. Meanwhile, out of the five decontamination methods evaluated in this study; the bleach method gave the poorest result in terms of growth yield and ability to reduce contaminants (10% and 43.3% respectively). This finding was however at variance with the assertion of Loretha *et al.* [9] that showed the bleach method as being the most

**Table 4:** Friedman ANOVA table (growth yields)

| | Simplified Concentration | Kudoh | Petroff | Oxalic | Bleach |
|---|---|---|---|---|---|
| Growth | 8(4.5) | 8(4.5) | 5(2.5) | 5(2.5) | 3(1.0) |
| Growth/contamination | 15(3.5) | 12(1.0) | 15(3.5) | 16(5.0) | 14(2.0) |
| Contamination | 7(1.0) | 10(3.5) | 10(3.5) | 9(2.0) | 13(5.0) |
| ER | 9.0 | 9.0 | 9.5 | 9.5 | 8.0 |
| $ER^2$ | 81.0 | 81.0 | 90.25 | 90.25 | 64.0 |

*Friedman test statistic, $Q_K = 36.3$; $P<0.05$*
*There is a significant difference in the growth yield of Mycobacteria by the different decontamination methods*

**Table 5.** Friedman ANOVA table (valuable/non- valuable)

| | Petroff | oxalic | Bleach | Simplified concentration | Kudoh |
|---|---|---|---|---|---|
| Valuable (Rank) | 20 (2.5) | 21 (4.0) | 17 (1.0) | 23 (5.0) | 20 (2.5) |
| Non valuable | 10 (3.5) | 9 (2.0) | 13 (5.0) | 7.0 (1.0) | 10 (3.5) |
| $ER_1$ | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| $ER_2$ | 36.0 | 36.0 | 36.0 | 36.0 | 36.0 |

*Friedman test statistic, $Q_K = 48.0$; $P<0.05$*
*There is a significant difference between valuable and non-valuable yield of Mycobacteria by the different decontamination methods*

## Discussion

Our findings show that the simplified concentration and Kudoh methods gave the best results considering pure growth yield of AFB. However, when the ability to reduce the level of media contaminants was evaluated, the simplified concentration method gave effective in the recovery of AFB and prevention of contaminants. The difference in our findings may be as a result of the fact these workers compared the bleach method with the oxalic acid, cetylpyrinium chloride, and cetyitrimethlammonium bromide methods; whereas, our study compared the bleach

with the Petroff's, oxalic, simplified concentration, and Kudoh methods. Therefore, future studies may require the comparison of more methods apart from the ones included in this study under the same laboratory setting.

In conclusion, our findings show that the simplified concentration method provided the best method of sputum decontamination for the recovery of AFB under our local setting. Apart from this, the Petroff's and oxalic methods are not suitable for field use due to the centrifugation required. Finally, despite these findings, further studies are required to explore mechanisms of reducing the incubation period of sputum decontamination procedures under the simplified concentration method in order to accelerate diagnosis of TB in resource limited countries.

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