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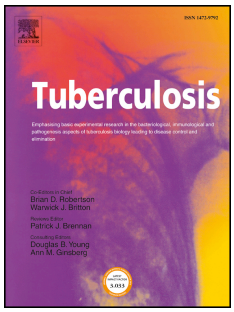

Centrifugation and decontamination procedures selectively impair recovery of important populations in *Mycobacterium smegmatis*

John A. Kennedy, Vincent O. Baron, Robert J.H. Hammond, Derek J. Sloan, Stephen H. Gillespie

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Centrifugation and decontamination procedures selectively impair recovery of important populations in *Mycobacterium smegmatis*.

*John A. Kennedy, Vincent O. Baron, Robert J.H. Hammond, Derek J. Sloan, #Stephen H. Gillespie

School of Medicine, University of St Andrews, Fife, United Kingdom

*Present address: John A Kennedy, School of Medicine, University of Edinburgh, Edinburgh, United Kingdom

Address correspondence to Stephen H. Gillespie at shg3@st-andrews.ac.uk

Abbreviations:

LR, Lipid rich, cells contain non-polar lipids

LP, Lipid poor, cells do not contain non-polar lipids

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Summary

Diagnosis and treatment monitoring of patients with tuberculosis (TB) requires detection of all viable mycobacteria in clinical samples. Quantitation of *Mycobacterium tuberculosis* (Mtb) in sputum is commonly performed by culture after sample decontamination to prevent overgrowth by contaminant organisms. Exponentially growing cultures have cells that predominately lack non-polar lipid bodies whereas stationary cultures have a predominance of cells with non-polar lipid bodies. This may reflect rapidly growing 'active' and non-replicating 'persister' sub-populations respectively in sputum from TB patients. We investigated the effect of decontamination on culture-based quantitation of exponential and stationary phase cultures of *Mycobacterium smegmatis* in an artificial sputum model. Exponentially growing populations were between 89 and 50 times more susceptible to decontamination than stationary phase cultures when quantified by most probable number and colony forming units. These findings suggest that decontamination selectively eliminates the 'active' population. This may impair diagnostic sensitivity, treatment monitoring, and compromise clinical trials designed to identify new antibiotic combinations with activity against all mycobacterial cell states.

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39 Keywords: mycobacteria, decontamination, dormancy, sample processing, culture

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1 Introduction

Detection of viable *Mycobacterium tuberculosis* (Mtb) from patient samples is fundamental for diagnosis and monitoring response to treatment. Culture techniques are the current gold standard and represent the most established methods (1).

Sputum decontamination is performed to reduce the number of non-mycobacterial species that might otherwise overgrow the Mtb and cause uninterpretable results. Sodium hydroxide (NaOH) combined with N-acetyl-L-cysteine (NALC) is the most commonly used technique: with NALC breaking up the sputum and NaOH reducing the risk of overgrowth by killing fast growing organisms. This moderately stringent treatment is known to reduce the number of mycobacteria recovered and could reduce the number of organisms below the limit of detection in paucibacillary specimens (2).

Loss of Mtb during decontamination also affects treatment monitoring. Studies of serial quantitative cultures on non-decontaminated sputum samples show that bacterial clearance from the sputum is best described by a biphasic curve: with rapidly growing Mtb eliminated quickly whilst non-replicating 'persister' cells are cleared slowly (3). Understanding the response of these different sub-populations may be key to identifying patients at a high risk of treatment failure, and to developing new drug regimens (4). It is known that when decontaminated samples are studied the characteristic biphasic pattern of response is lost (5). It is possible that the decontamination procedure selectively kills these bacterial sub-populations, highlighting the importance of investigating this effect.

Intracellular lipid inclusions within mycobacteria are associated with a non-replicating reversible state, decreased metabolic activity and increased phenotypic resistance to antibiotics (6, 7). From *in vitro* models, lipid rich (LR) cells predominate in late stationary phase cultures whilst lipid poor (LP) cells are more common during early logarithmic growth (8). Clinical studies using fluorescence microscopy to label intracytoplasmic lipid bodies with acid-fast bacilli on sputum smears have shown that patients who accumulate higher proportions of 'lipid body positive' Mtb cells during early treatment are at a higher risk of unfavorable outcomes. (9). Collectively these data suggest that LR cells *in vitro* and 'lipid body positive' cells in clinical specimens represent a similar, antibiotic tolerant mycobacterial phenotype, characterization of which may help explain treatment response. To assess the differential

elimination of LR and LP cells during antibiotic therapy it is essential to establish whether sputum decontamination selectively kills either bacterial sub-population.

This paper reports a study seeking to establish the impact of decontamination on the recovery of both exponential and stationary phase cultures of mycobacteria using *Mycobacterium smegmatis* as a model organism as this allowed the experiments to be performed rapidly. The use of an artificial sputum model allowed accurate quantitation of the effects of decontamination.

2 Results

2.1 Lipid bodies and decontamination

From three stationary cultures 277, 457 and 787 individual bacteria were counted. From three exponential cultures 782, 894 and 1190 bacteria were counted to calculate the LR and LP proportions. Fluorescence microscopy of Nile red stained *M. smegmatis* showed that stationary and exponential cultures contained 76.7% (95% Confidence intervals (CI) 70.6%-82.8%) and 29.7% (95% CI 6.9%-52.5%) of mycobacteria with non-polar lipid bodies, respectively.

2.2 Effect of artificial sputum and centrifugation

Artificial sputum had no effect on quantitation by MPN or CFU ($p=0.28$ and 0.34 respectively). Following centrifugation, the recovery rates decreased to 49.8% ($p= 0.015$) and 50.3% ($p= 0.0034$) for MPN and CFUs.

2.3 Stationary versus exponential inocula

Percentage recovery of bacteria from the stationary and exponential cultures following sample decontamination is shown in Figure 1. There were significant differences between stationary and exponential inocula quantified by MPN and CFU ($p<0.001$ and $p<0.001$ respectively). Recovery was higher in the stationary inocula by a factor of 89 and 50 for MPN, and CFUs respectively.

3 Discussion

Improving diagnostic tools for TB requires increased sensitivity to detect small numbers of bacteria in clinical samples. Understanding the effect of antibiotics in improving TB chemotherapy requires accurate quantitation of all Mtb populations in sputum samples collected at baseline and during

treatment. Whilst the gold standard for diagnosis and treatment monitoring remains mycobacterial culture, laboratory processing of culture samples is complex. Steps such as centrifugation and decontamination may affect Mtb recovery but data on the consequences of these are limited. The existence of bacterial subpopulations in differing metabolic states, identified by variable lipid content in clinical Mtb samples is increasingly recognized (7, 9). This paper uses different quantitative bacteriology techniques to describe the impact of sample processing with sodium hydroxide on the recovery of mycobacteria.

Our most important finding was that sample decontamination with NaOH, designed to eliminate non-mycobacterial cells, in combination with centrifugation, depletes mycobacterial recovery by up to 90%. Although it was known that NaOH treatment reduced mycobacterial viability we have extended this observation by showing that NaOH treatment has a different effect on mycobacteria depending on their cell state (Figure 1). Our data clearly show poorer recovery of viable mycobacteria from exponential (1-day old) cultures spiked into artificial sputum than from stationary (7-day old) cultures (Figure 1). As exponential cultures are mainly LP, whilst stationary phase cultures are mainly LR, it follows that LP bacteria are more vulnerable to NaOH and are selectively killed during decontamination.

As our model of sample processing was based on artificial sputum we performed initial experiments to show that the artificial sputum had no confounding effect on the recovery of mycobacteria. A secondary finding of these experiments was to confirm the prior results of Yoshimatsu et al., that centrifugation results in a loss of approximately half of the inoculum (10). Previous studies have also shown that further increasing the time or centrifugation force does not improve bacterial recovery (11). It follows that centrifugation is optimized but there is still considerable loss of cells, which are predicted to be lipid rich, which may diminish the sensitivity of sputum culture. Work by den Hertog *et al.* demonstrated that older Mtb cultures have a lower buoyant density which predicts a poorer recovery by centrifugation (12). However this does not explain our post decontaminations results which had a poorer recovery of the exponential culture.

Poor recovery of LP mycobacteria has implications for diagnosis. Patients with low bacterial loads of predominately LP cells could be falsely rendered culture negative. The lower bacterial load would also be smear negative and therefore undetected by conventional techniques. These cases may only be

identified by diagnostics methods, which do not require sample decontamination e.g. Gene Xpert MTB/RIF (13).

When LP bacteria are almost completely eliminated by decontamination, no assessment of the differential effects of antibiotics on this bacterial sub-population is possible. This may explain why studies using decontamination prior to quantitative culture have reported monophasic bacillary elimination (5) whilst studies using non-decontaminated samples have described a biphasic response (3). For the first time this paper provides evidence that the monophasic response it an artifact of the decontamination process that causes disproportionate loss of exponentially growing mycobacteria.

There are several limitations to the work described here. The experiments were conducted using artificial sputum model with *M. smegmatis* and there are differences in lipid content between mycobacterial species. Further studies should validate these results on samples containing *M tuberculsosis*. We used 'exponential' and 'stationary' phase cultures to generate LP-predominant and LR-predominant inocula respectively. Varying culture age may influence NaOH susceptibility of bacteria for reasons which are unrelated to any differential effect on LR and LP cells in clinical specimens Nevertheless, this paper provides the clearest evidence to-date that NaOH has a greater killing effect on LP populations of mycobacterial cells. Sputum decontamination, combined with centrifugation may sacrifice diagnostic sensitivity and compromise the ability to accurately monitor elimination of all bacterial populations during antibiotic therapy for tuberculosis.

3.1 Conclusion

This study has demonstrated for the first time that decontaminating mycobacterial samples has a differential effect on dormant and active sub populations. This has implications for monitoring response to treatment and therefore to the development of novel therapeutic regimes.

4 Materials and methods

4.1 Generating exponential and stationary cultures

Mycobacterium smegmatis (NCTC 8159) was used for all the experiments. A 1-day-old *M. smegmatis* culture created an exponential phase and a 7-day-old culture was used as a stationary phase culture. To create the exponential culture a flame sterilized nichrome loop was used to select a single colony from

Middlebrook 7H10 for inoculation in Middlebrook 7H9 with 0.05% Tween 80. This was incubated at 37˚C in a static incubator for 7 days reaching an OD_{600} of between 1.0 and 1.5. The exponential culture was prepared by pipetting 1 µL from the stationary culture into 20 mL of fresh media and incubated for approximately 24 hours until an OD_{600} of 0.05 was obtained.

4.2 Nile red staining and fluorescence microscopy

The proportion of mycobacteria within a culture containing non-polar lipid bodies was assessed with Nile red staining based on previously published methods (8). In brief 1 µL of Nile Red solution at 250 µg/mL dissolved in dimethyl sulfoxide was added to 100 µL of bacterial suspension in phosphate buffered saline (PBS). This was incubated at room temperature in the dark for 10 minutes and then washed twice by centrifuging at 20,000 *g*, for 3 minutes, discarding the supernatant and resuspending the pellet in PBS. The bacteria were heat fixed to a microscopy slide and examined with a Leica DM5500. An L5 filter cube with an excitation of 480/40 nm and emission 527/30 nm allowed visualization of Nile Red fluorescence from a non-polar lipid environment. Nile Red fluorescence from a more polar lipid environment were imaged using a TX2 filter cube, which had an excitation of 560/40 nm and an emission of 645/75 nm. Bacteria were manually counted from images generated from microscopy. Bacteria were counted as LR if fluorescence was detected with the L5 filter. This was then calculated as a percentage of the total bacteria identify using the TX2 filter.

4.3 Artificial sputum and centrifugation assessment

To determine whether the artificial sputum caused bacterial clumping that would confound bacillary quantitation, preliminary work was done in with *Mycobacterium smegmatis.* In brief, artificial sputum medium was prepared using mucin, electrolytes, egg yolk emulsion and amino acids as per the protocol of Sriramulu et al (14). A stationary inoculum was prepared as outlined above. The bacterial load was then quantified by most probable number (MPN) and CFUs with Middlebrook 7H9 with 0.05% tween 80 and 7H10 to establish a baseline. From the stationary culture, 20 µL was inoculated into 9.98 mL of artificial sputum in a 50 mL falcon tube, thoroughly vortexed and immediately a sample was taken for re-quantitation by MPN and CFUs to determine the effects of the artificial sputum.

The effect of centrifugation on bacterial recovery was assessed by adding 40 mL of PBS to the *M. smegmatis* spiked sputum and centrifuged at 3,000 *g* for 20 minutes at 4˚C. The supernatant was

discarded and the pellet was re-suspended in 1 mL of PBS. The bacterial load of the pellet was quantified by MPN and CFUs to determine the effects of centrifugation.

4.4 Decontamination

From the exponential culture 1 mL was inoculated into 9 mL of artificial sputum and 20 µL from the stationary culture was inoculated into 9.98 mL of artificial sputum this was performed to obtain equal starting inoculum sizes. Both stationary and exponential spiked sputum samples were mixed with an equal volume of 2% NaOH, 1% NALC and 2.9% sodium citrate and briefly vortexed. These were incubated at room temperature for 15 minutes and neutralized with 30 mL of PBS. Following centrifugation at 3000*g* for 20 minutes at 4°C the supernatants were discarded and then pellets were re-suspended in 1 mL of PBS. The pellets were quantified by MPN and CFUs. These steps were repeated in biological quadruplicate.

4.5 Bacteriological methods

The principle behind the MPN assay is to prepare replicate dilutions of the sample to identify the dilution beyond which there is no growth. The statistical analysis of the MPN is calculated based on the proportion of culture-positive replicates of this dilution. MPN assays were performed in a 96 well plate by inoculating 20 µL of each 10 fold dilution in Middlebrook 7H9 with 0.05% Tween 80 this was repeated for a total of 5 times. MPN counts were calculated according to the U.S. Food and Drug Administration procedure (15). Colony forming units were serially diluted and from these dilutions 10 µL was plated in triplicate onto Middlebrook 7H10. These were incubated at 37°C and were read daily for 10 days.

4.6 Statistical analysis

Mycobacterial recovery was defined as the proportion of the initial inoculum, which could be quantified after sample processing, expressed as a percentage. CFUs and MPN values were analyzed using 2 tailed, unpaired Student's T-tests in Microsoft Excel (version 14.3.1). Graph analysis was performed using Prism (version 7.04).

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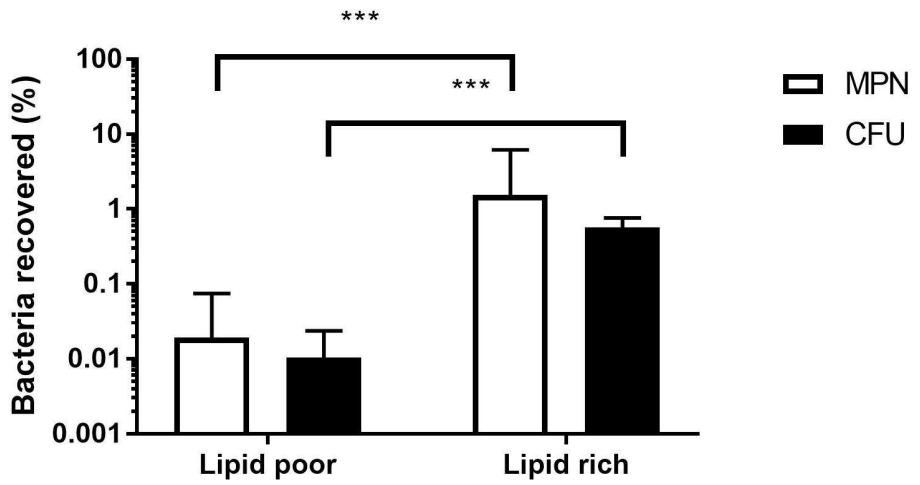

Figure 1. Effect of sodium hydroxide decontamination on the recovery of mycobacteria.

The error bars represent two standard deviations of the quadruplicates. * Indicate p < 0.001 for Student's T-test**

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