A DISPOSABLE MICROFLUIDIC CHIP FOR DETECTION OF INFLUENZA TYPE A IN CLINICAL SPECIMENS INTEGRATING RNA ISOLATION, REVERSE TRANSCRIPTION, AND CONTINUOUS FLOW PCR

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ABSTRACT

Detection of influenza type A infection in clinical specimens is demonstrated in a thermoplastic disposable microfluidic device by integrating viral RNA extraction and reverse transcription – polymerase chain reaction (RT-PCR). The accuracy of the microfluidic assay was evaluated with 146 human respiratory specimens collected at two clinical sites in Boston, MA, USA. The total assay time is less than three hours which is comparable to bench-top RNA extraction and RT-PCR methods and considerably faster than the gold standard diagnostic four day viral culture method. We demonstrate a new highly sensitive and specific integrated device for influenza A in clinical specimens.

KEYWORDS: Microfluidic, Diagnostics, Influenza, Virus, Clinical Sample, Point-of-care

INTRODUCTION

A point-of-care (POC) influenza diagnostic test is highly desirable as an alternative to the current clinical tests that include either rapid but inaccurate immunoassays or slow and labor intensive but accurate viral culture and direct fluorescent antigen (DFA) tests. Currently viral culture remains the gold standard. However, RT-PCR based methods are gaining wider acceptance for managing high case loads such as during the recent 2010 pandemic as the only rapid diagnostic method with comparable or better accuracy [1]. The Centers for Disease Control and Prevention (CDC, Atlanta, GA) recommended the CDC Realtime RT-PCR Protocol for Detection and Characterization of Influenza for use by clinical and state labs during the height of the pandemic, publicly available at http://www.who.int/csr/resources/publications/swineflu/realtimetypepcr/en/index.html. We employed their RT-PCR primer design and probe chemistry to develop and evaluate our integrated nucleic acid test chip with human respiratory specimens from local clinics.

THEORY

Nucleic acid based diagnostics for “real” human clinical samples must include a sample preparation step to isolate the target DNA or RNA template. Solid phase extraction (SPE) by silica is a rapid and simple method for purification of nucleic acids. A chaotropic salt (often in combination with detergents) both lyses the viral particle to liberate nucleic acids and promotes adsorption of DNA and RNA to the silica SPE column. The nucleic acid can be further purified and eluted from the silica for downstream amplification by PCR for detection [2]. To integrate RNA isolation and RT-PCR, some important considerations were made including a serpentine channel design, surface passivation with bovine serum albumin (BSA) and polyethylene glycol (PEG) to prevent loss of reagents and template to the chip surface, and simple (valve less) thermal and fluidic control in a single layer chip. Finally, in silico PCR channel designs were made and empirically validated for various thermal profiles for the denaturation, annealing, and extension steps of PCR [3].

EXPERIMENTAL

Nasopharyngeal aspirate and nasal swab samples were collected from patients into viral transport medium (VTM) at two clinical sites from 2008 – 2010 (Figure 1a). The microfluidic assay was performed in a 70 mm x 25 mm x 1.4 mm cyclic olefin polymer (Zeonex 690R, Zeon Chemicals) chip with three parts, μSPE, RT, and PCR (Figure 1b). The microfluidic assay was tested with 73 influenza A positive specimens and 73 influenza A negative specimens. Each specimen was tested individually in a single use disposable device with 100 μl of sample in 300 μl of lysis buffer (2M guanidine thiocyanate, 67% 2-propanol, 6 μg carrier RNA). Viral RNA purified in the μSPE channel was eluted, mixed with RT-PCR reagents in the reaction inlet, and flowed into the RT channel. The RT cDNA product was amplified by continuous flow PCR at a flow rate of 0.6 μl/min for 30 cycles of 95°C and 60°C with each cycle being 40 sec/cycle. Each RT-PCR reaction was a 50 μl reaction containing 13.5 μl of reagents and primers from Qiagen’s One-step RT-PCR kit – 4 μl one-step RT-PCR enzyme mix, 1X Q solution, 1X One-step buffer, 1mM additional MgCl₂, 1 mM each Universal Influenza InfA-F and InfA-R primers (sequences in CDC protocol above), 400 μM dNTPs, 0.015% w/v BSA, and 0.75% w/v PEG8000.
Figure 1: Experimental overview of specimen processing in the integrated chip. (a) Patient nasopharyngeal swab or aspirate which may contain influenza A virus is collected in viral transport medium (VTM). (b) RNA is extracted from the VTM sample in an SPE column and amplified by RT-PCR on-chip as shown in a picture of the fabricated device.

The experimental chip set-up is shown in Figure 2. The PCR product was collected and analyzed by gel electrophoresis off-chip by either a 2100 Bioanalyzer (Agilent, CA) or 12% polyacrylamide gel electrophoresis (PAGE). A 106 bp band indicated a positive reaction for the M1 gene target.

Figure 2: Experimental set-up of the integrated chip. Two chips situated on thin film heaters are shown connected to a dual barrel syringe pump.

The chip assay and rapid immunoassays (Xpect Flu A & B kit (Remel, KS) and BinaxNOW Influenza A & B kit (Inverness Medical, NJ)) were compared to tube real time RT-PCR. The tube RT-PCR method was also used to characterize the viral load of all samples (cDNA copies/ml (c/ml)) and to determine the type of influenza, A or B. All tube RT-PCR reactions were performed on RNA extracted using the Qiagen QiaAmp Viral RNA Mini Kit.

RESULTS AND DISCUSSION

The lower limit of detection of the chip was determined to be \(10^3 \text{ to } 10^4\) cDNA c/ml for clinical samples and a laboratory reference strain of influenza, A/PR/8/34 (ATCC, VA), respectively. The c/ml determination was made using standard methods of absolute quantitation with a standard curve of serially diluted plasmid DNA with one copy of the M1 target sequence per plasmid. The chip assay had a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 96% with corresponding 95.9% sensitivity and 100% specificity. The sensitivity of the chip decreased with lower viral loads (Table 1).

We did not observe a difference between the two types of specimens (aspirate vs. swab) with regard to performance in the chip in terms of the end-point PCR product yield concentration. The end-point PCR product ranged from 43 pg/µl – 1.5 ng/µl with the majority (77%) at 0.1 – 1.0 ng/µl. A representative gel image of chip processed influenza positive and negative specimens is shown in Figure 3. The end-point PCR product concentration tended to be greater in samples with higher viral loads (one-way ANOVA, p<0.0001). However, this relationship was strongest at the highest viral loads of \(10^6 \text{ to } 10^7\) c/ml (two tailed t-test, p<0.05) and weaker at lower viral loads (two tailed t-test, p>0.05). This combined with the accuracy of the chip at the spectrum of viral loads suggests the performance of the chip may be improved for lower viral load specimens.
Table 1. Sensitivity and specificity of the integrated NAT chip for influenza A for decreasing specimen viral loads

<table>
<thead>
<tr>
<th># specimens</th>
<th>False Negatives</th>
<th>Specimen Virus load (copies/ml)</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>$10^0$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>$10^9$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>$10^8$</td>
<td>100</td>
<td>100</td>
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<td>0</td>
<td>$10^7$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>$10^6$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
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<td>2</td>
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<td>100</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>$10^4$</td>
<td>95.8</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>$10^3$</td>
<td>95.9</td>
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</tbody>
</table>

Though this may be required for certain chronic viral infections with periods of immune suppressed viral replication such as HIV or HCV, acute respiratory infections such as influenza rarely have low viral loads (less than $10^5$ c/ml) in the symptomatic phase of infection when POC testing would be most applicable. The sensitivity of the chip assay proved to be sufficient in our study to detect influenza type A infection in the nasopharynx.

CONCLUSION
The new test reported here is a major improvement over current rapid POC immunoassay tests and comparable in sensitivity and specificity to viral culture and tube RT-PCR assays for influenza diagnostics. Finally, this device offers several advantages over tube RT-PCR and RNA isolation assays including portability, ease of use, minimal steps and low cost.

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REFERENCES

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Figure 3: Results of chip testing by 12% PAGE. Specimens infected with influenza A produced a 106 bp RT-PCR product (left gel). Negative specimens were negative for the 106 bp size band (right gel). (-)C is water only through the device; (+)C is a lab reference influenza A sample; L is a DNA ladder (Fermentas, GeneRuler Low Range DNA Ladder). Three influenza positive and three negative specimens are shown.