**Programmable digital microfluidic assay for simultaneous detection of multiple anti-microbial resistance genes**

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ABSTRACT

The rapid emergence of antimicrobial resistant bacteria requires the development of new diagnostic tests. Nucleic acid based assays determine antimicrobial susceptibility by detecting genes that encode for the resistance. In this study, we demonstrate rapid and simultaneous detection of three genes that confer resistance in bacteria to extended spectrum β-lactam and carbapenem antibiotics; CTX-M-15, KPC and NDM-1. The assay uses isothermal DNA amplification (Recombinase Polymerase Amplification, RPA) implemented on a programmable digital microfluidics (DMF) platform. Automated dispensing protocols are used to simultaneously manipulate 45 droplets of nL volume containing sample DNA, reagents and controls. The droplets are processed and mixed under electronic control on the DMF devices with positive amplification measured by fluorescence. The assay on these devices is significantly improved with a Time to Positivity (TTP) twice that of the benchtop assay.

**INTRODUCTION**

The emergence of antibiotic resistant bacteria is considered one of the greatest threats to human health as the infections are becoming increasingly common 1. The spread of resistance mechanisms has led to the continuous evolution of the β-lactamase enzymes against β-lactam antibiotics 2. The production of extended spectrum β-lactamases (ESBLs) in Gram-negative bacteria is an example of a spreading problem fuelled by exposure of bacteria to cephalosporin antibiotics. Amongst the class A enzymes, the most common ESBL are those of the CTX-M family 3. This gene has spread world-wide and is now found across at least 26 bacterial species 4,5. Of the CTX-M family, CTX-M-15, encoded by *bla*CTX-M-15, is currently the most prevalent within the UK, whilst CTX-M-14 is more common in other parts of the world. Given the widespread emergence of ESBLs, carbapenems have been considered as the last resort antibiotic, for the treatment of a range of multidrug resistant bacterial infections 6,7,8. However, carbapenem resistance is also emerging, dramatically limiting treatment options. There are several carbapenemase enzymes, including the *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo- β -lactamase (NDM). KPC is one of the most effective carbapenemases that can inactivate all basic β-lactams, cephalosporins and carbapenems, but is partially inhibited by inhibitors such as clavulanic acid and tazobactam 6. Spread of KPC has been driven in part by highly successful, multidrug-resistant strains of *K.pneumoniae*, although the gene is by no means limited to this pathogen. Conversely, NDM spread has been driven by the dissemination of plasmids, carrying the *bla*NDM-1 gene alongside a number of other antibiotic resistance markers 9. This dissemination is of concern for public health worldwide, given the occurrence of the gene in unrelated species and acquisition by *E.coli* which is one of the main community-acquired human pathogen. As carbapenems are the last resort for a number of multidrug resistant Gram-negative bacteria 7, a major cause of nosocomial and community bacterial infections, new methods are needed to maintain and extend the useful working life of this class of antibiotics. Current tests to determine whether bacteria are killed by antibiotics are based on standard culture of bacteria in the presence of an antibiotic. These indicate susceptibility to different antibiotic classes and can be used to direct therapy, but are slow and take up to 72 hours. Therefore, there is a clear need to develop rapid and sensitive tests to determine antibiotic resistance, both as part of the diagnosis and management of infection and to provide a rational basis for the appropriate prescription of antibiotics.

Nucleic acid (NA) analysis is a rapid and sensitive method that can directly identify the genes that confer resistance. It is particularly useful for slow growing or non-culturable microorganisms and also for the detection of point mutations 10. Polymerase chain reaction (PCR) is a well-known NA based amplification assay. It is generally performed in centralised labs with expert users and specialized equipment; this limits its utility for Point of Care diagnosis. In the absence of rapid results, treatment will often use broad spectrum antibiotics on an empirical basis, which may contribute to increased drug resistance. The emergence of multi-drug-resistant (MDR) strains of bacteria is increasing the need for rapid determination of antimicrobial susceptibility 11, and nearly 200,000 people die every year from MDR tuberculosis (TB) alone 12. Multiplexed PCR assays for simultaneous detection of resistance genes have been developed 13,14,15, but although PCR is the gold standard its use as a point-of-care diagnostic tools has been limited by the requirements for accurate temperature cycling. Therefore, isothermal DNA amplification methods that do not require thermal cycling are gaining popularity. One such technique is Recombinase Polymerase Amplification (RPA), which is a simple and robust method of amplifying DNA 16. It works with a wide range of sample matrices and requires only 2 primers. RPA uses recombinase enzymes for primer annealing and unwinding DNA. Real-time amplification of product can be monitored using a fluorescent readout using *exo* probes, a complimentary oligonucleotide that has a tetrahydrofuran (THF) moiety located between a fluorophore and a quencher. Exonuclease III in the reaction mix cleaves the THF once the probe has bound to the target sequence emitting fluorescence 17.

There is a growing literature describing the development of isothermal amplification techniques for nucleic acid based rapid diagnostics. However, there are very few reports of multiplexed detection of different target genes on the same device. Notable examples include Czilwik *et al* who developed a centrifugal microfluidic platform “LabDisk” and demonstrated multiplex PCR to identify four different microorganism (*Staphylococcus warneri*, *Streptococcus agalactiae*, *Escherichia coli* and *Haemophilus influenza*) in a small volume serum sample 18. They used nested PCR and amplified DNA from each organism from a 200L serum sample and 20L PCR reaction volume chamber. Choi *et al* also used a centrifugal platform to demonstrate a triplex isothermal RPA assay for identification of three different organisms in contaminated milk, using a 2.6L amplification volume 19. The LoD of the system was 4 cells. In an alternative approach, Kersting *et al* designed a simple chip capable of simultaneous multiplex isothermal amplification of DNA using RPA on a solid surface. Sample was continuously pumped across the surface of a 8.3L reaction chamber, and they were able to amplify specific target sequences from three pathogens *Neisseria gonorrhoeae*, *Salmonella enterica* and methicillin-resistant *Staphylococcus aureus* (MRSA) using genomic DNA 20. They demonstrated detection in 20 minutes and a LoD of extracted DNA from between 10 and 100 cfu. Crannell *et al* demonstrated a triplex assay using RPA to detect DNA from the protozoa *Giardia, Cryptosporidium,* and *Entamoeba* using a lateral flow readout with limits of detection in the hundreds of copies 21. Recently a simple microfluidic device was demonstrated by Renner *et al* that can identifies ESKAPE bacterial pathogens using RPA assays.22. They utilized a vacuum degassed PDMS cartridge with 16 reaction chambers that contained lyophilized RPA assays. A portable electronic reader and end-point fluorescence detection was used to identify the pathogen.

Digital microfluidic (DMF) technology provides an alternative method for performing fully programmable automated microfluidic assays using nL volume liquid droplets. DMF exploits the phenomenon of electrowetting-on-dielectric (EWOD) to move liquids 23. Droplets of liquid can be manipulated in two dimensions on the surface of an array of microelectrodes. Droplets with a wide range of volumes can be manipulated, and processes such as dispensing, splitting, mixing can all be accomplished using appropriate electrode patterns. Digital microfluidics has been used for many molecular assays 24,25 and since it requires very small volumes this often improves assay performance. Despite its flexibility, very few multiplexed assays have been demonstrated on DMF devices. Hua *et al* demonstrated a two-plex PCR by cycling droplets repeatedly in a loop between two zones of different temperature. They showed that DNA from *MRSA* and *Streptococcus pneumoniae* could be detected in real time with single copy sensitivity 26. More recently Prakash *et al* demonstrated a chip for the detection of Influenza A virus and Influenza B virus in clinical samples using RT-PCR 27.

Traditional “passive” DMF devices have a relatively small number of fixed hard-wired electrodes and the droplet manipulation operations (e.g. movement, shape) are limited to these electrodes patterned on the device. A new generation of DMF devices controlled by Thin Film Transistor (TFT) electronics has recently been developed 28,29. These Active Matrix AM-EWOD systems have many thousands of individually addressable electrodes enabling the simultaneous and independent manipulation of numerous droplets in 2-dimension. They provide a high level of flexibility in defining droplet size and shape. The AM-EWOD devices also incorporate electrical droplet sensing functions to monitor the position and size of droplets in almost real-time. These programmable DMF devices are therefore ideally suited for complex analytical processes providing a highly flexible platform capable of performing assays with nanolitre volume droplets.

Previously, we have demonstrated the enhancement in gene detection time and limit of detection as compared to benchtop RPA assays on the AM-EWOD platform29. In this paper, we further exploit the programmable capability of the active matrix DMF technology to amplify DNA extracted from antibiotic resistant bacteria in a triplex assay. All procedures for aliquoting, dispensing and mixing samples were performed on the device. Specifically, the triplex assay identifies and quantifies the three key genes responsible for antimicrobial resistance in gram-negative microorganisms in a mixed sample droplet in a short time window. These genes are NDM-1, KPC and CTX-M-15 that encode for carbapenemases and an ESBL respectively. The genes were identified using isothermal amplification methods and a fluorescent readout.

**METHODS**

**1. Digital Microfluidic (DMF) Device**

The TFT DMF device is shown in Fig 1A and has been described in detail elsewhere 28,29. It comprises an array of 96 x 175 individually addressable circuit elements or pixels, each of 200 μm x 200 μm. Adjacent electrodes are separated by a gap of 10 μm giving a total active area of the array of 7.37 cm2. Immediately adjacent to the high-resolution array are nine fluid input structures with reservoir electrodes that define a path for dispensing fluid onto the main array. The electrodes for the input are formed in the same ITO layer as the main array and are controlled by the TFT circuitry in the same way as the main array and consists of 7 electrodes with a total size of 3 mm x 9 mm. Each pixel also contains a sensor for measuring the capacitance of the droplet, as described previously 28. An image from the array of sensors shows the size and position of droplets on the array. Image processing techniques have been developed to measure the size of the droplets from the output sensor image, as described in 28. The actuation pattern can be re-written 50 times per second and a sensor image obtained 30 times per second. The sensor thus facilitates real time feedback of droplet size and position. A temperature sensor is also integrated onto the TFT substrate to regulate the temperature of the built in heater when used for DNA amplification chemistry.

**Control electronics and software**

A Printed Circuit Board (PCB) supplies the voltage and timing signals to drive the TFT electronics. Control firmware (VHDL) and application software (C#) were custom designed for automated control of droplets. A set of droplet operations including move, merge, split, dispense and mix is implemented by software through a Graphical User Interface. Droplet operations are pre-programmed by the user and implemented with real time feedback from the sensor. Droplets can also be manipulated through ‘click and drag’ operations on the sensor image. The complete DMF device consists of the TFT backplane and a top ITO plate, both of which are coated with Cytop. The two glass substrates are separated by a spacer, typically 125 μm. The space between the two plates (top and bottom) is filled with dodecane.

The thin film electronics layers up to and including ITO electrodes were fabricated using the Sharp CG Silicon TFT manufacturing process. An insulation layer of Al2O3 (300 nm) was deposited by Atomic Layer Deposition (ALD) over the electronics and a hydrophobic top layer formed by spin coating a thin layer (approximately 80 nm) of Cytop (Asahi Glass, Japan). This cytop coating was stripped and replaced for each new DNA assay.

**Bacterial culture and plasmid extraction**

Plasmids were extracted from an overnight culture of control strains based on *E.coli* Top10 using QIAprep Spin Miniprep Kit (Qiagen, UK) using the manufacturer’s protocol. The bacteria strains contains the plasmid pACYC184 carrying the ESBL or carbapenemase genes together with 300 to 500 bp upstream DNA, on a BamHI and XbaI fragment. The eluted plasmid was quantified on a Qubit® fluorometer using assay reagent.

**Benchtop RPA assay**

Primer sets within the *blaCTX-M-15*, *blaKPC* and *blaNDM-1*genewere designed and tested in multiple combinations. The sequences for the primers and probes used in the study are available on request from Public Health England. The commercial TwistAmp® Exo kit (TwsitDx, UK) was used for real time RPA assays, following the manufacturer’s instructions. Lyophilised RPA proteins were reconstituted with rehydration solution, forward and reverse primers, a fluorescent exo-probe and DNA samples containing plasmids with CTX-M-15, NDM-1 or KPC gene. In each 50 μL reaction, the final concentrations of primers was 0.42 μM and CY5 or Quasar 670® labelled probe was 0.12 μM, respectively. A 5 μl aliquot of the plasmid extracted from bacteria was added to this mix. Each RPA reaction mix was transferred to the well of a black polystyrene 96-well plate. Amplification was initiated by adding magnesium acetate to a final concentration of 14 mM and mixing vigorously. The plate was transferred to a GloMax microplate reader (Promega, UK) set to 39 °C and the fluorescence measured at 1 min intervals for 30 minutes.

**DMF RPA assay**

For real-time RPA assay on the DMF platform master droplets of reagents and sample (each with approximate volumes of 2 μL) were loaded directly onto the reservoir electrodes (Fig 1B). Tween® 20 (molecular biology grade, Sigma Aldrich, UK) was added to all the reagents to a final concentration of 0.1% v/v to reduce the surface tension of the droplets. As shown in the figure, the first three reservoir electrodes were loaded with three different RPA reaction mixes, with primers and probes specific to CTX-M-15, NDM-1 and KPC gene. The other reservoir electrodes were loaded with sample (plasmid), nuclease free water (no template control, NTC) and magnesium acetate. The top substrate electrode was then clamped onto the TFT backplane, creating a sandwich structure with a well-defined cell gap (125 ± 1 μm) between the two glass substrates. The cell gap was filled with dodecane while the reservoir electrodes are actuated to pin down the droplets. Pre-programmed electrode actuation sequences were used to perform the assay. The sequence was designed so that droplets do not cross over each other during manipulation, thus circumventing any issues of cross contamination between reaction droplets. The protocol was as follows. First the required number and volume of droplets were dispensed from the input reservoirs. The volume ratios are set to 4:1:1 for the RPA reaction mix, sample and magnesium acetate, respectively. The RPA reaction mix (RPA proteins, rehydration solution, primers and probe specific to a given gene) were prepared so that the final component concentrations in the reaction droplet were identical to those in the 50 μL benchtop assay.

The number of activated pixels on the array defines the size of each daughter droplet. The daughter droplets (5 for each RPA reaction mix, 15 magnesium acetate, 9 DNA droplets (triplicate) and 6 NTC droplets) were dispensed from the reservoir electrodes with the following volumes: RPA reaction mix = 180 nL (6 × 6 elements); DNA, NTC and magnesium acetate = 45 nL (3 × 3 elements). After dispensing, the daughter droplets were moved to pre-determined regions on the array under direct software control (Fig 1B). Reagent and sample droplets were mixed using a programmed mixing sequence, which repeatedly shuttles the droplets back and forth to shorten the mixing time 29. The video (see ESI) shows the sequence of droplet dispensing and mixing, speeded up 10X. The dispensing of RPA reagents (green) was done at half the speed of the Magnesium Acetate (red) because the RPA is much more viscous. During mixing, the droplets had an approximately rectangular shape. The final reaction droplet volume was 270 nL. The RPA reaction droplets were first mixed with the DNA (or NTC) then with magnesium acetate (Fig 1B). The array is then heated to 39 °C to initiate the RPA reaction.

**Optical system and data analysis**

A custom wide-field fluorescence detection system was made to image the entire device as shown in Fig 2. Light from an LED is directed on the chip by a focussing lens (100 mm, f2.8) and a band pass filter (590-650 nm, Semrock, UK) to obtain the required wavelength for fluorophore excitation. The fluorescence emitted by the droplets was imaged by DSLR camera (Canon 5D, Mark III), fitted with a macro lens (55 mm focal length) and an emission filter (670-740 nm, Semrock, UK). The droplets were exposed for 3 seconds and fluorescence recorded for a 2.5 second window during the exposure. An image was obtained every 30 seconds over 30 minutes using a custom software that controls the camera and light source.

A custom MATLAB script was used to process and analyse the images to obtain fluorescence plots. First the positions of the droplets were selected manually before the images are batch processed. Droplet intensity data was recorded by taking the mean value of all pixels within the selected droplet region. The non-uniform light distribution produced by the source over the device area was determined individually for each image. This non-uniform distribution was calculated by multiplying the column vector of median values for each row in the image by the row vector of median values for each column in the image. Median values were used to avoid a significant impact on the background approximation from the droplets. The resulting image was then normalised. The original image was then divided by the approximated background to remove the non-uniformity. Fig S1 and S2 shows an example of filtered and unfiltered image of fluorescent dye droplets on EWOD.

**Results and discussion**

The sensitivity of the RPA assay for detection of the genes encoding CTX-M-15, NDM-1 and KPC was first assessed using the bench-top assay. A serial dilution of the three different plasmids was performed, with water as a negative control. Amplification curves (in triplicate) are shown in Fig S3. The assay was quantified through a measurement of the Time to Positivity (TTP), defined as the time at which the fluorescence signal crosses 3 times the standard deviation of the negative controls. As expected, the TTP increased with decreasing plasmid concentration and a plot of TTP against logarithm of DNA concentration is shown in Fig S2 D, where a log-linear relationship is seen, demonstrating the quantitative capability of the assay. As shown in the figure, all three genes can be detected in a similar time window. The benchtop assay can detect as few as 10 copies of CTX-M-15, NDM-1 and KPC within 25 minutes.

The specificity of the primers and probes for a given gene was also evaluated by amplifying samples without the specific genes present. Fig 3A shows amplification curves (in duplicates) for three different samples (CTX-M-15, NDM-1 or KPC) in a reaction mixture containing primers and probes for only the CTX-M-15 gene. No amplification was observed for NDM-1 and KPC, while the fluorescence intensity from the sample containing plasmid with CTX-M-15 increased exponentially after a lag phase. Similarly, Fig 3B and C demonstrates the high specificity of the assay to the genes encoding for NDM-1 and KPC. Time to positivity (TTP) for a sample containing 1,000 plasmid copies of CTX-M-15, NDM-1 and KPC was 13 ± 0.5, 16.7 ± 0.1 and 17.6 ± 0.8 minutes respectively.

Using the droplet dispensing sequence outlined in Fig 1, the three different genes were also amplified and detected using RPA on the DMF platform. Fig 4A shows three images of the AM-EWOD, showing fluorescence from the droplets for the triple gene assay at t = 0, t = 10 and t = 30 minutes (after completion). There are five reaction droplets for each assay, corresponding to sample (in triplicate) and NTC (in duplicate), totalling 15 droplets representing 15 independent reactions. Each final droplet volume is approximately 230nL.

As shown by the end-point fluorescence image, no DNA contamination occurred during droplet manipulation, as evidenced by the consistent lack of amplification in the no template control (NTC) reactions. The example reaction shown in Fig 4A used droplets with plasmids and genes encoding for NDM-1 and KPC, thus the reaction showed positive for those genes and negative for CTX-M-15. Fig 4B shows the amplification curves for the droplet reactions in Fig 4A obtained from the fluorescent images. The TTP for NDM-1 and KPC for 1,000 copies is 7.3 ± 0.3 and 7.4 ± 0.3 mins, respectively, which is twice as fast as the bench top assay consistent with previous observations 28.

Finally Fig 5 shows a series of images for samples with different combination of the three plasmids at 1,000 copies per droplet. The images consistently indicate that no amplification occurs in the droplet with the missing gene demonstrating the excellent specificity of the assay. The amplification curves for the reactions extracted from the fluorescence intensity data can be seen in Fig S4 and S5.

**Conclusions**

In this work, we have demonstrated an automated multiple gene detection system using digital microfluidics AM-EWOD devices with isothermal RPA DNA amplification. The programmable device was used to implement a triplex assay to amplify the three of the most prominent genes conferring resistance to cephalosporins and carbapenems in Gram-negative bacteria. Sample DNA, RPA reagents (including gene-specific primers and probes), magnesium acetate and NTC were all dispensed from the reservoir droplets. For each assay, 5 reaction droplets (3 positive and 2 NTC) per gene were generated and measured by mixing the droplets dispensed from the reservoirs. This entire sequence was done in parallel for all 15 reaction droplets. The assay is specific and sensitive, and can detect all genes with a LoD of 10 copies in approximately 25 minutes on benchtop. The assays on device are twice as fast as the same assay on benchtop demonstrating increased speed and sensitivity, as required for PoC diagnostics. Future development of an integrated sample preparation unit will lead to a complete system that could provide a rapid and PoC DNA detection system for multidrug resistant pathogens.

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