

Full Length Research Paper

## Alternative technique for culturing sputum for mycobacteria isolation: Feasibility, performance and effect on laboratory quality assessment - A technical note

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Received 2 December, 2014; Accepted 27 April, 2015

**Mycobacteria culture remains the cornerstone of tuberculosis diagnosis. Naturally contaminated samples need pre-inoculation processing but some economically challenged medical facilities may benefit from a simpler and cheaper sputum decontamination procedure. The aim of this study was to test a simple decontamination method lacking a centrifugation step to be used in conjunction with the culture on Löwenstein-Jensen medium. A total of 7446 sputum samples collected from 3229 patients were microscopically examined and then cultured on Löwenstein-Jensen medium using a simplified Petroff method. All positive cultures were confirmed by direct microscopic examination and biochemical identification. Culture and microscopic status and time to positivity were recorded. Mean and median times to culture and contamination rate were similar as compared to classical Löwenstein-Jensen culture method. Overall results suggest that the described modified of Petroff method may be used with adequate results in resource poor settings as the method does not require an aerosol safe centrifuge and relies on cheap, stable and readily available reagents.**

**Key words:** Culture, mycobacteria, tuberculosis, Löwenstein-Jensen, centrifugation.

### INTRODUCTION

Despite the advent of novel molecular techniques for mycobacterial identification, bacteriological examination is still the foundation of a successful tuberculosis control program (Kantor et al., 1998). Although microscopic

examination is quick to perform and almost universally available, mycobacterial cultures play a central diagnostic role given their improved sensitivity. Cultures may be even more useful in high prevalence or high resistance

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areas; however in these settings, it is usually less likely to benefit from adequate laboratory facilities due to economic factors.

Culture of spontaneous expectorated sputum, as the most readily available specimen for pulmonary TB diagnosis, generally includes a decontamination step before media inoculation. Various decontamination methods are available, the most widely used being: sodium hydroxide (2-4%) alone, sodium hydroxide N acetyl cysteine, oxalic acid, cetylpyridinium chloride–NaCl and Ogawa Kudoh approaches (Rivas et al., 2010).

The Ogawa medium does not require sample decontamination and it is hypothetically cheaper than Löwenstein-Jensen medium (Soto et al., 2009). However, this is not necessarily true if media are not made on-site (as laboratory requirements are high for media manufacturing); additionally, the Ogawa technique may also be less useful for drug sensitivity testing (DST) (C.D.C. and N.I.H., 2006).

Except for the Ogawa Kudoh approach, which is considered to be particularly suitable for resource limited settings, the decontamination stage is promptly followed by an inoculum concentration step, usually achieved by centrifugation (Yeboah-Manu et al., 2004).

This step is time consuming and requires an aerosol safe centrifuge which is a relative expensive piece of equipment and therefore may represent a strain for low resources settings (Babady and Wengenack, 2012). The use of a standard centrifuge is possible, but since centrifugation carries a significant biohazard risk (mainly by generating aerosols and droplet nuclei, either by careless manipulation or by unfortunate events such as tube breaking or spillage) the use of a safety centrifuge is recommended. Furthermore, biosafety level 3 has been recommended for this type of operations by the Center of Disease Control (C.D.C. and N.I.H., 2006).

The aim of this study was to test a simple decontamination method lacking a centrifugation step to be used with the Löwenstein-Jensen culture media. The rationale behind this approach was to implement a more flexible and cheap technique— a method which allowed for a concentration step if available, or skip it in a low resources setting, and which relies on largely available culture media.

This idea was brought about by necessity, as our laboratory was confronted by a similar problem— an aerosol safe centrifuge was not available for a short period of time.

## MATERIALS AND METHODS

The study group included 7446 sputum samples from 3229 patients (suspected for pulmonary tuberculosis or undergoing monitoring during treatment) admitted to our center between March 12<sup>th</sup> 2009 and June 12<sup>th</sup> 2009, which were evaluated to be of satisfactory quality as per the WHO criteria (Kantor et al., 1998) (mucoid/mucopurulent, volume more than 3 ml) and processed within 24 h of reception.

The study design and general purpose consent procedure was approved by the "Grigore T. Popa" University of Medicine and Pharmacy Iasi Ethics Committee (the 16<sup>th</sup> of November 2008 meeting).

Patient records/information was kept anonymous and de-identified prior to analysis by laboratory personnel thus following the relevant local personal data regulations.

Each sample underwent microscopical examination - first using an auramine – rhodamine stain; positive smears were Ziehl Nielsen restained and reexamined. Each sample was subsequently cultured on Löwenstein-Jensen media using a modified Petroff procedure as described below.

From each sputum sample, 3 ml were transferred to a flat bottom flask containing glass beads, using a sterile disposable Pasteur pipette and an equal volume of 4% NaOH solution was added; the sample was gently mixed and set to rest for 30 min at room temperature; the mix was brought to neutral pH using a 8% HCl solution and Brom Thymol blue as indicator. Aliquots of 0.2 ml were then inoculated on Löwenstein-Jensen for a total of three slants per sample. The slants were then incubated at 37°C, horizontally for the first 48 h and then standing-up for up to 60 days. Positive cultures were all confirmed by direct microscopic examination for AFB, and biochemical identification (reductase, catalase and niacin tests).

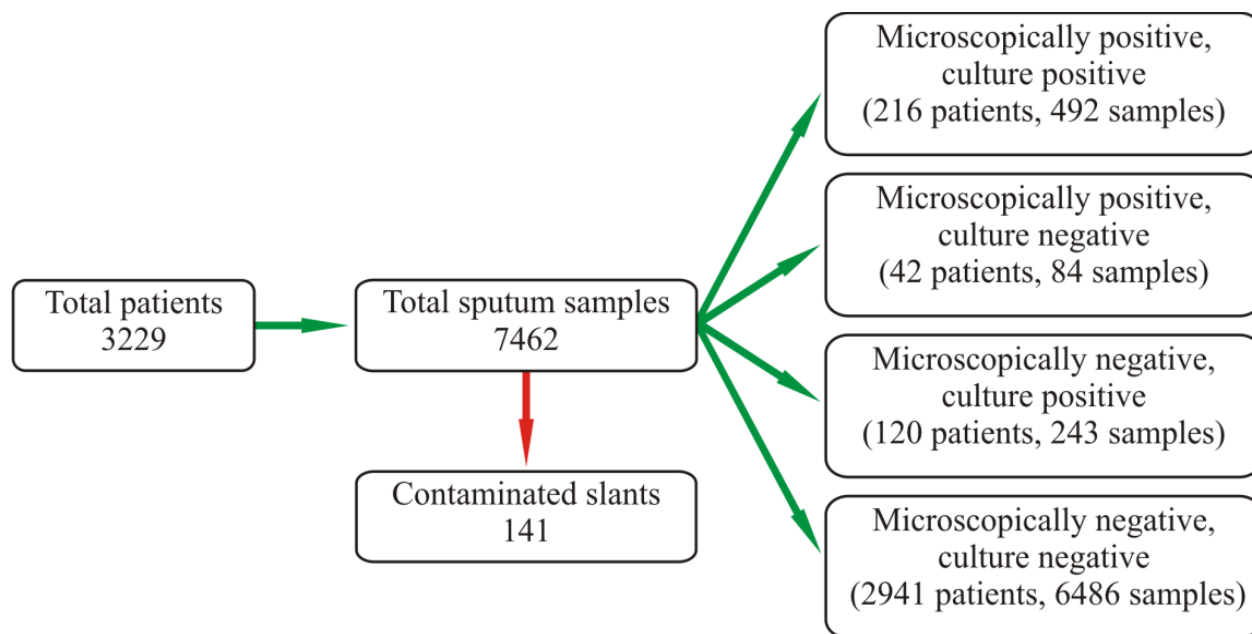
## RESULTS

From a total of 7446 samples, 819 (from 378 individuals patients) were either positive on direct microscopic examination alone (n=84, 10.3%, 42 patients) or associated with at least one non contaminated positive culture followed by positive identification of mycobacteria (n=735, 89.7%, 336 patients) (Figure 1).

There were 84 microscopically positive samples that had associated negative cultures – which all originated from 42 patients undergoing TB treatment at the two months milestone. There were 141 contaminated slants but in only two cases (which both were microscopically positive) no other culture was available as all corresponding slants were contaminated.

As far as culture positive results are concerned, from the total of 735 culture positive samples (from 336 patients), a total of 492 were microscopically positive (66.9% positive) and 243 microscopically negative (33.1%). These figures are similar to those from the regional tuberculosis register which reports a rate of 78.7% bacteriologically confirmed cases (76.6% were both culture and microscopically positive and 23.4% were microscopically negative, but culture positive) for the same time frame.

Mean time to culture positivity was 21 days (interquartile range 21-30 days). Median time to culture positivity (and interquartile ranges) according to the mycobacterial load in the sputum was 30 days (21-30 days) for microscopically negative, 21 days (21-23.25 days) for scarcely positive (less than 1+), and 21 days (21-27.75 days) for samples with 1+ positive sputum smears. All samples with 2+ and 3+ smears had positive culture results by 21 days. There were 141 contaminated slants with a 1.9% contamination rate, a figure implying adequate decontamination intensity for a Löwenstein-Jensen culture technique.



**Figure 1.** Study group structure – bacteriology status.

There were 6486 microscopically and culture negative samples (from 2941 patients). No definite data concerning the health status of these patients was available. However, we were not able to find any positive result in the local register for any patient with negative culture results in a three months' time window following the initial culture. Although this does not exclude false negative results, it may suggest a low value.

## DISCUSSION

Improving the diagnostic output of basic microbiology facilities has been previously explored (Ramos et al., 2010) from an economic point of view, at least as far as liquid media culturing was concerned. Different concentration methodologies have been tested- centrifugation vs. filtration with similar results; however, while filtration may be a cheaper alternative, it still bears an additional cost.

A similar rationale lead to a comparison between standard NaOH decontamination/centrifugation methods and direct inoculation using a MODS approach- significant differences were found in terms of sensitivity, 97 vs. 81% and contamination rate of 3 vs. 18% (Grandjean et al., 2008). This direct inoculation sensitivity loss cannot be ignored and should be evaluated considering the significantly increased contamination rate.

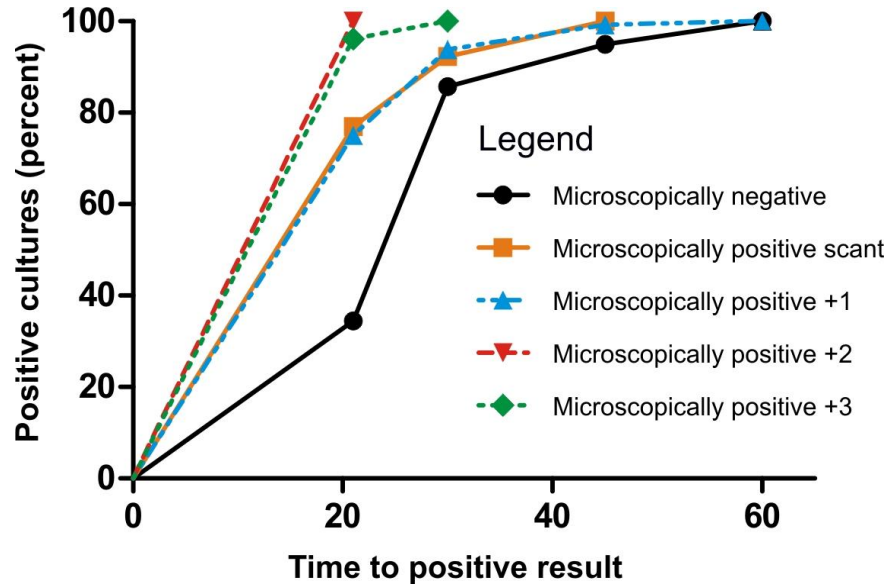
All these reports focused on broth cultures and to our best knowledge, there is little data available on alternative culturing methods involving Lowenstein Jensen medium, which is the main culture media available to resource limited settings (Satti et al., 2010).

Our data show that time to culture positivity is not significantly increased in low bacterial load samples (scant, +1 vs. +2, +3 for microscopy examination) suggesting a minimum sensitivity loss while using the modified culture protocol (Figure 2).

Our results also suggest that the described modified Petroff method may be used in conjunction with Lowenstein Jensen media with adequate results, in order to circumvent the need for an aerosol safe centrifuge, which has been previously considered a potential burden for financially challenged settings (Grandjean et al., 2008).

Sensitivity of this modified technique was not directly assessed in our study as comparism was not available. However, we may indirectly and roughly make an estimate using the proportion of sputum positive smears (confidence on this data is high as microscopy diagnosis was externally audited and considered to be 100% accurate for three years in a row) and considering the time to culture positivity data. Literature data evaluating the quality of direct sputum smear examination estimate that an excellent quality of direct examination generates around 65% microscopically confirmed cases out of all bacteriologically confirmed cases (Tanoue et al., 2002). This figure is close to the 70.3% value for our study group; a major sensitivity loss would have significantly increased the proportion of microscopically confirmed cases.

Forty six (12%) microscopically positive/culture negative cases may also suggest a lower sensitivity (possibly due to lack of concentration of the inoculum) but this data should be interpreted in the context of sample provenance (two months treatment milestone); the



**Figure 2.** Time to culture positivity stratified by microscopically assessed bacterial load.

possibility of non-viable MT should not be excluded. Along this line, there is somewhat controversial published data on sputum concentration (using the 2% NALC – 2% NaOH method) as having no significant impact on light microscopy results (Cattamanchi et al., 2009).

An extensive literature review article (Steingart et al., 2006) considered NaOH treated and centrifugated sputum to generate an increased microscopic sensitivity, estimating the role of centrifugation at around 7% based on somewhat old data (Cameron and Castles, 1945).

Considering these aspects, we cannot rule out a sensitivity decrease in the order of 10% or less. Therefore, this method may not be useful for low bacterial count samples such as gastric lavage, pleural fluids or samples from HIV infected individuals; these results should not be extrapolated to low quality sputum samples.

Contamination rate for our group was low, less than 2%, thus within the WHO recommended limits (Kantor et al., 1998). While contamination was not an issue for our setting, there are reports on significantly higher rates for alternative culture methods and this is probably to be expected if resources are scarce (Muyoyeta et al., 2009). Using decontaminant laced Lowenstein Jensen media might prove useful for these settings; PANTA, Heptatab and penicillin G proved to be effective as decontaminant additives to an added cost of less than 1 \$ per test (Kassaza et al., 2014).

Overall, the described concentration free method has some advantages as it does not require expensive equipment such as an aerosol safe centrifuge, it is less time intensive as compared to classical methods and only relies on cheap, stable and readily available reagents.

This peculiarity may allow for field culturing, thus significantly decreasing time to culture and also

contamination rate, even if the slants are later sent to a central facility, thus increasing the access to a referral center (Perkins et al., 2006).

This method makes use of the Lowenstein-Jensen media which is cheap, almost universally available, easily transported and can be used in conjunction with other methods.

## Conclusions

Our data suggest that this approach may be viable as a temporary measure in low resource laboratory settings at the putative price of a tolerable sensitivity loss.

## Conflict of Interests

The authors did not declare any conflict of interest.

## ACKNOWLEDGEMENTS

This research was supported by UEFISCS CDI from the project PN-II-ID-PCE-2011-3-0565. We thank dr. Daniela Diculencu from Department of Bacteriology, Clinic of Pulmonary Diseases, Iasi and Dr. Dumitru Cojocaru from Department of Biochemistry and Molecular Biology, "Alexandru Ioan Cuza" University, Iasi for critical suggestions concerning the protocol and the manuscript.

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