DIRECT DETECTION OF PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN BLOODSTREAM INFECTION BY PCR USING WIRE-GUIDED DROPLET MANIPULATION (WDM)

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ABSTRACT

Bloodstream infection (BSI), an expensive and deadly condition, burdens healthcare. Improved BSI treatment relies on rapid, point-of-care diagnosis of causative species and presence of plasmid-mediated drug resistance. We have designed, fabricated and assembled an apparatus for automated PCR amplification by wire-guided droplet manipulation (WDM). WDM minimizes thermal resistance and exploits convective heat transfer to increase reaction speeds. Molecular partitioning within the water-in-oil droplet enables PCR amplification with whole blood (WB) *in situ*. The V3 region of the 16s rRNA gene and a representative 4.884 kb plasmid were amplified by WDM from 10% WB. Optics has been designed for real-time detection.

KEYWORDS: PCR, bloodstream infection, 16s rRNA, molecular partitioning

INTRODUCTION

Bloodstream infection (BSI) affects 750,000 people/year, with 20-50% mortality [1] and $18,500 per hospital stay in the United States [2]. Delayed diagnosis creates the need for empirical, broad-spectrum antibiotic therapy, which may be unnecessarily broad or inadequately narrow [3] and creates a selective pressure towards development of antibiotic resistance [4]. Species identification from infected blood provides predictable information about chromosomally linked antibiotic resistance genes, but plasmid-mediated resistance can be unpredictable because plasmids can transfer between species [5]. Dangerous and increasingly common drug resistance genes are carried on horizontally transferable plasmids, such as *Klebsiella pneumoniae* carbapenemase (KPC) and β-lactamases [6]. Current laboratory practices may incorrectly diagnose the susceptibility of pathogens producing plasmid-mediated KPC, leading to inadequate patient isolation and increased morbidity. In response to the recent emergence of plasmid-mediated, broad-spectrum antibiotic resistance [7], rapid and point-of-care diagnostics must be developed by modification of molecular techniques.

Towards the aim of more complete diagnosis of BSI, including species and antibiotic resistance gene identification, we have designed, fabricated and assembled an apparatus for automated PCR amplification by wire-guided droplet manipulation (WDM) (Fig. 1). WDM offers unique characteristics that are highly desirable for rapid, point-of-care diagnosis in the hospital setting. Reaction speeds are greatly increased by continuous movement of the reaction droplet in direct contact with heated oil thereby minimizing thermal resistance and exploiting convective heat transfer. Molecular partitioning of nucleic acids and other PCR reagents from blood components, within the water-in-oil droplet, enable the PCR reaction with whole blood (WB) *in situ*. WDM is automated, reprogrammable and can be used to conduct a wide range of molecular biology techniques [8].

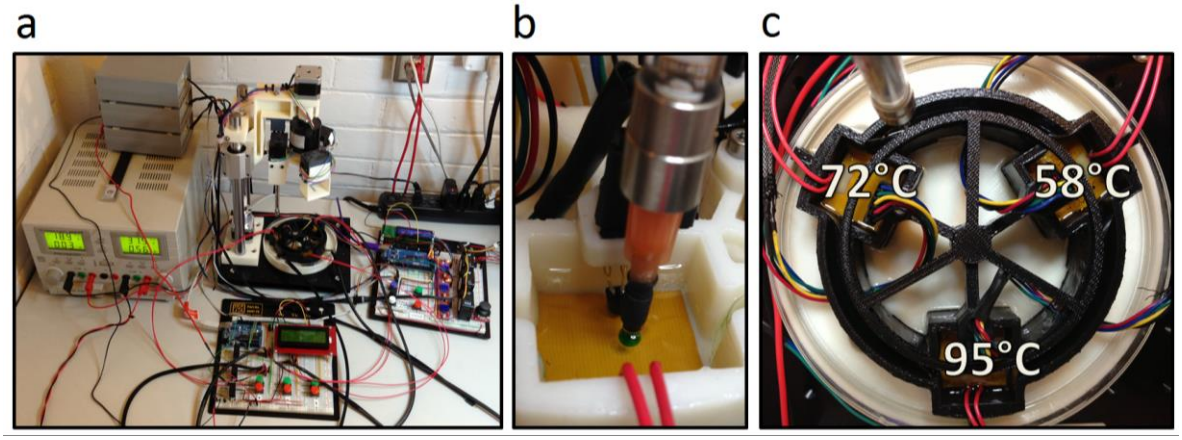

Figure 1: (a) The entire system includes a dual output power supply, mechanical robotic apparatus for syringe movement and droplet dispensing, heated oil baths, and electronic circuitry interfacing with reprogrammable microcontrollers. (b) Heat transfer by convection between the reaction droplet hanging from the syringe needle and the heated oil bath. (c) The reaction chamber consists of three independently heated oil baths at 95°C (denaturation), 50-60°C (annealing), and 72°C (extension).

EXPERIMENTAL

The WDM thermocycling apparatus, shown in Fig. 2a, contains a robotic positioning system to vertically translate and rotate a syringe. The syringe plunger is controlled by a linear actuator, which automatically dispenses a 5-10 μL droplet from a 15-guage blunt needle. During thermocycling, the droplet is serially dispensed into the three

independently regulated heated oil baths, which are maintained at one of the temperatures required for the phases of PCR—denaturation, annealing and extension (Fig. 1c). These temperatures are maintained within ±1°C of the set point and are monitored on a serial-enabled liquid crystal display (LCD). The entire system is powered by 20 V (for motors) or 45 V (for heaters), 2-3 A bench-top power supplies. The droplet hangs as a pendant drop (Fig. 1b) and is drawn into the needle between reaction phases, while the syringe is moving between oil baths through an oil-filled channel. Convective heating is ensured by continuously moving the droplet in the oil at a speed of 7.2 mm/s. The oil baths were set to 95°C, 52°C and 70°C for the amplification of V3 region of the 16s rRNA gene. The droplet spent 6.9 s, 10.5 s, and 18.3 s in the denaturation, annealing and extension baths, respectively. The PCR product was analyzed by gel electrophoresis to confirm the product length, and negative template control (NTC) reactions were conducted to ensure the integrity of the reaction.

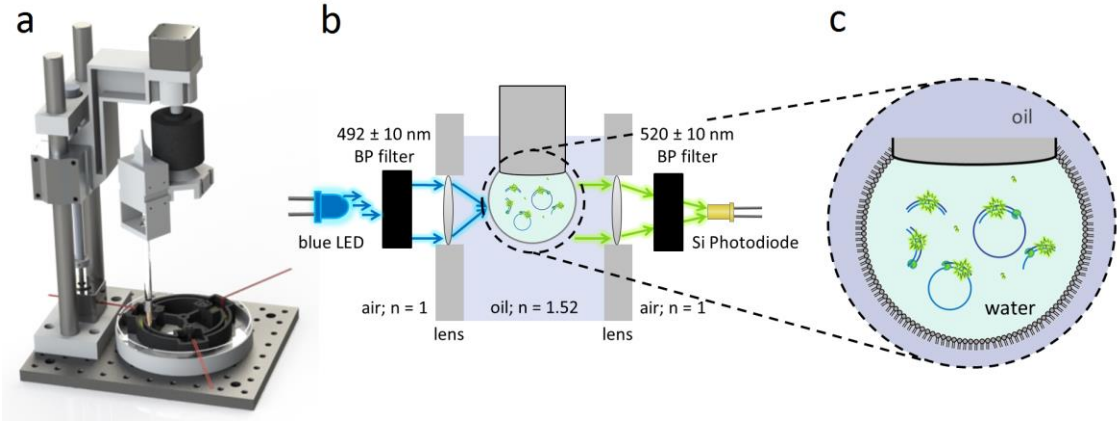

Figure 2: (a) CAD design of WDM thermocycling device. (b) Optical design for detection of fluorescent reporter excited by blue LED. (c) Molecular schematic illustrating the reactions within a droplet, where the fluorescent reporters bind to dsDNA amplicons from plasmid and the phospholipids from WB self-assemble at the oil-water interface.

RESULTS AND DISCUSSION

Amplification of the GAPDH gene with 10% whole blood (WB) was demonstrated at a speed of 8.67 s/cycle. The V3 hypervariable region of the 16s rRNA gene, which is commonly used for phylogenetic placement of clinically relevant bacteria [9], was amplified from purified *K. pneumoniae* genomic DNA (1 ng/µL) in under 20 minutes with 10% WB *in situ* in the sample, or 1% in the final PCR reaction volume (Fig. 3b). For pure genomic DNA, WDM performed inferior to conventional thermocycling. The addition of WB to the reaction mixture, acted as an inhibitor to conventional thermocycling, whereas WB increased the DNA yield by WDM thermocycling as evidenced by the increased band intensity seen in Fig. 3b. We created a representative plasmid by cloning a gene of interest into the pCR®-XL-TOPO® vector, yielding a 4.884 kb plasmid. We successfully and reproducibly amplified our gene of interest from the plasmid using WDM thermocycling with 10% WB *in situ* in the sample (or 1% in the final reaction volume). Conventional PCR failed to amplify the gene from the plasmid with WB *in situ*, while WDM PCR succeeded in amplifying due to molecular partitioning at the oil-water interface.

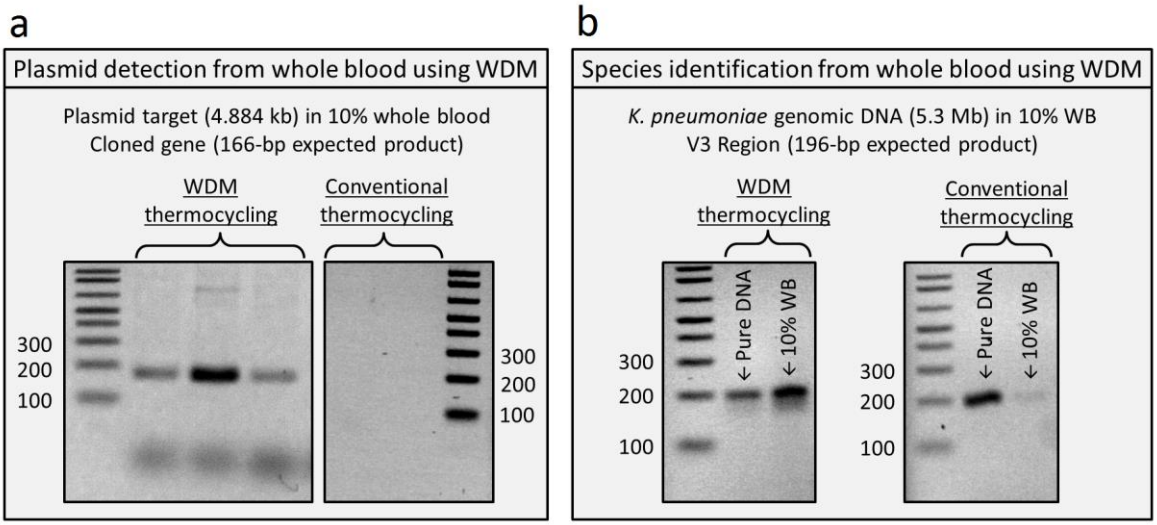

Figure 3: (a) Cloned gene amplified from plasmid target directly from WB. (b) 16s rRNA gene V3 hypervariable region amplified from K. pneumoniae genomic DNA directly from WB.

CONCLUSION

PCR by WDM has been shown to capably amplify our gene of interest from the 5.3 Mb *K. pneumoniae* genome as well as a 4.884 kb plasmid with whole blood *in situ*. This superior performance is owed to the unique convective heat transfer between heated oil and reaction droplet and molecular partitioning at the oil-water interface. WDM PCR can potentially be used to diagnose the species responsible for a patient's BSI but also to provide information about plasmid-mediated drug resistance, which is not exclusive to a single bacterial species.

Optics for the detection of real-time PCR chemistry has been designed. A blue LED excites at 492 nm and a photodiode or a digital camera detects the fluorescence emission at 520 nm. Lenses focus the light and bandpass filters remove unwanted light from the excitation and emission spectra. With the implementation of this optical design, our WDM thermocycling apparatus can be used to identify bacterial species as well as plasmid-mediated antibiotic resistance genes in real-time. The next clinically relevant step is amplification from a blood culture—bacteria growing in WB.

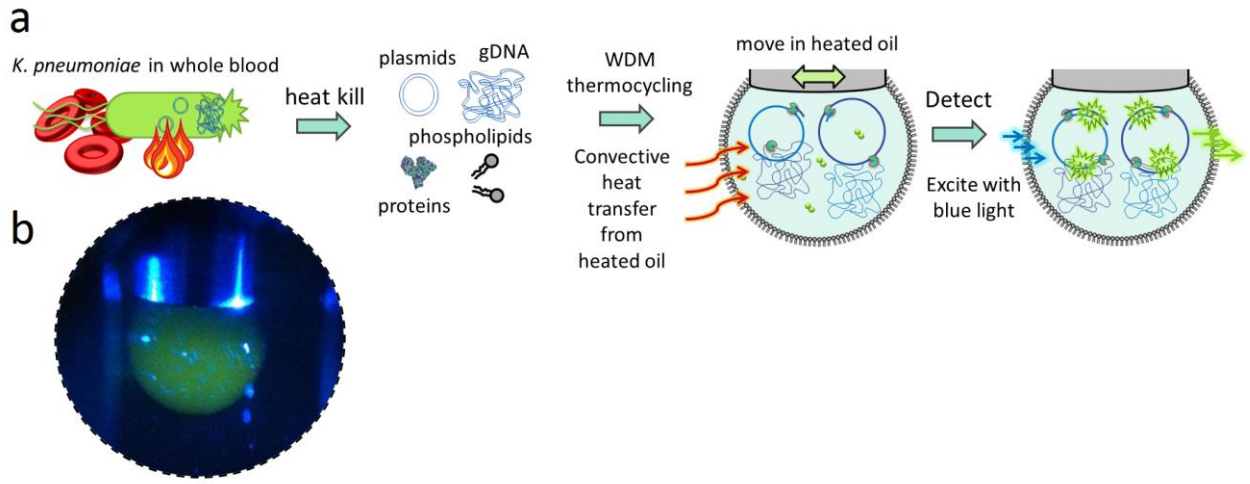

Figure 4: (a) Species identification and plasmid detection of bacteria in WB. (b) Picture of a droplet with DNA and SYBR green dye excited by blue LED.

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