Revisiting the Culture of Latent Stage *Mycobacterium smegmatis* on a Standard Agar Plate

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Abstract: Disc diffusion assay is a basic and initial test in the search for potential inhibitor against bacteria, including high pathogenic bacteria, i.e. *Mycobacterium tuberculosis. Mycobacterium smegmatis*, due to the characteristics of non-pathogenic, fast growing and can be handled by Biosafety Level 1 facility, is therefore widely used as the replacing model to study *M. tuberculosis.* However, *Mycobacterium* spp. can exist in active and latent stage by utilizes different metabolism pathway. Under the circumstances of nutrient or oxygen depletion, *Mycobacterium* spp. shift the energy generation cycle from tricarboxylic acid cycle (Krebs cycle) to glyoxylate cycle to progress into latent stage. It is challenging to obtain *Mycobacterium* spp. at latent stage. Here, we revisited the preparation of latent form *M. smegmatis*. We reported the medium, supplement and inoculum size to produce a reasonable lawn of *M. smegmatis* in a standard agar plate.

Keywords: *Mycobacterium smegmatis*, Latent stage culture, Medium and inoculum selection, Spreading method, Bacterial lawn for disc diffusion assay.

1. INTRODUCTION

Tuberculosis is still a public health concern in developing countries, affecting one-third of the world population. New drugs are needed due to the rise of multidrug resistant strains as well as total drug resistant strains. Biosafety Level 3 facility is must to study the causative agent, *Mycobacterium tuberculosis* (MTB). In order to minimize the risk of infection, *Mycobacterium smegmatis* is usually used at the preliminary stage for MTB drug discovery [1-5]. *M. smegmatis* is a well-known replacing model to study MTB [6-13] as this non-pathogenic strain is fast growing and can be studied with Biosafety Level 1 facility [14].

Mycobacterium spp. can exist in two phases during infection: the active and latent stages [15]. Both infections have the bacteria undergo different metabolism [16]. For instance, both stages have different energy generation cycle [17]. In active stage, *Mycobacterium* utilizes glucose as carbon source, generating energy via tricarboxylic acid cycle (Krebs cycle). Meanwhile, in latent stage *Mycobacterium* uses lipid as carbon source and generate energy via glyoxylate cycle to skip beta-oxidation steps during oxygen or nutrient depletion. Eradicating latent stage *Mycobacterium*, i.e. *M. tuberculosis* is as important as eliminating active stage tuberculosis as it can avoid "recurrent" infection. Thus, in order to obtain latent stage *Mycobacterium* spp., the culture media, supplement given and numbers of cell used are some of the influential factors. Here, we revisited and summarized the optimization of *M. smegmatis* latent stage culture in agar plate.

2. MATERIALS AND METHODS

2.1. Medium Preparation

Middlebrook 7H9 Broth with ADC Enrichment

7.85g ADC enrichment (Middlebrook Albumin Dextrose Catalase Supplement; BD Difco, USA) was dissolved and brought to 100ml with dH_2O and sterilized with Sartorius Minisart 0.2µm syringe filter. 4.7g Middlebrook 7H9 (BD Difco, USA) was dissolved and brought to 900ml with dH_2O, autoclaved and aseptically added with 100ml of the earlier prepared ADC enrichment.

Middlebrook 7H10 Agar with OADC Enrichment.

7.9g OADC enrichment (Middlebrook Oleic Albumin Dextrose Catalase Supplement; BD Difco, USA) was dissolved and brought to 100 ml with dH_2O and sterilized with Sartorius Minisart 0.2µm syringe filter. 19.0g Middlebrook 7H10 (BD Difco, USA) was dissolved and brought to 900 ml with dH_2O, autoclaved and aseptically added with 100ml of the earlier prepared OADC enrichment. Middlebrook 7H10 agar was then plated in 90mm (diameter) polystyrene Petri dishes.

M9 Minimal Broth

11.28g M9 minimal media (BD Difco, USA) was dissolved and brought to 1 L with dH_2O before autoclaving. 2ml 1 M $MgSO_4$ and 0.1ml 1 M $CaCl_2$ have

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then added aseptically. (i) 0.4% glucose, or (ii) 0.4% acetate was also added accordingly.

M9 Minimal Agar

11.28g M9 minimal media (BD Difco, USA) and 3.75g agar powder were dissolved and brought to 1 L with dH_2O before autoclaving. 2ml 1 M $MgSO_4$ and 0.1ml 1 M $CaCl_2$ have then added aseptically. (i) 0.1% glucose, or (ii) 0.1% acetate, or (iii) 0.4% glucose, or (iv) 0.4% acetate was also added accordingly. M9 minimal agar was then plated in 90mm (diameter) polystyrene Petri dishes.

2.2. Media Selection

Mycobacterium smegmatis strain mc^2155 (ATCC 700084) was purchased from American Type Culture Collection (ATCC). *M. smegmatis* stock culture store in -80^oC was used to grow in (A) Middlebrook 7H9 broth with ADC enrichment (37^oC, 200 rpm, 3 days), (B) M9 minimal broth with 0.4% glucose (37^oC, 200rpm, 6 days), and (C) M9 minimal broth with 0.4% acetate (37^oC, 200rpm, 4 days). 10µl of each culture was spot inoculated on M9 minimal agar plate supplemented with (i) 0.1% glucose, or (ii) 0.1% acetate, or (iii) 0.4% glucose, or (iv) 0.4% acetate as the carbon source. M9 minimal media with glucose and acetate represents environment for active stage and latent stage, respectively, for *Mycobacterium*.

2.3. Inoculum Size Selection

The inoculum which showed the highest cell density on the M9 minimal agar plate best was subsequently batch cultured in 20ml media with the same condition (37^oC, 200rpm, 3 days). The inoculum was then either diluted with dH_2O or concentrated by centrifugation to obtain a cell density of OD_{600nm} of 0.1, 1.0, 1.5, 2.0 and 2.6. These inoculums with different cell density were then spread on M9 minimal agar with 0.1% acetate.

2.4. Spreading Method Selection

Inoculum of OD_{600nm} 0.1, 1.0, 1.5, 2.0 and 2.6 was spread on M9 minimal agar with 0.1% acetate by, (i) dipped cotton swab in the inoculum and spread directly on the agar plate, (ii) 100 µl inoculum was pipetted to the agar plate and spread with cotton swab pre-dipped with M9 minimal broth, and (iii) 100µl inoculum was pipetted to the agar plate and spread with L-shape disposable plate spreader.

3. RESULTS AND DISCUSSION

In general, disc diffusion assay supposed to have inoculum with turbidity equivalent to 0.5 McFarland standard spread on agar medium, applying paper disc soaked with test extracts or antibiotic and observing the zone of inhibition. Initially, we used this general guideline but could not obtain a lawn of *M. smegmatis*. Thus, we revisited and carried out some modification to address a few suspected factors such as the carbon source, the inoculum size, and spreading method.

3.1. Effect of Medium

M. smegmatis was cultured in (A) Middlebrook 7H9 with ADC enrichment (represented active mycobacterium), (B) M9 minimal media with 0.4% glucose (control, active mycobacterium), and (C) M9 minimal media with 0.4% acetate (represented latent mycobacterium). Inoculum from (A), (B) and (C) was then plated on M9 minimal agar with different carbon source, i.e. (i) 0.1% glucose, (ii) 0.1% acetate, (iii) 0.4% glucose and (iv) 0.4% acetate. Figure **1** shows the growth of inoculum (A) to (C) on M9 minimal agars. All inoculums (A) showed growth in M9 minimal agars. The best culture was observed on M9 minimal agar with 0.4% glucose as this medium was its optimum growth condition (sufficient concentration of glucose as the favourite carbon source). The cell density was very much lower in 0.4% acetate compared with 0.4% glucose because *M. smegmatis* has shifted the metabolic pathway to use acetate, which was a less favourable carbon source. The cell density in 0.1% glucose was also lower compared to 0.4% glucose. However, no significant difference in cell density was observed between 0.1% glucose and 0.1% acetate as 0.1% glucose has limited carbon source while 0.1% acetate caused the shift metabolic pathway. Inoculums (B), representing active stage *Mycobacterium* in minimal media, showed lower growth compared with active mycobacterium inoculum in optimum media. Inoculum (B) was unable to grow in M9 minimal agar with 0.4% acetate and 0.1% glucose. Surprisingly, the growth was observed in M9 minimal agar with 0.1% acetate, indicating that the percentage of lipid carbon source could affect the growth of latent mycobacterium. Inoculum (C), representing the latent form *Mycobacterium*, was unable to grow in all agar plates. This could be due to the insufficient of cell density. Inoculum of latent stage *Mycobacterium* should be able to grow in lipid carbon source but might need longer growing time (> 4 days), in order to obtain cell density that was able to show growth like inoculum (B).

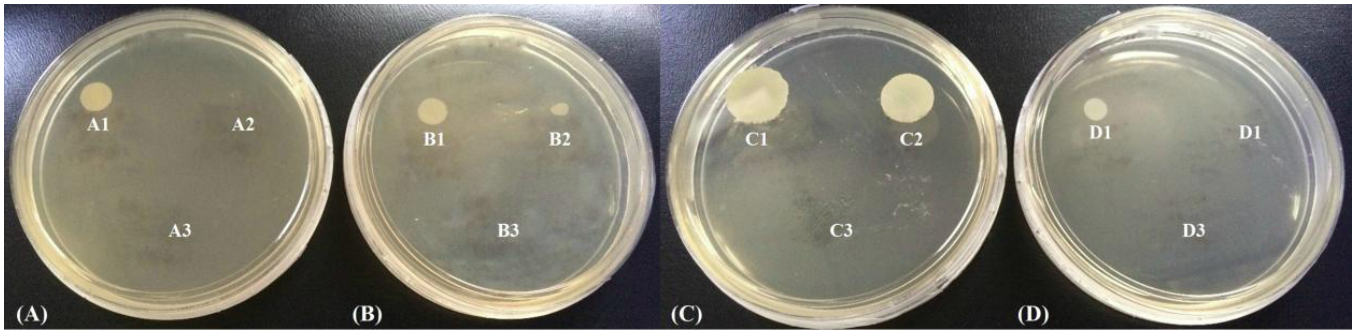

Figure 1: Spot inoculation of *M. smegmatis* on M9 minimal agar with (**A**) 0.1% glucose, (**B**) 0.1 acetate, (**C**) 0.4% glucose, and (**D**) 0.4% acetate. A1-D1 are inoculums from Middlebrook 7H9 with ADC enrichment; A2-D2 are inoculum from M9 minimal media with 0.4% acetate; A3-D3 are inoculums from M9 minimal media with 0.4% glucose.

As overall, inoculum (A) is the best to be used for preparing latent stage *M. smegmatis* due to its ability to grow well when the carbon source shifted from glucose into acetate. This is due to the fact that culturing *M. smegmatis* in optimum media took 3 days prior to harvest but culturing *M. smegmatis* in M9 minimal media will need at least than 6 days. Therefore, seed culture of *M. smegmatis* that growth in optimal media would be more time and cost effective in the consequence works. In addition, M9 minimal agar with 0.1% acetate would be more suitable to culture *M. smegmatis* compared to M9 minimal agar with 0.4% acetate. On the other hand, M9 minimal agar is meant to be supplied with carbon source prior use. The media is to be supplied with 0.4% carbon source according to the standard preparation protocol. However, other studies showed that *M. smegmatis* can be grown with as little as 0.1% carbon source [18]. After the comparison between 0.1% and 0.4% of carbon source (glucose or acetate), results showed in agreement with the above-mentioned studies that 0.1% carbon source performed better in the case of *M. smegmatis*. Thus, inoculum from Middlebrook 7H9 with ADC enrichment was used as seed culture to grow *M. smegmatis* on M9 minimal agar with 0.1% acetate to obtain the latent stage. It is, however, only media selection was not sufficient to obtain bacterial lawn on M9 minimal agar.

3.2. Inoculum Size Selection

Inoculums of different OD were spread on the agar plates with three different spread plate methods. Regardless of spread plate method, all agar plates with inoculum OD_{600nm} 0.1 and 1.0 did not show growth of *M. smegmatis* (Figure **2**). Inoculum OD_{600nm} 0.1 (equivalent to McFarland standard of 0.5) is a general standard for bacterial lawn preparation for disc diffusion assay.

However, the recommended cell density with McFarland standard is usually for *Escherichia coli*. *E. coli* has a relatively fast doubling time where OD_{600nm} 1.0 can be gained within 4 hours [19]. On the other hand, *M. smegmatis* need at least 3 days to obtain OD_{600nm} 1.0. Inoculum OD_{600nm} 1.5, 2.0 and 2.6 showed

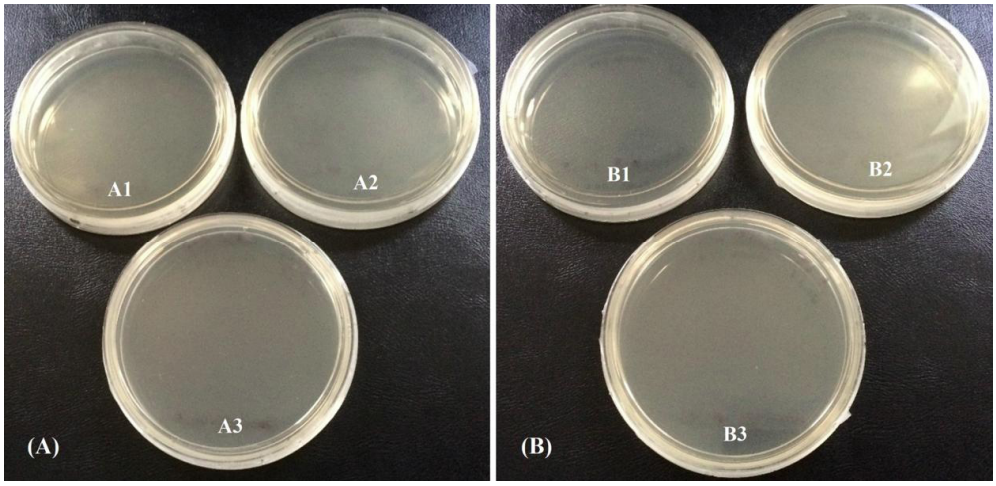

Figure 2: The culture of *M. smegmatis* (on M9 minimal agar with 0.1% acetate) with cell density (**A**) OD_{600nm} 0.1, and (**B**) OD_{600nm} 1.0. No growth was observed after 3 days of incubation at 37°C.

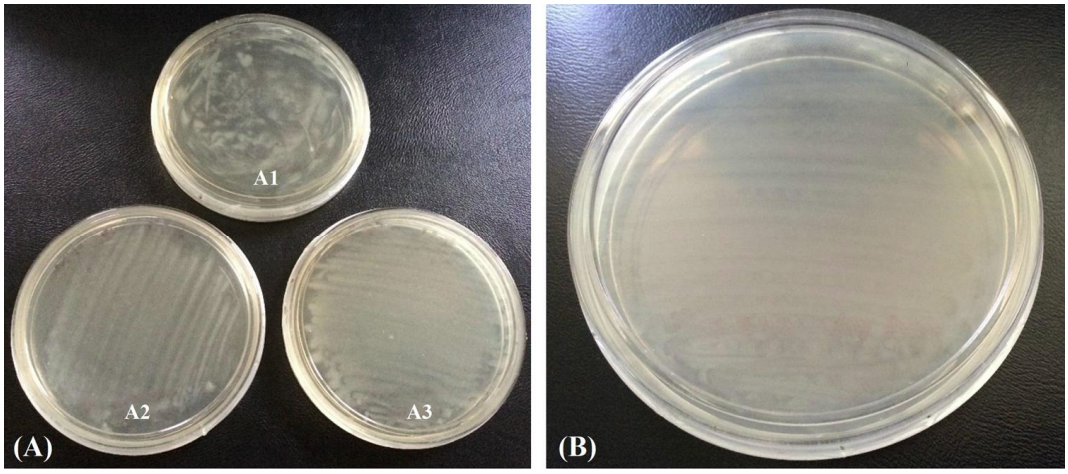

Figure 3: (**A**) The culture of *M. smegmatis* (on M9 minimal agar with 0.1% acetate) with cell density of OD_{600nm} 2.0 and 2.6 with different spreading methods (A1- 100µl inoculum OD_{600nm} 2.0 spread with plate spreader; A2- 100µl inoculum OD_{600nm} 2.0 spread with pre-dipped cotton swab with M9 minimal media). (**B**) The lawn of *M. smegmatis* (on M9 minimal agar with 0.1% acetate) produced from 100µl inoculum OD_{600nm} 1.5 spread with pre-dipped cotton swab with M9 minimal media.

growths on M9 minimal agar with 0.1% acetate. However, the lawn produced from inoculums OD_{600nm} 2.0 and 2.6 were too thick and might influent the actual result for disc diffusion assay. Therefore, with OD_{600nm} 1.5 is a more suitable cell density to be used for bacterial lawn production for disc diffusion assay.

3.3. Effect of Spreading Method

Inoculum OD_{600nm} 0.1 and 1.0 also did not show any growth regardless of spreading methods. Spread 100µl inoculums OD_{600nm} 2.0 and 2.6 with L-shape disposable plate spreader showed uneven lawn (Figure **3**). The cotton swab that was directly dipped and spread on agar caused most of the cells being retained in the cotton, thus fewer cells were transferred to the plate. This was evidenced from the spread of inoculum OD_{600nm} 2.0 with direct dipped and spread method, no growth was observed. Pre-dipped cotton swab with M9 minimal broth and spread 100µl inoculums OD_{600nm} 1.5 produced lawn that is more uniform (Figure **3**). Therefore, inoculum OD_{600nm} 1.5 that was spread using pre-dipped (with M9 minimal broth) cotton swab would the most suitable for disc diffusion assay.

In conclusion, bacterial lawn preparation prior to disc diffusion assay is an established technique and widely used for antimicrobial susceptibility testing for common bacterium such as *E. coli*. As per *M. smegmatis*, the detailed methodology for disc diffusion assay is limited. This work revisited and addressed challenges in order to obtain bacterial lawn for latent stage *M. smegmatis* prior to disc diffusion assay. The factors that affected the bacterial lawn for *M. smegmatis* were including the concentration of carbon source (acetate) in the media; inoculum size as well as spread plate method. These factors could easily be overlooked when only the general guidelines of the disc diffusion assay were followed. It is expected that this work could benefit more works in preliminary antimycobacterial drug screening for the discovery of new tuberculosis drug.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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