

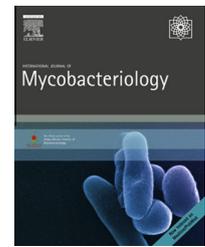


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Original article

## Use of RODAC plates to measure containment of *Mycobacterium tuberculosis* in a Class IIB biosafety cabinet during routine operations



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### ABSTRACT

**Objective/background:** Guidelines for the manipulation of *Mycobacterium tuberculosis* (MTB) cultures require a Biosafety Level 3 (BSL-3) infrastructure and accompanying code of conduct. In this study, we aimed to validate and apply detection methods for viable mycobacteria from surfaces in a BSL-3 MTB laboratory.

**Methods:** We evaluated phenotypic (Replicate Organism Detection and Counting [RODAC] plates) and molecular (propidium monoazide [PMA]-based polymerase chain reaction [PCR]) approaches for the detection of viable mycobacteria, as well as the effect of 70% ethanol applied for 5 min for disinfection against mycobacteria. For validation of the method, recovery of serial dilutions of *Mycobacterium bovis* bacillus Calmette–Guérin from glass slides was measured. Subsequently, we stamped surfaces in and around the biosafety cabinet (BSC) after different technicians had manipulated high bacterial load suspensions for routine drug-susceptibility testing in a Class II BSC.

**Results:** RODAC stamping could detect as few as three bacteria on slides stamped either 5 min or 60 min after inoculation. PMA-based PCR, tested in parallel, did not pass validation. Mycobacteria were still detected after 5-min disinfection with ethanol 70%. In the BSL-3, from 201 RODAC-stamped surfaces, MTB was detected in four: three inside a BSC—on a tube cap and on an operator's gloves—and one outside, on an operator's gown.

**Conclusion:** RODAC plates detect mycobacteria at low numbers of microorganisms. In addition, this method allowed us to show that 70% ethanol does not reliably kill mycobacteria when applied for 5 min to a dried surface, and that MTB bacilli may arrive outside a Class II BSC during routine practice, although the route could not be documented.

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## Introduction

Manipulation of *Mycobacterium tuberculosis* (MTB) cultures requires Biosafety Level 3 (BSL-3) infrastructure, equipment, and practices [1]. Use of liquid culture media and inoculating-loop manipulation increase the risk of creating aerosols, while only a few mycobacteria are sufficient to infect a laboratory worker [2,3]. The emergence of multidrug-resistant and extremely resistant strains requires particular attention to containment, both by the importance an infection may imply and by the increased volume of drug-susceptibility testing (DST) performed [4,5]. Despite the general recognition that BSL-3 measures effectively contain airborne, high-risk pathogens, there is little information on the concrete value of individual measures commonly incorporated in BSL-3 practices [6,7].

Culture-based tools are commonly used for detection of microorganisms in the environment. The presence of viable airborne particulates can be estimated using settling plates [8], whereas monitoring for surface-bound particulates can be done using Replicate Organism Detection and Counting (RODAC) plates, without the need for a decontamination step that could decrease the sensitivity of detection [9–14]. To allow for detection of slow-growing mycobacteria, a modified medium selective for preventing overgrowth by nonmycobacterial contaminants and enriched for supporting the growth of these fastidious microorganisms should be used [15]. Although this method has to date not been validated for mycobacteria, RODAC recovery rates described for other bacteria are highly variable; for example, for *Listeria monocytogenes*, the recovery rate is only 4.15%, and for *Clostridium difficile*, it is dependent on the medium used, and ranges from 0.3% to 45.8% [16,17].

Molecular methods are powerful and fast tools to detect traces of genetic material [18]. In particular, propidium monoazide (PMA)–polymerase chain reaction (PCR) is useful in this regard as it selectively amplifies DNA from viable bacteria [19]. This method has already been successfully applied to diagnose patients with drug-resistant MTB infection [20,21].

Surface decontamination of materials leaving the biosafety cabinet (BSC) is recommended, despite lack of evidence that MTB can be re-aerosolized from surfaces, and subsequently establish infection [1,22]. Where carried out, such decontamination should be done with a fast-acting disinfectant that does not affect bacilli inside culture tubes. Ethanol (70%) is used in some settings, however, data on its effectiveness are contradictory, with viable bacilli detected 1 min, 5 min, or 10 min after decontamination in two seminal studies [23,24].

Although BSL-3 standards call for a Class II cabinet (open, with laminar flow), this is based primarily on data from flow measurements rather than experimental evidence with bacterial detection, and these Class II cabinets are used in many laboratories handling MTB worldwide, in both high- and low-resource settings, where most laboratories do not meet all BSL-3 standards [25].

In this study, we evaluated both a culture-based and a molecular method for detection of mycobacteria from sur-

faces. We subsequently used the validated culture-based method to test the effect of ethanol decontamination of surfaces and to sample different surfaces in the environment after routine manipulation of MTB in a BSC Class II B1 [26].

## Material and methods

### Test strains

Validation experiments were performed using the non-pathogenic MTB complex (MTBC) *Mycobacterium bovis* bacillus Calmette–Guérin (*M. bovis* BCG; ITM-110295), which was cultured on home-made Stonebrink medium (Löwenstein–Jensen with pyruvate) for 3–4 weeks.

For quality control of media, we used the MTB reference strain (H37Rv; ITM-083715), *M. bovis* BCG (ITM-110295), and *Mycobacterium terrae* (ITM-110258) from the BCCM mycobacteria culture collection (Belgian Coordinated Collections of Microorganisms, Institute of Tropical Medicine, Antwerp, Belgium).

Because the sampling was part of a risk analysis in our TB laboratory, in accordance with our biosafety consultant, ethical approval was neither required for the BCCM samples nor for anonymous sampling in the BSL-3 laboratory, including those stamped on technicians' laboratory coats.

### RODAC method

RODAC plates were prepared using Middlebrook 7H11 agar, consisting in DIFCO Mycobacteria 7H11 agar (Becton Dickinson, Erembodegem, Belgium) supplemented with 2.1% Middlebrook oleic acid–albumin–Catalase–dextrose enrichment 10% [1.5% alpha-D(+)-glucose (Acros Organics, Geel, Belgium), 0.06% oleic acid (Sigma–Aldrich, Diegem, Belgium), 5% albumin bovine fraction V with pH modified at 6.3 (Acros Organics)], glycerol 0.5% (Merck, Overijse, Belgium), ticarcillin 50 mg/L, trimethoprim 10 mg/L, amphotericin B 10 mg/L, and colistin 200 mg/L (all antibiotics from Sigma–Aldrich). Each plate was prepared with 16 mL of medium, shaped as a parabolic meniscus, and stored at 4–8 °C for a maximum of 6 weeks. Each lot was validated by direct inoculation of one plate with MTB H37Rv or *M. bovis* BCG, and another one with *M. terrae*. Both plates should yield growth after 7–14-day incubation at 34–38 °C with 7.5% CO<sub>2</sub>.

To validate the recovery of mycobacterial bacilli from surfaces, a suspension (1 mg/mL) of *M. bovis* BCG was prepared [about 10<sup>6</sup>–10<sup>7</sup> colony-forming units (CFU)/mL according to positive control plates] with serial dilution until achieving 1 CFU on 3 different days (each starting from a different BCG subculture). Nonsterile microscopy slides were inoculated with 50 µL of those dilutions and placed in the BSC for subsequent stamping by the RODAC plates at different time points: within 10 s, about 5 min, or 60 min after pipetting. Each slide was stamped once, and the stamping covered about 1 cm<sup>2</sup>. At this point, slides had not yet completely dried except for the 60-min time point. The contact time between slide and RODAC plate was 1 s in the two first adjustment experiments and subsequently 1 min. Where mentioned,

slides were moistened with 50  $\mu$ L of sterile water and added after drying time. All BCG stamping validation experiments were performed in triplicate. Direct inoculation of the dilutions on a RODAC plate served as the positive control to calculate the capture rate with stamping. In the first experiment, an additional control was performed on Petri dishes with plain 7H11 medium. Colonies were counted after 28 days' incubation and considered negative at 10 weeks.

The capture rate was calculated as the mean number of colonies on the stamped plates, divided by the mean number of colonies on the directly inoculated RODAC plate of the respective bacterial suspension. When the number of colonies was >50/plate, it was categorized as 1+ (50–100 colonies), 2+ (100–200 colonies), 3+ (>200 colonies), or  $\infty$  (confluent).

In three different experiments, half of the slides were inactivated by adding 50  $\mu$ L of 70% ethanol (with 2.1% isopropyl alcohol; from Yvsolab, Turnhout, Belgium) after 5 min or 60 min, and subsequently stamped after 5-min ethanol contact.

### PMA-PCR method

A 1-mg/mL *M. bovis* BCG suspension was serially diluted in molecular-grade water and 10  $\mu$ L of each dilution (about  $10^3$  to <1 CFU) was incubated with PMA (Biotium, Hayward, CA, USA), for various combinations of incubation time (3–15 min) and PMA concentration (10–100  $\mu$ M). The suspensions were subsequently centrifuged for 20 min at 4000g, washed, and resuspended in Tris-EDTA buffer. All tubes were exposed to 400 + 230 W halogen light oriented at 45° at 20-cm distance for different durations (2–5 min). Negative controls were obtained by boiling part of the serial dilutions for 5 min before adding PMA, to kill all mycobacteria (internal validation). Mixtures of dead and live bacteria were obtained before PMA treatment by mixing boiled and nonboiled suspensions at ratios of 1:1, 5:1, and 25:1 at different microorganism concentrations.

All light-treated suspensions were lysed by overnight incubation with proteinase K, followed by boiling for 10 min. DNA was extracted following the modified Mangiapan method [27], which involved fishing DNA with a bead-bound probe specific for the 16S ribosomal RNA gene of MTBC (biotin-triethylene glycol [TEG]-CGTGGGTGATCTGCCCTGCAC and biotin-TEG-CGTCAATCCGAGAGAACCCGGA) and capturing bound DNA with streptavidin magnetic beads. PCR was performed with SYBR Green MTBC-specific primers for the 16S gene (GCGCTTTAGCGGTGTGGGATGA and TCGTCGCCTTGGTAGGCCGT). A DNA-positive control for the PCR and for the extraction was based on purified MTB H37Rv DNA at known concentration, and was used to validate the extraction and PCR method (without PMA). Sterile water was used as a negative control at each step.

### Sampling in the BSL-3 TB laboratory

Sampling was performed in the BSL-3 laboratory at the ITM Mycobacteriology Unit in Antwerp, Belgium. After completion of manipulations for DST, multiple places on the operator and in and around the BSC Class IIB were stamped by RODAC plates, with a 1-min contact time. Those DST manipulations,

considered at risk, were performed on routine or research samples growing MTB, using serial  $10^2\times$  and  $10^4\times$  dilutions from a 1-mg/mL bacterial suspension (approximately  $10^6$ – $10^7$  CFU/mL) for first-line DST, and  $10\times$  and  $10^3\times$  dilutions for second-line DST. As for the RODAC validation step, colonies were counted after 28 days' incubation, and considered negative at 10 weeks. Strains were identified as MTBC with the MPT 64 antigen test (SD Bioline, Yongin, Korea).

## Results

### Validation of the RODAC method

Growth of the positive control, the direct inoculum, was similar between the antibiotic-containing RODAC plate and Petri dish with plain 7H11 medium (Table 1). The CFUs obtained on control plates were in line with CFUs from routine positive control plates.

With a 1-s contact time between RODAC plates and the slides, the capture rate by stamping was not linear. Compared with direct inoculation, RODAC stamping with a 1-s contact time detected bacteria directly or after 5 min, but none were detected when stamping after 60 min, even after moistening (data not shown). When the contact time was increased to 1 min, the capture rate increased, especially at 60 min. Upon analyzing plates with countable numbers of colonies, the capture rate was found to be 60% (standard deviation [SD] 35%) after 5 min, and 32% (SD 38%) after 60 min (confidence interval 0–100% for both stamping times). Individual measurements are shown in Fig. 1A. In addition, a suspension with 2+ growth (on control) showed a similar growth when stamped after 5 min, and a 1+ growth when stamped after 60 min. No contamination was observed.

### Inactivation by ethanol

On average, slides inoculated with diluted *M. bovis* BCG grew seven colonies; addition of ethanol 5 min after inoculation reduced the colony growth to six (87%, SD 25%). Individual measurements are shown in Fig. 1B. When ethanol was added 60 min after inoculation, the number of colonies decreased from 1+ to 0–3 colonies (range) or, for low dilutions (3 and 14 colonies on average), to zero.

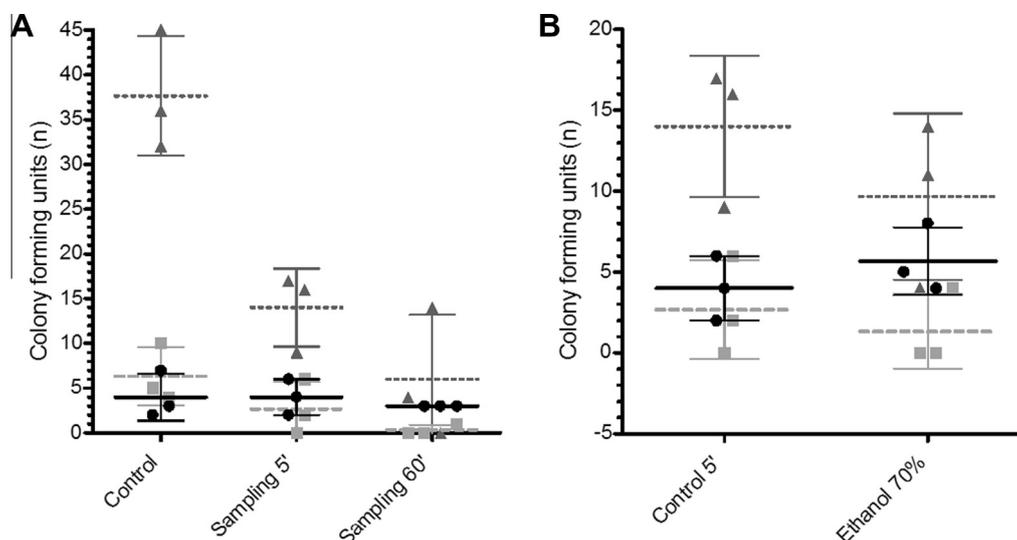
### Optimization of the PMA-PCR method

Linearity of the DNA extraction and quantitative PCR without PMA was established from  $10^1$  to  $10^4$  DNA copies (1 DNA copy/

**Table 1 – Medium controls.**

	7H11	RODAC
Dilution 1	1+	1+
Dilution 2	11	3
Dilution 3	1	1

Data are presented as the number of colonies growing 28 days after suspension inoculation on plates with 7H11 or RODAC medium. Serial dilutions 1:10. "1+" codes 50–100 colonies ( $n = 1$  for each). RODAC = Replicate Organism Detection and Counting.



**Fig. 1 – Validation experiments. (A) Stamping efficiency and (B) ethanol effect. Graph showing the number of colonies growing on each of the three RODAC plates on 2 days at the most discriminatory dilution ( $10^3 \times$  in 1 experiment and  $10^3 \times$  and  $10^4 \times$  on another day). Individual measures are represented by symbols: black circles (Series 1), light grey squares (Series 2), dark grey triangles (Series 3), whereas mean and standard deviation from triplicates are represented by horizontal lines: solid (Series 1), long-dashed (Series 2), or short-dashed (Series 3). (A) Control represents the direct inoculation on a RODAC plate; Sampling 5' and Sampling 60' are from RODAC plates stamped after 5 min and 60 min, respectively, with a 1-min contact time. (B) Control 5' represents RODAC plates stamped after 5 min, without adding ethanol. Ethanol 70% was added 5 min after bacterial inoculation on a microscopy slide, and stamped 5 min after ethanol addition. RODAC = Replicate Organism Detection and Counting.**

bacterium). Detection of <10 bacteria was possible, but not consistently: five of the ten experiments had a signal for the theoretic point of one bacterium. Although PMA has a theoretical ability to improve discrimination between viable and nonviable bacteria, we observed that PCR signals from dead bacteria were not completely inhibited after reaction with PMA, whereas PCR signals from live bacteria were inhibited in proportions similar to dead bacteria after reaction with PMA.

#### Sampling in BSL-3 TB laboratory with RODAC plates

On 30 different days, after one of eight different technicians had just completed manipulations for DST of MTB, we sampled from a number of different locations, including the technician's double-gloved little finger, both (gowned) forearms and thighs, two positions on the arm rest of the BSC, and the caps of inoculated MTB culture tubes (MGIT, LJ, or 7H11). In total, 201 RODAC plates were stamped. The exact locations are shown in Fig. 2. Fig. 2 also shows how the little finger is used during DST manipulations. The schedule of sampling is summarized in Table 2.

From those 201 stampings, five RODAC plates were positive on different days: two colonies and 1+ growth, respectively, on two plates stamped on little fingers, one colony from a tube cap (on the same day as the finger plate which grew 2 colonies), one colony from a forearm, and one colony from a thigh. All plates grew MTBC, except the positive RODAC plate from the forearm, with one colony that failed to grow

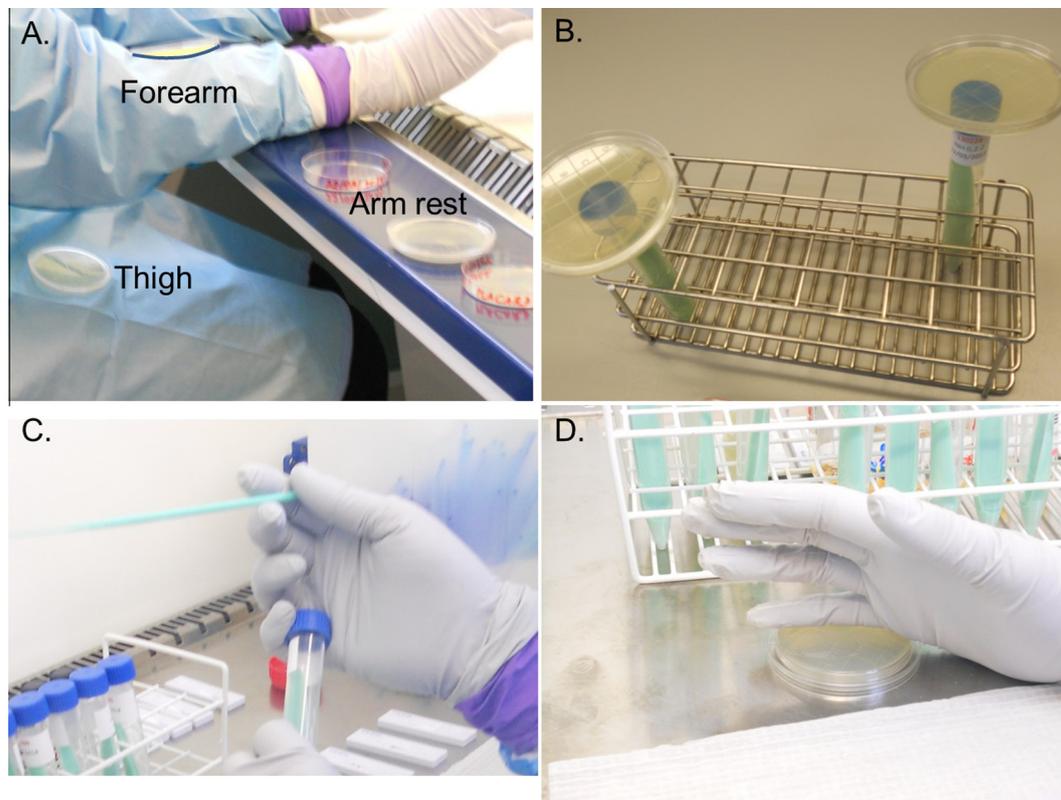
on subculture and could not be further identified. No contamination by non-mycobacteria was observed.

#### Discussion

The assessment of TB biosafety by monitoring the TB laboratory environment for the presence of viable mycobacteria is hampered by the absence of an established methodology and scanty data [6,7]. We developed an approach to assess the presence of mycobacteria on surfaces in the TB laboratory, and present data on the efficacy of decontamination with 70% ethanol.

Stamping with RODAC plates using a 1-min contact time showed a high sensitivity on glass, down to 3 CFU, yet with a wide confidence interval for capture rate; some replicates could not be cultured, suggesting that stamping at least three plates is necessary to evaluate the absence of viable mycobacteria. Sensitivity is presumably increased by extending sampling to multiple surfaces.

Our capture rate with MTBC appears to be in line with results obtained with other bacteria, detecting bacteria at a variable rate up to 50% [16,17]. Unlike *C. difficile*, our results on positive controls suggest that antibiotics used for selectivity did not have a detectable inhibitory effect on mycobacterial growth [28]. This finding suggests that a specific liquid growth medium could be developed for inoculation from non-decontaminated swabs, potentially shortening growth time [29,30]. As a different application, this medium might be tested in settings where TB cultures present a high contam-



**Fig. 2 – Locations of RODAC plates. (A) Stamping on the forearm, lap, and arm rest. (B) Stamping on tube caps. (C) The manner in which the little finger is used during the manipulation to open tube caps. (D) Stamping of the little finger. RODAC = Replicate Organism Detection and Counting.**

ination rate, not sufficiently managed when following the World Health Organization recommendations [31].

Our RODAC approach was considered sufficiently sensitive and selective to detect mycobacteria on surfaces in the BSL-3 TB laboratory, and therefore could be used for biosafety assessment in TB laboratories worldwide. In our case, we applied the RODAC method to operators (technicians) and the environment following DST, in which high concentrations of bacteria (1 mg/mL, about  $10^6$ – $10^7$  CFU/mL) are manipulated in liquid medium. This experiment took place in a routine environment that respects all current recommendations for TB manipulation: technicians had been trained on biosafety and specific techniques according to the good laboratory practices in place in our ISO 15189-accredited (BSL-3) laboratory, and the BSC is checked and maintained every 6 months according to recommendations. It is not clear whether the MTB colony on the technician's gown arrived directly by aerosol from the (open front) Class II BSC, or by contact with a contaminated finger, which might occur if unnoticed contaminated gloves are not removed inside the BSC prior to taking one's hand out to retrieve additional material not stored in the BSC. Even when an (contaminated) outer glove is removed inside the BSC, inner gloves may also be contaminated by colonies on the tube caps when specimens are removed from the BSC. Laminar flow might also have been disturbed by movements around the BSC, like incubator or laboratory door opening [12]. In our case, stamping on the arm rest, at risk of contamination in case of an airborne leak, was always nega-

tive, making airborne contamination, which would constitute a higher biosafety risk, less likely. Contamination on the laboratory gown represents an additional risk of transporting mycobacteria away from the BSC, with potential reaerosolization when removing the gown.

The present results prompted us to retrain our staff on always removing the outer gloves upon taking hands out of the BSC, even if only briefly. In addition, a customized closed-front Class II BSC has been installed in our BSL-3 laboratory. A closed-front BSC would better contain mycobacteria at the source, dramatically reducing the potential risk of any direct escape of aerosols [32]. Moreover, using wrist cuffs and double gloves reinforces removal of the outer gloves before exiting the arm sleeves. Such an approach may provide an alternative to a negative pressure laboratory in settings with limited resources, with priorities focused on a well-functioning BSC, its maintenance and calibration, training, and its periodic reinforcement [25].

Detection of bacteria on laboratory coat, gloves, and tubes also showed that RODAC stamping with our adapted medium works on surfaces other than glass, even if capture rate may be different.

PMA had been shown to be a promising approach to identify DNA from live bacteria, including mycobacteria [19–21]. Previous results were however from experiments with large numbers of bacteria, and PMA was used to distinguish between dead and living bacteria, as an alternative to DST. Our results suggest that detecting scarce live mycobacteria

**Table 2 – Stampings with RODAC plates in the BSL-3 laboratory.**

Stamping date	Tube cap	Finger	Forearm	Thigh	Arm rest
February 26, 2013			2	2	2
March 1, 2013			2	2	2
March 5, 2013			2	2 (1 with 1c)	2
March 7, 2013			2	2	2
March 13, 2013	1		2	2	2
March 22, 2013	1		2	2	2
March 27, 2013	1				
March 29, 2013	1				
August 9, 2013	8	4 (1 with 1+)			
August 12, 2013	3	1			
August 20, 2013		1			
August 22, 2013	9	1			
August 23, 2013		3			
August 30, 2013	3	1			
September 12, 2013	7				
September 26, 2013		1			
October 7, 2013		1			
October 14, 2013		2			
November 15, 2013	4				
March 24, 2014	1	1	2	2	2
March 25, 2014	1	1	2	2	2
March 26, 2014	3	3	6	6	6
March 28, 2014	1	1	2	2	2
March 31, 2014	1	1	2	2	2
April 2, 2014	2	2	4 (1 with 1c)	4	4
April 3, 2014	1	1	2	2	2
April 4, 2014	1	1	2	2	2
April 7, 2014	1	1	2	2	2
April 8, 2014	1	1	2	2	2
May 6, 2014	1 (1c)	1 (2c)	2	2	2
Total	52	29	40	40	40

Data are presented as number of plates for each stamping on the different days. After exploratory stamping outside the BSC and detection of one bacteria, we extended the stamping to the inside material, and later obtained one global stamping on different operators (technologists), including for each of the following: one tube cap, the used little finger, left + right forearm, left + right thigh, and two places on arm rest. Positive plate [and respective number of colonies (c)] are indicated in italics in brackets.

BSC = biosafety cabinet; BSL = biosafety level; RODAC = Replicate Organism Detection and Counting.

among a dead mycobacterial population is much more difficult, and PMA-PCR does not appear to be suitable for environmental monitoring.

In line with the results of Best et al. [23], 70% ethanol does not appear to be effective at killing mycobacteria, although our sample size is too small to draw firm conclusions.

## Conclusions

In conclusion, we validated an approach—mycobacterium-specific RODAC stamping—that can be readily applied in TB laboratories to monitor biosafety. Our results further call into question whether open Class II BSC and current recommendations are sufficient to contain mycobacteria at the source in a routine environment, particularly when handling high-risk specimens. While ethanol did not completely kill mycobacteria, further experiments are required to compare disinfectants suitable for decontaminating culture tubes without affecting their content's viability.

## Conflicts of interest

None declared.

## Acknowledgments

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G.D., P.R., B.C.d.J., and L.R. designed the study; D.v.S. developed the RODAC medium; G.D., E.N., and K.F. performed the laboratory experiments; G.D., B.C.d.J., and L.R. wrote the article; all authors approved the final version.

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