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Short communication

Simultaneous detection of hemagglutinin and neuraminidase genes of novel influenza A (H7N9) by duplex real-time reverse transcription polymerase chain reaction



Yan Li, Tao Wu, Xian Qi, Yiyue Ge, Xiling Guo, Bin Wu, Huiyan Yu, Yefei Zhu, Zhiyang Shi, Hua Wang, Lunbiao Cui*, Minghao Zhou*

Key Laboratory of Enteric Pathogenic Microbiology, Ministry of Health, Institute of Pathogenic Microbiology, Jiangsu Provincial Center for Disease Control and Prevention, Nanjing 210009, China

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A novel reassortant influenza A (H7N9) virus emerged recently in China. In this study, a duplex real-time reverse transcription polymerase chain reaction (rRT-PCR) assay was developed for the simultaneous detection of hemagglutinin (HA) and neuraminidase (NA) genes of H7N9 influenza viruses. The sensitivity of the assay was determined to be 10 RNA copies per reaction for both HA and NA genes. No cross-reactivity was observed with other influenza virus subtypes or respiratory tract viruses. One hundred and forty-six clinical and environmental specimens were tested and compared with reference methods and were found to be consistent. The assay is suitable for large-scale screening due to short turnaround times and high specificity, sensitivity, and reproducibility.

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Since February 2013, a novel reassortant influenza A (H7N9) virus has emerged in China (Gao et al., 2013), which has become a global public health concern (Horby, 2013; Uyeki and Cox, 2013; Wu et al., 2013). Human infections with H7 influenza viruses (H7N2, H7N3, and H7N7) have been reported (Hirst et al., 2004; Nguyen-Van-Tam et al., 2006). Unlike H5N1 viruses, infection with H7N2, H7N3, and H7N7 often cause mild to moderate symptoms in humans, and most of those infections are associated with virus outbreaks in poultry (Belser et al., 2009). A total of 133 human infections including 43 fatal cases have been reported as of July 4, 2013 (WHO, 2013a). The novel H7N9 virus has acquired some characteristic mutations that facilitate viral recognition of human-type receptors and efficient replication in mammals (Kageyama et al., 2013). The early and rapid detection of the virus in patients or in environmental samples is crucial for preventing virus transmission, and for initiating treatment as soon as possible. Detection methods for this novel virus are urgently needed in a large number of public health laboratories. Real-time reverse transcription polymerase chain reaction (rRT-PCR) is a powerful tool for the sensitive and specific detection of viruses. An rRT-PCR protocol

(WHO TaqMan assay) for the detection of H7N9 viruses was provided by the WHO Collaborating Center for Reference and Research on Influenza at the Chinese National Influenza Center on April 8, 2013 (WHO, 2013b). Another rRT-PCR assay was also developed for the specific detection of H7N9 (Corman et al., 2013). Both of these assays are single rRT-PCR methods that detect hemagglutinin (HA) and neuraminidase (NA) genes, respectively. In this study, a duplex TaqMan rRT-PCR assay was developed which can detect the HA and NA genes of the novel H7N9 virus simultaneously.

The forward primer and the probe for the detection of the HA gene are the same as those described in the WHO protocol. One degenerate base was introduced into the reverse primer to minimize nucleotide mismatches. In order to design primers and probes for the detection of NA genes, available N9 gene sequences from GenBank and the Global Initiative on Sharing Avian Influenza Data (GISAID) were aligned using the CLUSTALX multiple alignment program (Thompson et al., 1997). Primer and probe sequences were designed and analyzed for secondary structure formation, G+C content, primer dimer formation, hairpin formation, and their compatibility with multiplex PCR using the software Primer Express 3.0 (Applied Biosystems). Primers and probes were synthesized by Sangon (Shanghai, China) (Table 1).

RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The duplex TaqMan rRT-PCR assay was conducted using the SuperScript III

* Corresponding authors at: Institute of Pathogenic Microbiology, Jiangsu Provincial Center for Diseases Control and Prevention, 172 Jiangsu Road, Nanjing 210009, China. Tel.: +86 25 83759372; fax: +86 25 83759372.

E-mail addresses: lbcui@jscdc.cn (L. Cui), zmh@jscdc.cn (M. Zhou).

Table 1
Primers and probes used in the duplex rRT-PCR assay for the detection of the novel H7N9 virus.

| Primer/Probe | Sequence (5'–3') | Position | References |
|-------------------------|------------------------------------|----------------------|------------|
| H7-Forward | AGAAATGAAATGGCTCTGTCAA | 468–489 ^b | WHO (2013) |
| H7-Reverse ^a | GGYTTTTTCTTGATTTTTATAGACTTAG | 550–521 ^b | |
| H7-Probe | FAM-AGATAATGCTGCATTCGCCGAGATG-BHQ1 | 495–519 ^b | This study |
| N9-Forward | CAAACGGAACAATACAGATAGGT | 422–445 ^c | |
| N9-Forward | TGTACACTGTGGGCGGTGAT | 499–480 ^c | |
| N9-Probe | JOE-CCAGTATCGGCCCTGATAAGCT- BHQ1 | 447–469 ^c | |

FAM, 6-carboxyfluorescein; JOE, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; BHQ1, black hole quencher.

^a Modified from the protocol provided by the WHO.

^b GISAID accession number: EPI439507.

^c GISAID accession number: EPI439509.

Platinum One-Step Quantitative RT-PCR System (Invitrogen). The final optimized reaction mixture consisted of 10 μ L of 2 \times reaction buffer, 0.4 μ L SuperScript III RT/Platinum Taq Mix, 0.8 μ M each of H7 and N9 primers, 200 nM each of H7 and N9 probes, and 4 μ L RNA templates. Duplex rRT-PCR was performed on a Rotor gene 3000 (Corbett) with the following conditions: 50 $^{\circ}$ C for 20 min, followed by 95 $^{\circ}$ C for 2 min, then 45 cycles of 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 45 s. Single rRT-PCR assays were performed according to the protocol provided by the WHO. Fluorescence was recorded at 60 $^{\circ}$ C. The specimen was considered positive if the C_t value was ≤ 38.0 .

The analytic sensitivity of the duplex TaqMan rRT-PCR assay was compared with the WHO TaqMan assay and a commercial single H7N9 rRT-PCR kit (bioPerfectus technologies, Taizhou, China) with a 10-fold dilution series of a nasopharyngeal aspirate (NPA) from a patient infected with the H7N9 virus (approximately 4.8×10^6 copies of the viral genome/mL). Each dilution series was tested in the duplex rRT-PCR assay in triplicate. The analytic sensitivity of the duplex TaqMan rRT-PCR assay for the detection of both H7 and N9 genes was a 10^{-4} dilution (4.8×10^2 copies/mL), which was comparable to that of the commercial H7N9 rRT-PCR kit for detecting both H7 and N9 genes (4.8×10^2 copies/mL), and that of the WHO TaqMan assay for detecting the H7 gene (4.8×10^2 copies/mL). However, it is more sensitive than the WHO TaqMan assay for detecting the N9 gene (4.8×10^3 copies/mL). To determine the actual detection limit (number of copies per reaction) of the duplex TaqMan rRT-PCR assay, *in vitro* RNA transcripts of HA and NA genes from the H7N9 virus were prepared with T7 RNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions using influenza A/Nanjing/1/2013 (H7N9) RNA as a template. The synthetic RNA transcripts were then purified, quantified, and mixed in equimolar amounts, and diluted ten-fold from 10^7 to 10^0 RNA copies/reaction. RNA templates were then tested in the duplex TaqMan rRT-PCR assay. Each concentration was tested in triplicate. The actual detection limit of the assay was found to be approximately 10 copies of synthetic RNA per reaction for both H7 and N9 genes (Fig. 1).

The specificity of the duplex rRT-PCR assay was determined with a panel of respiratory tract viruses including influenza virus A (seasonal H1N1, H3N2, 2009 pandemic H1N1, H5N1, novel H7N9, and H9N2 subtypes), influenza virus B, human rhinovirus, human enterovirus, human respiratory syncytial virus, human parainfluenza virus (types 1, 2 and 4), human metapneumovirus, human coronavirus (229E, NL63, HKU1, and OC43), and human adenovirus. All tested samples were negative except for the novel H7N9 virus.

Intra-assay variation was assessed by testing three separate dilution series (10^7 – 10^0 copies/reaction) of RNA standards within a single run. Inter-assay variation was determined by testing each dilution of the RNA standard in triplicate on three different days. Since only one test for the H7 gene obtained a C_t value (41.2) at the highest dilution (10^0 RNA copies/reaction), we recorded the C_t values that were positive for RNA ranging from 10^1 to 10^7 copies per reaction and calculated the coefficients of variation (CV) and

confidence intervals (CI) separately for each RNA dilution. The mean CVs of intra-assay variability for H7 and N9 genes were 2.05% (95% CI, 0.78–3.32%) and 2.08% (95% CI, 0.69–3.47%), respectively. The mean CVs of inter-assay variability for H7 and N9 genes were 1.85% (95% CI, 0.07–3.63%), and 1.98% (95% CI, 0.14–3.82%), respectively. These results suggest that the duplex TaqMan rRT-PCR assay was sufficiently reproducible.

To evaluate the performance of the duplex rRT-PCR assay, a reference standard was established that combined the results from the viral culture, WHO TaqMan assay and the commercial H7N9 rRT-PCR kit. A sample was determined to be positive for H7N9 virus when at least two of the three reference methods were positive. For the WHO TaqMan assay and commercial H7N9 rRT-PCR kit, a sample was determined to be positive for H7N9 virus when both H7 and N9 genes were detected. A total of 146 specimens including 102 clinical specimens from suspected patients (13 tracheal swabs or wash and 89 nasopharyngeal swabs) in hospitals and 44 environmental specimens in living animal markets (22 poultry cage swabs, 6 poultry water, and 16 poultry feces) were tested using the duplex rRT-PCR assay and reference methods (Table 2). When the results of the duplex rRT-PCR were compared with the combined results of the viral culture, WHO TaqMan assay and commercial H7N9 rRT-PCR kit, they were found to be identical. Both methods identified 27 specimens that were positive for the H7N9 virus. Two clinical specimens and one environmental specimen which tested

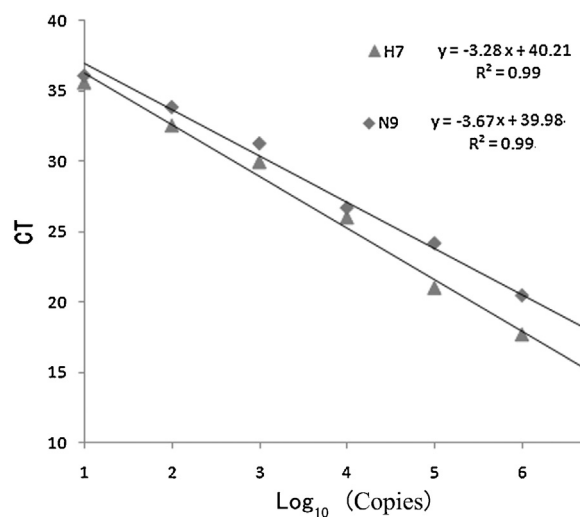


Fig. 1. Sensitivity and dynamic range of the duplex rRT-PCR assay for detection of H7N9 virus. Serial 10-fold dilutions of viral RNA standard (from 10^1 to 10^7 copies) were plotted against the threshold cycle (C_t). The specimen was considered positive if the C_t value was ≤ 38.0 . The minimum detection limit of the assay was found to be approximately 10 copies per reaction for both H7 and N9 genes. The coefficient of determination (R^2) and the equation of the regression curve (y) were calculated. \blacktriangle , H7 gene; \blacklozenge , N9 gene.

Table 2
Comparison of the duplex rRT-PCR assay with viral culture, WHO-recommended TaqMan assay, and the commercial rRT-PCR kit for detecting novel H7N9 virus.

| Methods | No. of clinical specimens (%) | | No. of environmental specimens (%) | |
|------------------------|-------------------------------|-----------|------------------------------------|-----------|
| | Positive | Negative | Positive | Negative |
| Viral culture | 13 (12.7) | 89 (87.3) | 3 (6.8) | 41 (93.2) |
| WHO TaqMan assay | 20 (19.6) | 82 (81.4) | 4 (9.1) | 40 (91.9) |
| Commercial rRT-PCR kit | 22 (21.6) | 80 (78.4) | 5 (11.4) | 39 (88.6) |
| Duplex rRT-PCR | 22 (21.6) | 80 (78.4) | 5 (11.4) | 39 (88.6) |

negative using the WHO TaqMan assay were determined to be positive by both the duplex rRT-PCR assay and the commercial H7N9 rRT-PCR kit.

This novel H7N9 lineage has become a major public health concern. Although sustained person-to-person transmission of the virus has not been reported, there is a risk that mutations in the virus could facilitate this. In fact, the Asp701Asn substitution in the PB2 gene associated with mammalian adaptation is present in human H7N9 isolates (Chen et al., 2013). Molecular diagnostic assays that can specifically detect H7N9 viral RNA are powerful tools to monitor H7N9 virus infections, investigate risk factors, and can provide important information such as the duration of viral shedding, the infectious period, genetic variability, potential virulence, and the spectrum of clinical illness (Uyeki and Cox, 2013). Two currently available rRT-PCR assays are not duplexed and must amplify two to three gene segments separately to identify H7N9 viruses (Corman et al., 2013; WHO, 2013b). Moreover, the WHO TaqMan assay was less sensitive for the detection of N9 genes compared with H7 genes, which makes it difficult to interpret the results when detecting low copies of viral RNA. Although no other H7 viruses are known to circulate in human populations, we could not exclude the possibility of new reassortant viruses emerging in the future, as co-infection with two viruses has been observed recently (Zhu et al., 2013). Therefore, samples were only scored as positive for H7N9 virus when both H7 and N9 genes were detected. The high sensitivity, specificity, and reproducibility of the duplex rRT-PCR assay described in this study was found to be similar to the single rRT-PCR assay, indicating that the duplex RT-PCR assay can serve as a substitute method for the current single rRT-PCR method. In this study, both H7 and N9 genes were detected simultaneously in one tube which provides a cost- and time-saving method for the detection of novel H7N9 viruses and is suitable for large-scale screening purposes. It should be noted that this assay is optimized for human samples, and therefore care must be taken in the interpretation of the results, since H7 or N9 avian influenza viruses from other lineages can also be detected by this assay, especially when applied to environmental specimens. If only H7 genes were detected while N9 genes was not, the result could be explained by the presence of other H7 AIV lineages in the samples, such as H7N2 and H7N7. Likewise, if only N9 genes was detected while H7 genes were not,

the result could be explained by the presence of other N9 viruses in the samples, such as H3N9 and H6N9.

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