



Disposal of the large volume of sputum positive for *Mycobacterium tuberculosis* by using microwave sterilisation technology as an alternative to traditional autoclaving in a tertiary respiratory care hospital in Delhi, India

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SUMMARY

Background: Everyday, tuberculosis hospitals collect enormous amount of sputum containing viable *Mycobacterium tuberculosis* bacilli, the disposal of which is a challenging task. Chemical (5% phenol) and physical (autoclaving) disinfection methods involve cost, space and cause further environmental degradation. Over the years, use of microwave for sterilisation of biomedical waste has become widespread. However, its efficacy to sterilise large volume of *M. tuberculosis* positive sputum has never been investigated.

Aim: To evaluate the effectiveness of microwave in sterilising large volumes of *M. tuberculosis* positive sputum samples.

Methods: 226 sputum samples positive for *M. tuberculosis* were checked by Ziehl-Neelsen staining and liquid culture (MGIT™) both before and after microwaving. χ^2 test was performed, and p-value <0.05 was considered significant.

Findings: Before microwaving, samples containing acid fast bacilli (AFB) and live *M. tuberculosis* bacilli were 93.8% and 95% (\approx 94.7%) respectively; which came down to 14.2% (32) and <1% (\approx 0.9%) in post microwave. In the 32 post-microwave AFB positive samples, bacilli appeared apoptotic, decreased in size, fragmented, loosely arranged and were easily missed as stain artefacts. Their beaded appearance was not appreciable. Background pus cells were of smaller size, did not take up methylene blue stain properly, and multilobed nuclear material was missing.

Conclusion: The study shows efficacy of microwave as an alternative sterilisation method for large volume sputum samples containing *M. tuberculosis* bacilli. Microwave can become an effective sterilisation method, especially for isolated tuberculosis care centres in countries which struggle for disposal of sputum, the biomedical waste.

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Introduction

Biomedical waste (BMW) management is a crucial aspect of any health care facility [1]. Nowadays, various BMW management guidelines [2,3] lay stress on the treatment of all infectious waste at the time of generation itself. For example, any blood component is initially decontaminated with 1% Sodium Hydroxide solution for 20 minutes before being discarded [4]. Similarly, sputum is to be sterilised by either autoclaving or chemically treated with 5% phenol before being discarded [4]. However, often, these wastes are generated in huge quantity or in such a condition where it is not feasible to decontaminate them at the site of generation itself. In big tuberculosis care hospitals, sputum is collected every day in copious volume. Sputum from pulmonary tuberculosis cases is dense, viscid, and sticky and has a spoiled smell. It is often teeming with *Mycobacterium tuberculosis* bacilli and may even be produced in large amount by active cases [4]. Therefore, handling and disposal of such BMW poses an administrative and environmental challenge.

Currently, available physical and chemical methods for sputum disinfection have both certain advantages and disadvantages [5]. Five per cent phenol, one of the commonest low-cost chemical disinfectants used, exhibits low biodegradability and is considered a health risk [6]. Moreover, the discarding process of this phenol becomes a challenge in itself. Autoclaving, whose setup requires space, money and rigorous quality check, is feasible only when the collection is not in significantly high amount. Furthermore, the pre-vacuum purge of autoclave air may contain live bacilli and is thus not feasible during sterilisation of semi-solid samples like sputum [7]. Very often, the use of HEPA filters and gravity cycle autoclave is limited because of the cost involved. It is to be emphasised *M. tuberculosis* is a biosafety level 3 organisms and belongs to Category C of Critical Biological Agents for public health preparedness by Centre for Disease Control and National Institute of Health [8]. Not even a single *M. tuberculosis* organism should go live into the environment after the BMW treatment because of its multi-drug resistant (MDR) and extended drug resistance (XDR) nature. Therefore, better and safer disinfection methods are required to replace them.

In the last few decades, the use of microwave radiation has gained much popularity in the medical field [9,10]. This technology is used widely for sterilisation of both laboratory items (media, plastic Petri dishes, towels and contaminated plates) [10,11] and BMW (scalpel, femoral head and dental implant sterilisation) [12,13]. Although studies have shown microwave effectiveness in killing common non-mycobacterial organisms [11,14], there is a lack of conclusive evidence about its ability to sterilise *Mycobacterium tuberculosis* bacilli, particularly when present in a large volumes of semi-solid clinical samples like sputum.

National Institute of Tuberculosis and Respiratory Diseases (NITRD), New Delhi, India, is a premier tuberculosis care institute in India. It has separate sputum sterilisation processes for the laboratory and wards. Since sputum samples received in the laboratory are very small in volume, they are first sterilised by autoclaving and then discarded. In the wards, the hospital encourages every sputum-producing patient not to spit in the open, but to collect sputum in steel cups provided free of cost. On average, around 350–400 cups, amounting to 30–35 litres

sputum, are collected daily. It is a tremendous task to decontaminate such large amounts of sputum. The established method using 5% phenol would amount to using a large volume of chemical and thus more downstream environmental pollution. Likewise, the induction of a dedicated autoclave was not viable because of space and cost issues. Therefore, the hospital is currently following a very crude disposal method wherein phenol mixed sputum is poured from steel cups into a bigger steel vessel, mixed with water, heated for 15–20 minutes and then drained off in hospital drain lines for further treatment in the effluent treatment plant. It is done with the knowledge that *M. tuberculosis* is killed at 60°C in 15–20 minutes [15]. Steel cups are later autoclaved and used again. Although this method is cheap and straightforward, it has many drawbacks. It requires a great deal of human manipulation and has abysmal compliance. When checked on a pilot basis, effectiveness to kill *M. tuberculosis* by this boiling method was not consistent every day.

OptiMazer™ 30 is a popular commercial microwave technology used for sterilisation of clinical biomedical waste. NITRD hospital has a dedicated 2540 MHz, 1500-Watt Microwave OptiMazer™ 30 machine. It is an automatic machine with digital display, inbuilt temperature sensors and pre-set time duration mechanisms for each stage of the sterilization cycle. It has an inbuilt mechanism which can adjust for brief power interruptions and thus does not abort the cycle if power supply is temporarily lost (a possible issue in low income countries). NITRD uses this machine for sterilization of routine (non-sputum) BMW. However, its efficacy was never determined to decontaminate large volumes of phenol mixed sputum samples. Therefore, the present study was conducted to evaluate the effectiveness of Microwave as an alternative method to autoclaving for sterilising large volumes of sputum with viable *M. tuberculosis* for safe disposal.

Methods

The prospective study was conducted in the Department of Medical Microbiology, NITRD, which has a national reference laboratory for tuberculosis diagnosis. A total of 226 consecutive sputum filled cups were collected over a four month period from patients diagnosed with active pulmonary tuberculosis. The diagnoses of these patients had already been confirmed by Ziehl-Neelsen (ZN) staining and cartridge-based nucleic acid tests (CBNAAT, Xpert MTB/RIF™). Every morning a new steel cup filled with 50 ml 5% phenol was given to patients and the previous days' sputum filled cups were collected back. Sputum was collected irrespective of the age, sex and previous or current comorbid conditions of the patient. Only naturally expectorated and discarded sputum was collected; patients were never asked for or were induced for sputum expectoration. To ensure no change in sputum collection habits and thus avoid any bias, patients collected sputum in routinely used steel cups and were never given plastic cups.

From every sputum filled cup, a small portion was processed by a NALC/NaOH [16] method; then tested by ZN staining and liquid culture-based method of MGIT (Mycobacterium growth Indicator tube) (BD BACTEC™ MGIT™) to appreciate structure and viability of *M. tuberculosis* bacilli respectively. A viability check was done because all of these patients were receiving antitubercular drug therapy, and the possibility of dead bacilli



Figure 1. The cups used in the study. (A) Microwavable plastic cups, (B) Steel cups.

being expectorated in sputum could not be ignored. After this, the sputum was poured from each steel cup into a separate microwavable plastic cup and microwaved in the OptiMazer™ 30 machine according to the manufacturer's guidelines (Figure 1); steel and plastic cups were later sterilized by autoclaving and used again. The machine first sprinkled water over these loosely closed plastic cups and then operated at 2450 MHz, 1.5 KW to heat the entire load to 100°C. Holding temperature and time for sterilisation were set at 100°C for 30 minutes. The steps followed were according to the manufacturer's guidelines for routine BMW. Free falling loose lids over these plastic cups ensured proper steam and radiation effect. Spores of *Bacillus atrophaeus* (HiMedia Laboratories Pvt. Ltd.) were used as controls and kept alongside the sputum filled cups in the centre of the machine. Now, a small portion of this microwaved sputum was processed by NALC/NaOH [16] method; stained by ZN stain and cultured by MGIT to appreciate radiation-induced changes in the structure and viability of *M. tuberculosis* bacilli. Approximately 350–400 plastic cups were microwaved each day in 4–5 batches. Control ampoules were checked for any colour change.

All sample processing and handling techniques were done by professionals trained for *M. tuberculosis* culture under bio-safety cabinets strictly according to Culture and Drug Susceptibility Testing guidelines (C & DST) published by Central Tuberculosis division [17], India. No ethical approval was conceived because the work was done on discarded sputum samples which were intended for disposal, and there was no change in sputum collection procedures for patients.

Statistical Analysis was done using SPSS version 20 (IBM®, New York, NY, USA). Chi-square test was performed, and p-value of <0.05 was considered indicative of a statistically significant difference.

Results

Out of 226 non microwaved sputum samples (Table I) collected from active pulmonary tuberculosis cases, 94.7% (214/226) were culture-positive for *Mycobacterium tuberculosis* on MGIT; however, this came down to a mere 0.9% (2/226) in post microwave samples (Table II) ($\chi^2 = 16.02$, $p < 0.05$). Similarly, before microwaving, 93.8% (212/226) sputum samples were positive for acid-fast bacilli on Ziehl-Neelsen staining; but only

Table I

Sputum sample analysis before microwaving (n=226).

	MGIT positive	MGIT negative	Total
ZN smear positive	204 (90.3%)	8 (3.5%)	212 (93.8%)
ZN smear negative	10 (4.4%)	4 (1.8%)	14 (6.2%)
Total	214 (94.7%)	12 (5.3%)	226 (100%)

14.2% (32/226) in post-microwave samples ($\chi^2 = 12.23$, $p < 0.05$).

The 32 AFB smear-positive post microwave sputum samples had altered image of bacilli. Compared to pre-microwaved sputum samples, where AFB was of beaded appearance and present in clumps on a background of clearly appreciable pus cells (Figure 2), AFB in post-microwaved sputum samples were of decreased size (both length and thickness), fragmented, were not in clusters and could be easily missed or ignored as stain artefacts. Also, their beaded appearance was not appreciable. The pus cells in the background were of smaller size, did not take up methylene blue stain properly, and their multilobed nuclear material was either missing or fragmented (Figure 3).

Discussion

This study was done to evaluate the effectiveness of microwave technology as an alternative sterilising method to traditional autoclaving for large volume sputum samples when collected from active pulmonary tuberculosis cases. In the present study, sputum samples were treated in an OptiMazer™ 30 with 2450 MHz, 1.5 KW microwave radiation for 45 minutes and then checked for *Mycobacterium tuberculosis* bacilli presence by Ziehl-Neelsen (ZN) staining and for viability by inoculating in highly sensitive MGIT™ liquid culture media. The percentage of sputum samples containing live *M. tuberculosis* bacilli came down from 95% ($\approx 94.7\%$) in non-microwaved samples to <1% ($\approx 0.9\%$) in post microwaved samples ($\chi^2 = 16.02$, $p < 0.05$). Similar studies done for evaluating microwave effectiveness on non-mycobacterial organisms such as *Escherichia coli* [18,19], *Clostridium perfringens* [20], *Staphylococcus aureus* [21,22], *Salmonella* [23,24], and *Listeria* spp [25] have reported 99.99% reduction in organism count. Although quantitative studies for *M. tuberculosis* count could not be done in this study, 94% decrease in number of AFB positive sputum samples does show that microwave is useful in sterilising a large volume of sputum samples.

Radiation induces apoptosis in the cell [26,27]. The decrease in the percentage of sputum samples containing live bacilli was due to apoptotic changes occurring at the cellular level as is evident by altered size and increased fragmentation of AFB and the surrounding pus cells (Figure 3). A similar study

Table II

Sputum sample analysis after microwaving (n=226).

	MGIT positive	MGIT negative	Total
ZN smear positive ^a	2 (0.9%)	30 (13.3%)	32 (14.2%)
ZN smear negative	0	194 (85.8%)	194 (85.8%)
Total	2 (0.9%)	224 (99.2%)	226 (100%)

^a All acid-fast bacilli had altered morphology.

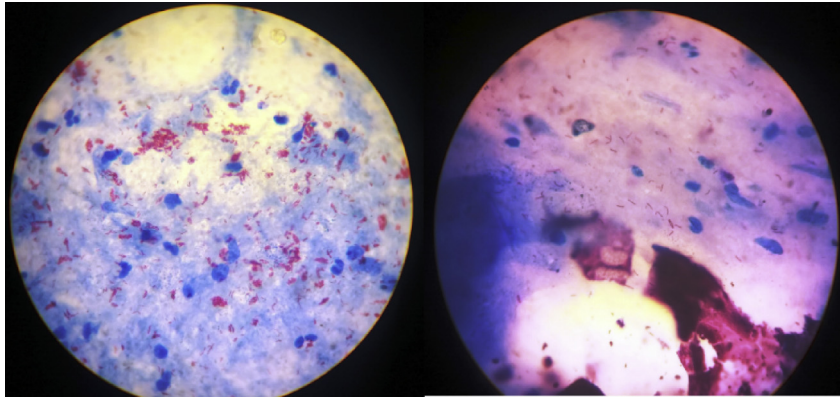


Figure 2. Pre-microwave ZN staining images showing AFB in clusters with large multilobed Pus cells in the background.

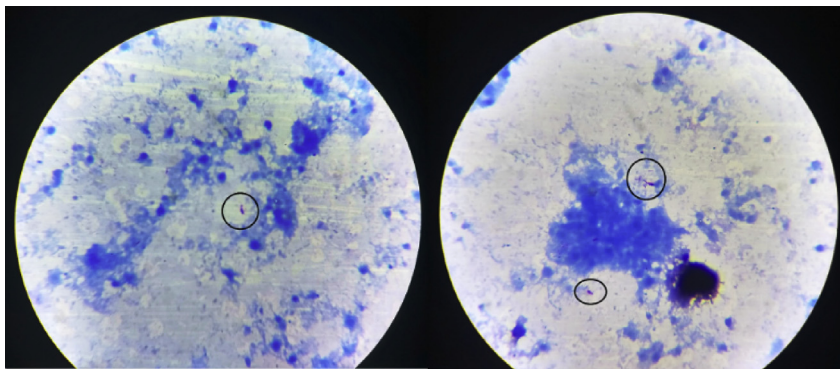


Figure 3. Post-microwave ZN staining images showing fragmented AFB of small size, and not present in clusters. In the background are the small Pus cells without multilobed nuclei.

done by Kakita et al. [28] on microwaved *Lactobacillus* phage PL-1 particles also reported similar changes and proved that the effect of irradiation is more profound at the level of DNA chain. In the current study, radiation appears to break nuclear material of cells as is evident by altered nuclear material of background pus cells. However, whether this physical disruption also occurred in *M. tuberculosis* could not be proved.

The present study is more of an observational study wherein an attempt has been made to evaluate the effectiveness of commercially available microwave radiation technologies (OptiMazer™ 30) in sterilising *M. tuberculosis* positive sputum samples, especially when present in large volume. Complementing data by showing the linear relationship between microwave radiation dose and its effect on microbial load was not attempted because of lack of resources. Although the lack of resources and technological expertise did limit the use of high-end molecular techniques in knowing the exact level of apoptotic changes in *M. tuberculosis* DNA, the basic microbiological techniques of staining, culture and microscopy implemented do complement the data presented. To the best of our knowledge, this is the first study from a selected urban tuberculosis/respiratory speciality hospital of a developing country and provides an insight into the effectiveness of a commercially available microwave technology (OptiMazer™ 30) which is already being used for routine BMW while dealing with large volume sputum samples. Autoclaving issues such as long heating periods and heat loss can be bypassed by using microwave technology which runs in quick batches of small

duration. This can become an effective sterilisation method, especially for isolated tuberculosis care centres in countries which struggle daily for disposal of sputum and biomedical waste.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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