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LETTER TO THE EDITOR

Development of the real-time RT-PCR detection system for determination of pandemic influenza A (H1N1) virus

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Pandemic influenza A virus 2009 H1N1 (pH1N1) initially called as the swine flu virus was recognized in Mexico (*1*) and since then, it has affected the global public health-care system (*2*). PCR primer sets used for the identification of seasonal influenza virus was not applicable for the identification of current pH1N1 (*3*). Development of a reliable PCR detection system with high sensitivity for the identification of pH1N1 virus is needed, although some PCR methods for the identification of pH1N1 have been already published (*46*). In this study, we developed a simple, relatively fast, and reliable real-time RT-PCR (rRT-PCR) system that was based on the previously published guidelines established by WHO (*3*). The detection system allowed rapid screening of the clinical samples containing influenza A virus and was suitable for the subsequent identification of pH1N1 subtype.

The virus strains used in this study included seasonal (H1N1, H3N2, B), swine (H1N1, pH1N1) and avian influenza viruses isolated from humans (H5N1, H9N2). The viruses were propagated in MDCK cells or embryonated eggs and viral titers were determined using the hemagglutinin assay. Viral suspension in volume 100 μl was used for the extraction of viral RNA using automatic machine M48 (Qiagen). Transcribed matrix (M), hemagglutinin (HA), and nucleoprotein (NP) RNAs of influenza virus A/Califonia/9/2009 (H1N1) were prepared using the Riboprobe *in vitro* transcription system (Promega). RNA copy number was determined according to the method reported earlier (*7*). For identification of the virus, we used primers and probes targeting M gene of influenza A virus (*8*), NP gene of swine influenza A virus, and HA gene of pH1N1, respectively. Sequence homology analyses were performed between the candidate primers and reference isolates including the avian, swine, and seasonal influenza viruses. The degenerate bases should replace those bases, which present incompatibility in the individual primer and probe sites. Two primer sets (SWH1-1~2) targeting the HA gene and 4 primer sets (SWNP-1~4) targeting the NP gene were selected as candidates. To test the efficiency of primer and probe sets, we used a cross-detection method to examine reference viruses using QuantiTect™ Probe RT-PCR Kit (Qiagen). Firstly, RT reaction was completed by 1 cycle at 50°C for 30 mins. Next, target genes were amplified by 1 cycle at 94°C for 15 mins and 45 cycles at 94°C for 15 secs, 55°C for 30 secs, and 72°C for 30 secs, each. The results showed that the primer and probe sets did not cross-react with the seasonal or avian influenza viruses except for the SWH1-2 and SWNP-4 sets that presented cross-reaction with the strains 1 and 3 of H3N2 virus, respectively. In addition, the SWH1-1 and SW-NP1 primer sets were effective enough for the identification of swine influenza H1N1 and pH1N1 viruses due to the relatively low Ct value. The selected primer and probe sets for identifying the pH1N1 included InFluA (F: 5′-GACCRATCCTGTCACCT CTGAC, R: 5′-GGGCATTYTGGACAAAKCGTCTACG, Probe: 5′-FAM-

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Abbreviations: CV = coefficient of variation; HA = hemagglutinin; HAU = hemagglutinin assay unit; M = matrix gene of influenza virus; NP = nucleoprotein; pH1N1 = pandemic influenza A virus 2009 (H1N1); rRT-PCR = real-time RT-PCR

TGCAGTCCTCGCTCA CTGGGCACG-BHQ1), SWNP-1 (F: 5′-TCMGACATGCGAACRGAAGTT, R: 5′-GGGYT CGTTGCCTTTTCGT, 5′-FAM-CCAGAAGATTTGTCCT TCCA-BHQ1) and SWH1-1 (F: 5′-ACATTCGAAGCAACT GGAAA, R: 5′-GTRTTRCAATCGTGGACTGG, Probe: 5′-FAM-TCCATTGCGAAKGCATATCTCGG-BHQ1). Three specific detection reactions for influenza virus were performed to identify influenza A, swine influenza A, and pH1N1 virus. Each primer set was processed into a reaction mixture with reverse transcriptase and thermostable DNA polymerase. The experiment was performed by the Chinese Beijing Kinghawk Pharmaceutical Co. that specializes in rRT-PCR diagnosis. The sensitivity of the combined detection system for pH1N1 virus was evaluated by detection of *in vitro* transcribed RNA (10-fold serially diluted) and viral RNAs that were extracted from strain 2 of H1N1 viruses A/swine/China/216/1991 with titer of 32 HAU/50 µl and A/Califonia/9/2009 with titer of 64 HAU/50 µl. Samples were incubated at 50°C for 30 mins, then at 94°C for 3 mins, and thermal-cycled for 45 cycles at 94°C for 10 secs and 55°C for 40 secs, each. The results showed that the sensitivity of 3 primer sets could be detected at 5~50 copies or 10^{-5} diluted swine influenza virus RNA or 10^{-7} diluted novel influenza virus RNA.

The intra assay reproducibility was validated by the coefficient of variation (CV) for Ct value of 10-fold dilutions from 10^{-2} to 10^{-6} of H1N1 virus RNA (A/swine/china/216/1991) tested by 4 different detection groups A, B, C, and D. For SWNP-1, the sensitivity of group C was at 10^{-6}, which was higher than the sensitivity of group A, B, and D by 10^{-5}. For SWH1-1, group A and C had similar sensitivity that was at 10^{-5} compared to the sensitivity of group B and D at 10^{-4} (Table). To simulate positive samples with a wide range of concentrations, the CV for SWNP-1 was calculated using four dilutions (10^{-2}-10^{-5}) of the A/swine/China/248/1991 H1N1 viruses. The CV of SWNP-1 for each dilution was 3.66, 3.45, 2.73, and 1.97%, respectively. Additionally, the CV of SWH1-1 was 2.96, 2.26, and 1.03%, what corresponded to 10^{-2}, 10^{-3}, and 10^{-4} dilution, respectively.

Table. Ct values of reproducibility test

Variation	Group A	Group B	Group C	Group D
SWNP-1				
10^{-2}	18.75	19.5	18.82	17.83
10^{-3}	22.93	23.87	23.9	22.24
10^{-4}	27.17	27.97	28.34	26.68
10^{-5}	30.71	31.94	31.83	32.06
10^{-6}	–	–	34.2	–
SWH1-1				
10^{-2}	19.46	20.56	20.05	19.27
10^{-3}	23.96	25.24	24.89	24.43
10^{-4}	28.48	28.88	29.14	28.59
10^{-5}	31.97	–	33.00	–
10^{-6}	–	–	–	–

For clinical validation, 37 clinical samples including throat swab (n = 17), nasopharyngeal swab (n = 17), nose swab (n = 1), and nasopharyngeal aspirate (n = 2) were detected by both the developed rRT-PCR system and viral isolation with MDCK cell culture. The results showed that 32 out of 35 rRT-PCR positive samples (91.43%) were positive also by virus isolation. Two remaining samples tested by the developed rRT-PCR and viral isolation were negative in both tests. All samples were positive on ribonuclease P gene that indicated the absence of false-negative results. Additionally, 368 clinical throat swab samples consisted of 4 groups were examined by the developed rRT-PCR system. No positive samples were detected in the groups of people with close contact to pH1N1 cases without influenza like symptoms (n = 152), patients with fever without contact history (n = 48), and healthy people (n = 80). InfluA-positive samples (n = 102) for seasonal influenza were identified using specific primers provided by CDC. Finally, 109 clinical throat swab samples were detected by our developed assay and WHO-released rRT-PCR detection system (*9*). The results showed that 33 samples were influenza A positive by both WHO-released and newly developed detection system. Further, 24 out of 29 samples positive by present developed SWNP-1 primer set were positive also by the WHO-released swInfA primer set. Curiously, 24 samples that were positive by the presently developed SWH1-1 primer set were negative by the WHO-released swH1 primer set. The negative results by both detection systems were obtained in 76 samples. The consistent ratios of the 3 primer sets between WHO-released and developed detection systems are 100%, 95.41%, and 77.98%, respectively.

The presented data suggested that the developed rRT-PCR assay is highly specific and sensitive. The InFluA primer set can detect selected influenza A viruses including avian, seasonal, swine, and pH1N1 viruses. The SW-NP1 and SWH1-1 primer sets can react just with the swine and pH1N1 influenza virus. Additionally, our test has good reproducibility as shown by the low CV. The suitability of the rRT-PCR test described in this study as a diagnostic tool for pH1N1 virus was confirmed by testing clinical samples. It is obvious that presently developed system can identify pH1N1. Besides, there is a high consistency between the WHO-released and the developed detection systems. Moreover, the newly developed system could compensate for the lower sensitivity of WHO-released H1 identification.

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References

1. Centers for Disease Control and Prevention (CDC), MMWR Morb. Mortal Wkly Rep. 58, 400–402, 2009.
2. Cox A, Guglielmetti P, Coulombier D. Euro Surveill, 14, pii: 19397, 2009.
3. World health organization. http://www.who.int/csr/disease/swineflu/swineflu_guidance_labs_20090425.pdf, 2009.
4. Pabbaraju K, Wong S, Wong AA, J. Clin. Microbiol. 47, 3454–3560, 2009. doi:10.1128/JCM.01103-09
5. Poon LL, Chan KH, Smith GJ, Clin. Chem. 55, 1555–1558, 2009. doi:10.1373/clinchem.2009.130229
6. Wang R, Sheng ZM, Taubenberger JK, J. Clin. Microbiol. 47, 2675–2677, 2009. doi:10.1128/JCM.01087-09
7. Fronhoffs S, Totzke G, Stier S, Mol. Cell Probes 16, 99–110, 2002. doi:10.1006/mcpr.2002.0405
8. Wen LY, Xu H, Lan Y. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 20, 24–26, 2006.
9. Fouchier RA, Bestebroer TM, Herfst S, J. Clin. Microbiol. 38, 4096–4101, 2000.