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fast track

Molecular Detection of Human H7N9 Influenza A Virus Causing Outbreaks in China

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BACKGROUND: A novel subtype of influenza A virus (H7N9) was recently identified in humans. The virus is a reassortant of avian viruses, but these human isolates contain mutations [hemagglutinin (HA) Q226L and PB2 E627K] that might make it easier for the virus to adapt to mammalian hosts. Molecular tests for rapid detection of this virus are urgently needed.

METHODS: We developed a 1-step quantitative real-time reverse-transcription PCR assay to detect the novel human H7N9 virus. The primer set was specific to the hemagglutinin (*HA*) gene of the H7N9 viruses currently causing the outbreak in China and had mismatches to all previously known avian or mammalian H7 HA sequences. In addition, the assay was evaluated using influenza A viruses of various genetic backgrounds and other negative controls.

RESULTS: The detection limit of the assay was approximately 0.04 $TCID_{50}$ (median tissue culture infective dose) per reaction. The assay specificity was high and all negative control samples, including 8 H7 viruses not closely related to the human H7N9 virus, tested negative.

CONCLUSIONS: The established assay allows rapid detection of the novel human H7N9 virus, thereby allowing better pandemic preparedness.

Influenza A virus is a negative-sense RNA virus with 8 gene segments. The natural reservoir of the virus is believed to be of avian origin. With the exception of bat influenza virus recently detected in bats, all other hemagglutinin (HA)[4] (H1–16) and neuraminidase (NA) (N1–9) subtypes can be detected in aquatic fowls *(1)*. Sporadic transmissions of avian influenza viruses from birds to mammals occur. Some of the viruses from these zoonotic transmission events can become established in the new host populations by acquiring de novo mutations and/or gene reassortments. The H1N1 pandemic of 2009 is a good example in which the pandemic virus was a result of multiple reassortments between avian, human, and swine influenza viruses in pigs *(2)*.

The recent detection of H7N9 virus in humans is of great concern. This novel human subtype was first reported on March 31, 2013. At the time of preparation of this manuscript (May, 2013), there were 128 confirmed H7N9 cases and 24 deaths in eastern China. Sequence analysis suggested that the virus was a reassortment generated from avian viruses circulating in wild birds and ducks *(3)*. H7 influenza viruses can be broadly classified into 3 major lineages, the Eurasian (EA), American, and equine lineages. The HA segment of the human H7N9 virus has emerged from the EA lineage. The H7N9 virus has been detected in poultry and environmental samples obtained in wet markets *(4)*, suggesting that H7N9 virus-infected poultry might be one of the sources of human infections. Strikingly, some of these human H7N9 viruses have mutations that are known to facilitate avian viruses to adapt into mammalian hosts *(3)*. Although epidemiological data do not suggest that the virus can achieve sustainable transmissions between humans, cases caused by limited human-to-human transmissions still cannot be completely ruled out *(5)*. Preliminary experimental data also indicate that the virus is still susceptible to NA inhibitors *(6)*. Thus, early identification of H7N9 patients might be critical to the effectiveness of clinical treatments. In this study, we report the development of a rapid diagnostic test for this novel pathogen.

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[4] Nonstandard abbreviations: HA, hemagglutinin; NA, neuraminidase; EA, Eurasian; GISAID, Global Initiative on Sharing All Influenza Data; RT-PCR, reverse-transcription PCR; $TCID_{50}$, median tissue culture infective dose; BSL3, biosafety level 3.

Materials and Methods

SEQUENCE ANALYSIS AND PRIMER DESIGN

Full-length HA sequences of human H7N9 influenza virus (A/Shanghai/1/2013, A/Shanghai/2/2013 and A/Anhui/1/2013) were obtained from the Global Initiative on Sharing All Influenza Data (GISAID) (http://platform.gisaid.org/). Full-length H7 HA sequences derived from influenza viruses of EA (n = 276), American (n = 371). and equine (n = 22) lineages were downloaded from a public database (Influenza Virus Resource, http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html; data downloaded on April 8, 2013). The downloaded sequences were edited and aligned by BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide frequency at each position of our target sequences was analyzed using WebLogo (http://weblogo.berkeley.edu/logo.cgi). A phylogenetic tree was constructed using MEGA5 *(7)*. The primers and probe sequences were modified from those reported by Tsukamoto et al. *(8)*, with the degenerated primers used in the previous report changed to sequences that were purely specific for the human H7N9 influenza virus.

RNA SAMPLES AND QUANTITATIVE REVERSE-TRANSCRIPTION PCR ASSAYS

Viral RNA from human H7N9 influenza viruses was extracted using a commercial RNA extraction kit, following the manufacturer's instructions (QIAamp Virus RNA Mini Kit, Qiagen). To perform quantitative 1-step reverse-transcription PCR (RT-PCR) assays specific for the human H7N9 virus, viral RNA was amplified by TaqMan EZ RT-PCR core reagents (Applied Biosystems) in a 7500 Sequence Detection system. Briefly, 1 μL purified RNA was amplified in a 25-μL reaction containing 2.5 U rTth DNA polymerase (Applied Biosystems), 5 μL of 5× EZ buffer A, 300 μmol/L of each dNTP (except 600 μmol/L for dUTP), 3 mmol/L manganese acetate, 0.25 U AmpErase UNG, 0.2 μmol/L forward primer (5′-ATAGATAGCA GGGCAGTTGG-3′, corresponding to nucleotides 916–935 of the HA-encoding sequence), 0.2 μmol/L reverse primer (5′-GATCAATTGCCGATTGAGTG-3′, complementary to nucleotides 1137–1156 of the HA-encoding sequence), and 0.2 μmol/L probe (5′ FAM-CCYTCYCCYTGTGCRTTYTG-BHQ1-3′, corresponding to nucleotides 1096–1115 of the HA-encoding sequence). Reactions were first incubated at 50 °C for 2 min, followed by 60 °C for 30 min. After a 5-min denaturation at 95 °C, reactions were thermal cycled 40 times (94 °C for 20 s, 60 °C for 1 min). Serially diluted plasmid DNA containing the target sequence derived from influenza virus A/Anhui/1/2013 was used as the positive control. RNA extracted from avian H7 influenza A viruses (American H7 lineage, n = 1; EA H7 lineage, n = 7), influenza viruses of other HA subtypes (H1–H6 and H8–H12, n = 1 for each subtype), human respiratory samples from patients with seasonal influenza virus infection (n = 5), and human respiratory samples from patients with other respiratory infections (n = 5) were used as negative controls in the test. In addition, the RT-PCR assay was also subjected to an end-point dilution test and the results analyzed by a probit regression model in SPSS (n = 8) *(9)*.

A degenerated primer set (pan-EA H7 forward: 5′-ATMAATAGCAGRGCARTRGG; pan-EA H7 reverse: 5′-GATCWATTGCHGAYTGRGTG-3′) previously shown to be broadly reactive to H7 HA sequences from the EA lineage *(8)* was also evaluated by use of the above RNA samples. With the exception of primers, all ingredients used in this quantitative RT-PCR assay, including the detection probe, were identical to the one described above. The pan-EA H7 HA reactions were first incubated at 50 °C for 2 min, followed by 52 °C for 30 min. After a 5-min denaturation at 95 °C, reactions were thermal cycled 40 times (94 °C for 10 s, 52 °C for 20 s, and 60 °C for 32 s).

Results

PRIMERS AND PROBE SPECIFICITY

A total of 672 full-length H7 HA sequences, including 3 human H7N9 HA sequences, were used in the primer design. The newly developed primer set was designed to be specific for the human H7N9 viruses, but with several mismatches to all H7 HAs from different lineages (Fig. 1, asterisks). With the exception of human H7N9 viral sequences, viral H7 HA sequences that had the closest match to the primer set contained a minimum of 2 mismatches at the forward primer (positions 4 and 18, n = 19). The degenerated probe established by Tsukamoto et al. *(8)* was found to be broadly reactive to EA H7 HA sequences, including those from human H7N9 viruses (Fig. 1).

We also studied a pan-EA H7 primer set designed in a previous study *(8)*. This degenerated primer set was found to match with a great majority of EA H7 HA (data not shown). However, the forward primer was found to have 2 mismatches to the human H7N9 sequences (at positions 4 and 18). Further testing of this primer set also suggested that this primer set failed to detect H7N9 (see below).

EVALUATION OF H7N9 DETECTION ASSAYS USING VIRAL RNA SAMPLES

Viral RNA samples extracted from different human H7N9 isolates (A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013) were tested by the newly es-

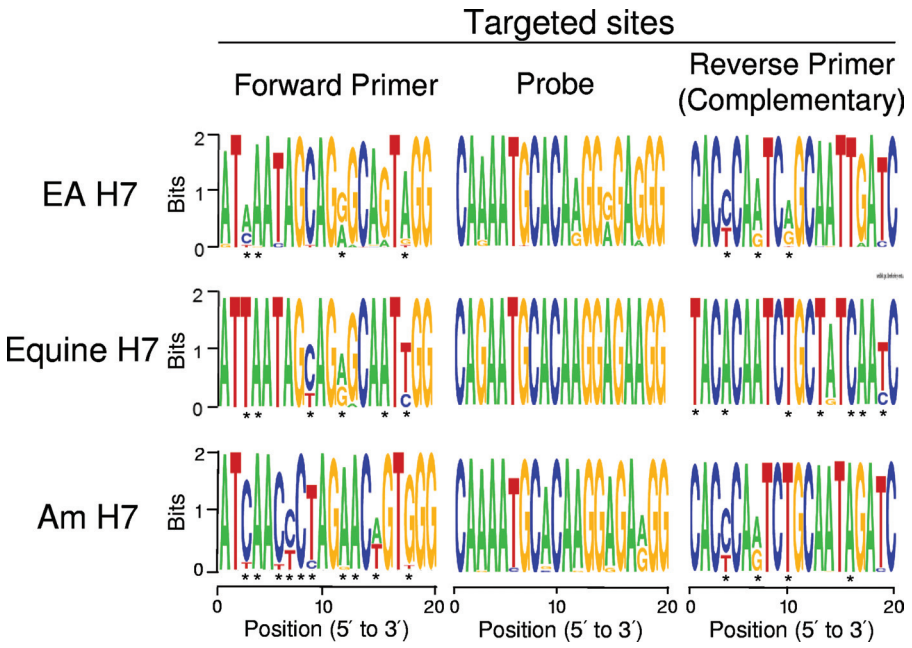

Fig. 1. Nucleotide sequence alignment of EA (upper panel), equine (middle panel) and American (Am) (lower panel) lineages of H7 HA-encoding sequences.

The sequence conservations of all the analyzed sequences in the targeted sites (forward primer, reverse primer, and probe) are graphically summarized in Weblogo format (http://weblogo.berkeley.edu/logo.cgi). The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of the nucleic acid at that position. Asterisks indicate the positions at which the majority of viral sequences would have a mismatch to the primers for human H7N9 detection.

tablished RT-PCR assay. As expected, these human H7N9 isolates were detected by the assay (Fig. 2). In addition, we used a number of control samples to evaluate the analytical specificity of our assay. In particular, RNA samples extracted from other H7 viruses were tested (EA lineage= 7 and American lineage = 1). Among these tested H7 control samples, 4 were phylogenetically related the human H7N9 and 2 of these samples had 3 mismatches to our primers (forward primer, positions 4 and 18, 5′-to-3′ orientation; reverse primer, position 14, 5′-to-3′ orientation; Fig. 3, blue asterisks). None of these H7 control samples was found to be positive in the test (Fig. 2). In addition, RNA samples extracted from other viral HA types (n = 11) and respiratory samples, either with seasonal influenza virus (n = 5) or other respiratory pathogens (n = 5), were all negative in the test. These results indicated the newly established assay to be highly specific to the human H7N9 virus causing the current outbreak in China.

We also used the same panel of the RNA samples to evaluate the performance of the pan-EA H7 quanti-

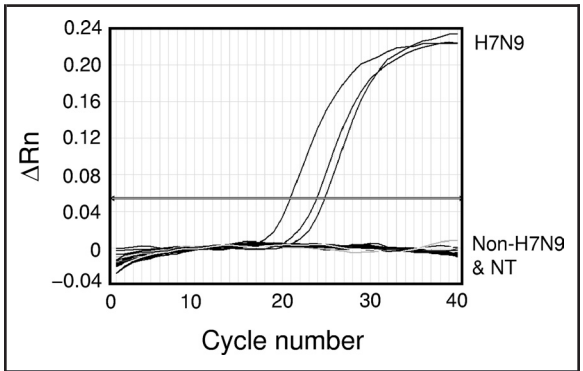

Fig. 2. Real-time RT-PCR assay for the human H7N9 virus.

Signals of RNA samples extracted from human H7N9 isolates (A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013), other H7 viruses, and no template (NT) control are shown as indicated. Rn, normalized reporter.

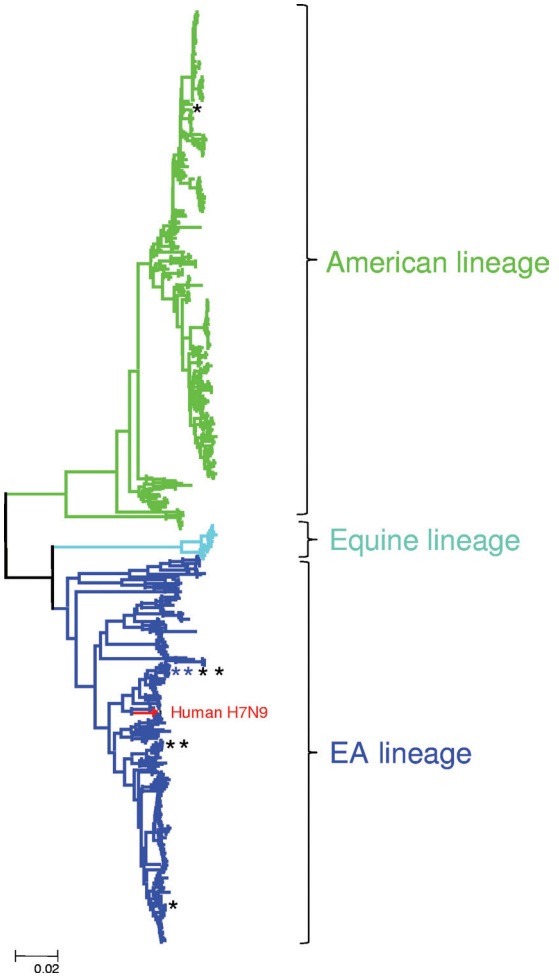

Fig. 3. Phylogenetic analysis of influenza viruses of H7 subtypes.

Phylogenetic trees were constructed by the neighbor-joining method in MEGA (http://www.megasoftware.net/). Branches that are of EA, American, and equine lineages are in blue, green, and cyan, respectively. Branches for the human H7N9 virus are in red. The approximate positions of H7 viral samples used as negative controls are highlighted by asterisks. Bars show the estimated genetic distances of these viruses. Control H7 samples having the closest match to the H7N9-specific primers are highlighted by blue asterisks.

tative RT-PCR assay *(8)*. As expected, all the EA H7 control samples were found to be positive in the test. However, even when a low annealing temperature was used in the reverse transcription and PCR amplification steps, none of the human H7N9 samples was detected by this pan-EA H7 assay.

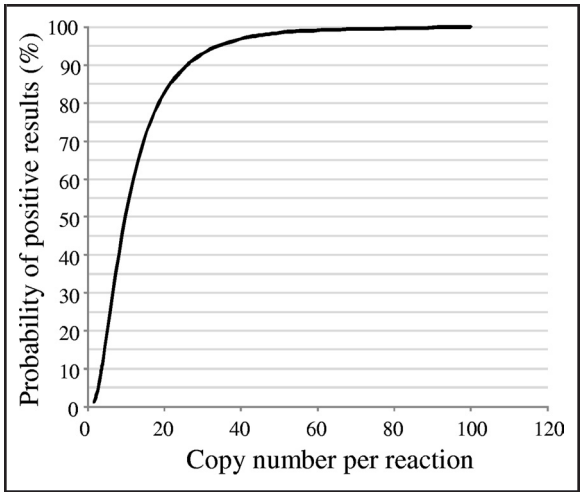

Fig. 4. Probit regression curve of real-time RT-PCR assay for human H7N9 virus.

END-POINT DILUTION TEST AND PROBIT REGRESSION MODEL

A 10-fold dilution series of viral RNA extracted from the human H7N9 isolate (A/Anhui/1/2003) was tested by the newly established quantitative RT-PCR assay. The detection limit of the test was found to be approximately 0.04 median tissue culture infective dose ($TCID_{50}$) per reaction. In addition, we used the quantitative RT-PCR assay to test serially diluted plasmid DNA containing the full-length HA of the H7N9 virus. The newly established assay was efficient (efficiency, 82%; standard curve slope, −3.85; $R^2 = 0.98$) and had a dynamic range of at least 8 orders of magnitude (10^8 to 10 copies/reaction). We also used probit regression analysis to study the data generated from these standard reactions *(9)*. Our preliminary work suggested that the target concentration at which >95% of the assay could be expected to yield positive results was 34 copies per reaction (Fig. 4).

Discussion

In this study, a 1-step real-time RT-PCR assay was developed for detection of the novel H7N9 virus currently causing human infections in China. The assay was highly specific for this virus and did not cross-react with other H7 viruses from the EA and other lineages (Fig. 2). The turnaround time of the whole process for samples is about 3 h, including 2 h for the 1-step RT-PCR assay. These results suggest that the established assay should be suitable for screening H7N9 viruses in human samples. In addition, this test might also be useful as a screening test for detecting this specific H7N9 virus in poultry (see below).

Using viral RNA and DNA plasmid as standards in our test, we found the detection limit of the assay to be approximately 0.04 $TCID_{50}$ and the 95% probit value of test to be 34 copies per reaction. Assuming the H7N9 virus has replication kinetics similar to those of other human influenza virus infections *(10)*, our assay should be analytically sensitive enough to identify patients who have active virus replications. Nonetheless, additional evaluation of this assay using clinical samples from H7N9 patients is needed.

Among the first 3 human H7N9 sequences available in the GISAID, there are some variations in the globular head region of the HA. Although these HA sequences are still highly similar (overall sequence identity >99.2%), the virus is likely evolving rapidly. and there might be multiple introductions of avian H7N9 viruses from animals to humans. Our primers and probe targets are located at the region that is genetically more stable than the region at the globular head of HA. Our assay should, at least in theory, be able to detect viruses closely related to the human H7N9 viruses. As we were developing the assay, additional RNA sequences (n = 12) from H7N9 viruses detected in human, poultry, and environmental samples were released to the database in GISAID and all of these newly released HA sequences still matched 100% with the primer and probe sequences from our study. One should, however, note that the diversity of human/avian H7N9 viruses causing the current outbreak in China is yet to be defined. Thus, the performance of our quantitative RT-PCR for other human H7N9 viruses or their immediate precursor(s) in animals needs to be investigated further.

Similar to other HA subtypes, there is a huge nucleotide variation in H7 HA sequences. Our RT-PCR assay described here is highly specific to the human H7N9 influenza virus, but it is essential to have assays that can broadly react with HA of the same subtypes. The previously developed pan-EA H7 assay would be an important tool to identify EA H7 viruses other than those closely related to the human H7N9 virus *(8)*. We have confirmed that the pan-EA H7 assay can be broadly reactive with multiple EA H7 viruses, except the current human H7N9 virus. Assays of this kind would be useful to identify previously known EA H7 viruses circulating in avian populations. In addition, there have been a number of incidences of human cases caused by EA H7 avian viruses with other NA subtypes *(11)*. Therefore, these previously developed pan-H7 HA assays would still be useful to identify possible human cases caused by other EA H7 viruses in this or other geographical locations *(12)*. Nonetheless, we recommend that human samples suspected to contain nonseasonal human influenza virus be referred to a WHO reference laboratory for further testing.

Accidental release of human pathogens from the laboratory poses a huge public health concern. Since the human H7N9 virus is regarded as a biosafety level 3 (BSL3) pathogen, we intentionally generated a DNA plasmid containing the H7 HA gene as a positive control in our test. We reasoned that this might help minimize the handling of this BSL3 pathogen in laboratories that do not have the recommended biosafety facilities. In addition, this would also help distribute this positive control to laboratories at different geographical regions in a more cost-effective and robust manner. This DNA plasmid control is available upon request.

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