Application of Replicate Organism Detection and Counting Method (RODAC) in Measuring *Mycobacterium Tuberculosis* Contamination in High Burden Laboratories

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Abstract

Background: Technicians working in high burden tuberculosis (TB) laboratories pose a higher risk of being infected by *Mycobacterium tuberculosis* from clinical samples. Contamination control is mandatory to detect the release of bacteria into the working environment and to minimize the risk of exposure to the workers. The contamination measurement is rarely performed due to the lack of standard methodology. This study optimized and applied a unique culture-based method named Replicate Organism Detection and Counting (RODAC) plates to assess the presence of *M. tuberculosis* contaminant in the laboratory with high burden of clinical samples.

Methods: RODAC was applied on twenty working surfaces in the Mycobacteriology Laboratory of Universitas Padjadjaran. The results of RODAC were compared with DNA-based detection from the same working surfaces using in-house IS6110 real-time PCR (IS6110-qPCR). The detection limit of the RODAC plate was 19.6 CFU mL^{-1}.

Results: From all working surfaces tested, two distinct colonies were found on RODAC plate stamped on the Ziehl-Neelsen staining basin. Those colonies were identified as *M. tuberculosis* and non-tuberculous mycobacteria (NTM), as confirmed by the MPT64 antigen test and the presence of acid-fast bacilli. IS6110-qPCR detected the presence of *M. tuberculosis* DNA in ten sampling points, including the ZN staining basin, incubators, and microscopy areas. IS6110-qPCR detected more working surface contamination versus RODAC. However, it was noted that RODAC, which was a culture-based method, detected live bacteria, while PCR could not distinguish between live and dead bacteria.

Conclusion: The application of the RODAC plate is more suitable for monitoring the contamination of live bacteria in the working environment and to inform a proper corrective action.

Keywords: Contamination control, IS6110, *Mycobacterium tuberculosis*, qPCR, RODAC

Introduction

Laboratory technicians are known to pose a higher risk of *Mycobacterium tuberculosis* infection from processing clinical samples. A retrospective study shows that the incidence rate of developing tuberculosis (TB) in either skilled or unskilled laboratory technicians is the highest compared to non-laboratory workers, achieving 422 cases per 100,000 people each year[1] despite the rigid quality control (QC) in TB laboratories. Therefore, national government bodies and the World Health Organization (WHO) have published biosafety guidance to minimize the risk of TB laboratory-acquired infections.[2,3]

Detailed minimum biosafety precautions for various types of TB diagnostics, such as Ziehl–Neelsen (ZN) staining, culture, molecular testing, and QC, have been published. The existing QC protocol for the laboratory mainly revolves around contamination prevention,[4–6] test result reliability, and external quality assessment,[7–9] facilities airflow assessment and calibration,[2,4,6] as well as cross-contamination assessment.[9,10] However, concrete measurements of the biosafety risk of exposure to *M. tuberculosis* in TB laboratories

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are rarely done due to the lack of standard methodology,[11] so individual laboratories should improvise to ensure biosafety. This study was performed to optimize and apply a unique culture-based method named Replicate Organism Detection and Counting (RODAC) plates to assess the presence of *M. tuberculosis* contaminant in the laboratory with a high burden of clinical samples.

Culture-based method using RODAC plate is designed to monitor common bacterial contamination, but also works on *M. tuberculosis* contamination detection. This method is useful in identifying surface contaminants with a capture rate between 38–60%, depending on the recovery time.[11] RODAC can also detect the release of viable bacteria to the environment, and unlike the gold standard polymerase chain reaction (PCR) based tests which can detect both dead and viable bacteria. This experiment was conducted to prove RODAC effectivity compared to a PCR-based test to detect contamination in twenty surface areas in the TB research laboratory in Universitas Padjadjaran. The experiment results are used as the basis for improving a standard protocol for biosafety and biosecurity in a high burden TB laboratory.

Methods

The experiment was conducted in the Mycobacteriology Laboratory of the Faculty of Medicine, Universitas Padjadjaran. The study did not use human samples for bioanalysis. Therefore, no ethics statement was needed in conducting the experiments.

The workflow was described in Figure 1. The laboratory consisted of four units: 1) sputum processing and primary culture area; 2) media preparation area; 3) incubator area; 4) observation (microscopy) area (Table 1 and Figure 2).

M. tuberculosis H37Rv (ATCC 27294™) was used in all optimization steps and as a positive control for the experiment. The bacterium was inoculated in a solid Ogawa medium for 21 days prior to the experiment.

Middlebrook 7H11 (DIFCO) for RODAC was prepared with slight modification from the original protocol by Daneau et al.[11] Briefly, Mycobacteria Middlebrook 7H11 agar was sterilized and supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment, 0.06% oleic acid, 5% albumin, and 0.5% glycerol. Premixed polimixin B, amphotericin B, nalidixic acid, trimethropim, azlocilin (PANTA) were used to inhibit other bacteria's growth. The media were then poured into RODAC plates, 16 mL each, and stored at 4°C for a maximum 4 weeks. The inhibition effect of PANTA on *M. tuberculosis* growth was observed by inoculating reference strain H37Rv on RODAC plates supplemented with PANTA 2%.

An inoculum of one McFarland of M. tuberculosis H37Rv was prepared and serially diluted up to 10^{-6}. Each 10^{-1} to 10^{-6} dilutions were then inoculated onto RODAC plates and incubated at 37°C for 28 days. Total plate

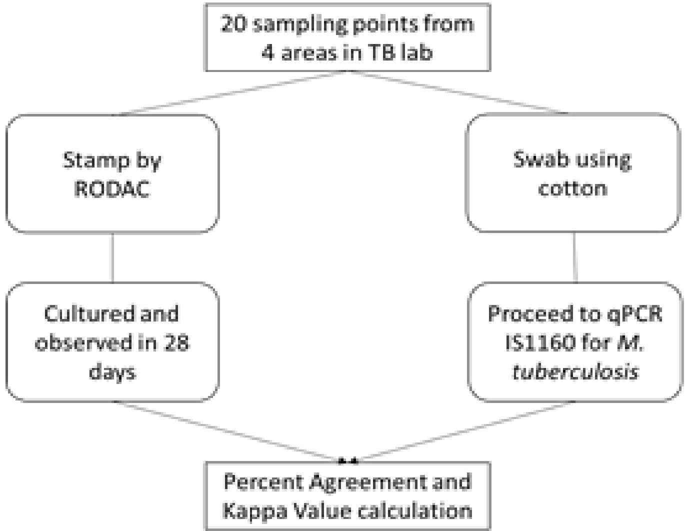

Figure 1 Sampling and Detection Platforms for Contamination Check

Table 1 Sampling Areas and Points

A. Sputum processing and primary culture area	B. Media Preparation Area
1. Class II BSC type 2A before culture	1. Media preparation bench
2. Interior surface of class II BSC type 2A during culture	2. Floor
3. Exterior surface of class II BSC type 2A during during	3. Workbench
4. Class II BSC type 2A 5 minutes after disinfection	4. 37°C incubator
5. Technician's gloves during culture assay	
6. Lab gowns (forearm and thigh)	C. Incubator Area
7. Floor	1. Floor
8. Workbench	2. Sample processing bench
9. ZN staining basin	3. 37°C incubator
	D. Observation Area
	1. Inverted microscope
	2. Light microscope
	3. Floor
	4. Microscope bench

counts were performed at the end of the incubation period to determine the assay's detection limit.

Sampling was done on various surfaces in 4 areas in the mycobacteriology laboratory (Table 1 and Figure 2). RODAC plates were stamped on surfaces in the sampling area, with a one-minute contact time,[11] including the class II Biosafety Cabinet (BSC) type A2 before, during, and after performing sputum processing and primary culture; lab gowns; ZN staining basin; and the microscopes. The plates were then incubated at 37°C and observed for 28 days. The presence of mycobacteria growth on the media was confirmed through ZN staining and MPT64 antigen test (SD Bioline, Yongin, Korea).[12] A swab technique using sterilized cotton swabs was performed

Figure 2 Sampling Points Layout. A) Sputum Processing and Primary Culture Room, B) Media Preparation Room, C) Incubator Room. D) Observation Room

Table 2 Colonies Grown on RODAC Plate Stamped on AFB Staining Basin's Surface

Morphology	ZN	MPT64	Species
	Positive	Positive	MTB
	Positive	Negative	NTM

Note: ZN= Ziehl–Neelsen, MPT64= Mycobacterium protein tuberculosis, MTB= *Mycobacterium tuberculosis*, NTM= Non-Tuberculous Mycobacteria

on the same surface areas for IS6110-qPCR analysis, which covered around 12 cm^2, similar to the diameter of the RODAC plate. The swabs were then dipped in phosphate buffer saline (PBS) solution and stored at 4°C prior to deoxyribonucleic acid (DNA) extraction.

DNA from swab samples was extracted using a QIAmp DNA mini kit (Qiagen, USA) as instructed by the manual. Seven microliters of extracted DNA was used as a template for IS6110-qPCR for M. tuberculosis in a twenty microliter reaction.[13] IS6110-qPCR reaction was performed using Platinum qPCR Supermix-UDG (Invitrogen, USA) in iQ5 Thermal Cycler (Biorad Laboratories, Inc.). The quality of the reactions was carefully controlled by including positive, negative, and internal controls for every reaction.

Results

At the end of the incubation period, the growth of *M. tuberculosis* on RODAC plates was observed at dilution 10^{-1} to 10^{-5} and no growth was observed at dilutions 10^{-6}. Based on the number of colonies on the most diluted series, it was found that the detection limit of the RODAC plate was 19.6 CFU mL^{-1}.

The growth of bacteria was observed on the RODAC plate stamped at the ZN staining basin. Two distinct colonies were seen on the plate. Both colonies were subjected to ZN staining to see the presence of acid-fast bacilli and MPT64 antigen detection to identify the *M. tuberculosis* complex. One colony was identified as *M. tuberculosis* complex and the other colony as non-tuberculous mycobacteria (NTM) (Table 2). No growth was detected on RODAC plates stamped on other sampling surfaces. *M. tuberculosis* DNA was detected at 10 sampling points, mostly in the areas where ZN staining activities were carried out (3 areas), incubators (2 areas), and observation rooms (2 areas). The results of the RODAC plates and IS6110-qPCR were shown in Table 3.

Discussion

A robust protocol for microbial risk assessment in high burden TB laboratories is nonexistent due to the wide variety of diagnostic levels and scopes per laboratory.[14] Therefore, the individual laboratories must improvise the way to control the release of pathogenic *M. tuberculosis* into the environment despite good microbiological practice (GMP) has been applied. RODAC plates are commonly used for other microbial risk assessments, which inspired us to evaluate the reliability of this method as an alternative for assessing high burden TB laboratory. After optimization of the medium (by using Middlebrook 7H10, which is selective for Mycobacteria), we determined its least limit of detection. It was 19.6 CFU mL^{-1} equivalent to conventional mycobacterial cultures ranging from 10–100 CFU mL^{-1}.[15] Twenty sampling points in the TB laboratory were then stamped by RODAC plates and were swabbed for further qPCR tests.

RODAC plates are moderately sensitive to detect *M. tuberculosis* contaminants on the working surfaces. The bacteria captured at the staining basin were confirmed as *M. tuberculosis* and NTM,[16,17] which is commonly

Table 3 Comparison between RODAC Plates and IS6110-qPCR

Sampling Points	RODAC	IS6110- qPCR
Bacterial inoculation room		
Class II BSC type IIA		
Before culture (A1)	-	DNA detected
Work surface during culture (A2)	-	-
Arm rest during culture (A3)	-	-
After disinfection(A4)	-	-
Technician's gloves (during culture, A5)	-	DNA detected
Lab gown (after culture, A6)	-	-
Floor (A7)	-	-
Workbench (A8)	-	DNA detected
ZN staining basin (A9)	Growth detected	DNA detected
Media preparation room		
Media preparation laminar (B1)	-	DNA detected
Floor (B2)	-	-
Workbench (B3)	-	DNA detected
Liquid culture incubator (B4)	-	DNA detected
Incubation room		
Floor (C1)	-	-
Workbench (C2)	-	-
Solid culture incubator (C3)	-	DNA detected
Microscopic observation room		
Inverted microscope (D1)	-	-
Light microscope (D2)	-	DNA detected
Floor (D3)	-	DNA detected
Workbench (D4)	-	-

Note: RODAC= Replicate organism detection and counting, BSC= Biosafety cabinet, ZN= Ziehl–Neelsen, qPCR= quantitative polymerase chain reaction, DNA= deoxyribonucleic acid

found as environmental bacteria. It explains the possibilities of improper disposal or disinfection of staining waste material into the ZN staining basin and bacterial decay from the microscope slide to the basin during the staining process, which is still alive. The result of this experiment is very important, given alive bacilli can infect laboratory workers. By knowing the results, we have improved our decontamination process, restricted access during the staining process, and personal protective equipment to ensure biosafety.

Known for its higher sensitivity in *M. tuberculosis* detection,[13,18,19] qPCR of insertion sequence IS6110 was also performed in this experiment. Lower cycle threshold (Ct) values, indicating a higher load of *M. tuberculosis* DNA, were detected in areas that include processing of clinical samples and positive culture, such as ZN staining basin, light microscope, incubators, biosafety cabinets, and technician's gloves during the sputum culture process. The only exception is the ZN staining basin which still contained alive bacteria. Most probably, the DNA detected by IS6110 came from dead cells, as evidenced by undetected growth on RODAC plates. Positive qPCR with no growth on RODAC plates indicates DNA remains from dead or non-viable bacteria located on the surfaces which have been killed by routine surface disinfection or room sterilization using a UV lamp. Referring to these results, room sterilization and proper waste/working surface disinfection are important to assure laboratory biosafety.

Our study was limited by the performance of the RODAC plate in a research-based high burden TB laboratory. The detection limit of the RODAC plate still needs to be improved so that it can capture a lower number of live mycobacteria in other clinical environments, such as hospital wards, outpatient clinics, and others.

Possible routes of contamination are contaminated droplets, gloves, plate or inoculation tube surface, and aerosols from the open front of Class II BSC[11] despite being calibrated routinely. Proper disinfection and UV irradiation of work surfaces, BSC maintenance and calibration, and refresh

training for the laboratory technician must be given a special attention in maintaining laboratory biosafety, especially in high burden laboratories.[20] RODAC plates are suitable for biosafety assessment in TB laboratories on a routine basis, even though there is still a lack of data regarding the previous applications.

As a simple routine assessment for high burden setting laboratory, RODAC usage is advised due to its reproducible and fast ability to detect the release of viable bacteria. Several studies reported the application of the method in different clinical settings, such as on the surfaces in the patient-care compartment of ambulances.[21] Another study applied RODAC for recovery of multidrug-resistant organisms, 780 environmental surfaces in 63 rooms of patients on contact precautions in four intensive care units at one hospital.[22] Although those studies applied RODAC plates to detect the presence of other live bacteria, not TB. Further studies in the application of TB clinics can also be considered to detect the release of aerosol containing bacteria from patients' coughs. It is recommended to complement it with a PCR test as a support to monitor the spread of environmental DNA contamination.

To conclude, the application of RODAC plate is more suitable for monitoring the contamination of live bacteria in the working environment, and to inform a proper corrective action. Further studies are needed, not only in a larger number of sampling points and laboratory settings but also in a wider variety of comparing the method to gain more data on RODAC reliability.

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