Development of Rapid Immunochromatographic Test for Hemagglutinin Antigen of H7 Subtype in Patients Infected with Novel Avian Influenza A (H7N9) Virus

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Abstract

Background: Since human infection with the novel H7N9 avian influenza virus was identified in China in March 2013, the relatively high mortality rate and possibility of human-to-human transmission have highlighted the urgent need for sensitive and specific assays for diagnosis of H7N9 infection.

Methodology/Principal Findings: We developed a rapid diagnostic test for the novel avian influenza A (H7N9) virus using anti-hemagglutinin (HA) monoclonal antibodies specifically targeting H7 in an immunochromatographic assay system. The assay limit of detection was 10^{3.5} pfu/ml or 10^3TCID50 of H7N9 virus. The assay specifically detected H7N9 viral isolates and recombinant HA proteins of H7 subtypes including H7N7 and H7N9, but did not react with non-H7 subtypes including H1N1, H3N2, H5N1, H5N9, and H9N2. The detection sensitivity was 59.4% (19/32) for H7N9 patients confirmed by RT-PCR. Moreover, the highest sensitivity of 61.5% (16/26) was obtained when testing H7N9 positive sputum samples while 35.7% (5/14) of nasopharyngeal swabs and 20% (2/10) of fecal samples tested positive. No false positive detection was found when testing 180 H7N9 negative samples.

Conclusions/Significance: Our novel rapid assay can specifically detect H7 HA antigen, facilitating rapid diagnosis for prevention and control of the on-going H7N9 epidemic.

Introduction

The novel H7N9 avian influenza virus was first isolated from three patients with severe lower respiratory tract disease of unknown cause in China in March 2013 [1]. As of November 6th, 2013, a total of 139 cases of H7N9 infection had been laboratory-confirmed, causing 45 deaths in mainland China and 1 in Taiwan [2,3]. As of January 28 of 2014, more than 100 new H7N9 cases have been reported. H7N9 was initially detected in the Yangtze River Delta, and area encompassing Shanghai, Zhejiang, Anhui and Jiangsu provinces, but has since spread to more than 12 provinces and cities including Beijing, Hebei, Shandong, Henan, Jiangxi, Hunan, Fujian, Guangdong and Taiwan, indicating a continuing epidemic of H7N9 infection in humans. Severe lower respiratory tract infection was predominant in human H7N9 infection, and a relatively high mortality of about 32.4% (45/139) was observed as of November 6th, 2013 [1,2,4,5].

Human infection with subtype H7 avian influenza A viruses had rarely been described and never reported in China before 2013. These infections may have been overlooked due to mild symptoms or asymptomatic infection in humans and low pathogenicity in poultry [6–9]. Before the 2013 outbreak of H7N9, a highly pathogenic avian influenza A(H7N7) infection was reported to result in one fatality in the Netherlands in 2003 [10,11].

The novel H7N9 virus is a reassortant of triple avian influenza viruses and has genetic markers that can bind strongly to human-like receptors, and are known to be associated with improved replication of avian influenza viruses in mammals [1,4,6]. Therefore, the possibility of human-to-human transmission cannot be ruled out [12]. Although no sustained human-to-human transmission of H7N9 virus has been confirmed [1–4], one case of transmission between a father and daughter has been identified in Eastern China by genetic comparison of viral isolates [12].

The hemagglutinin gene sequence suggests that the novel H7N9 virus may be a low pathogenic avian influenza virus, and has been found in poultry including pigeons and chickens [4]. However, the extent of distribution of asymptomatic infection of H7N9 virus in
human and domestic poultry is unclear partly due to the lack of well-evaluated testing kits.

Diagnosis of H7N9 infection relies on isolation and culture of H7N9 virus from patient samples, requiring a biosafety level 3 laboratory equipped with real-time reverse-transcription PCR analysis (RT-PCR) capabilities, and well trained technical personnel. Real-time RT-PCR assays for specific detection of H7N9 virus were established shortly after the initial outbreak [13], and in May and July, 2013 the Chinese Food and Drug Administration (CFDA) approved three PCR kits for specific detection of H7N9 virus [14]. However, so far no well-evaluated rapid immunoassays have been approved. Considering the high mortality rate and the possibility of human-to-human transmission of the novel H7N9 virus, a simple rapid diagnostic screening assay is critical for monitoring this epidemic in both humans and animals. Such a test is urgently required for rapid diagnosis and early antiviral treatment of patients infected with H7N9, and may facilitate detection of asymptomatic infections. Here we report the development and evaluation of a simple lateral flow assay for rapid detection of hemagglutinin (HA) H7 subtype antigen.

Materials and Methods

Ethics Statement

The study protocol and informed consent documents were reviewed and approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. All participants provided written informed consent.

Monoclonal Antibodies and Influenza Virus Proteins

The H7-directed monoclonal antibodies C1 (clone 1175-18C4) and C2 (clone 2113-3B7), and recombinant HA proteins of influenza A viruses H1N1, H3N2, H5N1, H7N7 and H7N9 (A/Shanghai/2/2013) were purchased from Immune Technology Corp. (NY, USA). Monoclonal anti-H7 antibodies were obtained by DNA immunization of animals with plasmid DNA containing the full length of HA gene of H7N7 virus (H7N7/England/268/96) (Genebank No. AF028020). In addition, recombinant HA proteins of H7N9 (A/Anhui/1/2013) and H7N7 were kindly provided by Dr. Ling Chen of the State Key Laboratory of Respiratory Diseases (Guangzhou, China) while inactivated H1N1, H5N9, and H9N2 virus lysates were obtained from South China University of Technology (Guangzhou, China).

Influenza Viruses

Influenza A (H7N9) (A/Anhui/1/2013) was kindly provided by the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (Beijing, China). Another 4 H7N9 virus isolates were provided by the State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China). All H7N9 viruses were obtained by propagating clinical specimens in the allantoic sac and amniotic cavity of 9-to-11-day-old specific pathogen-free embryonated chicken eggs for 48 to 72 h at 35°C. The virus titers (TCID50 or pfu/ml) of H7N9 samples were determined as described earlier [15].

Quality Control Panel

The quality control panel for the H7N9 immunoassay was created by the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China and consisted of 7 dilution samples from one H7N9 positive sample and 20 H7N9 negative samples.

Clinical Samples

A total of 99 samples including throat swabs, sputum and feces were collected from 32 patients with laboratory-confirmed H7N9 infections. The average age of these patients was 62±16 years (ranging from 36 to 86 years), and 62.5% (20/32) were male. In addition, throat swab and sputum samples were also collected from 180 patients with fever or influenza-like symptoms that were not infected with H7N9. The average age of these patients was 43±17 years (ranging from 21 to 81 years), and 48.9% were male. Of the H7N9 negative patients, twelve suffered from influenza.

Table 1. Analytic sensitivity and specificity of rapid diagnostic test for recombinant hemagglutinin derived from influenza viruses.

| Proteins tested | Virus strains            | Concentration(ng/ml) | RDT results* |
|-----------------|--------------------------|----------------------|--------------|
| Recombinant HA  | A/ Shanghai/2/2013(H7N9) | 1                    | +            |
|                 |                          | 10                   | ++           |
|                 |                          | 100                  | +++          |
|                 |                          | 1000                 | +++          |
| Recombinant HA  | A/ Anhui/1/2013(H7N9)    | 1                    | +            |
|                 |                          | 10                   | ++           |
|                 |                          | 100                  | +++          |
|                 |                          | 1000                 | +++          |
| Recombinant HA  | A/ Netherlands/2/19/03   | 1                    | –            |
|                 |                          | 10                   | +            |
|                 |                          | 100                  | ++           |
|                 |                          | 1000                 | +++          |
| Recombinant HA  | A/ Hubei/1/2010(H5N1)    | 1000                 | –            |
| Recombinant HA  | A/ Victoria/361/2011(H3N2)| 1000                 | –            |
| Recombinant HA  | A/ California/06/2009(H1N1)| 1000                 | –            |

*Signal intensity was determined by comparing with a standard color chart and categorized as weak (+, C7–9), moderate (+++, C4–6) or strong (+++, C1–3) positivity, respectively. “–” indicates a negative test result.

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including 7 patients with confirmed H1N1 infection. Six H7N9 negative patients tested positive for measles, 10 patients for TB, and 15 patients for mycoplasma.

### Table 2. Rapid Diagnostic Test Quality Control Panel.

| Code | Virus strains | Subtypes | Virus titer (TCID₅₀) | HA titer | RDT results* |
|------|---------------|----------|----------------------|---------|--------------|
| P1   | A/Anhui/1/2013 | H7N9     | 10⁷                  | ++      |              |
| P2   | A/Anhui/1/2013 | H7N9     | 10⁶                  | ++      |              |
| P3   | A/Anhui/1/2013 | H7N9     | 10⁵                  | ++      |              |
| P4   | A/Anhui/1/2013 | H7N9     | 10⁴                  | +       |              |
| P5   | A/Anhui/1/2013 | H7N9     | 128                  | +++     |              |
| P6   | A/Anhui/1/2013 | H7N9     | 12.8                 | ++      |              |
| P7   | A/Anhui/1/2013 | H7N9     | 1.28                 | +       |              |
| N1   | A/California/7/2009 | H1N1Pdm | 32                   | –       |              |
| N2   | A/Perth/16/2009 | H3N2     | 128                  | –       |              |
| N3   | B/Chongqing Yuzhong/1384/2010 | BV | 128                  | –       |              |
| N4   | B/Guangdong Luohu/1512/2010 | BY | 128                  | –       |              |
| N5   | A/Chongqing Yuzhong/108/2007 | H1N1 | 32                   | –       |              |
| N6   | B/Heilongjiang Hulan/116/2010 | BV | 16                   | –       |              |
| N7   | B/Shanghai Luwan/173/2011 | BY | 32                   | –       |              |
| N8   | B/Guangdong Luohu/1512/2010 | BY | 128                  | –       |              |
| N9   | B/Shanxi Belling/127/2008 | BY | 16                   | –       |              |
| N10  | B/Gansu Chengguan/1118/2008 | BY | 64                   | –       |              |
| N11  | B/Hubei Wujiang/158/2009 | BV | 32                   | –       |              |
| N12  | A/Fujian Tongan/196/2009 | H3N2 | 128                  | –       |              |
| N13  | A/Brisbane/10/2007 | H3N2 | 128                  | –       |              |
| N14  | A/Hubei Jiangan/1139/2009 | H3N2 | 32                   | –       |              |
| N15  | A/Yunnan/1145/2005 | H3N2 | 128                  | –       |              |
| N16  | A/Hubei Beihu/1143/2011 | H3N2 | 64                   | –       |              |
| N17  | A/Liaoning Huanggu/1183/2007 | H1N1 | 256                  | –       |              |
| N18  | A/Jiangxi Donghu/312/2006 | H3N2 | 128                  | –       |              |
| N19  | A/Anhui Baobei/137/2008 | H3N2 | 64                   | –       |              |
| N20  | A/Guangdong Nongan/SWL112/2010 | H1N1Pdm | 128                  | –       |              |

* Signal intensity was determined by comparing with a standard color chart and categorized as weak (+, C7–9), moderate (+++, C4–6) or strong (+++, C1–3) positivity, respectively. “−” indicates a negative test result.

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### Table 3. Detection Limit of Rapid Diagnostic Test and Real Time RT-PCR for H7N9 isolates.

| H7N9 virus isolate | Source | Dilution titers | Labs tested | LOD of RDT | Ct of RT-PCR |
|--------------------|--------|-----------------|-------------|------------|--------------|
| Isolate 1          | Zhejiang | 10⁻¹–10⁻⁵      | Zhejiang*   | 10⁻⁴      | 31.36        |
| Isolate 2          | Zhejiang | 10⁻¹–10⁻⁵      | Zhejiang*   | 10⁻⁴      | 27.21        |
| Isolate 3          | Zhejiang | 10⁻¹–10⁻⁵      | Zhejiang*   | 10⁻⁵      | 29.67        |
| Isolate 4          | Zhejiang | 10⁻¹–10⁻⁵      | Zhejiang*   | 10⁻⁴      | 26.52        |
| A/Anhui/1/2013     | Anhui   | 10⁻¹–10⁻⁷ TCID₅₀ | Beijing #1*  | 10⁻² TCID₅₀ | 30.00        |
| A/Anhui/1/2013     | Anhui   | 10⁻⁵–10⁻⁵ pfu/ml | Beijing #2*  | 10⁻⁷ pfu/ml | 29.00        |
| A/Shanghai/1/2013  | Shanghai | 10⁻⁵–10⁻⁵ pfu/ml | Beijing #2*  | 10⁻¹⁵ pfu/ml | 29.00        |

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### Lateral Flow Immunoassay

H7 directed C1and C2 monoclonal antibodies were used as capture and detector antibodies respectively in the lateral flow immunoassay. C2 (20 ug/ml) was conjugated with 0.01% colloidal gold and sprayed on glass fiber. C1 (0.8 mg/ml) and goat-anti-mouse...
Table 4. Rapid Diagnostic Test of clinical samples from influenza A(H7N9) positive patients.

| Samples    | No. of Patients | Ct of PCR | RDT detection |
|------------|----------------|-----------|---------------|
| Sputum     | 26             | 26.3±4.6  | 16            |
| Throat swab| 14             | 35.0±5.0  | 5             |
| Feces      | 10             | 35.5±5.3  | 2             |
| control line did not appear within 15 min, the test was considered invalid. The signal intensity of the test lines was compared with a standard color chat consisting of 9 grades: weak (+), moderate (++), and strong (+++) positivity represent signal intensity of C7 to C9, C4 to C6 and C1 to C3, respectively.

Real-time RT-PCR

Real-time RT-PCR was carried out using primers and protocols previously described [13]. Briefly, a 25 μl reaction was set up containing 5 μl of template RNA, 12.5 μl of 2 X RT-PCR master mix, 1 μl of 25 X RT-PCR enzyme mix, 0.5 μl of Probe (20 μM), 0.5 μl of each of the primers (40 μM) and 5 μl of RNase-free water. Thermal cycling conditions consisted of 45°C for 10 min, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s, 60°C for 45 s. H7N9 viral RNA and non-H7N9 viral RNA were used as positive and negative controls, respectively while RNase free water was used as blank control.

Results

Analytic Sensitivity and Specificity

We created a panel of recombinant HA proteins with which to determine the specificity of our novel lateral flow immunoassay rapid diagnostic test (RDT). The panel included recombinant HA derived from two N7N9 viruses, A/Shanghai/2/2013 and A/Anhui/1/2013; one H7N9 virus A/Netherlands/219/03; one H5N1 virus, A/Hubei/1/2010; one H3N2 virus, A/Victoria/361/2011; and one H1N1 virus, A/California/06/2009. The limit of detection (LOD) for H7N9 virus HA was 1 ng/ml, and for the H7N7 virus was 10 ng/ml (Table 1). No cross-reactions were observed when testing 1000 ng/ml recombinant HA proteins of H1N1, H3N2, H5N1 (Table 1), or 1000 ng/ml of viral lysates of H1N1, H5N9 and H9N2 viruses (data not shown).

The analytic sensitivity and specificity of the novel RDT was further probed using a quality control panel including a series of dilutions of one H7N9 positive sample and 20 H7N9 negative clinical samples (Table 2). All 7 dilutions of H7N9 sample tested positive by our RDT, and no false positive results were observed for the 20 H7N9 negative samples including inactive H1N1, H3N2 and influenza B viruses (Table 2).

The LOD of our RDT was further characterized by testing a series of diluted H7N9 virus culture supernatants with known TCID50 or pfu/ml (Table 3). Samples with low levels of H7N9 virus (10^3 TCID50 or 10^3.5 pfu/ml) tested positive by our assay (Table 3).

Sensitivity and Specificity when Testing Clinical Samples

A total of 99 samples including throat swab, sputum and fecal samples from 32 laboratory-confirmed H7N9 patients were tested in parallel with both real-time RT-PCR and our RDT immunoassay. The detection sensitivity of our immunoassay was 59.4%
together, our H7 specific assay was more sensitive than the influenza A(H7) viruses [16]. Taken Baas’s results and our study less sensitive for H7N9 virus than for the seasonal or other from 1x10⁵ to 1x10⁵.5 TCID₅₀/mL or 22–24 Ct values, indicating found that the LOD of five of the six RDTs for H7N9 virus ranged their ability to detect H7N9 virus (A/Anhui/01/2013). They RDTs by detecting nucleoprotein antigen of influenza viruses for the low detection limit of about 30 Cts by real-time RT-PCR, our our RDT assay detected 33.3% (33/99) of H7N9 infections PCR, 59.4% (19/32) tested positive by our RDT. Furthermore, all throat swab and sputum clinical specimens from non-H7N9 patients also tested negative.

According to the best of our knowledge, this is the first rapid immunoassay that has been evaluated with H7N9 clinical samples for detection of HA antigen of H7 subtypes. This RDT can be used for rapid diagnosis and epidemiological study of H7N9 infection, which can be subsequently confirmed by virus culture and PCR.

Our study is limited by the small number of clinical samples from H7N9 patients because when we were developing the assay, less than 150 H7N9 patients have been confirmed H7N9 positive since the outbreak. Therefore, the detection sensitivity and specificity of this assay must be further evaluated when more H7N9 patient samples are available. In addition, our rapid assay can only detect HA antigen of H7 subtypes. For correct diagnosis and subtyping of H7N9 virus, neuraminidase (NA) detection should be integrated into the assay. The refinement and improvement of our RDT for detection of H7N9 virus are ongoing.

One interesting finding highlighted by this study is that the H7 directed monoclonal antibodies employed in this assay were very sensitive to and can specifically detect the H7N9 virus in clinical specimens. This observation raises the possibility of developing universal subtype specific assays using subtype specific antibodies. At present, 17 HA subtypes and 10 NA subtypes have been characterized, resulting in a total of 170 possible combinations of various HA and NA subtypes or recombinant influenza viruses [17]. If subtype specific assays could be developed and their usefulness could be approved, assays that are both sensitive and specific to possible new recombinant influenza viruses can be quickly developed in advance of their discovery. Such assays would provide important tools for prevention and control of new influenza epidemics.

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Author Contributions

Conceived and designed the experiments: ST. Performed the experiments: KK LC XZ CQ ZZ WH TJ. Analyzed the data: ST KK JW WL EED YS. Wrote the paper: ST KK EED.

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