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Detection and typing of human-infecting influenza viruses in China by using a multiplex DNA biochip assay

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Rapid identification of the infections of specific subtypes of influenza viruses is critical for patient treatment and pandemic control. Here we report the application of multiplex reverse transcription polymerase chain reaction (RT-PCR) coupled with membrane-based DNA biochip to the detection and discrimination of the type (A and B) and subtype (human H1N1, human H3N2, avian H5N1 and avian H7N9) of influenza viruses in circulation in China. A multiplex one-step RT-PCR assay was designed to simultaneously amplify the HA and NA genes of the four subtypes of influenza A viruses and NS genes to discriminate type A and B viruses. PCR products were analyzed by a membrane-based biochip. The analytical sensitivity of the assay was determined at a range of 2–100 copies/reactions for each of the gene transcripts. Eighty one clinical samples, containing 66 positive samples with evident seasonal influenza virus infections, were tested, which gives the clinical sensitivity and specificity of 95.5% and 100% respectively. For the avian influenza samples, 3 out of 4 H5N1 samples and 2 out of 2 H7N9 avian samples were correctly identified. We argue this method could allow a rapid, reliable and inexpensive detection and differentiation of human-infecting influenza viruses.

1. Introduction

Influenza virus belongs to the family *Orthomyxoviridae*, which is a common contagious pathogen that causes acute respiratory tract illness in humans. Influenza virus can be classified into three types (A, B and C) based on matrix and nucleoproteins (Peiris et al., 2009), of which Type A and B virus are prominent among the causes of severe respiratory illness. According to different antigenicity of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), influenza A virus can be further divided into 19 HA and 11 NA subtypes. Recently, H1N1, H3N2 subtypes of influenza A virus and influenza B virus are the most common subtypes causing annual influenza epidemics (Holmes et al., 2005; Petric et al., 2006; Yoon et al., 2014). Pandemics occurred sporadically in humans in recent decades, such as the swine-origin H1N1 in 2009. Moreover, hundreds of human severe infections caused by avian influenza viruses of H5N1 and H7N9 subtypes were reported in recent years, rais-

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ing the concerns about their potential pandemic threat. Although direct human-to-human transmission has not been reported, the mutations in H5N1 (Linster et al., 2014) and H7N9 viruses could make them transmit between human beings. Thus, development of a rapid and reliable diagnostic method for influenza virus surveillance is of critical importance to monitor epidemics and prevent a potential pandemic outbreak.

The traditional method to detect influenza virus includes virus culture, isolation and characterization by immunoassay (Storch, 2000; Wang et al., 2002), which usually takes 3–7 days (Ellis and Zambon, 2002). Other diagnostic methods employ rapid point-of-care test (Baas et al., 2013; Weinberg and Walker, 2005), real-time RT-PCR (Chen et al., 2013; Fan et al., 2014; Kang et al., 2014; Kang et al., 2010) and multiplex RT-PCR combined with chip detection. Rapid point-of-care test is of simple use and enables rapid testing within 15 min, but generally has low sensitivity (Eggers et al., 2012; Hurt et al., 2007). Real-time RT-PCR usually detects 1 (sub)type of influenza virus in a single reaction (Kang et al., 2010; Kuo et al., 2014; Li et al., 2013; Wang et al., 2009). Multiplex RT-PCR simultaneously amplifying target genes of multiple influenza viruses. The PCR products are further detected by low-density microarray or xTAG technology (Luminex), which allows the simultaneous detection of numerous types and subtypes of influenza virus with relatively high sensitivity and specificity (Gall et al., 2009; Huang et al., 2009; Kessler et al., 2004; Tian et al., 2014; Townsend et al., 2006; Zou et al., 2007). Despite the prominent benefit of multiplex RT-PCR based technologies in influenza virus detection, the expensive instruments for printing probes and detecting signals of multiple viruses could impose a great financial burden for its wide use. So a rapid, multiplex and inexpensive virus detection method is expected to improve the management of patients and the control of transmission events.

To distinguish seasonal influenza A viruses of H1N1, H3N2, influenza B virus and avian influenza virus H5N1 and H7N9 prevalence in China, we designed a multiplex RT-PCR assay that was able to simultaneously amplify H1, H3, H5, H7, N1, N2, N9, NSA (Influenza A virus NS gene) and NSB (Influenza B virus NS gene) genes. To lower the cost, we used a membrane-based hybridization method integrated into an automatic instrument for RT-PCR products detection. The whole detection process takes about five hours.

In this study, we evaluated the performance of our method on 81 clinical specimens, and results were compared with Xpert Flu assay. Furthermore, we tested the method on 10 avian influenza virus samples by comparing to the gene sequencing results. The membrane-based biochip assay is expected to provide a rapid, sensitive, liable and low-cost technique for identification and subtyping of influenza A and influenza B viruses.

2. Material and methods

2.1. Primers and probes design

All the sequences of human influenza A virus H1, H3, H5, H7, N1, N2, N9, NS genes and human influenza B virus NS gene prevalence from 2008 to 2013 were collected from Influenza Research Database (IRD, http://www.fludb.org). Partial sequenced genes were removed. For each target gene, Sequences were aligned using ClustalX 2.0 (Larkin et al., 2007), and then a degenerate sequence was generated according to multiple sequence alignment result by an in-house python script. Highly conserved regions were selected for multiplex PCR primer design using JPCR (Kalendar et al., 2011). Probes were designed in the context of corresponding amplicons. In order to eliminate the nonspecific cross-reactivity between subtypes, the primers and probes were matched against the database contained all the other non-target influenza sequences. Primer with miss match sites more than 5 and probe with miss match sites more than 7 were considered to be good hits. The reverse primers were labeled with biotin at 5′ termini and probes were tailed by poly(T)10 and amine groups (NH_2) at 5′ termini to facilitate immobilized on the membrane chip. Primers and probes were synthesized by Shanghai Sangon Biotech.

2.2. RNA transcript preparation

Influenza virus gene fragments for the 9 targets were cloned into the pET-30a vector (Novagen brand; EMD Madison, WI) containing T7 promoter. The sequences used for cloning were A/Guangdong/01/2009 (H1), A/Acre/15093/2010 (H3), A/Bangladesh/207095/2008 (H5), A/Fujian/1/2013 (H7), A/Aalborg/INS132/2009 (N1), A/Alabama/01/2011 (N2), A/Fujian/1/2013 (N9), A/Boston/82/2009 (Flu A NS), and B/Alabama/02/2010 (Flu B NS). RNA transcripts were in vitro synthesized using the T7 RiboMax Large Scale RNA Production System (Promega, Madison, WI, USA) and quantitated by Qubit RNA BR Assay Kit in the Qubit 2.0 Fluorometer to determine the copy number (Life Technologies, Grand Island, NY, USA).

2.3. Clinical samples and avian influenza samples

A total of 81 clinical nasopharyngeal swab samples were collected by Beijing Chaoyang hospital. These samples were detected positive for H1N1 (n = 33), H3N2 (n = 16), influenza B (n = 17) and negative (n = 15) by Xpert Flu (Cepheid, Sunnyvale, CA). Samples were shipped at 4 °C, and stored frozen at −80 °C.

10 avian influenza samples were provided by Yangzhou University. Due to the high pathogenicity of avian influenza virus, nucleic acids were extracted and then reverse transcripted to the first-strand cDNA in a Biosafety Level III laboratory at Yangzhou University. cDNA samples were shipped at 4 °C and stored frozen at −20 °C.

2.4. Nucleic acid extraction, multiplex RT-PCR

Viral RNA was extracted from 280 μl of clinical samples using QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. A multiplex one-step RT-PCR was carried out using viral RNA as template. Nine pairs of primers that capable of specifically amplifying the gene of H1, H3, H5, H7, N1, N2, N9, NSA, NSB and the universal primer uni-12 targeted the conserved 3′-teminal 12 bp oligonucleotide of each gene segment of influenza virus were dissolved at 20 μM with RNase-free water and mixed. The SuperRT One Step RT-PCR kit (Cwbio, Beijing, China) was used for amplification. To set up the amplification reaction, 5 μl RNA template was mixed with 12.5 μl 2 × RT-PCR buffer, 0.5 μl RT-PCR enzyme, 5 μl primer mix, 5 units RNase inhibitor, and RNase-free water to bring the final volume to 25 μl. The thermal profile was as follow: 30 min at 45 °C; 2 min at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C; 5 min at 72 °C. The sequences of each specific primer were listed in Table 1. The PCR products were labeled with biotin during amplification.

For multiplex PCR, 5 μl of cDNA sample was mixed with 15 μl PCR mixture, containing 5 μl primer mix and 10 μl of Multiplex PCR MasterMix (Cwbio, Beijing, China). The thermal profile was 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C; 5 min at 72 °C.

2.5. Preparation of membrane chip

A total of 11 oligonucleotide probes (Table 1) including 9 specific probes and 2 probes for positive and negative control were diluted to 20 μM with $NaHCO_3$ buffer (0.5 M, pH 8.4), 0.2 μl of each