International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Sputum-Spotted Solid Matrix Designed to Release Diagnostic-Grade *Mycobacterium tuberculosis* DNA Demonstrate Optimal Biocontainment Property

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Abstract: ***Background*: Biosafety quotient of a reagent-coated cellulose matrix designed to release Mycobacterium tuberculosis (Mtb) DNA from sputum deposited onto it was determined. *Method*: Thirty-seven sputum samples infected with Mtb and 5 sputum samples from healthy individuals were processed and spotted onto a chemically coated cellulose matrix named the TBSend cards. Live Mtb bacilli were attempted for rescue from the spotted TBSend cards by washing them in phosphate buffer saline under mild shaking conditions. No live Mtb bacilli could be detected by the BACTEC™ MGIT 960™ TB System from both Mtb infected and Mtb non-infected sample-spotted TBSend cards when they were washed at two different time-points namely, 1 minute and 15 hours from the time of spotting of the cards with processed sputum. *Result*: The study consolidated published findings that chaotropic agents, which is an active component of the TBSend card module, have cent per cent bactericidal properties with regard to Mtb. *Conclusion*: Mtb infected sputum mixed with a biological cell-lysing solution and spotted onto a cellulose matrix coated with chaotropic salts effectively eliminates all live pathogen and can be used as a bio-safe Tb sputum transportation, storage and DNA-release device for Mtb NAAT.***

Keywords: TBSend card, M. tuberculosis, Biosafety, Biocontainment.

1. Introduction

There has been a dramatic improvement in approach for addressing health threat posed by Tuberculosis (TB) disease following implementation of the Millennium Development goals which aimed to reduce to half, the worldwide mortality and morbidity caused due to TB between the period of 1990 and 2015. Despite this tremendous global effort, TB continues to be a major cause of mortality and morbidity arising from a single infecting pathogen across the world[1].

The transmission of this pathogen is dictated by the environment, type of host and several other factors. For infections that arise due to direct transmission of the pathogen, it is necessary to understand the potential for infection of the index patients in the disease zones[2,3]. The rate of infection from a single patient depends on individual characteristics, with some people infecting a larger number of uninfected persons while others, infecting a fewer number of them or none[4].

TB infection occurs when the bacilli come out from an infected patient and get dispersed in the air and eventually reach the alveoli of another human host. The defence mechanism of the host however phagocytizes the pathogen using its alveolar macrophages that constitute its innate immune defence mechanism[5]. However, this infection progresses towards disease when few of the microbes escape the innate defence and replicate actively inside the macrophages. They then migrate to other cells in the vicinity that include epithelial as well as endothelial cells and soon reach significantly high microbial burdens[6].

The process of coughing by infected patients releases the highest quantum of droplets of various sizes[7]. It is therefore imperative that great precautions are taken while handling sputum of patients suffering from TB as this is one of the potent sources of spread of the disease[8,9].

Mycobacteria bacilli has the potential to retain viability for several months on dry surfaces. It has been demonstrated that *M. bovis* can survive on moisture-free surfaces at 4-degree C. Similarly, *Mtb* has been shown to survive in cockroach faeces for 8 weeks, on carpets for 19 days, on dry wood for more than 88 days, in wet as well as dry soil for over 4 weeks and in the environment in general for over 74 days, especially when the bacilli is protected from intense light sources[10,11].

Transportation of sputum for diagnostic purposes has significant potential for spread of the disease and is an underestimated cause for dissemination of the pathogen within people engaged in the process who eventually become potential secondary spreaders of the disease. An Indian study revealed that only around 33% of doctors in the private sector use proper and prescribed methods of transportation of sputum for its testing[12]. In yet another study it was found that only half of the patients surveyed followed appropriate sputum transportation practices[13]. Therefore, safe sputum disposal and transportation is a key factor for proper control of spread of this disease[14] along with activities to enhance awareness among common man and the medical fraternity which together play a crucial role in TB control strategies[15].

In this study, we demonstrate biocontainment quotient of a solid-matrix-based sputum transportation, storage and DNA-release device called the TBSend card and its potential to address biosafety concerns when used for room temperature transportation, extended period of storage at non-refrigerated condition and processed to release diagnostic-grade DNA for Nucleic Acid Amplification Testing (NAAT) including its cartridge-based variants (CB-NAAT).

2. Materials and Methods

Collection of clinical samples

Smear-positive sputum from presumptive TB patients (n = 37) were used in this study. All samples were collected from walk-in patients (3 ml) visiting the Microcare laboratory and Tuberculosis Research Centre (Surat, Gujarat), a TB testing facility accredited by the Central TB Division, Ministry of Health, Government of India. Samples were collected between the period of 2017-2019 in four batches, namely batch 1, 2, 3 & 4 and comprising 12, 18, 7 and 5 sputum samples respectively. Batch 1, 2 and 3 consisted of TB infected sputum samples while batch 4 consisted of sputum from healthy individuals.

Processing of sputum samples

Sputum samples were processed following standard bacteriological methods. Briefly, 1 ml of a sputum sample was treated by adding equal volumes of N-acetyl-L-cysteine–sodium hydroxide solution followed by an incubation of 20 minutes. The solution was then neutralized by adding phosphate buffered saline and centrifuged. The sediment was suspended in 1 ml volume of sterilized phosphate buffer saline and used for preparation of smear for microscopy as well as for inoculation into the culture medium[16]. Liquid cultures were performed using the BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD). Time to culture positivity was recorded as the period in number of days between the inoculation of a sample and detection of growth of *Mycobacteria*.

Loading the TBSend card with sputum

TBSend cards, routinely used for spotting processed sputum followed by its storage and extraction of DNA, were used in this study. To load the card with sputum, around 600 µL of sputum sample was processed by mixing equal volume of Spotting buffer (supplied with the kit), vortexing for 30 seconds and incubating at room temperature for 10 minutes. Around 1.2 ml of this sample-buffer solution was spotted onto the TBSend card using a disposable pipette (provided with the kit).

Rescue of MTB bacilli from TBSend card spotted with TB infected sputum

After (a) 1 minute and (b) 15 hours from the time of spotting of the TBSend card with processed TB-positive sputum, the circular sputum-spotted card was removed from the container using the flexible handle attached to it and placed into a wide mouth bottle prefilled with 3 ml of phosphate buffer, saline, mixed well and incubated for 15 minutes on a rotary shaker set to function at a speed of 75 revolutions per minute. Post incubation, a volume of 1 ml of the resultant "washed" buffer was transferred onto a sterile tube, processed in a way similar to that for sputum and used to run a BACTEC™ MGIT 960™ TB System for detection of live Mtb bacilli.

Each of the 42 sputum samples were divided into 3 aliquots, namely A, B and C, each consisting of 1 ml of sputum. Aliquots A and B were processed and used to spot two TBSend cards labelled Card A and B respectively while aliquot C was used to inoculate growth media for running the BACTEC™ MGIT 960™ TB System.

All Card A were spotted on day 1 at 19 hours while Card B, on day 2 at 9:59 hours respectively. The process for release of probable live Mtbbacilli from Card A & B if any, was initiated at 10 hours on day 2 by washing the spotted cards as described above and used to inoculate growth tubes of the BACTEC™ MGIT 960™ TB System.

Experimental design

For each TB positive sputum sample, three different BACTEC™ MGIT 960™ TB runs were conducted. In the first two runs, namely that for card A and B, the inoculum used were card-washed buffers retrieved after (a) 1 minute and (b) 15 hours of spotting of the TBSend card with sputum respectively. The third run (card C) was direct inoculation of sputum to run the BACTEC™ MGIT 960™ TB System after its necessary pre-inoculation processing as suggested by the manufacturer (Figure 1).

The study was approved by the Institutional Review Board of Nirmal Hospitals (Nirmal/HPL/Ethics/001) and performed in accordance with the principles of the Declaration of Helsinki. Written informed consents were obtained.

3. Results

Sputum samples from 58 individuals were screened using smear microscopy technique and 37 were found to be smear positive for Mtb. Sputum samples collected from 5 healthy individuals were smear-negative.

All sputum samples positive by smear microscopy were processed on the BACTEC™ MGIT 960™ TB System and found to be positive for Mtb. However, the time to culture positivity varied from sample to sample. Five sputum samples which were collected from healthy individuals did not show any growth in culture after an incubation of 42 days.

TBSend card-washed buffers taken after 1 minute and 15 hours from spotting of TB positive sputum onto it did not show any Mtb growth in culture. This included all 37 sputum-positive samples as well as 5 sputum negative samples collected from healthy individuals. The results are summarized in Table 1.

4. Discussion

In the domain of infectious diseases that are transmitted by air, Mtb is a classical archetype and an extremely potent causative agent for the disease of tuberculosis[17]. In the case of human tuberculosis, the primary source of infection is other humans suffering from this pulmonary disease.

Zoonotic tuberculosis, mainly arising out of cattle with *M. bovis* being the pathogen of cause do carry significance but its contribution to the cause of human tuberculosis is low and at 1.4% only[18].

The diagnosis of tuberculosis classically depends on detection of Mtb pathogen by culture method. Acid fast bacilli or AFB smear is an economical and inexpensive method for TB diagnosis but cannot differentiate nontuberculous mycobacteria (NTM) from Mtb. The culture method of detection of Mtb is more sensitive compared to AFB smear method but the results are generated after several weeks making it a time-consuming protocol[19].Compared to these two methods, Nucleic acid amplification tests or NAATs has now evolved as a rapid and sensitive technology for diagnosis of TB and is also effective in distinguishing NTMs[20]. A large number of studies have highlighted the advantages of NAAT in controlling TB which primarily include unnecessary treatment of the disease[21,22], shorter time period and reduced delays in initiation of treatment[23,24].

In India, observation suggests that detection of drug resistant TB, which was earlier dominated by the culture method has now gradually shifted towards molecular drug susceptibility testing (Molecular-DST). Several NAAT protocols has been approved by the World Health Organization (WHO) which include line probe assays (LPA), namely GenoType® MTBDRplus VER 2.0 (first-line LPA) and GenoType® MTBDRsl VER 2.0 (second line LPA), manufactured by Hain Lifesciences, Nehren, Germany, Xpert® MTB/RIF and MTB/RIF Ultra assay (Cepheid, Sunnyvale, USA) and Truenat™ MTB-Rif Dx assay (Molbio Diagnostics, Goa, India)[25].

The Line Probe Assay from Hain Lifesciences has been adopted In India by the NTEP programme and is the platform of choice for detecting drug resistant TB in direct smear-positive sputum samples and cultured Mtb bacteria obtained from smear negative samples[26]. Data suggest that in India, in the year 2018, around 3,46,282 and 72,748 samples were tested using first- and second-line LPA as compared to only 16,399 samples being tested by culture method for detection of drug resistant TB[27]. This hints at the scalability of adopting NAAT for detection of drug resistant tuberculosis with the LPA testing platform serving as a model. However, it may be noted that the use of LPA is limited to the national and intermediate reference laboratories and a few other certified laboratories in the country which are equipped with high end diagnostic facilities and adequately trained operators that are not present in Designated Microscopy Centres in India[27]. This gap raises potential logistic challenges as it involves transportation of highly infectious sputum from remote and geographically challenging areas to the central testing laboratories and poses potential biosafety concerns.

TBSend card, developed by Wobble Base Bioresearch Private limited, India (Patent Application number: 201621024943 dated July 20, 2016) is a proprietary reagent coated cellulose matrix housed in an airtight container that can be used for spotting Mtb infected sputum. For applying sputum on the card, it is mixed with an equal volume of spotting buffer at a ratio of 1:1 and the resultant mixture is poured onto the card. Dried, Mtb infected sputum-spotted cards retain the target pathogen DNA for a long period of time (>6 years) and can release it on demand by simply suspending it in a buffer and incubating it for 15 minutes for CB-NAAT and can also be purified for other NAAT which are based on classical real time fluorescent PCR, end point PCR, Line probe assays and Next generation sequencing platforms, using a hybrid spin column (Patent application No. 201921017045 dated April 29, 2019) that is specially designed for this and similar other DNA purification applications.

Some of the crucial components of TBSend card module are the spotting buffer and the reagents used to coat the card which comprises of strong chaotropic agents and nonionic surfactants having hydrophilic polyethylene oxide chain and aromatic hydrocarbon groups with potential to lyse cells as well as an antifungal agent. Chaotropic salts are co-solutes that break down the network of hydrogen bonds between the water molecules and effectively lower down the stability of proteins by reducing the hydrophobic effect[28].

Most of the commercial nucleic acid extraction kits are known to contain strong chaotropic salts. They are more effective than traditional cell lysis buffers that comprise NaCl, EDTA and Tris buffers as the primary components[29].

In a study by Clinghan *et al.*, (2013)[30] the authors demonstrated that two popular DNA extraction kits, namely NucliSENS easyMAG (bioMérieux, Boxtel, the Netherlands) and Qiagen QIAamp DNA mini kit (Hilden, Germany) effectively inactivated Mtb cells rendering them biocontainment-safe for handling purposes. For this, clinicals strains of Mtb were incubated in NucliSENS and Qiagen lysis buffer, both of which contained chaotropic salt as the primary cell lysing agent and then inoculated onto LJ media-containing slant-tubes and MGIT growth tubes. No growth was observed after 6 weeks in either of the samples.
In this study, the resource population was carefully constructed in order to involve a majority number of TB positive sputum samples in the study. All sputum samples found positive by smear microscopy also demonstrated growth in the BACTEC™ MGIT 960™ TB System. As expected, the days to positivity from the date of inoculation for each sample varied depending upon the quantum of live inoculum that went into the growth tubes for each of them.

TBSend card spotted sputum cards washed with phosphate buffer by incubating for 15 minutes in a rotary shaker did not release any live bacilli. This was demonstrated by nil growth in culture after an incubation of 42 days, the maximum recommended by the manufacturer of BACTEC™ MGIT 960™ TB System to call a sample as TB positive.

The most widely used medium for transportation of Mtb culture is the Middlebrook 7H9[31]. However, preparation of this media is cumbersome owing to expensive components, need for proper media-preparing infrastructure and trained manpower. Further, its composition being generically enriched makes it prone to easy contamination either during its preparation or transportation. It is now proven that

temperature of storage rather than the suspension media affects the viability of *Mtb* bacilli[32]. Hence a suspension media devoid of growth supplements is more suitable for transportation of *Mtb* rather than Middlebrook 7H9 media that promote fastidious growth.The inherent buffering capacity of phosphate buffer saline that restrain *Mtb* bacilli from replicating and accumulating toxic byproducts is ideal for extended survival followed by retrieval of *Mtb* bacilli[33].

Two categories of TBSend cards were used in this study. In the first (Card A), the TB-sputum spotted cards were washed in phosphate buffer 1 minute after spotting while in the second (Card B), it was washed after 15 hours of spotting with TB infected sputum. In the former, the card was moist and the time lapsed with the sample on the card was less (1 minute) compared to the other (card B) where the card dried up due to a comparatively longer time lapse of 15 hours. In both the cases no live Mtb microbes were detected by culture method indicating that Mtb cell viability was lost at least 1 minute after deposition of the processed samples onto the card if not earlier.

The complete absence of any live Mtb on the TBSend cards spotted with processed, TB positive sputum is well anticipated. The spotting buffer to which sputum samples were mixed at a ratio of 1:1 consisted of a mixture of strong chaotropic salts among other components, all in soluble form. On the other hand, the cellulose matrix with which the processed sputum (mixed with spotting buffer) came into contact during the spotting process too had chaotropic salts and non-ionic detergents that played crucial role in lysing of cells. This study therefore further consolidated the finding of Clinghan *et al.*, (2013)[30] and established the fact that chaotropic agents effectively inactivates live Mtb bacilli.

From this study, the authors conclude that once Mtbinfected sputum is spotted on to a TBSend card following prescribed instructions, it achieves optimal biosafety quotient and can be labelled as biosafe and non-hazardous from Mtb contamination.

Acknowledgements

This study was supported by Grand Challenge-TB Control (GCTBC) grants, namely GCTBC/C2P1/2015/09/30/01 & GCTBC/C2P2/2017/04/01/01 and funded by Biotechnology Industry Research Assistance Council (BIRAC) of the Government of India, United States Agency for International Development (USAID), and Bill & Melinda Gates Foundation. IKP Knowledge Park (Hyderabad, India) was the implementation partner for these programmes.

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Table 1: Summary of data obtained from processing of the resource population that comprised 37*M. tuberculosis* infected sputum and 5 sputa from healthy individuals.

Sr. no.	Sample ID	Smear Microscopy	BACTEC™ MGIT 960™ TB System					
		Sputum	TBSend card A (1 minute)		TBSend card B (15 hours)		Direct Sputum	
		Result	Growth	Days of incubation	Growth	Days of incubation	Growth	Days of incubation
1	WM-2	Positive	No Growth	42	No Growth	42	Growth	27
2	WM-3	Positive	No Growth	42	No Growth	42	Growth	23
3	WM-4	Positive	No Growth	42	No Growth	42	Growth	18
4	WM-26	Positive	No Growth	42	No Growth	42	Growth	29
5	WM-28	Positive	No Growth	42	No Growth	42	Growth	24
6	WM-29	Positive	No Growth	42	No Growth	42	Growth	29
7	WM-35	Positive	No Growth	42	No Growth	42	Growth	27
8	WM-40	Positive	No Growth	42	No Growth	42	Growth	29
9	WM-48	Positive	No Growth	42	No Growth	42	Growth	28
10	WM-49	Positive	No Growth	42	No Growth	42	Growth	28
11	WM-50	Positive	No Growth	42	No Growth	42	Growth	32
12	WM-57	Positive	No Growth	42	No Growth	42	Growth	22
13	WM-59	Positive	No Growth	42	No Growth	42	Growth	25
14	WM-60	Positive	No Growth	42	No Growth	42	Growth	27
15	WM-64	Positive	No Growth	42	No Growth	42	Growth	28
16	WM-109	Positive	No Growth	42	No Growth	42	Growth	17
17	WM-121	Positive	No Growth	42	No Growth	42	Growth	26
18	WM-124	Positive	No Growth	42	No Growth	42	Growth	17
19	WM-128	Positive	No Growth	42	No Growth	42	Growth	21
20	WM-129	Positive	No Growth	42	No Growth	42	Growth	21
21	WM-166	Positive	No Growth	42	No Growth	42	Growth	11
22	WM-181	Positive	No Growth	42	No Growth	42	Growth	20
23	WM-209	Positive	No Growth	42	No Growth	42	Growth	38
24	WM-213	Positive	No Growth	42	No Growth	42	Growth	29
25	WM-232	Positive	No Growth	42	No Growth	42	Growth	36
26	WM-313	Positive	No Growth	42	No Growth	42	Growth	11
27	WM-353	Positive	No Growth	42	No Growth	42	Growth	21
28	WM-354	Positive	No Growth	42	No Growth	42	Growth	22
29	WM-355	Positive	No Growth	42	No Growth	42	Growth	22
30	WM-356	Positive	No Growth	42	No Growth	42	Growth	29
31	WM-357	Positive	No Growth	42	No Growth	42	Growth	35
32	WM-358	Positive	No Growth	42	No Growth	42	Growth	18
33	WM-360	Positive	No Growth	42	No Growth	42	Growth	25
34	WM-361	Positive	No Growth	42	No Growth	42	Growth	8
35	WM-362	Positive	No Growth	42	No Growth	42	Growth	18
36	WM-363	Positive	No Growth	42	No Growth	42	Growth	25
37	WM-368	Positive	No Growth	42	No Growth	42	Growth	31
38	WM-24	Negative	No Growth	42	No Growth	42	No Growth	42
39	WM-85	Negative	No Growth	42	No Growth	42	No Growth	42
40	WM-107	Negative	No Growth	42	No Growth	42	No Growth	42
41	WM-110	Negative	No Growth	42	No Growth	42	No Growth	42
42	WM-125	Negative	No Growth	42	No Growth	42	No Growth	42

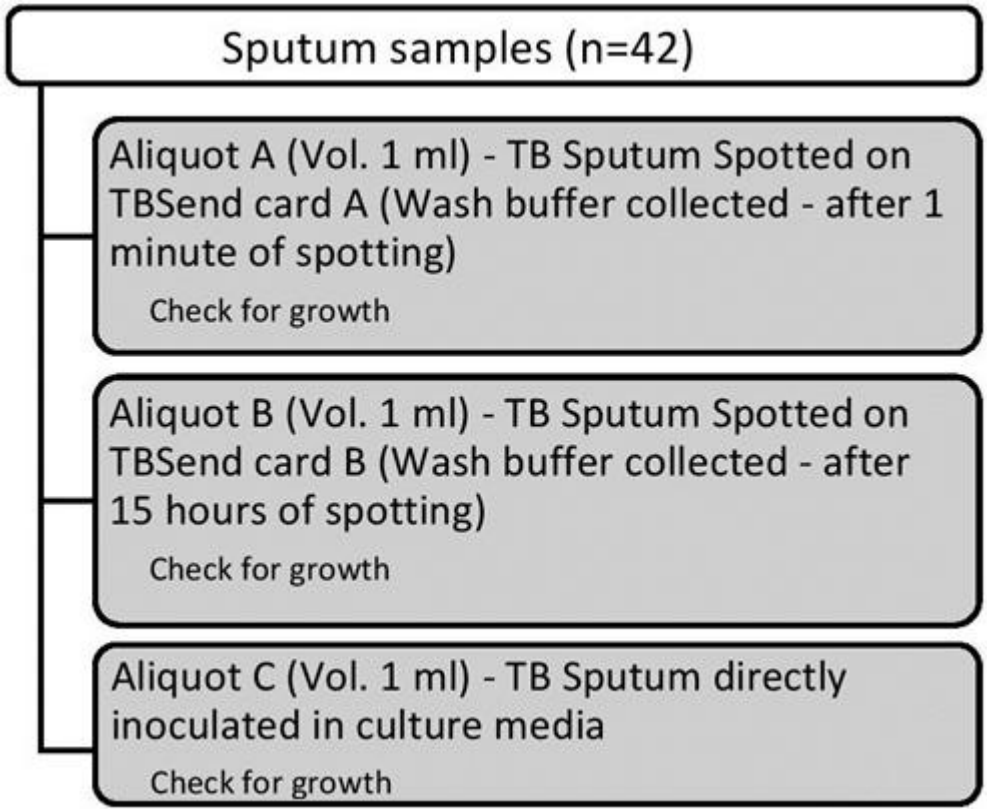

Figure 1: Study design for estimating infectivity of TB-sputum spotted TBSend using BACTEC™ MGIT 960™ TB System as the live TB bacilli detection platform.