# High Volume Biological Sample Processing using Microwaves

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***Abstract*** — This paper describes the design and optimization of a 10ml cartridge for patient sample processing using a 3.5GHz (empty resonant frequency) $TM_{010}$ cylindrical microwave cavity. The cartridge has been designed to augment a novel approach for the rapid diagnosis of *M.tuberculosis* (the causative agent of Tuberculosis) which uses the direct application of microwaves to a bacteria containing sample to release pathogen specific DNA. The target bacterial DNA is then captured and recovered using magnetic nanoparticles coated with pathogen specific DNA probes. Excitation parameters were optimized using three surrogates for *M.tuberculosis*, namely *M.smegmatis*, *M.abscessus*, and *M.bovis* suspended in water and simulated sputum. The paper also explores the mechanism of microwave mediated DNA release from bacteria using scanning electron microscopy. Examination of bacteria exposed to microwaves at power levels known to mediate the release of DNA reveals no obvious signs of permanent cell disruption, suggesting that a more subtle interaction is taking place. Finally, the presence of microwave liberated *M.bovis* DNA was able to be detected at a level of sensitivity comparable to that achieved using microscopy.

## I. INTRODUCTION

Rapid diagnostic techniques are imperative for the effective treatment of diseases. The development of techniques that are both inexpensive and do not require extensive technical knowledge are especially important for use in environments with low resources and limited access to specialist facilities.

In the UK, tuberculosis(TB), caused by a bacterium called *Mycobacterium tuberculosis*, is considered a disease of the past which does not affect modern life. However, in some parts of the world including areas in Africa and Asia, this disease still runs rife throughout the population. In 2021 the World Health Organisation recorded 10.6 million cases of TB worldwide causing 1.6 million deaths[1]. Latent Tuberculosis Infection (LTBI) (which occurs in persons infected with *M. tuberculosis* but without symptoms) is estimated to infect around 25% of the global population[2]. While LTBI is not immediately problematic, the carrier being relatively healthy and unable to infect others[3], it can be activated if bacterial growth outruns the ability of the host's immune system to control the pathogen. This can occur when the host develops an immunodeficiency disorder (Graves disease[4], Hashimoto thyroiditis[5], rheumatoid arthritis[6], Crohn disease[7]) or is infected with HIV. Studies have reported that a concurrent HIV infection in LTBI patients results in 30% of individuals developing active tuberculosis, as opposed to 5-10% in HIV negative patients[8].

Furthermore, multi drug-resistant strains of TB (MDR TB) have emerged in recent years, the most common of which are resistant to isoniazid and rifampin (first-line drugs used to treat TB). Even more resistant strains have emerged known as extensively drug-resistant (XDR) TB which are resistant to isoniazid and rifampin and at least two other common TB drugs[9]. Rapid diagnosis is key to ensuring that the patient receives the antibiotics, which are more likely to be effective against the strain with which they are infected.

Currently, the most common diagnostic techniques for TB are direct microscopy, chest x-ray, bacterial culture and the tuberculin skin test. The techniques are inexpensive but require technical knowledge, are relatively insensitive, and can take a considerable amount of time (6-8 weeks[10]) to generate a result. A number of rapid, DNA-based diagnostic techniques have been developed (COBAS TaqMan MTB[11], Loop-mediated isothermal amplification (LAMP)[12], Gene Xpert TB assays[13], GenoType Line-Probe Assays etc.[14]) which generate a result in approximately 2 hours[15]. These methods offer a huge improvement in time to diagnosis over the two more common methods and boast higher sensitivity and specificity rates (76.5% and 100%, respectively, for the TB LAMP assay[16]). They are, however, expensive and labour intensive, requiring an additional purification stage which further increases the time to diagnosis.

Here, a novel diagnostic approach is being developed which uses microwaves to rapidly release single stranded (ss) DNA from bacteria. Pathogen specific DNA sequences are then captured by magnetic nanoparticles coated with target specific DNA probes (shown in Fig.1).

The initial prototype system employed metallic triangular structures inside a conventional microwave oven to focus the electric field onto a small sample (500$\mu$l)[17]. This method was able to process and detect the presence of *C. difficile* in complex organic samples in less than a minute. Its major limitation, however, was that the input power transfer efficiency into the sample which was found to be less than 0.1%.

To improve matters, a secondary system was created using solid state electronics and single mode microwave cavities.

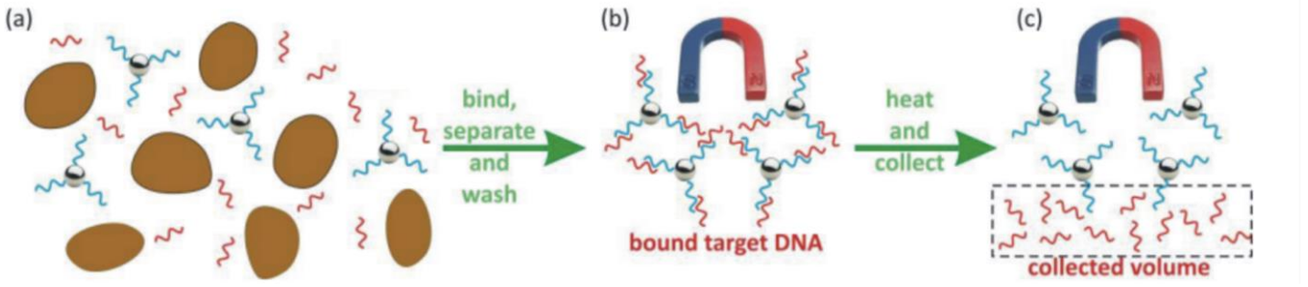



FIG. 1. Demonstration of DNA purification technique from patient samples using a) microwave excitation and b) magnetic nanoparticle capture and c) separation

This system used samples placed inside a 0.25ml Eppendorf tube to couple to the microwave electric field within the cavity. This enabled the microwave power to decrease from $1kW_{rms}$ to around $10W_{rms}$ whilst maintaining the same rapid ssDNA release (this is referred to as the Eppendorf method). The major limitation of this approach is the small sample volume (0.2-0.25ml) that can be processed. The average volume of a sputum sample produced by a TB patient is 2-5ml,[18] meaning that multiple iterations of microwave processing would have to be undertaken to process the entire sample. Processing the entire sample in a single microwave excitation would greatly simplify the process and would increase sensitivity. This paper outlines the design of a cartridge able to process up to 10ml of sample volume in a single excitation and the optimization of ssDNA release from Mycobacterial cultures using this new cartridge.

## II. EXPERIMENTAL METHODS

### Microwave System:

The schematic circuit diagram of the microwave system is shown in *Fig.2*.

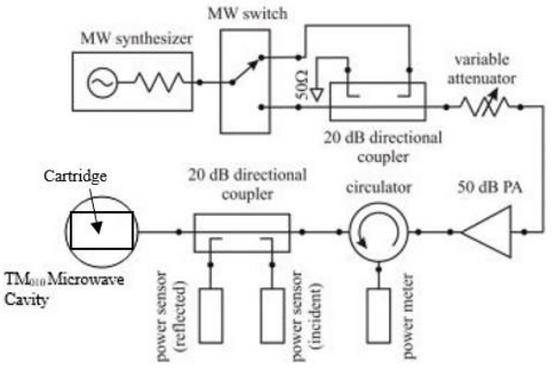



FIG. 2. Circuit diagram for the microwave extraction system used for experiments in this paper, referred to as the **PMA** (portable microwave applicator)

The operation of this circuit is straight forward. The output of the microwave generator is fed through a 50dB power amplifier and then fed into a $TM_{010}$ cylindrical microwave cavity resonant at 2.5GHz (for an Eppendorf tube excitation). An adjustable coupling loop is used to impedance match the cavity at resonance to the 50Ω system impedance, ensuring maximum power transfer into the cavity and its cartridge load. The input and output power are measured by power sensors attached to the circulator, which also prevents any reflected power entering the output of the amplifier. Measurements taken with these sensors are used to calculate the voltage reflection coefficient (S11 parameter) of the system throughout the experiments, so that the power delivered can be monitored.

It is important to note here that due to the inherent limits of certain components within the circuit, the output has a bandwidth of 2-4GHz. Images of the $TM_{010}$ cylindrical cavity used and 0.25ml eppendorf tube are shown in *Fig.3*.

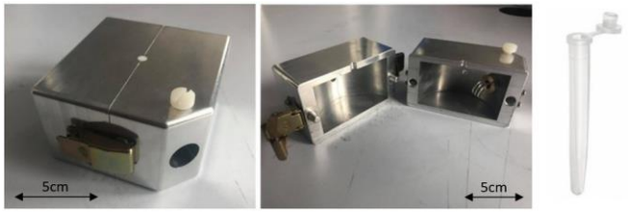



FIG. 3. Aluminium $TM_{010}$ cylindrical microwave cavity (Left/Middle). 250$\mu$l Eppendorf tube (right, not to scale)

### General Methods:

The rapidly growing mycobacterial strains (M.smegmatis and M.abcessus) used in these studies were incubated in LB broth for 3 days and centrifuged at 4000g for 10 mins. The cells were washed three times using phosphate buffered saline(PBS) and re-suspended in PBS.

The slow growing M.bovis BCG was incubated in 10ml of 7H9 broth media (Becton Dickinson and Company) incubated for 2 weeks at 37°C in a shaking incubator (MaxQTM 440, Thermo Scientific), at 200 rpm. The resulting cells were washed three times in PBS and re-suspended in PBS.

A simulated sputum mixture was prepared to mimic the vis-

cosity of regular sputum. This consisted of autoclaved 1% solution of methyl cellulose in water[19]. The solution was not mixed thoroughly to aid/mimic the non-uniformity of sputum.

## III. EPPENDORF METHOD

As the validity of the Eppendorf method had been demonstrated previously it is used as a control against which the larger cartridge design can be tested. To ensure a similar level of ssDNA release from the target bacteria, a microwave electric field of a similar strength and uniformity is required within the sample. The creation of a near uniform microwave electric field is important as it will negate the scenario whereby some target bacteria remain intact at a point within the sample but the DNA has been released but fully destroyed in another.

*Fig. 4* shows a COMSOL simulation of an Eppendorf tube sample with our $TM_{010}$ cavity and *Fig. 5* shows a plot of the electric field strength within the sample. This field is generated from a 12W input power into the cavity (used as standard in previous experiments[20]).

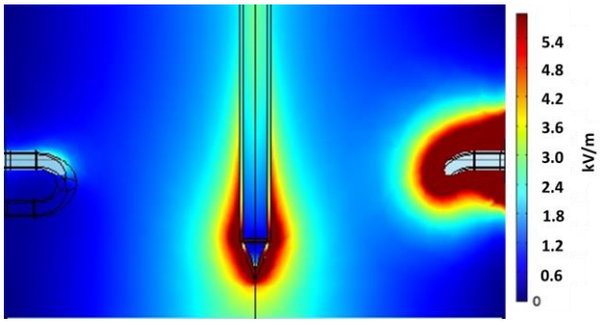



FIG. 4. COMSOL simulation of Electric field distribution for Eppendorf tube sample inside $TM_{010}$ Cylindrical Cavity. The cavity is excited using a 12W power input from the loop on the right hand side, which couples to the mode's magnetic field around the inner cavity perimeter.

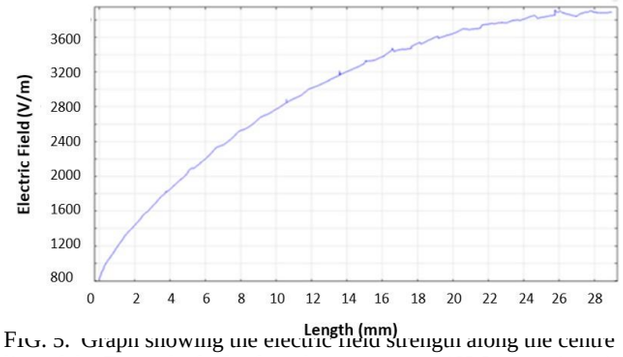



FIG. 5. Graph showing the electric field strength along the centre line of the Eppendorf tube from bottom to top (12W power input).

The simulation gives a range of electric field between 0.8 - 3.8 $kVm^{-1}$, yielding an average field strength of 2.3 $kVm^{-1}$. These values give a baseline to aim for in simulations involving samples of larger volume.

## IV. 10ML CARTRIDGE DESIGN

The electric field in the Eppendorf tube is created as shown in *Fig. 6*.

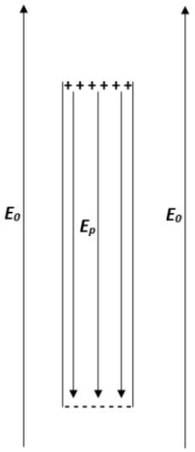



FIG. 6. Demonstration of Electric Field generation inside Eppendorf Tube Sample.

Here:

- $E_0$ is the applied electric field
- $E_p$ is the depolarizing electric field caused by the depolarisation charges induced on the sample surface whose planes are perpendicular to $E_0$ (denoted by the +/- charges in Fig. 6).
- $E_1$ is the resultant electric field in the sample. For long, thin samples relative to the electric field.

As the sample is long, thin and oriented parallel to the direction of the electric field, the depolarisation charges (created by dielectric materials such as water) generated at the top and bottom of the sample are relatively small and have a minimal effect on the electric field. The electric field (assuming maximum power transfer) inside the sample can be estimated using the following equation:

$$E_1{}^2 \approx \frac{P_{in}}{\pi f_0 \varepsilon_2 \varepsilon_0 V_s} \qquad (1)$$

Careful tuning of the coupling loop with the sample in place results in S11 < -20dB. The cavity quality factor drops from ~10000 when empty to around ~200 when sample loaded, this justifying the assumption used in Eq.(1).

Assuming a standard excitation (explained in the Eppendorf method section) and taking the numerical values of: $P_{in}$ = 12W, $f_0$ = 2.5GHz, $\boldsymbol{\varepsilon}_2$ = 10 (for water @2.5GHz), $V_s$ =

$2.5 \times 10^{-7}$.

Gives: $E_1 = 8.3 \times 10^3 Vm^{-1}$

The $TM_{010}$ cylindrical cavity used has internal dimensions of r = 4.6cm and h = 4cm, to give an unloaded operating frequency of 2.5GHz (although the height is fairly inconsequential). To maintain keep this operating frequency for a 10ml sample volume requires a modification to the aspect ratio of the sample cartridge, since preserving the aspect ratio of the Eppendorf tube would result in an unfeasibly long cavity. To keep the same depth of the sample cartridge (and thereby use the same cavity for excitation) the radius of the sample needs to be increased. This is simulated in *Fig. 7*.

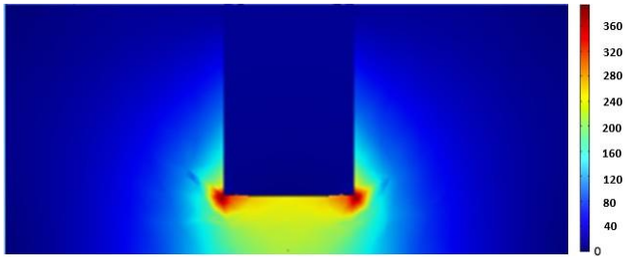



FIG. 7. Simulation of electric field (V/m) within 10ml sample tube. The internal electric field is reduced to almost zero.

As *Fig. 7* clearly demonstrates, simply increasing the radius of the sample tube is not a usable solution. As the surface area at the top and bottom of the sample is increased the effect of the depolarisation charges increase drastically and cause the internal electric field to reduce to a few % of the applied field ($10^2 Vm^{-1}$ range).

The solution lies in simply rotating the sample orientation through 90°to be perpendicular with the applied electric field. The creation of the electric field in this orientation is shown in *Fig.8.*

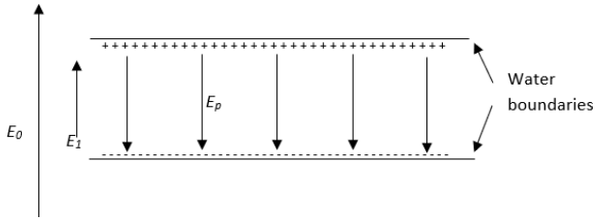



FIG. 8. Demonstration of Electric field generation inside sample orientation perpendicular to the electric field.

This orientation has been used in the past for analysis rather than excitation as it results in lower electric field strengths within the sample but a higher level of uniformity[21]. For this application multiple of these sample volumes can simply be stacked on top of each other to increase the volume but giving approximately the sample electric field strength and uniformity in each level. The resultant field should still be high enough to cause DNA release from the target bacteria.

Reusing *Eq.1* we can estimate the electric field within a 10ml sample ignoring (for the moment) reduction caused by depolarisation charges.

The parameters are entered as previously, except with a larger volume:

- $P_{in} = 12W$
- $f_0 = 3.5GHz$
- $\varepsilon_2 = 10$ (for water @2.5GHz)
- $V_s = 1 \times 10^{-5} m^3$

giving an electric field value of: $E_1 = 1.1 \times 10^3 Vm^{-1}$

An electric field reduction of only a factor of 5 should be sufficient to still cause DNA release from target bacteria.

It is important to note the reduction in frequency caused by the sample. Through standard perturbation theory the reduction in frequency caused by the addition of a lossy sample can be calculated.

To give a more accurate idea of the electric field inside the sample cartridge a model was developed using the "stack" approach and simulated.

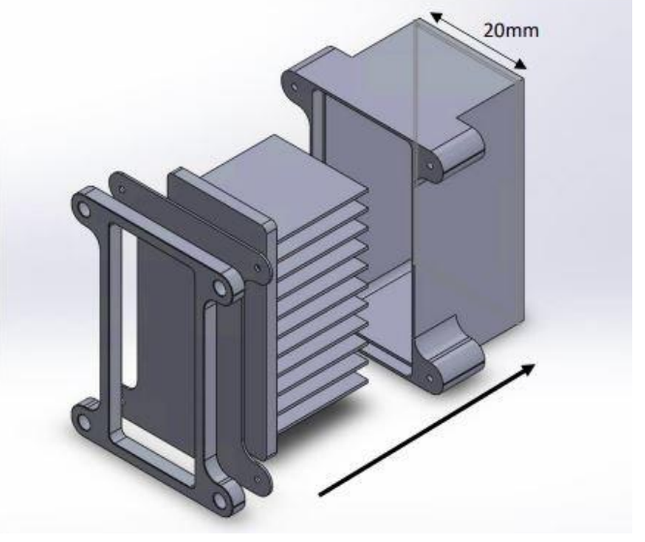



FIG. 9. 10ml Stack cartridge modelled in SolidWorks. Parts from left to right: clamp, seal, lid, body. The height of the cartridge is 40mm.

The cartridge operation is simple. The sample is placed in the body of the cartridge and then the lid is fitted to create horizontal partitions. The cartridge is then made waterproof (important to protect the user and to eliminate cross contamination of samples) by the placement of a seal over the top of

the lid held in place by the clamp and 4 nylon screws. The full simulated model is shown in *Fig. 10*.

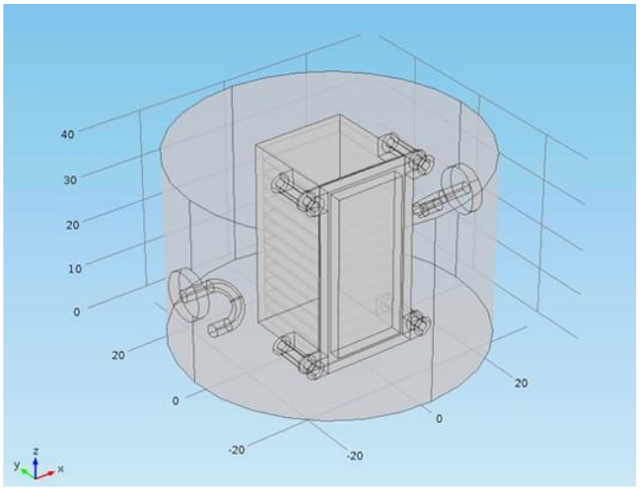



FIG. 10. Model of 10ml Stack Cartridge inside $TM_{010}$ Cylindrical Cavity

An initial simulation using a 2.5GHz cavity showed the operating frequency dropping well below the 2GHz minimum of the PMA. The cavity was redesigned to have an unloaded operating frequency of 3.5GHz. The model above shows the 10ml cartridge inside this 3.5GHz cavity.

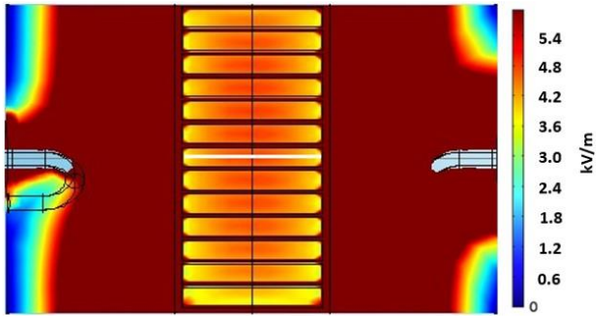



FIG. 11. Simulation of electric field inside 10ml stack cartridge. The cartridge is full with a water sample. The cavity is excited using a 12W power input from the loop on the right hand side. The white line is the line along which data points were taken for *Fig. 12*

The simulation gives an electric field range of $700Vm^{-1}$ - $980Vm^{-1}$ (giving an average of $840Vm^{-1}$). By comparison with the Eppendorf method, the electric field strength within the sample has dropped by a factor of around 3 (from $\sim 2.3kVm^{-1}$ to $\sim 0.84kVm^{-1}$) but the uniformity (difference between the lowest and highest field strength) of the electric field has increased by around a factor of 10 (from $\sim 3kVm^{-1}$ to $\sim 0.3kVm^{-1}$). In the following experiments, the effect on the DNA release caused by the loss of electric field will be mitigated by optimizing the excitation time.

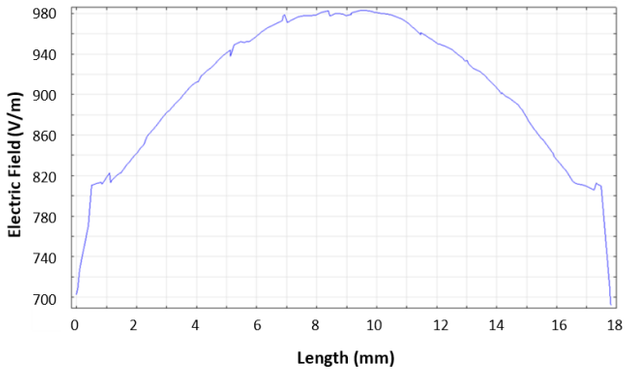



FIG. 12. Graph showing electric field strength along a horizontal line in the middle section of the 10ml cartridge from left to right (demonstrated by the horizontal white line on *Fig. 11*).

## V. OPTIMIZATION OF SSDNA RELEASE FROM BACTERIA

The ultimate aim of this approach is to detect the presence of pathogenic bacteria in a patient sample as quickly as possible meaning the conditions which maximize the release of pathogen specific ssDNA must be identified.

10ml of water or simulated sputum sample containing $1 \times 10^8$ CFU/ml of either *M.smegmatis*, *M.abscessus* or *M.bovis* BCG were decanted into the 10ml cartridge. Samples were then excited for varying periods of time using a 12W power level input. The amount of ssDNA release was determined using the Qubit ssDNA Assay Kit(Invitrogen) and Qubit 3.0 fluorometer (Invitrogen).

If it is assumed that the total energy dissipation within the sample will scale linearly with the volume to yield an equivalent ssDNA release, the length of time needed for excitation can be estimated. The optimal parameters derived for the Eppendorf method are 12W for 20 seconds with a 40% duty cycle. Multiplying directly the ratio of volume (0.25ml : 10ml, ×40) by length of time for excitation (40 x 20s) then multiply by 40/100 to remove the duty cycle (or 100% duty cycle) gives a value of 320 seconds (or 5 mins and 20 seconds) assuming 100% transfer efficiency into the sample.

The ssDNA concentration values were determined every 120 seconds, up to 960 seconds excitation time, and compared with results obtained from non-microwaved samples. Power transfer efficiency did not drop below 90% for any iteration.

The data presented in figs 13 and 14 has been corrected by subtracting the results at t = 0s, for each mycobacteria respectively. It clearly demonstrates that the maximum ssDNA release occurred after 480 seconds (or 8 minutes) for all samples. The data for *M.abcessus* in simulated sputum

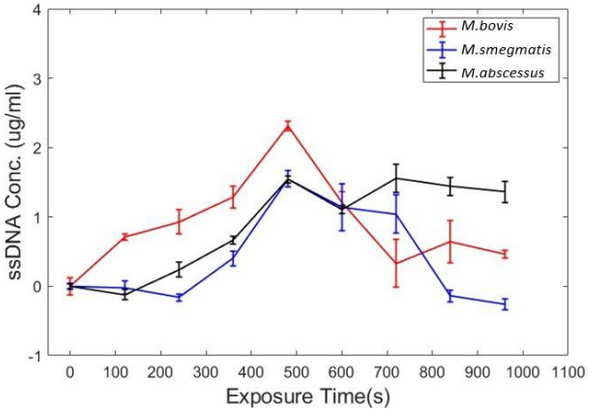



FIG. 13. Microwave mediated ssDNA release using 10ml Stack cartridge from $10^8$CFU/ml sample of various Mycobacteria in molecular grade water. The data has been corrected by subtracting each respective t=0s value. The error is shown as 1 standard deviation away from the mean value ($N = 3$).

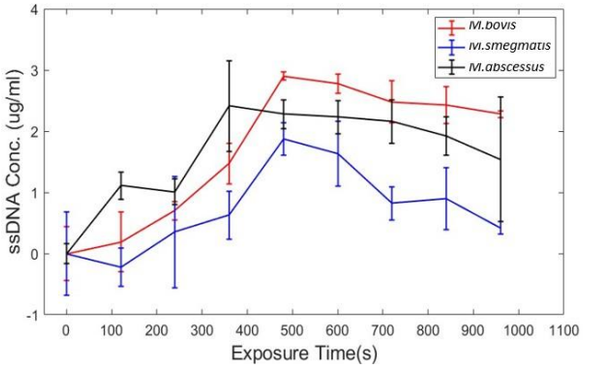


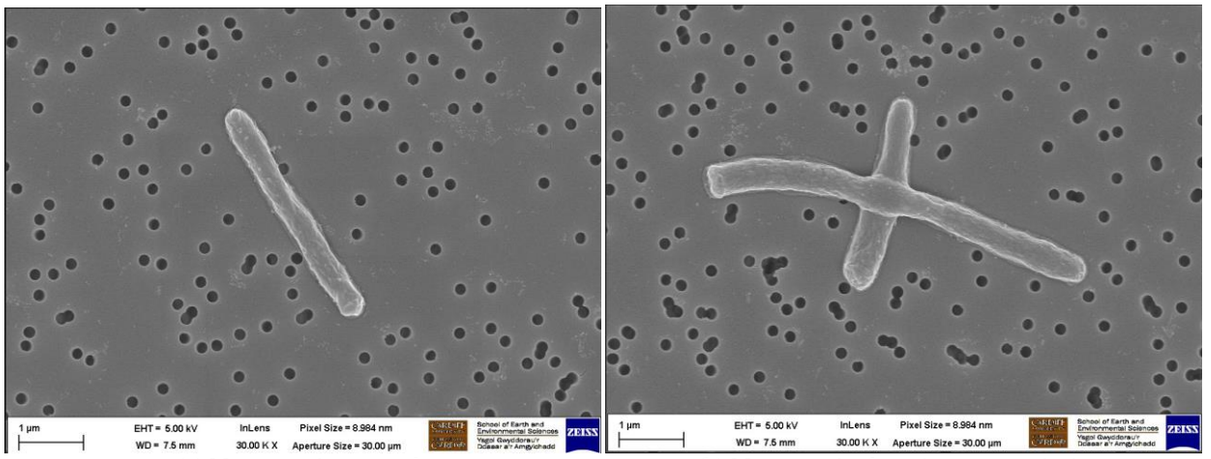

FIG. 14. Microwave mediated ssDNA release using 10ml Stack cartridge from $10^8$CFU/ml sample of various Mycobacteria in simulated sputum. The data has been corrected by subtracting each respective t=0s value. The error is shown as 1 standard deviation away from the mean value ($N = 3$).

showed that the ssDNA concentration peaked after 6 minutes and remained constant until the 8 minute mark. Establishing an 8 minute excitation period for a 10ml sample size is therefore justified.

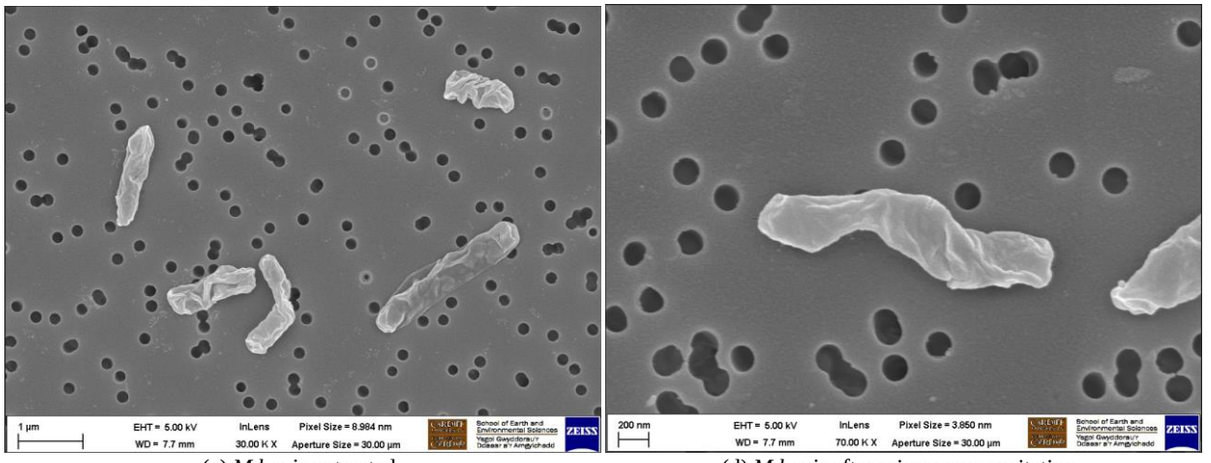
The simulated sputum data also demonstrated a greater spread of ssDNA concentration compared to the data obtained from samples suspended in water, suggesting that the non-uniform nature of the simulated sputum resulted a non-uniform release of ssDNA.

## VI. SCANNING ELECTRON MICROSCOPE IMAGING OF MYCOBACTERIA

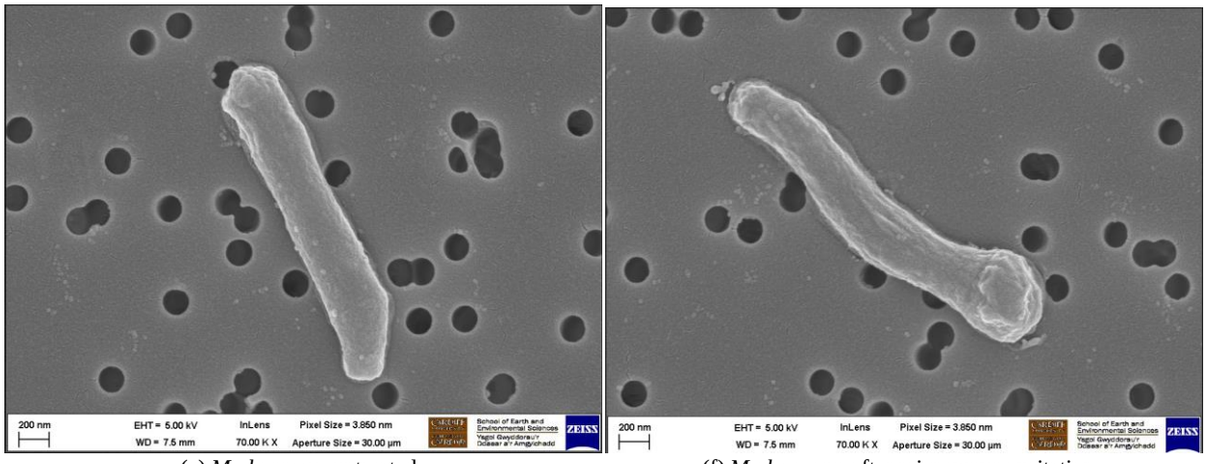
The mechanisms behind the release of DNA from bacteria following exposure to microwaves is not well understood. It was initially suggested that the microwaves fully lysed the bacteria, however, this was subsequently shown to be incorrect. Evans et al. reported that while exposure to the microwave power levels employed in this study had little effect on the viability of the test micro-organism, which included *M.Smegmatis*, it did cause a transient increase in cell wall permeability to latex particles labelled with fluorescent dyes[20]. It is possible that this increase in permeability due to microwave interaction allows DNA to escape the cell and that it is "released" DNA which is measured post microwave exposure. It is not possible to take real-time footage of individual bacteria cells during microwave excitation so a series of SEM images was obtained of the different species of mycobacteria pre and post microwave excitation to look for evidence of structural damage (Fig. 18). It is clear from the images that the microwave excitation has no visible effect on any of the bacterial strains (indeed the only conclusion that can be drawn with any certainty is that *M.bovis* seems more susceptible to the dehydration method used in preparation for SEM, as the bacteria are more shrivelled). These results mirror the earlier study of Evans et al suggesting that the increased levels of extracellular ssDNA are the results of transient disruption of cell wall structure. The viability of the bacteria was also tested pre and post microwave excitation (using the optimal exposure time/level for DNA release) with no measurable difference.




(a) *M.smegmatis* untreated (b) *M.smegmatis* after microwave excitation




(c) *M.bovis* untreated (d) *M.bovis* after microwave excitation




(e) *M.abscessus* untreated (f) *M.abscessus* after microwave excitation

FIG. 15. Samples of *M.smegatis*, *M.bovis*, and *M.abscessus* were sputter coated with c. 20 nm thickness of AuPd to ensure conductivity using a BIORAD SC500 coater. Samples were imaged using a the In-Lens secondary electron detector on a Zeiss Sigma HD FEG-SEM in the School of Earth and Environmental Sciences, Cardiff University, under high vacuum conditions. A beam energy of 5 kV was used with a 30 $\mu$m diameter final aperture and a working distance of 7.5 mm. The magnification of images a), b) and c) is x30K and the magnification of images d), e) and f) is x70K.

## VII. DETECTION OF PATHOGEN SPECIFIC TARGETS

The final experiment in this paper determines the ability of DNA probes with specificity for a region of DNA within the IS1801 gene which is unique to *M.bovis*, to distinguish the bacterium from other Mycobacterial species. This was achieved by combining microwaved based DNA extract with DNA capture using DNA probe functionalized magnetic nanoparticles (MP). The MP/target DNA complex was detected by a send target DNA specific probe labeled with an enzyme, horseradish peroxidase (HRP) in a process known as enzyme linked oligonucleotide sandwich hybridisation assay (ELOSHA)[22].

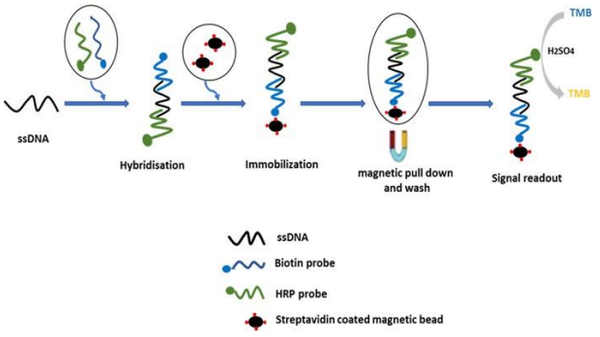



FIG. 16. A cartoon describing the various steps in the target DNA capture and detect process

As can be seen in Fig. 17, *M.bovis* generated a significantly stronger signal (p-value < 0.05, one way ANOVA) than the other non-*M.bovis* isolates when suspended in PBS (all isolated were suspended at $10^8$ CFU/ml).

The limit of detection of the combined microwave/ELOSHA system was determined using different concentrations of *M.bovis* suspended in PBS. As can be seen in fig. 17 a significant (p-value < 0.05) signal was observed at a concentration of $10^4$ CFU/ml as determined using a one way (ANOVA) test.

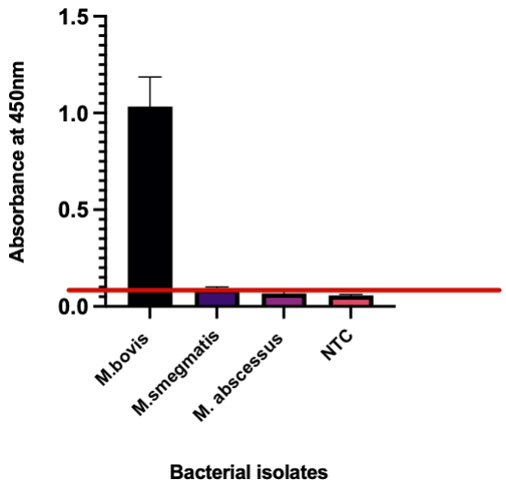



FIG. 17. **Detection of *M.bovis* using a combination of microwaves and ELOSHA.** *M. bovis* BCG str. Pasteur 1173P2, *M. smegmatis* Mc2155(S2) and *M. abscessus* ATCC 19977 suspended in PBS ($10^8$ CFU/ml) were microwaved and probed with magnetic nanoparticle and IS1801 specific probes. The presence of target specific DNA was determined by the intensity of the colour change due to the action of HRP. A positive cut value-off value for the assay was determined as the mean optical density (OD) plus three x3 mean values of the mean value of *M.smegmatis* which is indicated by a red horizontal line. NTC = No template control. Data represents mean of triplicate experiment ± standard error.)

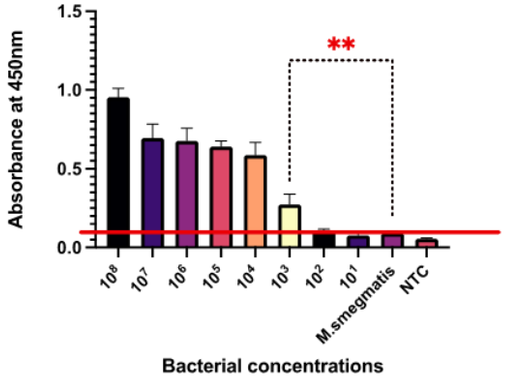



FIG. 18. **The limit of detection of the combined microwave and ELOSHA assay for *M.bovis*** A culture of *M. bovis* BCG str. Pasteur 1173P2, was serially dilute in water to generate a concentration range from $10^8$ CFU/ml to $10^1$ CFU/ml. Each bacterial concentration was analyzed for its ability to generate a signal following processing. A positive cut value-off value for the assay was determined as the mean optical density (OD) plus three 3x standard deviation of the mean value of *M. smegmatis* Mc2155(S2) (at $10^8$ CFU/ml) as indicated by a red horizontal line. NTC = No template control. Data represents mean of triplicate experiment ± standard error. ** demonstrates the lack of significant difference between the $10^8$*CFU/ml M.Smegmatis* control and the $10^3$*CFU/ml M.bovis* measurement .

## VIII. CONCLUSION

A novel cartridge model for the microwave excitation of 10ml aqueous samples has been designed, tested, and optimised by altering the microwave parameters using a range of Mycobacterial species as surrogates for M.tb. A power level of 12W for an 8 minute excitation with no duty cycle was identified as the optimum for the release of ssDNA. Preliminary characterization studies suggest that the release of this DNA is due to the transient disruption of the bacterial cell wall. In proof of principal studies the presence of microwave liberated M.bovis DNA was detected using our in house ELOSHA assay. In its current configuration the assay is as sensitive as microscopy but not as sensitive as PCR ($10^2$ CFU/ml) so work is in progress to increase the sensitivity of the assay and reduce the sample processing time.

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