A DISPOSABLE DNA AMPLIFICATION PLATFORM FOR THE DETECTION OF *CLOSTRIDIUM DIFFICILE* INFECTED STOOL SPECIMENS

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ABSTRACT

We present a disposable DNA amplification platform for helicase-dependent isothermal amplification (HDA) designed for the detection of *Clostridium difficil*e in human stool specimens. This platform consists of a low-cost, thermoplastic reaction chip, a pair of commercially available toe warmers commonly used by skiers as a chemical heater, and styrofoam cups as insulators. The platform is capable of maintaining the optimum temperature for HDA at 65 °C ± 2 °C for more than 55 min. When compared to reactions performed using thermocycler, this system has a comparable DNA detection limit ($1.25x10^{-2}$ pg) using cheap, readily available materials with no need for electricity.

KEYWORDS: Helicase-dependent amplification (HDA), Disposable, *Clostridium difficile*, Stool, Thermoplastic

INTRODUCTION

Each year, over 9.5 million deaths are caused by infectious diseases, nearly all occurring in developing nations [1]. *C. difficile*-associated diarrhea (CDAD) is the most common nosocomial infectious diarrhea in the developed world and was chosen as a test organism for this study. In resource-poor countries where electricity, financial support, and skilled workforces are inadequate, there is a growing interest in the development of appropriate, easy-to-adapt diagnostic technologies that can rapidly and accurately identify pathogens. Here we describe a disposable DNA amplification platform capable of conducting an isothermal HDA assay on human stool specimens infected with *C. difficil*e, thereby eliminating the need for complicated thermocycling. In combination with emerging readout technologies [2, 3], the system could greatly impact the accessibility of molecular assays for application in resource-poor settings.

THEORY

As shown in Fig.1, the system consists of a low-cost, thermoplastic microfluidic chip with three reaction chambers, a pair of toe warmers as an isothermal heat source for the HDA reaction, and two Styrofoam cups as a passive temperature control system. The toe warmer consists of a polypropylene bag containing iron powder that oxidizes in an exothermic reaction and produces heat when exposed to oxygen in the air. Therefore, by controlling air supply in the Styrofoam cups, the temperature as well as the duration of the reaction can be manipulated. In our experiment, temperature control during a given experiment was governed by the number of 1 mm diameter vent holes, punched on opposite sides of a Styrofoam cup. To initiate the HDA reaction process, first two toe warmers were activated by the removal of their protective films. Next, the microfluidic chip, preloaded with the reaction mixture, was sandwiched between the warmers, and the entire assembly was placed inside the cup. Lastly, the Styrofoam cup was capped with another cup to complete the isothermal chamber. The intra-cup temperature was monitored using a thermocouple attached to the microfluidic reaction chamber. As shown in Fig.1 (c), 55 vent holes resulted in a near steady-state temperature of 65 ˚C, the optimum temperature of the HDA reaction that was maintained for longer than 55 min.

EXPERIMENTAL

The microfluidic chip was constructed from six layers of cyclic olefin polymer (COP) film. The reaction chamber (15 × 2.5 × 0.75 mm) is able to hold an HDA reaction mixture with a volume of 25 µl. In this work, a tissue culture cytotoxicity assay was used as the gold standard assay. A QIAamp DNA stool mini kit was used to extract stool genomic DNA following the manufacturer's protocol. In this paper, 5 *C. difficile* positive (toxin A) and 3 negative were tested in chip to demonstrate the efficiency of our disposable DNA amplification platform. An HDA reaction was set up using an IsoAmp III Universal tHDA kit from Biohelix (Beverley, MA) in order to determine if the patient samples positive by the gold standard cytotoxicity test would also test positive by HDA. *C. difficile* genomic DNA from strain 630 (ATCC, Manassas, VA) was used as a positive control. The primers used to amplify a 110 bp product were: *C. difficile tcdA* forward primer (SpeI) 5'-A CTA TAC TAG TGA TGT TGA TAT GCT TCC AGG TAT TCA C-3' and reverse primer (AatII) 5'-GAA TGA CGT CTA TCA TTT CCC AAC GGT CTA GTC CAA T-3'. The primers contain SpeI and AatII restriction enzyme sites to allow HDA amplicons

to be gel isolated, cloned and sequenced to verify their identity. In brief, 2.5 µl of DNA template was added to a 25 µl reaction mix containing 1X annealing buffer II, 40 mM NaCl, 4 mM $MgSO_4$, 1.75 mM of IsoAmp® dNTP, 0.2 µM *C. difficile tcdA* forward primer, 0.1 µM reverse primer, and 12.75 µl nuclease free water. To determine the limit of detection for *C. difficile* genomic DNA using our HDA assay, we spiked known concentrations of *C. difficile* genomic DNA into DNA extracts from *C. difficile* negative stool and performed HDA reactions. Each 25 µl reaction contained 5 µl of DNA. Half of that (2.5 µl) was human specific DNA extract from negative stool extracted samples with an average concentration of 50 ng/µl. The other half contained titrations of ATCC genomic *C. difficile* specific DNA at known stock concentrations (125 pg to 2.5×10^{-3} pg). The master mix was then split to perform both on-chip and in-tube HDA reactions to determine the detection limit of each method. The in-tube HDA reactions were performed in triplicate using an ABI 2720 at 65 °C for 30 min. The on-chip HDA reaction was performed using our disposable, toe warmer-styrofoam cup temperature controller at 65 °C for 30 min. All experiments were repeated 2-3 times to confirm the results.

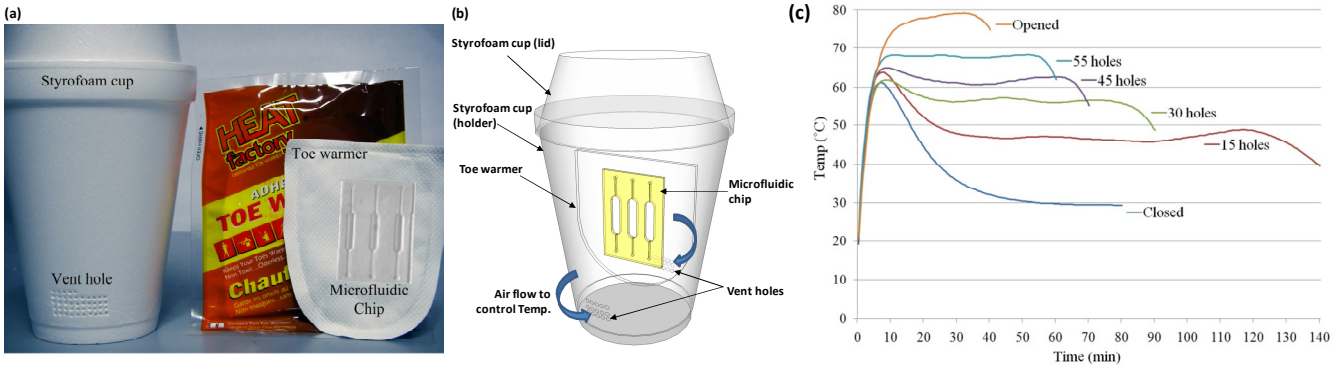

Fig. 1 *(a) Toe warmer and styrofoam cup as DNA amplification platform. (b) The number of vent holes on the sides of styrofoam cup controls the direct air supply that controls the temperature of the reaction chamber. (c) Temperature stability inside the Styrofoam cups with 15, 30, 45, and 55 holes punched on both sides (Each hole is 1 mm in diameter).*

RESULTS AND DISCUSSION

As shown in Fig. 2(a), polyacrylamide gel analysis confirms that the toe warmer-Styrofoam cup platform was effective at amplifying the appropriate *tcdA* products from both stool DNA samples (#1, #2, #3, #4 and #5) and commercial genomic *C. difficile* DNA, but no product was generated in the negative control (nuclease free water) reaction. In addition, as shown in Fig. 2(b), none of the 3 cytotoxicity negative stool samples (#6, #7 & #8) amplified a product. The HDA on-chip reaction results were consistent in each case with the corresponding reaction in-tube (Fig. 2(c)) and cytotoxicity assay. Both the on-chip and in-tube reaction generated a primer-dimer product of 76 to 90 bp (Fig. 2). It has been found that the HDA reaction often exhibits non-specific amplification, which can sometimes lead to a long non-specific product that can be confused with target products [4, 5].

For each concentration of *C. difficile* genomic DNA, the HDA reaction was repeated at least 6 times to compare the limit of detection of the on-chip and in-tube HDA reactions (Fig. 3). We found that at high *C. difficile* DNA concentrations (above 0.125 pg), the limit of detection for on-chip HDA was comparable to in-tube HDA reactions. However, as the amount of *C. difficile* DNA decreased to 2.5×10^{-2} pg, the in-tube *vs.* on-chip results diverged. In the tube reactions, below 2.5×10^{-2} pg, not all of the replicates resulted in amplified product. For the chip-based reactions, this lower limit was 1.25×10^{-2} pg. Neither the in-tube or on-chip HDA reaction could detect *C. difficile* genomic DNA below 10^{-3} pg/µl (amount in reaction is 2.5×10^{-3} pg). From the gel result of on-chip amplification (Fig. 3), we found that a higher amount of *C. difficile* in the reaction (shown in chip 4 & 5 in Fig. 3) didn't always show darker bands than those contained a smaller amount (shown in chip 6 in Fig. 3). It might be because there are variations in temperature control, pipetting and sample collecting. Thus, the disposable DNA amplification platform performed similarly to the standard tube reactions in this case.

CONCLUSIONS

We developed a platform composed of a microfluidic chip, toe warmers and Styrofoam cups to perform isothermal DNA amplification without electrical power. By punching various numbers of holes in the cup, the airflow was controlled so that the desired temperature could be maintained. We performed isothermal amplification of DNA extracted from patient stool samples, and specific identification of *C. difficile* was achieved based on HDA for toxinA. The results were consistent with the gold standard cytotoxicity assays for *C. difficile* in stool. We also demonstrated that our disposable platform functions comparably to a standard method using a thermocycler for temperature control with similar detection limits for *C. difficile* DNA in patient samples. This work is a step toward the fabrication of an inexpensive, handheld, point-of-service disposable

diagnostic assay for infectious diarrhea. Continuing work includes integration of on-chip stool extraction and readout to make a completely handheld device.

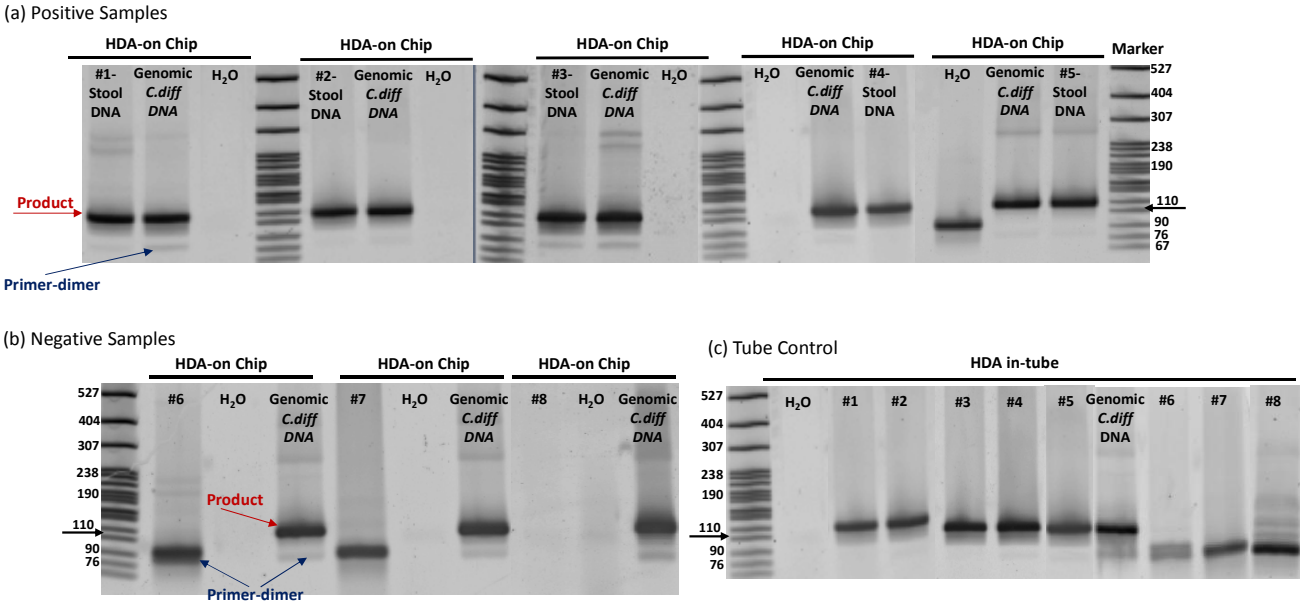

Fig.2 *Gel electrophoresis analysis of the HDA on-chip amplicons using 12% polyacrylamide gel with MspI digested pBR322 as marker: (a) five positive human stool DNA samples that are affected by C. difficile; (b) 3 negative human stool DNA samples that are not affected by C. difficile; (c) HDA in-tube as a control.*

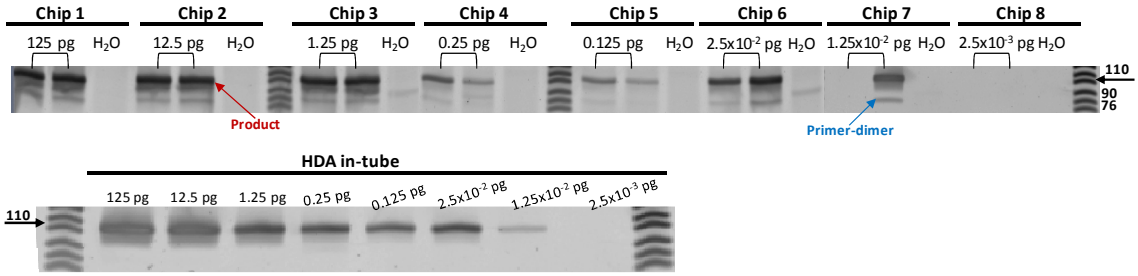

Fig.3 *Sensitivity of HDA on-chip vs. in-tube reaction: HDA mixture with series amounts (125 pg – 2.5×10^{-3} pg) of C. difficile purified genomic DNA as the template. Then HDA was performed both on-chip (via toe warmers and Styrofoam cups as the heater) and in-tube (via thermocycler). The product yields were compared using polyacrylamide gel.*

ACKNOWLEDGEMENTS

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