Integrated Lab-on-a-Chip Influenza Diagnostic Designed for Low Cost Manufacturing

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ABSTRACT

In this paper we describe a completely integrated lab-on-a-chip for genetic analysis of bacteria or viruses. The chip was designed for low cost manufacturing in thermoplastics by choice of low cost materials (thermoplastics) and designing for injection molding and for minimal assembly. To reduce the complexity of the disposable chip, all active components were removed from the microfluidic chip and incorporated into an instrument. We present the integrated chip and associated instrument for detection of bacteria and viruses. In particular, here we demonstrate the on-chip RT-PCR and detection using the CDC proprietary assay for influenza A.

Keywords: lab-on-a-chip, microfluidics, influenza, infectious disease diagnostics

1 INTRODUCTION

The field of infectious disease diagnostics has traditionally relied upon culture for identification of pathogens, which is a time-intensive process. Thus, clinicians typically make drug therapy choices based on clinical presentation, but in the absence of pathogen identification. Advances in clinical diagnostics have enabled some truly rapid tests (e.g. dipstick immunoassays), but these tests typically lack the sensitivity required for most disease states. The field of clinical diagnostics has been increasingly relying on molecular techniques, but traditional PCR requires time-intensive sample preparation by skilled technicians and dedicated laboratory space. As such, molecular diagnostic tests are typically batched in the clinical laboratory and the effective turn-around-time is 12-24 hours. In cases of suspected pathogens that are easily spread (and thus require additional isolation precautions) or that are life-threatening, and when identification of the pathogen informs drug therapy choices, a point-of-care diagnostic would greatly improve the standard of care. Influenza is an example of such a pathogen, highlighted by the H1N1 influenza pandemic. The outbreak has overwhelmed laboratories, particularly because of the required use of PCR-based testing to differentiate the sub-type.

In a normal year, the human population is infected by both influenza A and influenza B viruses. In the United States, this usually results in approximately 36,000 deaths each year, with the largest numbers of casualties among people over 65 years of age and very young children [1]. The type and number of infections in any given year are used to formulate the vaccine for the following year, so it is possible that an emerging strain would not be "covered" by the widely distributed vaccine, as was the case for the H1N1 swine flu. Moreover, the highest risk individuals (neonates and immuno-compromised individuals) are not eligible for the vaccine as it is a live vaccine. The incubation period for influenza in an adult is about two days, and an infected person is contagious about one day before the onset of symptoms. Complications of the flu are common and include pneumonias resulting from the primary infection and secondary bacterial infections. The infected individual sheds virus for several days; infected children are contagious for up to 10 days. Immune compromised individuals can shed virus for many weeks. Influenza has been associated with encephalopathy, transverse myelitis, Reye syndrome, myositis, myocarditis, and pericarditis.

When a child comes into the emergency department in respiratory distress, s/he must be placed in isolation until confirmed as to not having a highly infectious respiratory infection. Typically, the physician will order lab work to diagnose if the child has RSV, influenza A or B, or parainfluenza 1, 2, or 3 (Figure 1). Depending on the outcome of the lab work, the child will be prescribed different drug therapies. If the child is diagnosed with influenza within the first 48 hours of onset of symptoms, neuraminidase inhibitors, such as oseltamivir (Tamiflu), will be prescribed as it has been shown to lessen the course of disease by 1.5 days, but only if administered in that initial time window [2].

To address these challenges and provide a truly rapid test that meets the demands of high sensitivity and

specificity, many have proposed to develop a point-of-care molecular diagnostic that automates the sample preparation, nucleic acid amplification, and detection in a low cost miniaturized lab-on-a-chip format. The benefits of this format include reduced costs (less technician time, no need for specialized facilities, less reagent use), improved reproducibility, and fast results. However, much of the work in this field has not yet resulted in a completely integrated lab-on-a-chip or a design that is truly low cost to manufacture.

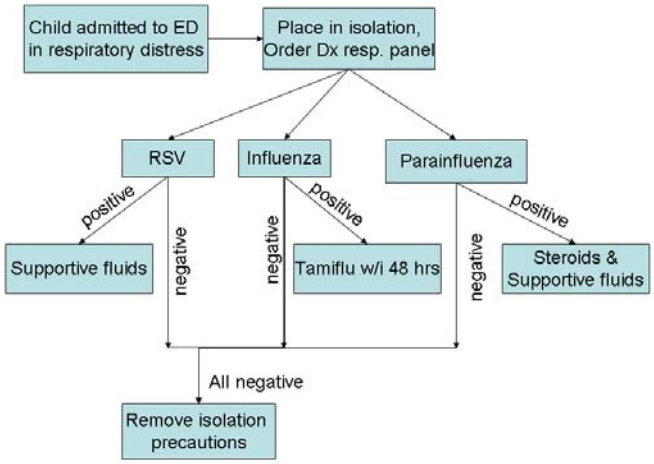

Figure 1: Clinical decision making for child in respiratory distress with respect to drug therapy and isolation precautions

To vastly improve the treatment of infectious diseases, a point-of-care diagnostic that can quickly identify the causative agent and inform drug therapies is needed. The current point-of-care diagnostics lack the sensitivity and specificity required by most disease states. In the case of influenza, it is critical to differentiate between swine flu and other strains of flu, which can only be done by genetic testing (currently unavailable at the point of care). To meet this challenge, we continue to develop a completely automated and miniaturized low-cost lab-on-a-chip diagnostic (disposable chip and instrument) that isolates nucleic acids from raw samples, amplifies the signal via PCR, and then detects the target amplicon via fluorescence. The chip uses a porous polymer monolith technology embedded in a low cost plastic disposable. The chip was designed to be manufactured at low cost, as all active components (pumps, valves, heaters, optics) are located in the instrument and the channels are in a planar geometry that allows the chips to be manufactured by injection molding. Although this is a platform technology that would be useful for many different clinical situations, we are developing the technology for influenza diagnosis.

2 MATERIALS AND METHODS

The microfluidic chips were manufactured as previously described [3]. Viral RNA was purified from *in vitro* cultured influenza A (VR-1469, ATCC, Manassas, VA). Virus was cultured in MDCK cells (ATCC CCL-34). Titers were estimated using hemagglutinin (HA) assays, and serial dilutions of virus in buffer were made. RNA from the virus dilutions was extracted using a commercial RNA extraction kit (QIAamp®, Qiagen Inc., Valencia, CA). Viral RNA was amplified using a proprietary one-step quantitative RT-PCR assay from the United States Center for Disease Control (CDC). The protocol was transferred under a material transfer agreement under the condition that we would not distribute the details of the protocol. We ported the CDC assay to the microfluidic chip without modification except to increase the concentration of BSA (an additional 0.3%) to help block the additional surface area in the chip with respect to a microtiter plate well. The instrument was operated as previously described [3], but with modified temperatures to correspond to those required for the RT-PCR.

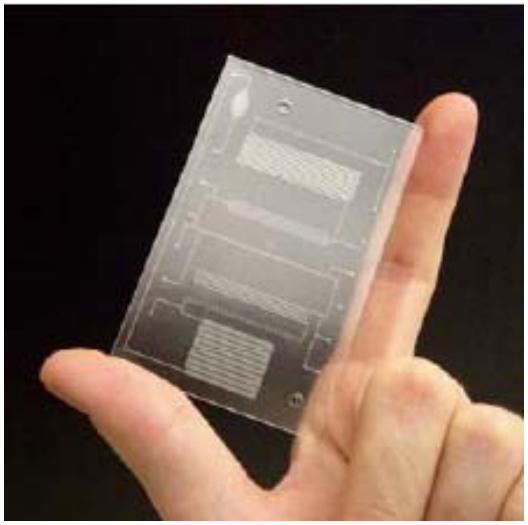

Figure 2: Plastic disposable lab-on-a-chip for detection of pathogens from liquid samples.

3 RESULTS AND DISCUSSION

3.1 Microfluidic chip

We have developed a completely integrated lab-on-a-chip and associated instrument for the detection of pathogens from liquid samples [3]. The system conducts lysis, nucleic acid isolation and concentration, reverse transcription polymerase chain reaction (RT-PCR), and end-point fluorescent detection of the target amplicon. To enable truly low-cost manufacture of the single-use disposable chip, we designed the plastic chip in a planar format (Figure 1) without any complicated active components to be amenable to injection molding and utilized a novel porous polymer monolith (PPM) embedded with silica that has been shown to lyse bacteria and isolate the nucleic acids from clinical samples [4-8]. The chip has been injection molded in Zeonex, which is a thermoplastic

with a high melting temperature to allow PCR, a good UV transmissibility for UV-curing of the PPM, and low auto-fluorescence to allow fluorescence detection of the amplicon.

3.2 Prototype Instrument

We built a prototype instrument (Figure 3) to automate the control of the fluids, temperature cycling, and optical detection with the capability of accommodating various chips designs [3]. We employ a remote valve switching method that enables fluid control without valves or pumps on the chip; they are instead located in the instrument. The remote valve switching takes advantage of the inherent incompressibility of liquids such that once all the fluid lines are full, the fluids in the chip can be driven by remote pumps and switched by remote valves.

The instrument has the capability of thermal cycling for PCR. The thermal heating of the PCR channel was accomplished by placing a ceramic heater in direct contact with the chip. The cooling was achieved by blowing room temperature air on the cooling fin located on the heater's underside. The chip was insulated on the opposite side of the chip from the heater to minimize heat loss and maintain close to uniform temperature throughout the PCR mixture.

The detection of end-point fluorescence was realized with an optical spectrometer. The detection optics consist of an excitation source and a detector, both located underneath the chip and oriented 90 degrees from one another. A broadband mirror is also positioned above the chip, to reflect more of the emission to the detector. The excitation source is an LED with a center wavelength of 470 nm, which is filtered through a short-pass filter with a cutoff wavelength of 500 nm. To enable the greatest flexibility in fluorescence detection, we chose to integrate a spectrophotometer as our detector. The spectrophotometer measures light intensity for a broad range of wavelengths. The software can either collect a whole spectrum of optical data or a single wavelength such as 525 nm (the peak emission of FAM, the dye used here).

3.3 On-Chip RT-PCR and Detection

In our previous work, we demonstrated the function of the chip for detecting *B. subtilis* markers starting from either genomic DNA or from *B. subtilis* bacterial cell culture [3]. In both of these examples, DNA was isolated from the starting sample by mixing the sample with chaotropic agents (guanidium thiocyanate) and by passing the sample over a porous polymer monolith (PPM) embedded with silica particles. The PPM acted to both mechanically lyse the bacterial cells and to capture the nucleic acids. The column was washed and then the purified nucleic acids were eluted and mixed with a PCR master mix before being shunted to the PCR well on-chip. Since the CDC assay for influenza A was a one-step RT-PCR assay, we were able to use the same chip design as was previously developed for DNA targets. The PCR master mix was simply changed to a one-step RT-PCR master mix and the thermal cycling protocol changed to include an initial step at 50°C to allow for the reverse transcription of the RNA to DNA, before continuing on with the thermal cycling for the PCR.

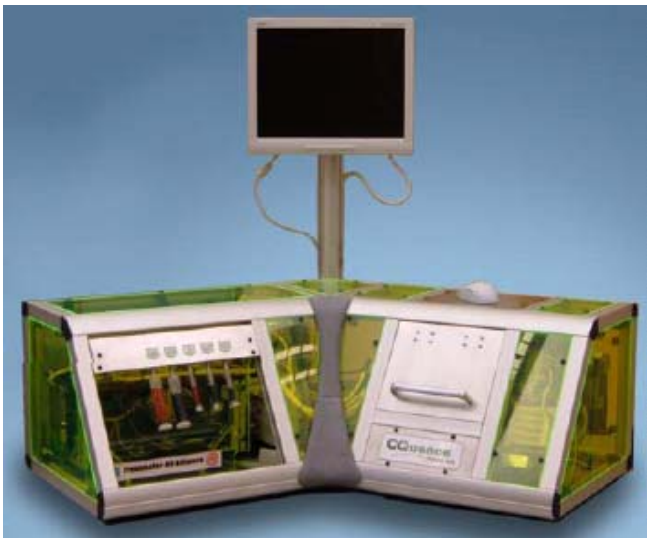

Figure 3: Prototype instrument for running various lab-on-a-chip disposables and protocols.

An RT-PCR mix with and without 0.25ng/μL influenza RNA was mixed and run sequentially on the chip. The negative control (no RNA) was run first. Then, the chip was cleaned with a 15% Clorox bleach and 2% detergent (7X-O-Matic, ICN Biomedicals, OH, USA) wash and rinsed with diethylpyrocarbonate (DEPC)-treated water. Finally, the chip was loaded with the RT-PCR mix containing the RNA. The instrument automatically measured the fluorescence from the Taqman assay at the end of the PCR. The positive control optical signal with the baseline subtracted is shown in Figure 4.

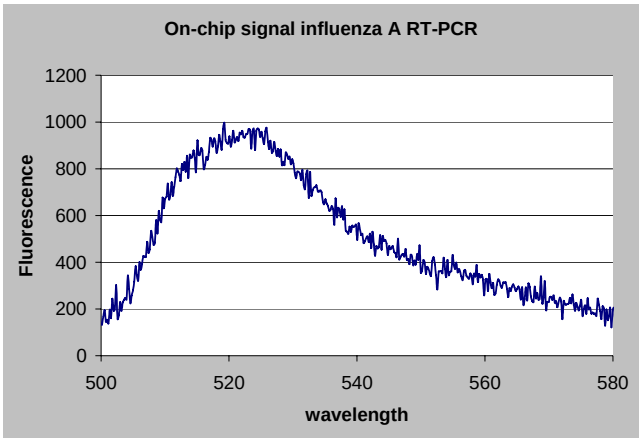

Figure 4: End-point optical measurement from influenza on-chip RT-PCR. The baseline data (fluorescence of RT-PCR mix before thermal cycling) is subtracted.

After on-chip RT-PCR, the contents of the detection well were collected and analyzed by gel electrophoresis to confirm the presence of the amplicon (~100 bp). The gel was stained with Sybr green I (Molecular Probes, Eugene, OR). As shown in Figure 5, the anticipated product was observed in the positive control sample. As is typical for PCR assays, primer-dimer products are also present in the amplified sample however because the on-chip assay is a Taqman assay they do not contribute to the optical signal. One should note however, that we often observe increased side reactions on-chip as compared to off-chip controls, presumably due to interactions with the sidewalls of the chip. We continue to optimize the on-chip reaction to minimize these side reactions.

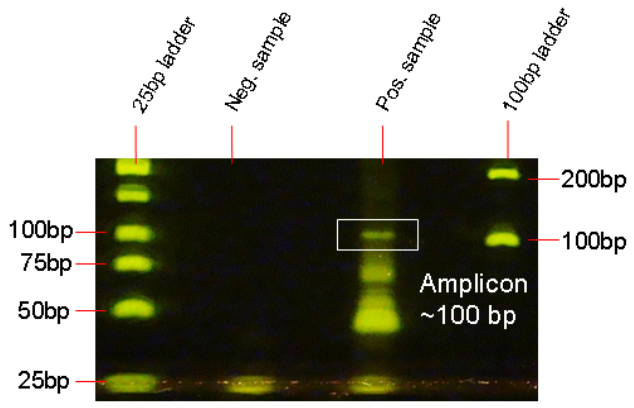

Figure 5: Electropherogram of RT-PCR product (white box) on chip from a negative control (no viral RNA) and a positive control (0.25 ng/μL RNA).

4 CONCLUSIONS

In this work we present a fully automated instrument and low cost disposable lab-on-a-chip for detection of pathogens in liquid samples. Because of the urgent need for point-of-care influenza diagnostics and sub-typing, we have focused on developing this technology for influenza diagnosis. Working with the CDC, we have ported a clinically proven RT-PCR assay onto the microfluidic format. The ability to quickly adapt a new PCR assay onto the microfluidic format will be an important attribute to any point-of-care test aimed at the diagnosis of an emerging or seasonally variable pathogen, such as influenza. In the future, we will assess the sensitivity and specificity of the assay with nasopharyngeal aspirates collected from patients suspected of influenza infection.

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