DEVELOPMENT OF SmartAmp2-BASED TECHNOLOGY FOR RAPID DETECTION OF THE 2009 PANDEMIC INFLUENZA A/H1N1 VIRUS

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ABSTRACT

After the outbreak in Mexico in April last year, the 2009 pandemic influenza A/H1N1 virus spread among humans worldwide. We have developed a SmartAmp2-based method to detect the 2009 pandemic influenza A/H1N1 virus. The method comprises reverse transcriptase and isothermal DNA amplification reactions in one step, where RNA extraction and PCR reaction are not required. Application of the SmartAmp2-based method utilizing an exciton-controlled hybridization-sensitive fluorescent primer enabled us to specifically detect the HA segment of the 2009 pandemic influenza A/H1N1 virus within 40 min. The clinical usefulness of this method has been evaluated by clinical studies.

KEYWORDS: SmartAmp, influenza, H1N1, pandemic, detection, clinical application

INTRODUCTION

The 2009 pandemic (pdm) influenza A/H1N1 virus, a new strain of virus identified in Mexico in April 2009, caused outbreaks at both local and global scale with severe consequences for human health and the global economy [1]. On June 11, 2009, the World Health Organization declared that the infections caused by the new strain had reached pandemic proportion. The genomic segments of the new strain were most closely related to swine viruses. However, unlike most avian and swine A influenza viruses that can result in sporadic human infection via animals to human transmission, but lack the ability of human to human transmission, the swine-origin 2009 pdm H1N1 influenza A virus showed a strong ability to transmit from human to human.

In the case of infection with the 2009 pdm H1N1 influenza A virus, many of patient groups such as the elderly, the immune-compromised and those with underlying chronic conditions such as asthma or chronic obstructive pulmonary disease were vulnerable to complication that resulted in mortality [2]. Although WHO has most recently announced the post-pandemic period, there are still concerns that the 2009 pdm virus may mutate or re-assort with existing influenza viruses giving more virulence when it returns. In fact, the 1918 Spanish flu pdm virus was relatively mild in its first wave and acquired more virulence when it returned in the winter. Thus, preparedness on a global scale against a potential more virulent strain is highly recommended. Currently-used rapid influenza diagnostic tests (lateral flow immuno-chromatographic tests) detect influenza viral nucleoprotein antigen, however they do have a relatively low sensitivity with about 30% of false negative results. While PCR is a sensitive method, it requires RNA extraction and reverse transcriptase (RT) reaction steps. Therefore, it is crucially needed to develop a simple, rapid, and highly sensitive method that enables clinical detection of the 2009 pdm influenza A/H1N1 virus.

In order to respond to such global needs, we have most recently developed the SmartAmp2-based method targeting the HA segment of the 2009 pdm influenza A/H1N1 virus.

THEORY

(1) Influenza A viruses belong to the family Orthomyxoviridae, containing a genome composed of eight segments (i.e., PB1, PB2, PA, HA, NP, NA, M, and NS) of single-stranded negative-sense RNA [3]. The HA segment RNA encodes haemagglutinin protein that is critical for binding to cellular receptors and fusion of the viral and endosomal membranes. Based on the NCBI Influenza Virus Resource, we decided to target one region in the HA segment RNA, since the sequence has been found to be little mutated but specific for the 2009 pdm influenza A/H1N1 virus as compared to other influenza A viruses, such as seasonal H1N1 and H3N2.

(2) SmartAmp2 is based on the concept that DNA amplification itself is the signal for detection of a specific target sequence. Differing from widely-used PCR, the SmartAmp2 reaction is isothermal DNA amplification [4,5]. This is a great advantage for our rapid detection of influenza A viruses, since we can make RT and isothermal DNA amplification reactions in one reaction tube (Fig. 1). The process of SmartAmp2-based DNA amplification requires five primers: turnback primer (TP), boost primer (BP), forward primer (FP) and two outer primers (OP1 and OP2) (Fig. 2). To design primer sets for the reaction, algorithms specific to SmartAmp2 primer design have been developed. Primers are selected based on these algorithms using optimal melting temperature and product size range. The genomic sequence between the annealing sites of TP and FP primers is the target region that will be amplified by the SmartAmp2 reaction.

(3) We have most recently developed exciton-controlled hybridization-sensitive fluorescent primers [6,7], named “Exciton Primers”, which significantly enhance the signal/noise ratio. Exciton Primers function as sequence-specific...
After hybridization to complementary sequences, Exciton Primer provides sequence-specific fluorescent signal for real-time monitoring of amplification reactions [7]. Exciton Primers show high signal strength with low background leading to a superior specificity and sensitivity compared to SYBR Green I (Fig. 3).

**Figure 1:** Comparison of RT-PCR and SmartAmp methods for detection of the 2009 pdm H1N1 influenza A virus.

**Figure 2:** Primers for SmartAmp reaction.

**Figure 3:** Chemical structure of the “Exciton Primer”

### EXPERIMENTAL

Primers for the SmartAmp2 reaction were optimized by using the standard reaction mixture and cDNA encoding the partial sequence of the HA segment. Once the SmartAmp2 primer set was optimized, we tested the RT-SmartAmp2 reaction medium that contained reverse-transcriptase and Aac DNA polymerase as well as SmartAmp2 primers and synthesized RNA encoding the partial sequence of the HA segment. By reducing the copy number of the synthesized RNA in the reaction mixture, we determined the minimal detectable level.

To handle actual swab samples, we developed a simple pre-treatment procedure. Swab samples were found to contain hitherto unknown substance(s) that potentially inhibited the RT-SmartAmp reaction.

### RESULTS AND DISCUSSION

The SmartAmp2-based detection method, thus developed, was proven to be specific to the 2009 pdm influenza A/H1N1 virus. No cross-reactivity was observed to other influenza viruses, such as seasonal H1N1, H3N2, and B type (Fig. 4). The minimal detectable level for the HA segment of the 2009 pdm influenza A/H1N1 virus was estimated to be $\sim 10^2$ copies of RNA in the RT-SmartAmp2 reaction mixture (25 µl). When the isolated 2009 pdm influenza A/H1N1 virus ($10^7$ pfu/ml) was diluted by $x 10^3$, $x 10^4$, and $x 10^5$, the RT-SmartAmp2 method could detect the virus in those diluted samples (Fig. 4A).

**Figure 4:** Cross-reactivity test with the 2009 pdm A/H1N1 (A), seasonal A/H1N1 (B), A/H3N2 (C), and type B (D).
To evaluate our method, we conducted clinical research in Japan during the pandemic period of October 2009 to January 2010. Swab samples were acquired from a total of 383 outpatients at seven hospitals located in Tokyo, Chiba, and Osaka areas. The 2009 pandemic influenza A/H1N1 virus in the swab samples was detected by the SmartAmp2-based method, and the corresponding results were subsequently compared with data of rapid influenza diagnostic tests and viral genome sequence analysis. By the SmartAmp2-based method, we could detect the 2009 pandemic influenza A/H1N1 virus in patients’ swab samples even in early stages (< 12 h) after viral infection.

CONCLUSION

The SmartAmp2-based detection method provides a simple and practical tool to rapidly detect the 2009 pandemic influenza A/H1N1 virus. We are presently developing micro-fluidics systems for SmartAmp2 reactions which can be applied for clinical practices when the second pandemic wave emerges.

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REFERENCES


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