**A novel microfluidic dielectrophoresis technology to enable rapid diagnosis of *Mycobacteria tuberculosis* in clinical samples.**

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**Running title: Dielectrophoresis can diagnose *Mtb***

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**Abstract**

To achieve the global efforts to end Tuberculosis (TB), affordable diagnostics suitable for true point-of-care (POC) implementation are required to reach the ‘missing millions’. Additionally, diagnostics with increased sensitivity and expanded drug susceptibility testing (DST) are needed to address drug resistance and to diagnose low-bacterial burden cases.

The lab-on-a-chip technology described herein used dielectrophoresis (DEP) to selectively isolate *Mycobacterium tuberculosis (Mtb)* from sputum samples, purifying the bacterial population ahead of molecular confirmation by multiplex qPCR. After optimisation using a panel of 50 characterised sputum samples, the performance of the prototype was assessed against the current gold standards, screening 100 blinded sputum samples using characterised and bio-banked sputum provided by Foundation for Innovative New Diagnostics (FIND).

Concordance with culture diagnosis was 100% for smear negative samples and 87% for smear positive samples. Of the smear positive samples, the high burden sample concordance was 100%. Samples were diagnosed based on visual assessment of the DEP array and by multiplex qPCR assay.

The results described herein demonstrate the potential of the CAPTURE-XT® technology to provide a powerful sample preparation tool that could function as a front-end platform for molecular detection. This versatile tool could equally be applied as a visual detection diagnostic, potentially associated with bacterial identification for low-cost screening or coupled with an expanded PCR assay for genotypic DST.

**Introduction**

Tuberculosis (TB) presents a multifaceted challenge to diagnostics which impedes treatment access and sustains global transmission 1. Pulmonary tuberculosis, the most common presentation of the *Mycobacterium tuberculosis* (*Mtb*) infection in humans, is primarily diagnosed from analysis of sputum samples 2. Traditionally, smear microscopy is used with Ziehl-Neelsen staining to specifically highlight acid-fast bacterium 3.

Mycobacterial burden is measured as grades of smear positivity (3+ to scanty) which is used to evaluate disease severity and associated infectiousness of the patient. While this methodology is low-cost and requires minimal laboratory facilities, sensitivity is poor with a detectable limit of 104 bacilli/ml of sputum and the quality of the diagnostic can vary between site and operators 4,5. More recent improvements to smear microscopy have been implemented including fluorescent cell-labelling with the auramine stain, but lower burden infections still remain undiagnosed 6. For higher sensitivity interrogation of so-called ‘smear negative’ samples, culture has remained the WHO-recommended methodology 7. The ‘time to culture positivity in liquid culture’ system has long been used to predict patient outcome. Sputum samples are first decontaminated to eliminate competing, faster growing commensal bacteria and then incubated in growth medium to determine the presence of *Mtb* 8. While undisputedly the most sensitive diagnostic with limits of detection from 1-10 bacilli/ml of sputum, the slow growth rate of *Mtb* and requirement of biosafety level 3 facilities to effectively perform this method of diagnosis remains a limitation, particularly in resource restricted settings 9.

Meeting the Sustainable Development Goals for Tuberculosis requires the design and implementation of new technologies to address the key bottlenecks in TB control and elimination 10. Novel diagnostic technologies are required to improve case detection, thereby reaching the “missing millions”, estimated in 2019 to be 2.9 million people globally 11,12. Cepheid’s GeneXpert MTB/RIF® assay and the GeneXpert MTB/RIF Ultra is the WHO’s main recommended rapid diagnostic test for detection of TB and rifampicin resistance as other more portable technologies are currently not available to provide accurate and rapid diagnosis 13. There is still a need for rapid, accurate and robust TB diagnostic tests suitable for use at the point of care 14, with high sensitivity for children and patients with HIV co-infection (WHO 2021).

The imperative for these much-needed advances will not just be based on increased sensitivity of detection in new assay formats for pathogen-specific DNA assays, but equally on developing *in vitro* diagnostic (IVD) platforms applicable to high-endemic, under-resourced areas of the world 15. This requires devices that are portable and not dependent on infrastructure, yet suitable for use with difficult to manipulate sputum samples and capable of providing purified samples to test, as well as enhanced sensitivity of detection 16.

Herein we describe a prototype microfluidic chip-based system that can process solubilised sputum from suspected TB patients, capture *Mtb* bacilli for visual analysis (as a substitute for smear microscopy) and provide a purified sample for molecular confirmation by qPCR and ultimately for drug-susceptibility analysis.

CAPTURE-XT® technology employs dielectrophoresis (DEP) separation of *Mtb* bacilli from the patient sample allowing isolation and differential purification of the bacilli from other organisms and impurities that can mask detection or interfere with molecular analysis 17. DEP exploits the relative polarizability of a particle and the medium in which it exists to manipulate the particle’s movement. The DEP force is governed by properties of the particle of interest and the medium in which it exists (sample or buffer) such that a ‘capture field’ can be generated by applying a voltage to an array of electrodes interfaced with the solution in which the particles are suspended. Since the system was first described, DEP has been the subject of numerous studies and coupled with microfluidics for application as biosensors, environmental sensors and in the development of medical diagnostics. Such work has been extensively covered in reviews 18,19,20.

DEP therefore represents a novel and as yet untested approach to TB diagnosis that could provide a test with improved performance for low bacterial burden sputum samples, such as smear “scanty” or smear negative (but culture positive) samples - an area of current diagnostic inaccuracy and low performance. A prototype microfluidic DEP purifier was used here ahead of a qPCR assay to assess DEP performance against gold standard TB diagnostics on a blinded set of 100 human sputum samples provided by the Foundation for Innovative New Diagnostics (FIND).

**Methods**

**Samples**

Aliquots (0.2 - 0.5 ml) of human sputum were provided by FIND from a biobank repository (Zeptometrix Corporation with ethical approval from 100 patients. Samples were previously comprehensively characterised and relevant patient history recorded). Gold standard diagnostics were used for determination of the patients’ disease status including at least liquid/solid culture and smear microscopy (Ziehl Nielsen) 21. Sputum samples originated from South Africa, Peru and Vietnam and were provided blinded with a 12-digit unique identifier. Sputum samples were stored at -20°C until required for analysis. An additional 50 samples were provided with known smear and culture status for use in protocol optimisation. The 50-sample panel consisted of 20 negative (smear negative; culture negative); 9 scanty (smear negative; culture positive) and 21 positive (smear positive graded between 1+ to 3+; culture positive). The blinded data set was predominantly made up of negatives and scanty/smear negative-culture positive samples by request, as these are the most challenging samples to confirm as positive for Mtb for any TB diagnostic.

**Sample processing protocol**

500 µl sputum sample was thawed at room temperature for 5 minutes before the addition of 500 μl thinning buffer (2% DTT (Sigma, Germany), 1% Tween 80 (Calbiochem, UK), 1% Triton-X100 (Sigma), 20 mM EDTA pH7 (Sigma) in sterile deionised water). Samples were vortexed for 5 minutes and incubated at 40°C for 90 minutes before addition of 3 µl Baclight cell stain mix (Invitrogen, USA). Samples were then incubated at 40°C for a further 30 minutes before the addition of 24 ml sterile deionised water. Samples were vortexed for 5 minutes and filtered through 8 µm nucleopore filter (Whatman Sigma) and then subjected to probe sonication (Rinco ultrasonics UK Ltd, London) at 70% amplitude for 25 seconds in 5 second intervals. All samples were processed in a biosafety level 3 laboratory.

**Microfluidic dielectrophoresis (DEP) device and platform**

The principles of dielectrophoresis (DEP) are based on a phenomenon originally described by Pohl, *et al.* 22,23. Figure 1 shows a schematic of particles (bacteria) attracted to the edges of the electrodes generating the electrical field.

DEP electrodes (Au/Ni) were fabricated onto poly(methyl methacrylate) (PMMA) substrate with microfluid channels defined in SU-8 photoresist (Epigem, UK). Fluidics were designed such that eight parallel channels controlled the flow of solution across the electrodes from a single inlet and converged to a single outlet for simple fluid handling. Fluidic connections were made from the microfluidic device using standard connectors (IDEXX) and machined mounts. Devices were flushed with sterile deionised water to void air before sample was introduced. Electronic connections were made by pogo and BNC connectors (RS components) to a function generator (Textronix AFG1022) and signal amplifier (Tabor 9250). A sinusoidal AC current was established (10 MHz, 1 Vpp) from two channels, connecting to two independently circuited electrode arrays on the DEP device - Channel 1 (CH1) and Channel 2 (CH2) respectively.

Two iterations of the DEP control platform were used simultaneously with comparable performance. The first system, built with standard laboratory equipment utilised an BX53F microscope (Olympus, Japan) with image acquisition and processing via Image J (version 1.51, www.imagej.net/software/fiji, accessed Jan 2017 24). Fluid control was completed via a syringe pump (Cole-Palmer, UK). The second iteration moved towards integration of control components within a bespoke platform. Platform components and imaging systems (Thor labs ltd, UK) alongside pressure driven fluidics (Fluigent, France) were controlled through a single user interface in µManager software 25.

In both iterations, filter sets compatible with excitation/emission of 485/498 nm were used to detect the presence or absence of bacilli attracted to the edges of the electrodes and images captured using a R3 camera (Retiga, USA). Bacilli in the thinned sputum samples were stained with BacLight™ Bacterial viability stain, following manufacturer’s instructions (Thermofisher, USA).

Under DEP, bacilli are attracted to areas of high field density created by arrays of interdigitated electrodes, powered by an AC signal (Figure 1). In order to increase the processing capacity of the test devices, fluidics were designed to allow dual-phase isolation. For initial capture and volume reduction, the sample was split across 8 parallel channels within which the bacilli are collected and immobilised onto primary electrode arrays until the sample has run near to completion. At the end of the sample volume, the DEP fields are de-activated, releasing the bacteria back into the convergent flow streams for secondary capture and concentration onto a single (secondary) electrode bed where their presence can be confirmed by fluorescence microscopy. Once visual analysis is complete, the DEP field is again de-activated, releasing the bacteria into the flow stream for elution from the microfluidic device. The purified sample can then be used for molecular analysis. The process is outlined graphically in Figure 2.

Processed sputum was loaded onto the syringe pump and flowed through the device to establish a linear flow velocity of 25 – 35 μL/min across the primary capture fields. The electrode bed was activated (CH1) with a 10 MHz, 1 Vpp current and remained active until the sample had processed to 90%. CH1 was deactivated, terminating the field from the primary capture electrode and, simultaneously, the secondary capture electrode was activated (CH2). After visual analysis of the secondary electrode (100X magnification with GFP filter) the presence or absence of bacilli was noted and recorded photographically (Retiga R3 camera with µManager software). Where capture/time was measured, the number of bacilli were counted using a countstack plugin in ImageJ software to count particles, compiled over one minute.

**Bacteriology: cultures and quantitation**

For optimisation of the system *Mtb*-negative sputum spiked with various mycobacterial strains were tested. *Mycobacterium tuberculosis*, strain H37Ra (ATCC, USA); *Mycobacterium smegmatis* MC2155 (ATCC) were kept as stock strains and stored in liquid nitrogen. These were cultured and serially transferred at weekly intervals (1 ml of 108 seeding culture added to 8 ml broth) in Middlebrook 7H9 (SLS, UK) supplemented with 10% albumin-dextrose-catalase (ADC) (Becton Dickinson, USA) and 0.2% glycerol. The cultures were incubated at 37oC. The cultures were serially transferred for five weeks before a fresh culture was started.

**Quantitative PCR Assay and Specificity testing**

A triplex quantitative PCR assay using Taqman probes was established for three specific *Mtb* targets: two established gene targets *IS6110* and *IS1081*, and *Rv1707* which was designed in-house. Primers and probes were designed using the Primer3plus programme 26 with emphasis on minimal amplicon size for rapid amplification. *Rv1707* was selected as an *Mtb*-specific locus while the multi-copy targets *IS6110* and *IS1081* were included to increase the sensitivity of *Mtb* complex detection. Bioinformatic analysis of the gene target primer pairs and probes by Basic Local Alignment Search Tool (BLASTN) was undertaken to confirm non-homology across species. Primers, probes, Tm values, amplicon size and copy number for each gene target are shown in Table 1. A Ct cut-off value of 38 was assigned based on the GeneXpert manual which designates any sample with Ct value of 38 or higher as negative.

An exclusivity testing panel consisting of non-tuberculous mycobacteria (NTMs) and a range of Gram-positive and Gram-negative bacteria was obtained from The Belgian Co-ordinated Collection of Microorganisms (BCCM) (Brussels Belgium), and from Prof. Tim Bull (St. George’s University of London). Each primer set was tested against the panel to determine the specificity of the primers. PCR was performed using Qiagen Multiplex PCR master mix, following the manufacturer’s instructions, with 1 ng DNA and 2 µM of each primer and probe per reaction. BioRad CFX cycling conditions were 95°C for 15 s, followed by 40 cycles of: 30 s at 94°C, 90 s at 57°C, and 60 s at 72°C, and finally a 10 min incubation at 72°C. Each DNA sample was tested both alone and spiked with TB DNA as an internal control. The PCR products were analysed electrophoretically using the Agilent 2100 Bioanalyzer (Supplementary Table 1).

For molecular analysis of the CAPTURE-XT® eluates, using the *IS6110* qPCR assay, CH2 was deactivated, terminating the secondary capture field and a 15 µl eluate was collected from the device outlet into 2 ml screw cap tubes. Several eluates were collected under different conditions such as during electrode activation, as well as a non-DEP sample, which was thinned sputum only. Device eluates and thinned sputum samples were subject to heat-kill via incubation in a 95°C water bath for 45 min to render samples safe to be taken out of the containment level 3 laboratory. Samples were then processed for cell lysis by the addition of 5 µl microlysis solution reagent (Clent Life Sciences, UK) before heat cycling (80°C – 15 min, 96°C – 2 min, 80°C – 4 min, 96°C – 1 min, 80°C – 1 min, 96°C – 0.5 min). 2 µl of eluate was used as template in qPCR as described with *IS6110* primers and probe.

**Discordance testing**

Following unblinding, samples which had been called incorrectly were labelled ‘discordant’ and were re-processed for repeat testing using a second aliquot of sputum. Higher capacity DEP devices (64 parallel channels rather than 8) were utilised to reduce processing time. Also, to increase specificity, the *IS6110* PCR was supplemented with *IS1081* and a *Mtb* specific target (*Rv1707*). The third aliquot was subject to culture in order to detect and confirm the presence of possible low levels of *Mtb* in the sample; this was achieved using selective Kirchner medium with calf serum (SLS) and antibiotic cocktail (Polymyxin B 200,000 U/L, Ticarcillin 100 mg/L, Amphotericin B 10 mg/L, Trimethoprim 10 mg/L) (Mast Laboratories, UK) or Middlebrook 7H9 media (0.2% glycerol, 10% OADC) and antibiotic cocktail (Polymyxin B 200,000 U/L, Ticarcillin 100 mg/L, Amphotericin B 10 mg/L, Trimethoprim 10 mg/L) (Mast Laboratories) at 37°C for 2-3 weeks following which the liquid culture was plated onto Middlebrook 7H11 Agar Plates (10% OADC, 0.5% glycerol plus antibiotic cocktail (Polymyxin B 200,000 U/L, Ticarcillin 100 mg/L, Amphotericin B 10 mg/L, Trimethoprim 10 mg/L) (Mast Laboratories) and incubated at 37°C for 3-4 weeks and assessed for colony growth.

**Analysis**

Statistically significant differences in Ct values between thinned sputum samples and post-DEP eluates were estimated using Student’s T-test, 2-tailed and paired.

**Results**

**CAPTURE-XT® device optimisation**

A real-time flow image of *Mtb* capture by the DEP electrodes is presented in Supplementary video S1. This shows the flow of solubilised sputum through the microfluidic chamber passing over the primary electrode bed and gradual trapping of bacilli onto the edges of the DEP electrodes and accumulating on the edges of subsequent electrodes from right to left as more sample is processed. The particle sizes vary; the smaller particles are captured bacilli (Figure 3). After saturation of the electrodes the electrical field is switched off and the bacteria are immediately released with a few remaining particles on the electrodes.

Initial experiments to optimise the DEP-mediated bacterial cell isolation were performed with *M. smegmatis*, suspended in 0.001X PBS solution as a bio-safe alternative to *Mtb*. To determine the required optimal capture conditions, AC signals of varying frequency were applied to the cells and their response to the field monitored visually. The position of the cells upon the electrodes was noted along with any additional phenomena observed and representative images are presented in Supplementary Figure 1 along with maxima counts (Supplementary Figure 1 E), indicating the suitability of varying frequency currents for the isolation of Mycobacteria. The capture efficiency was noted to be highest at 10 MHz and at 10 Vpp. These conditions were therefore selected for continued protocol development for *Mtb*.

**Sputum sample preparation optimisation**

In order to apply CAPTURE-XT® technology as a TB diagnostic tool, sputum samples must be compatible with the microfluidics without introducing chemicals or compounds which would affect the response of the bacterial cells to the DEP forces. As such, the commonly used mucolytic agents such as N-acetyl L-cysteine (NALC) and sodium hydroxide (NaOH) cannot be applied due to the salt concentration these chemicals contain. Initial optimisation of flow rate, field strength, time of processing and solute constituents were investigated using TB-negative human sputum spiked with cultured *Mtb* bacilli or *M. smegmatis*. Using fluorescent microscopy and quantitative image software, the efficiency of capture and release was investigated. Of the compounds screened, dithiothreitol (DTT) showed a consistent efficacy at reducing the viscosity of sputum that was enhanced by incubation at slightly elevated temperatures (40°C) and by the addition of detergents. Sputum optimal ‘thinning’ required the addition of 2% DTT, 1% Tween 80, 1% Triton X100, 20 mM EDTA in a 1:2 dilution with 5 min vortex and 120 min incubation at 40°C. Figure 4 demonstrates the improvements in capture and release with this protocol. The NALC thinning method resulted in a maximum capture of 36 bacteria over 150 seconds and did not release due to the viscosity. The optimised thinning method, however, captured over 1200 bacteria in a 250 second period (876 in 150 seconds) and release was 97%.

Control of the ionic content of the processed sample is also required as the response of the targets to the DEP field is affected. This effect was reduced by dilution with de-ionised water. The number of cells captured by DEP was negligible in un-diluted samples but was evident from a 1:5 dilution and essentially complete at in 1:50 dilution with no cells noted traversing the electrodes without capture. While 100% capture would be the goal, the capture efficiency at 1:25 dilution was optimal to minimise runtimes through the microfluidic device at linear flow velocity of 25 – 35 μL/min. An optimised set of conditions was thus established prior to testing with clinical sputum specimens from TB cases and controls defined by conventional microbiological gold standard methods.

**Confirmatory PCR assay design**

Following a positive visual diagnostic, indicated by the presence of bacteria upon the electrodes, molecular analysis was used to confirm the presence of *Mtb* bacilli as opposed to other organisms possibly present in the processed sputum. For this study, a qPCR assay was developed for three separate gene targets considered specific for *Mtb*: *IS6110*, *IS1081* and a bioinformatically determined *Mtb* specific target, *Rv1707*. Primer-probe combinations worked well either as individual assays or as a triple-plex assay with limits of detection of <10 bacteria (calculated as genome copies equivalence from added DNA). Primer sets were screened for cross-reactivity against a panel of organisms including most of the known *Mtb* complex (*Mtb*C), a wide range of slow-growing mycobacteria, and known oral cavity or sputa bacterial contaminants. No significant cross-reactivity was noted *in silico* during the primer design stage. All primers displayed good species specificity for *Mtb* when tested on purified DNA in PCR assays. Cross-reactivity in PCR assays with the panel of control organisms was assessed electrophoretically with no-cross reactivity except for *M. celatum* that reacts weakly with *IS6110* and *IS1081*, and *M. vaccae* that also showed weak cross-reaction with *IS1081*. The results of species specificity testing are summarised in Supplementary Table S1. For the initial evaluation of DEP purification with a human sample panel, the *IS6110* primer-probe set was selected for use.

**CAPTURE-XT®****evaluation with sputa from TB cases**

**Initial validation set**

Protocol optimisation and initial evaluation of the CAPTURE-XT® technology and workflow were performed using bio-banked sputum samples. Samples had been previously characterised using gold-standard TB diagnostics including culture, and smear microscopy. Analysis of these characterised samples using the DEP isolation method was completed with both a visual result, in the form of a microscopy image of the collection electrodes, and a molecular read-out from qPCR of the eluates. The 50 unblinded samples were for optimisation of the protocol to be used on the 100 blinded sample set, as well as to demonstrate efficacy. To perform quantitative analyses on the optimisation set, a dataset with controlled variables were pulled out using the following exclusion criteria:

* All samples must be run on the same chip type
* All samples must be thinned with the same thinning solution
* All samples must correlate with the known burden (e.g., if there was capture with a negative, or no capture with a 3+)
* All samples ran without the appearance of bubbles or interference from EP effects

After establishing these initial methodological variables, 17 sputum samples were used with the selected methodology to assess performance ahead of the blinded study. These were: 4 smear negatives; 6 smear negative culture positive (S-C+); 2 1+; 2 2+; and 3 3+ (Tables 2 and 3below). Note that FIND defined smear negative culture positive as scanty whereas the usual definition of scanty is smear positive/culture positive. For these unblinded samples we are examining smear negative/ culture positive.

Positive qPCR amplification of the *IS6110* target using sample eluates showed Ct values in approximate proportion to both the smear microscopy grade and the visual bacterial accumulation on the DEP electrodes (see Figure 5). There was a clear proportionality between Ct value and bacteriological load, estimated by smear status. The overall qPCR data for these smear-graded unblinded samples for a total of 17 samples are shown in Table 2. This semi-quantitative correlation between bacteria load and genome equivalents measured by qPCR confirms the potential suitability of this bacterial capture and purification methodology for use ahead of molecular diagnostic tests.

Initial concordance between gold-standard diagnostic criteria and the combined visual imaging/qPCR data for these 17 selected samples (above) is presented in Table 3. Good concordance was evident for the smear positive samples, graded from smear-negative/culture-positive to 3+.

Two of the ‘negative’ samples were strongly positive in the DEP run and were therefore excluded as they had, upon examination by FIND, been mislabelled (see Table 3 annotation 1). Concordance with qPCR for the smear-negative samples was 50% due to the qPCR results being borderline positive (Ct 37.7 and 37.6) and therefore did not reach the negative cut-off Ct (38) (see Table 3 annotation 2). However, these initial experiments demonstrate a proof of principle that DEP capture and visualisation, and quantitation by qPCR, shows concordance and a proportionality for the graded smear positive sputum samples.

*Mtb* confirmatory qPCR assays showed a significant difference between the thinned sputum samples’ Ct values and the post-DEP eluate samples. The average decrease in Ct from thinned sputum sample to post-DEP eluate for scanty, 1+, 2+ and 3+ sets was 0.8 (P = 0.02), 0.9 (P = 0.003), 1.34 (P = 0.02), and 0.9 (P = 0.007) respectively. This equates to a fold-change of 1.7, 1.9, 2.5, and 1.9, respectively. These data indicate that the bacterial particles visualised as being captured by DEP and then released are *Mtb* because there is enrichment with *Mtb* in the eluate compared to non-captured thinned sputum samples.

**Blinded sputum set analysis**

As the results from the unblinded samples had shown good correlation with the known bacteriological status of the samples, the developed methodology described was subsequently used for the analysis of a 100-sample set, provided without clinical information allowing a blind assessment of the technology against gold-standard diagnostics.

The 100 blinded sputum samples were processed using the optimised protocol. Images from visual analysis electrode arrays were captured at the end of each sample run and used to make the primary diagnostic call in a binary fashion as *Mtb* positive or *Mtb* negative. Eluates were collected following imaging for molecular analysis with *IS6110* qPCR. A sample with a Ct of <38 was considered positive, however, samples returning a Ct value between 35 and 38 was considered ‘borderline’. In this case the qPCR data was examined and a judgement of positivity was made and agreed by laboratory staff (i.e. shape of amplification curve and apparent titration of diluted samples). Where samples were discrepant between the visual DEP and PCR result such samples were classified as positive. Of the 100 samples tested, 66 were classified as positive, 27 as negative and 7 as borderline. Results were submitted to FIND for un-blinding and full clinical data were returned with concordance determination. An overall concordance of 86% was seen across positive samples, with 100%, 90% and 84% for 3+, 2+ and 1+ smear classifications, respectively. Concordance was lower for smear negative samples with 76% agreement between gold standard and the CAPTURE-XT® methodology.

**Discordance testing**To resolve the discordant samples, second aliquots from the same initial sputa samples were subject to repeat analysis. Discordance analysis was completed on a second iteration of the CAPTURE-XT® platform and higher capacity fluidic consumable in line with ongoing technology developments. To ensure sensitivity was maximised, the full 500 µl sputum sample was processed, rather than running to visual positivity. To improve qPCR specificity, the *IS6110* PCR was combined with *IS1081* and the *Rv1707* gene targets in a multiplex format. Completion of the repeat analysis returned improved results with an overall concordance of 93% for smear positive samples with three ‘smear scanty’ samples remaining false negatives. The concordance of smear negative/culture positive samples remained at 76.9%. The triplex PCR enabled improved discrimination of amplification from PCR artifacts and reduced false positive calls from 15 to 1. However, upon further investigation, this remaining one false positive sample that was classified originally as smear and culture negative returned positive after prolonged bacteriological culture in our lab, confirming the positive qPCR result. Overall, the DEP purification methodology returned a concordance of 76.9% for smear negative, culture positive samples, with an overall concordance for all culture positives of 87%. The sensitivity and specificity were 87% ± 16.9 and 100% ± 19.6 respectively. The final results of the study are presented in Table 4.

**Discussion**

The eradication of TB, as one of the Sustainable Development Goals set by the United Nations, requires not only tests with improved sensitivity but those with the potential to be used in near-patient settings, beyond the confines of laboratories and health care centres, where vast numbers of TB-infected people require them 10,12. The technology presented in this study demonstrates the potential of CAPTURE-XT® for the capture, release and subsequent detection by qPCR of *Mtb* from sputum samples. Overall, the prototype CAPTURE-XT® platform achieved a specificity of 100% and a sensitivity of 87%. In comparison, GeneXpert has been shown to have a specificity of 90.4% and a sensitivity of 78.2%, and 98.7% and 87.5% respectively for Ultra 11,29. Concordance rates between ‘gold standard’ diagnostics and DEP purification with *Mtb* specific qPCR assays were 100% for 2+ and 3+ smear positive samples. Lower bacterial burden samples (smear negative, culture positive), which are more likely to give false negative results in standard diagnostic tests, had a concordance of 76.9% in the DEP/qPCR assay 30. Interpretation of qPCR was improved when three targets were used. This could be due to the variable copy number of *IS6110* which can be present in 1-20 copies or indeed absent depending upon strain while *IS1081* and *Rv1707* are present in stable copy number of 6 and 1 respectively, producing a more reliable pan-strain test 31. The DEP/qPCR study described here used species-specific qPCR as a cross-validating assay to show efficiency of capture, purification and release of *Mtb* by DEP directly from solubilised sputum specimens. Also, it is worth noting here that the Ct value cut-off used in this proof-of-principle was 38. This chosen negative threshold value (Ct = 38) and the subjective decisions as to positivity for some of the low bacterial burden samples with Ct = 35 – 38 is a limitation of this study which had insufficient samples to determine a more quantitative estimation. While there is little consensus on what the cut-off value should be across different tests, the Ct value cut-off for GeneXpert is 38, which was why that value was chosen for this study. In addition, some samples which were deemed positive in the DEP system were, upon further inspection by FIND, indeed positives and had been erroneously labelled as negatives. This system, therefore, was able to detect *Mtb* bacteria which had previously gone undiagnosed. In fact, the qPCR result for several samples collected from thinned sputum only were negative, but post-DEP eluates were positive due to the concentrating effect of DEP, which means samples with very low burden are less likely to be counted as false negatives. Once optimised, this system could, therefore, have an increased sensitivity.

In this feasibility stage, all work was performed in a bio-safety cabinet, with connecting tubing which resulted in a large dead volume. These issues affect the speed and accessibility of the assay and reduced the potential DEP concentration effect through sample dilution. However, condensing the system into a portable front-end device would resolve these issues. The sample study presented here was limited to processing 500 µl of sputum. For improved sensitivity and an expedited time to result, devices with highly paralleled capture channels are being developed to increase throughput. Current microfluidic setup can capture the bacteria in a sample in less than one hour with a visual read-out for an initial diagnosis before qPCR analysis for low-burden samples and for drug resistance profiling. Refinements to aspects of the biological processing and device design are anticipated to result in significantly improved performance. For this study, bio-banked samples were gratefully utilised; however, prior storage at -80°C may have resulted in a loss of cell viability or affected the bacterial cell membrane required to elicit a response to dielectrophoresis. Repeated freeze-thawing reduced the viability of the samples in our hands (data not shown) 32. Furthermore, the effect of freezing must be considered with regards to the ease with which thinning can be achieved for microfluidic compatibility. To advance the performance analysis of this technology, fresh samples are required to better simulate a real-world scenario for point of care diagnostics. The fluorescent dye employed in this study was not specific for *Mtb* and therefore could have given false positives in a mixed sample. Using a *Mtb*-specific dye would improve the specificity of the visual analysis aspect of this technology 33. This assay could also be used in the future for stratification of treatment.

In summary, we have demonstrated, as a proof-of-principle using biobanked TB sputum specimens and an experimental DEP capture technology, that CAPTURE-XT® is a sensitive and specific platform for TB diagnosis. This has potential as a next generation TB diagnostic or as a “front end” sample preparation technology for visual and subsequent molecular detection techniques. In addition, the qPCR assay could easily include detection of markers for drug resistance, making this technology capable of personalised medicine.

**References**

1. Walzl G, McNerney R, du Plessis N, Bates M, McHugh TD, Chegou NN, Zumla A. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. Lancet Infect Dis, 2018, 18:e199–210

2. WHO. Global Tuberculosis Report 2019. 2019

https://www.who.int/publications/i/item/9789241565714. (accessed January 26, 2021)

3. Dzodanu EG, Afrifa J, Acheampong DO, Dadzie I. Diagnostic Yield of Fluorescence and Ziehl-Neelsen Staining Techniques in the Diagnosis of Pulmonary Tuberculosis: A Comparative Study in a District Health Facility. Tuberc Res Treat, 2019, 2019:1–6

4. Lombardi G, Di Gregori V, Girometti N, Tadolini M, Bisognin F, Dal Monte P. Diagnosis of smear-negative tuberculosis is greatly improved by Xpert MTB/RIF. PLoS One, 2017, 12

5. Singhal R, Myneedu VP. Microscopy as a diagnostic tool in pulmonary tuberculosis. Int J Mycobacteriology, 2015, 4:1–6

6. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins M, Aziz MA, Pai M. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis, 2006, 6:570–81

7. WHO. Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents Recommendations for HIV-prevalent and resource-constrained settings. 2006

8. Grandjean L, Martin L, Gilman RH, Valencia T, Herrera B, Quino W, Ramos E, Rivero M, Montoya R, Escombe AR, Coleman D, Mitchison D, Evans CA. Tuberculosis diagnosis and multidrug resistance testing by direct sputum culture in selective broth without decontamination or centrifugation. J Clin Microbiol, 2008, 46:2339–44

9. Balcha TT, Sturegård E, Winqvist N, Skogmar S, Reepalu A, Jemal ZH, Tibesso G, Schön T, Björkman P. Intensified Tuberculosis Case-Finding in HIV-Positive Adults Managed at Ethiopian Health Centers: Diagnostic Yield of Xpert MTB/RIF Compared with Smear Microscopy and Liquid Culture. PLoS One, 2014, 9:e85478

10. Falzon D, Migliori GB, Jaramillo E, Weyer K, Joos G, Raviglione M. Digital health to end tuberculosis in the Sustainable Development Goals era: achievements, evidence and future perspectives. European Respiratory Journal, 2017, 50 (5) 1701632

11. Mechal Y, Benaissa E, El Mrimar N, Benlahlou Y, Bssaibis F, Zegmout A, Chadli M, Malik YS, Touil N, Abid A, Maleb A, Elouennass M. Evaluation of GeneXpert MTB/RIF system performances in the diagnosis of extrapulmonary tuberculosis. BMC Infect Dis, 2019, 19:1069

12. WHO. Global tuberculosis report 2020, 2020. https://www.who.int/publications/i/item/9789240013131. (accessed January 26, 2021)

13. WHO. WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF. 2017

14. WHO. Global Tuberculosis Report 2018. 2018

https://apps.who.int/iris/handle/10665/274453. (accessed January 26, 2021)

15. Seki M, Kim CK, Hayakawa S, Mitarai S. Recent advances in tuberculosis diagnostics in resource-limited settings. Eur J Clin Microbiol Infect Dis, 2018, 37:1405–10

16. Rie A Van, Page-Shipp L, Scott L, Sanne I, Stevens W. Xpert ® MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: Hype or hope? Expert Rev Mol Diagn, 2010, 10:937–46

17. Hawkins BG, Lai N, Clague DS. High-sensitivity in dielectrophoresis separations. Micromachines, 2020, 11

18. Fernandez RE, Rohani A, Farmehini V, Swami NS. Review: Microbial analysis in dielectrophoretic microfluidic systems. Anal Chim Acta, 2017, 966:11–33

19. Hughes MP. Fifty years of dielectrophoretic cell separation technology. Biomicrofluidics, 2016, 10

20. Yang L. A Review of Multifunctions of Dielectrophoresis in Biosensors and Biochips for Bacteria Detection. Anal Lett, 2012, 45:187–201

21. Global laboratory initiative advancing TB diagnosis A publication of the Global Laboratory Initiative a Working Group of the Stop TB Partnership Mycobacteriology Laboratory Manual, 2014

22. Pohl HA, Hawk I. Separation of living and dead cells by dielectrophoresis. Science (80- ), 1966, 152:647–9

23. Pohl HA, Crane JS. Dielectrophoresis of Cells. Biophys J, 1971, 11:711–27

24. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012 97, 2012, 9:676–82

25. Edelstein AD, Tsuchida MA, Amodaj N, Pinkard H, Vale RD, Stuurman N. Advanced methods of microscope control using μManager software. J Biol Methods, 2014, 1:10

26. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res, 2007, 35:W71

27. Islam N, Wu J. Microfluidic transport by AC electroosmosis. J Phys Conf Ser, 2006, 34:356–61

28. Oh J, Hart R, Capurro J, Noh H. Comprehensive analysis of particle motion under non-uniform AC electric fields in a microchannel. Lab Chip, 2009, 9:62–78

29. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, Banada PP, Deshpande S, Shenai S, Gall A, Glass J, Krieswirth B, Schumacher SG, Nabeta P, Tukvadze N, Rodrigues C, Skrahina A, Tagliani E, Cirillo DM, Davidow A, Denkinger CM, Persing D, Kwiatkowski R, Jones M, Alland D. The new Xpert MTB/RIF ultra: Improving detection of Mycobacterium tuberculosis and resistance to Rifampin in an assay suitable for point-of-care testing. MBio, 2017, 8:1–12

30. Rieder HL, Lauritsen JM, Naranbat N, Katamba A, Laticevschi D, Mabaera B. Quantitative differences in sputum smear microscopy results for acid-fast bacilli by age and sex in four countries. Int J Tuberc Lung Dis, 2009, 13:1393–8

31. Alonso H, Samper S, Martín C, Otal I. Mapping IS6110 in high-copy number Mycobacterium tuberculosis strains shows specific insertion points in the Beijing genotype. BMC Genomics, 2013, 14:422

32. Shu Z, Weigel KM, Soelberg SD, Lakey A, Cangelosi GA, Lee KH, Chung JH, Gao D. Cryopreservation of Mycobacterium tuberculosis complex cells. J Clin Microbiol, 2012, 50:3575–80

33. Kamariza M, Shieh P, Ealand CS, Peters JS, Chu B, Rodriguez-Rivera FP, Babu Sait MR, Treuren WV, Martinson N, Kalscheuer R, Kana BD, Bertozzi CR. Rapid detection of Mycobacterium tuberculosis in sputum with a solvatochromic trehalose probe. Sci Transl Med, 2018, 10

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Table 1: PCR primers and probe sequences used in triplex qPCR assay.

# Copy number in *Mtb* H37Rv. Strains of *Mtb* have variable numbers of *IS6110*, ranging from 0 – 16.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Primer** | **Sequence** | **Tm (°C)** | **Amplicon size (bp)** | **Copy number#** |
| *RV1707* F | 5’-ATCATCATGACCCAGCTGGATC-3’ | 63.5 | 134 | 1 |
| *RV1707* R | 5’-GAGCTGACCAGAACCAGGAC-3’ | 59.8 |  |  |
| *IS6110* F | 5’-CGAACTCAAGGAGCACATCA-3’ | 60.0 | 135 | 16 |
| *IS6110* R | 5’-AGTTTGGTCATCAGCCGTTC-3’ | 60.1 |  |  |
| *IS1081* F | 5’-CTGCTCTCGACGTTCATCGCCG-3’ | 71.6 | 135 | 6 |
| *IS1081* R | 5’-GGCACGGGTGTCGAAATCACG-3’ | 70.1 |  |  |
|  |  |  |  |  |
| **Probe** |  | **Tm (°C)** | **Fluorophore** |  |
| *RV1707* | 5’-CGTCCTGCTGCTGGCTAGCG-3’ | 68.9 | HEX |  |
| *IS6110* | 5’-ACCGTCAGGGCATCGAGGTG-3’ | 68.0 | FAM |  |
| *IS1081* | 5’-GCGATGAGCGGTCCAATCAGCGCAA-3’ | 77.9 | TEXAS RED |  |

Table 2. qPCR Ct values for unblinded sputum samples graded bacteriologically. Mean and standard deviation (SD). N = number of samples included. S-C+= smear negative culture positive, Negative = smear and culture negative

|  |  |  |  |
| --- | --- | --- | --- |
| Smear status | Mean Ct | SD | N |
| Negative | 38.4 | 1.1 | 4 |
| S-C+ | 35.7 | 1.8 | 6 |
| 1+ | 27.5 | 1.0 | 2 |
| 2+ | 26.6 | 2.3 | 2 |
| 3+ | 24.2 | 2.0 | 3 |

Table 3: **Unblinded evaluation of test set of sputa**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| FIND smear result | Samples tested | DEP +ve | PCR +ve | Concordance with gold standard | |  |  |
| DEP | PCR |  |  |
| 3+ | 3 | 3 | 3 | 100% | 100% |  |  |
| 2+ | 2 | 2 | 2 | 100% | 100% |  |  |
| 1+ | 2 | 2 | 2 | 100% | 100% |  |  |
| S-C+ | 6 | 6 | 6 | 100% | 100% |  |  |
| Negative | 4 | 01 | 22 | 100% | 50% |  |  |
| 1Two negatives which had immediate, obvious capture were mislabelled and therefore excluded  2Ct values were 37.7 and 37.6 which are borderline positive by the set criteria | | | | | | | |

**Table 4. Final 100-sample test performance evaluation**Number of samples with classification by FIND. *Mtb* positive samples sub-divided by smear status and rating. Concordance calculated as percentage of samples for which the experimental protocol returned the same diagnostic outcome (*Mtb* positive or *Mtb* negative) as the FIND ‘gold standard’ diagnostics.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample classification** | | | **Samples tested** | **Correct diagnosis** | **Concordance (%)** |
| Total | | | 100 |  |  |
| *Mtb* negative | | | 29 | 29 | 100 |
| *Mtb* positive | | | 71 | 62 | 87 |
|  | smear negative/ culture positive (Total) | | 26 | 20 | 76.9 |
|  | smear positive (Total) | | 45 | 42 | 93 |
|  |  | Scanty/ 1+ | 25 | 22 | 88 |
|  |  | 2+ | 10 | 10 | 100 |
|  |  | 3+ | 10 | 10 | 100 |

**Figure Legends:**

**Figure 1: Schematic of end-on electrodes within the DEP chip (A) and the DEP fields within a microfluidic channel (B). A. Yellow circles denotes particle of interest e.g. bacteria. Blue arrows indicate sample under flow. The interdigitated electrode array has an alternating current applied, shown as black (negative) or white (positive) rectangles. B. Lines represent DEP field. Colour represents field strength where red denotes strong attractive forces.**

**Figure 2: *Mtb* isolation process flow: A) Sputum sample is thinned, stained, and connected to the fluidic system (1). Sample is directed through the microfluidic cassette, flowing over parallel arrays of interdigitated electrodes (2 and 3). B) The primary electrode arrays are activated, establishing a capture field, attracting the *Mtb* towards the primary electrode edges (2). C) When the sample is almost exhausted, the smaller, secondary electrode array is activated (3). The primary arrays are deactivated, releasing the bacteria from the capture fields into the flow. D) The *Mtb* are re-captured on the secondary electrode array which can be visualised by fluorescent microscopy (4). The presence of fluorescent foci indicates a positive result for TB infection. E) Deactivation of the secondary electrode array releases cells into the flow allowing collection in a small volume (as low as 5 µl) for molecular analysis (5).**

**Figure 3: Comparison of sputum vs bacteria. Top panel shows an electrode bank capturing a positive sputum sample, with both bacteria (in circles) and sputum debris (in squares) visible. Bottom panel shows bacteria isolated from medium only.**

**Figure 4: Particle counts pre- (A) and post- (B) *Mtb*-spiked sputum thinning optimisation. Pre-optimisation was with NALC only. Post optimisation was with the lysis buffer described herein, with a two-hour incubation at 40°C. Arrows indicate the time point at which the dielectric field was terminated and bacteria were released. Signal noise due to low capture numbers in the NALC-only run (left).**

**Figure 5: Representative samples from each of the smear microscopy and culture classifications of TB burden are presented with sample identification number in parentheses. Bacilli were clearly visible at the edges of electrodes, where the field strength is greatest, indicating successful cell capture from the sputum sample. *Mtb* smear negative, culture positive sputum showed only minimal captured bacteria. All samples demonstrated a low level of background non-specific captured debris. A correlation between smear positive grading and visual count of captured particles interpreted as *Mtb* bacilli was seen. Correlation between *Mtb* DNA quantification by species-specific qPCR was also seen with the degree of captured fluorescent particles on the DEP electrodes (Table 2). Images of negative and S-C+ samples are magnified (x 100, rather than x 40) for better clarity.**

*Supplementary video S1*

Video of capture and release from the primary electrodes. The camera is paused once the electrodes are saturated, and then restarted at t=45 secs to record the release.

*Supplementary Figure 1. M. smegmatis* capture was noted from frequencies of 1kHz. However, bubbles were quickly produced by the electrodes as a result of excessive Joule heating resulting in boiling of the solution (A). The number of bacterial cells captured increased in-line with frequency up to 1MHz. Visual analysis of capture highlighted that below 500kHz, capture is not resulting from DEP but rather is a product of AC electroosmosis 27,28 with cells suspended along the centre line of electrodes, appearing offset in the direction of solution flow that is from right to left in this case (B and C). Such an effect increases the amount of irreversible cell adhesion to electrodes and therefore is not optimal for the purposes of bacterial isolation, which requires efficient release upon field deactivation and is often associated with damage to the electrode surface with prolonged field activation. At 100kHz, cells continue to concentrate via AC electroosmosis. DEP capture of bacterial cells is visualised as fluorescent bacterial particles accumulating at the edges of the electrodes only (D). Maxima counts from post-capture images following activation of electrodes at varying frequency are shown in graph E. As the DEP response of a target organism is inextricably linked to its individual cellular dimensions and composition, fine optimisation of capture conditions was undertaken using *Mtb* (H37Ra), and capture efficiency was found to be 10 MHz and was therefore selected for continued protocol development for *Mtb*. (Graph F).

**Supplementary Table 1**: Summary of species-specificity of the newly designed TaqMan primer pairs using the exclusivity panel of organisms. *🗶 = no cross-reactivity ✓ = correct specificity*

|  |  |  |  |
| --- | --- | --- | --- |
|  | *RV1707*  (134bp) | IS6110  (135bp) | *IS1081*  (138bp) |
| M. tuberculosis complex |  |  |  |
| *M. tuberculosis (H37Rv)* | ✓ | ✓ | ✓ |
| *M. bovis (BCG)* | ✓ | ✓ | ✓ |
| *M. bovis* | ✓ | ✓ | ✓ |
| *M. africanum* | ✓ | ✓ | ✓ |
| *M. canetti* | ✓ | ✓ | ✓ |
| *M. microti* | ✓ | ✓ | ✓ |
| Slow-growing mycobacteria |  |  |  |
| *M. asiaticum* | Faint band | 🗶 | 🗶 |
| *M. avium subspecies avium (S4)* | 🗶 | 🗶 | 🗶 |
| *M. celatum* | 🗶 | ✓ | ✓ |
| *M. chimaera* | 🗶 | 🗶 | 🗶 |
| *M. gastri* | 🗶 | 🗶 | 🗶 |
| *M. gordonae* | 🗶 | 🗶 | 🗶 |
| *M. haemophilum* | 🗶 | 🗶 | 🗶 |
| *M. intracellulare (Type 7)* | 🗶 | 🗶 | 🗶 |
| *M. interjectum* | 🗶 | 🗶 | 🗶 |
| *M. kansasii* | 🗶 | 🗶 | 🗶 |
| *M. lentiflavum(Strain 1)* | 🗶 | 🗶 | 🗶 |
| *M. lentiflavum(Strain 2)* | 🗶 | 🗶 | 🗶 |
| *M. marinum* | 🗶 | 🗶 | 🗶 |
| *M. malmoense* | 🗶 | 🗶 | 🗶 |
| *M. scrofulaceum* | 🗶 | 🗶 | 🗶 |
| *M. shimoidei* | 🗶 | 🗶 | 🗶 |
| *M. simae* | 🗶 | 🗶 | 🗶 |
| *M. szulgai* | 🗶 | 🗶 | 🗶 |
| *M. terrae (Strain 1)* | 🗶 | 🗶 | 🗶 |
| *M. terrae (Strain 2)* | 🗶 | 🗶 | 🗶 |
| *M. ulcerans (strain 912)* | 🗶 | 🗶 | 🗶 |
| *M. xenopi* | 🗶 | 🗶 | 🗶 |
| Fast-growing mycobacteria |  |  |  |
| *M. abscessus* | 🗶 | 🗶 | 🗶 |
| *M. chelonae* | 🗶 | 🗶 | 🗶 |
| *M. flavescens* | 🗶 | 🗶 | 🗶 |
| *M. fortuitum* | 🗶 | 🗶 | 🗶 |
| *M. perigrinum* | 🗶 | 🗶 | 🗶 |
| *M. phlei* | 🗶 | Faint band | 🗶 |
| *M. porcinum* | 🗶 | 🗶 | 🗶 |
| *M. septicum* | 🗶 | 🗶 | 🗶 |
| *M. smegmatis MC2155* | 🗶 | 🗶 | 🗶 |
| *M. vaccae* | 🗶 | 🗶 | ✓ |
|  |  |  |  |
| Other bacteria potentially present in sputum: |  |  |  |
| *Acinetobacter baumannii* | 🗶 | 🗶 | 🗶 |
| *Citrobacter freundii* | 🗶 | 🗶 | 🗶 |
| *Corynebacterium striatum* | 🗶 | 🗶 | 🗶 |
| *Enterobacter cloacae* | 🗶 | 🗶 | 🗶 |
| *Escherichia coli* | 🗶 | 🗶 | 🗶 |
| *Hemophilus influenzae* | Faint band | ✓ | 🗶 |
| *Klebsiella pneumoniae* | 🗶 | 🗶 | 🗶 |
| *Moraxella catarrhalis* | 🗶 | 🗶 | 🗶 |
| *Pseudomonas aeruginosa* | 🗶 | 🗶 | 🗶 |
| *Staphylococcus aureus* | 🗶 | 🗶 | 🗶 |
| *Streptococcus agalactiae* | 🗶 | Faint band | 🗶 |
| *Streptococcus agalactiae( strain2)* | 🗶 | 🗶 | 🗶 |
| *Streptococcus mitis* | 🗶 | 🗶 | 🗶 |
| *Streptococcus pneumoniae* | 🗶 | 🗶 | 🗶 |
| *Streptococcus pyogenes* | 🗶 | 🗶 | 🗶 |

1ng, which is equivalent to 106 bacteria, was used as input DNA for the PCR and visual inspection for correct *Mtb*-specific amplicon size was used as the electrophoretic readout. It is important to note a more clinically relevant sample input would have, at most, 103 to 104 with mixed infections. qPCR was carried out on all 52 samples at both 1ng, and 0.01ng input DNA (results not shown here). The only strains that gave amplification at a clinically relevant sample input (0.01ng) were the TB complex strains. None of the other strains showed cross reactivity.