PCR for Detection of Oseltamivir Resistance Mutation in Influenza A(H7N9) Virus

Wei Wang,[1] Zhigang Song,[1] Wencai Guan,[1] Yi Liu, Xiaonan Zhang, Lei Xu, Jianhua Li, Zhenghong Yuan, and Yunwen Hu

Sensitive molecular techniques are needed for rapid detection of the R292K oseltamivir-resistant mutant of influenza A(H7/N9) virus strain to monitor its transmission and guide antiviral treatment. We developed a real-time reverse transcription PCR and single nucleotide polymorphism probes to differentiate this mutant strain in mixed virus populations in human specimens.

An outbreak of human infections with a novel reassortant avian-origin influenza A(H7N9) virus occurred in several provinces of China during March 2013 (*1*), This outbreak caused 137 laboratory-confirmed cases and 45 deaths as of October 2013 (www.who.int/csr/don/2013_10_24a/en/index.html). An unusually high proportion of severe cases and a high case-fatality rate have been observed for patients infected with this virus (*2*).

We reported emergence of an influenza virus with a mutation in the neuraminidase (NA) gene (R292K) and its association with severe clinical outcome in infected persons (*3*). Studies have shown that the NA R292K mutation can cause a high level of resistance to oseltamivir in influenza A(H7N9) virus (*4,5*). Thus, sensitive molecular techniques are needed for rapid detection of influenza virus with this mutation to monitor its circulation and transmission and guide antiviral treatment. In this study, we developed a single-nucleotide polymorphism (SNP) real-time reverse transcription PCR (RT-PCR) to differentiate NA 292K mutant virus from R292 wild-type virus in clinical samples.

The Study

The NA R292K assay has 2 reactions with 1 pair of primers. One reaction contained a FAM-labeled SNP probe specific for the 292K mutant strain and a second reaction contained a VIC-labeled probe specific for the R292 wild-type strain (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1364-Techapp1.pdf).

To assess the sensitivity of the assay, we constructed 2 plasmids that contained R292 wild-type virus or 292K mutant virus, respectively. Fragments of the NA gene inserted into the plasmids were amplified from nasopharyngeal swab specimens from 2 patients infected with influenza A(H7N9) virus and confirmed by using Sanger sequencing (online Technical Appendix). The 2 plasmids were serially diluted 10-fold (10^1–10^{11} copies) in sterile water and used to test the assay. The linear range of sensitivity was 10^2–10^8 copies. The lower limit of detection was 100 copies/reaction (3/3 reactions detected) for wild-type and mutant virus (Figure). However, the sensitivity of the duplex reaction containing both probes was 100-fold lower than that of each separate reaction.

Of 35 respiratory samples tested, 6 were infected with influenza A(H3N2), 2 with influenza A(H1N1) virus, 6 with influenza A(H1N1)pdm09 virus, 4 with parainfluenza virus, 4 with human rhinovirus, 4 with human coronavirus, 5 with influenza B virus, and 4 with respiratory syncytial virus. In addition, 6 other respiratory samples were virus negative. Cross-amplification was not observed during sample testing. Thus, the assay is highly specific for detecting the mutant NA gene of influenza A(H7N9) virus.

To test the performance of the assay when 292K mutant and R292 wild-type viruses were present in 1 sample, a series of mixtures containing the 292K plasmid and the R292 plasmid at copy numbers of 10^4 copies/reaction were prepared at the following ratios of mutant virus to wild-type virus: 2:98, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:5, and 98:2. The $\Delta C_t K - R$ of the mixture a ratio of 50:50 was used as the assay-specific normalization value in determination of the percentage of 292K mutant in mixed population as described by Liu et al. (*6*). The assay detected the 292K mutant in the mixture at a proportion of 2% of the 10^4 copies/reaction, and correct estimation of its proportion ranged from 10% to 98% (online Technical Appendix).

To validate the assay with clinical samples, we tested 11 paired nasopharyngeal swab specimens and sputum specimens obtained from 9 patients infected with influenza A(H7N9) virus who had various disease outcomes (Table). The time of sampling (mean 12.6 days, range 7–20 days) was at the end of treatment with an NA inhibitor (oseltamivir or peramivir) or afterwards. Eleven of 22 samples were positive for influenza A(H7N9) virus by a quantitative real-time RT-PCR described in a previous study (*3*). Seven of 11 samples had positive results in the R292K assay:

Author affiliation: Shanghai Public Health Clinical Center, Shanghai, China

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[1]These authors contributed equally to this article.

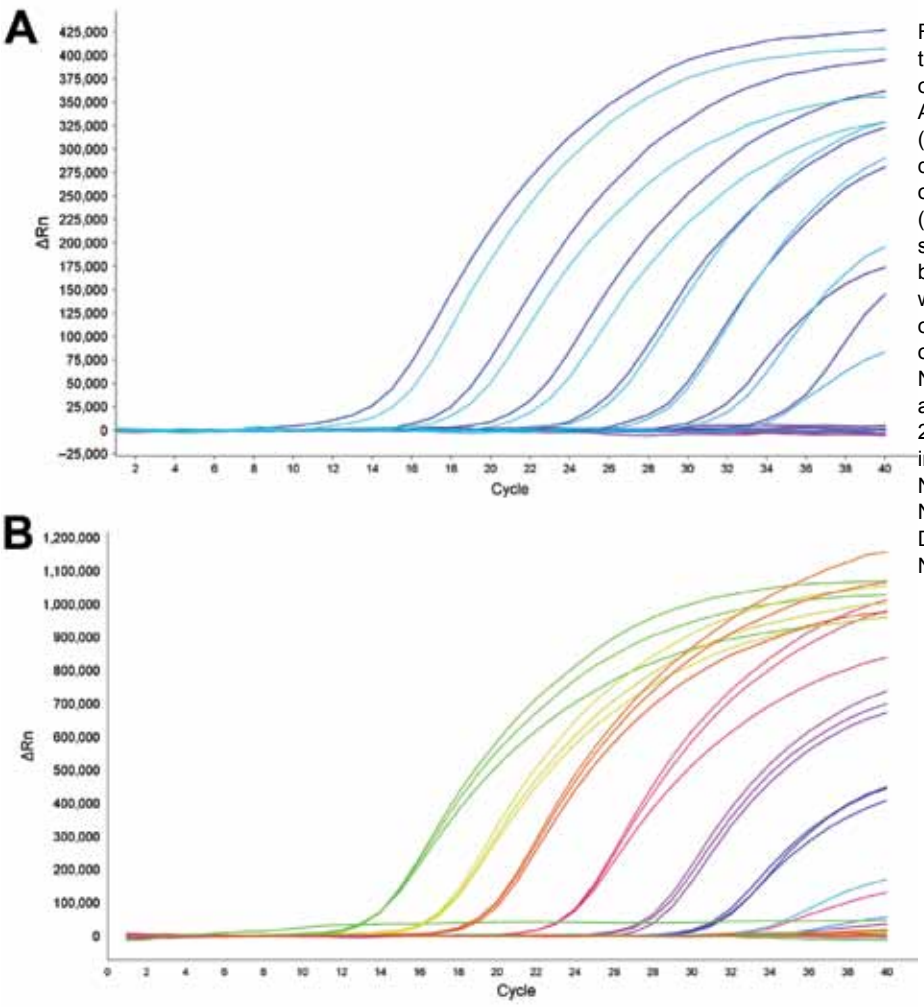

Figure. Dynamic range of reverse transcription PCR for detection of oseltamivir resistance in influenza A(H7N9) virus. Amplification curves (ΔRn vs. cycle number) for serial dilutions of plasmid with 292K (mutant) or R292 (wild-type) neuraminidase (NA) fragments. ΔRN is change in signal magnitude (reporter signal minus baseline signal). Assay dynamic range was linear at template concentrations of 10^2–10^8 copies/reaction. A) Detection of NA 292K mutant strain with probe N9-K: slope = -3.388, R2 = 0.997. Light and dark blue curves indicate probe NA 292K in duplicate wells. Violet curves indicate control wells. B) Detection of NA R292 wild-type strain with probe N9-R: slope = –3.672, R2 = 0.992. Different colored curves indicate probe N9-R in triplicate wells.

5 samples positive in the 292K assay and 2 samples positive in both assays. Four of 11 samples were negative in both assays. All 292K-positive samples were further confirmed as positive by Sanger sequencing of NA genes.

The 7 samples that contained the 292K mutant were obtained from 4 patients: 2 patients who died (patients 2 and 3) and 2 patients who recovered (patients 8 and 10). In our previous study, sequencing of the NA gene was not successful for the first throat swab specimens from patients 8 and 10 because of low viral load (*3*). However, in this study, the 292K mutant was found in sputum specimens from these 2 patients on days 7 or 15, respectively, after initiation of antiviral treatment. This finding suggested that influenza A(H7N9) virus mutated under the pressure of antiviral treatment, which led to failure of the virus to clear the lower respiratory tracts. These 2 patients, who were infected with the drug-resistant mutant virus, recovered from their diseases, which suggested that host immune response might play a major role in controlling the mutant virus.

Conclusions

Higher viral load in sputum samples indicated that there might be factors, including hemagglutinin (HA) binding preference, which favor greater replication in the lower respiratory tract. A similar phenomenon was observed in patients infected with the HA D222G mutant of influenza A(H1N1)pdm09 virus; this virus showed preferential replication in the lower respiratory tract and this infection was correlated with severe outcomes or deaths (*7*).

We have developed an SNP real-time RT-PCR for detection of a drug-resistant NA R292K mutant of influenza A(H7N9) virus. The sensitivity of the assay is lower than that of an HA7-specific real-time RT-PCR (i.e., 4 samples positive for influenza A(H7N9) virus were not detected by this RT-PCR). However, as a screening tool, this assay is sensitive, specific, fast, and inexpensive.

This assay had a detection threshold of 10% for a mutant strain in a mixed viral population, which is more sensitive than Sanger sequencing (detection threshold of 25% for a minor component in a mixed viral population) (*6*).

Table Detection of wild-type neuraminidase R292 and mutant 292K influenza A(H7N9) virus in clinical samples from 9 patients in China, by reverse transcription PCR*

Patient no.†	Outcome	Sample type	Time after oseltamivir treatment started, d	Time after oseltamivir treatment ended, d	292K:R292 ratio	Viral load, copies/mL‡
2	Died	NPS	9	−1	100:0	1.78×10^5
		S	9	−1	100:0	8.76×10^4
		NPS	10	−2	100:0	6.14×10^4
		S	10	−2	25.7:74.3	1.29×10^4
3	Died	NPS	13	3	–	ND
		S	13	3	–	3.03×10^3
		NPS	16	6	–	ND
		S	16	6	100:0	1.21×10^4
5	Died	NPS	7	−4	–	9.96×10^2
		S	11	0	–	ND
6	Recovered	NPS	16	−3	–	ND
		S	19	0	–	ND
7	Recovered	NPS	13	0	–	ND
		S	13	0	–	ND
8	Recovered	NPS	7	0	–	ND
		S	7	0	100:0	1.76×10^4
10	Recovered	NPS	11	−4	–	ND
		S	15	0	91.7:8.3	5.25×10^4
15	Died	NPS	20	4	–	ND
		S	20	4	–	5.62×10^3
17	Recovered	NPS	11	0	–	ND
		S	11	0	–	3.06×10^4

*NPS, nasopharyngeal swab specimen; S, sputum specimen; –, no ratio because samples had negative results; ND, not detected.
†Patient identification was identical with that used by Hu et al. (*3*). Patients 15 and 17 are initially reported in the current study.
‡Method used to determine viral load was reported by Hu et al. (*3*).

This assay will help clinicians monitor emergence of drug-resistant virus strains during treatment of patients with NA inhibitors to prevent persistent viral replication and severe inflammatory reactions.

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Dr Wang is an associate professor in the Pathogen Diagnosis and Biosafety Department, Shanghai Public Health Clinical Center affiliated with Fudan University, Shanghai, China. Her primary research interest is genomic features of respiratory pathogens.

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Address for correspondence: Yunwen Hu, Pathogen Diagnosis and Biosafety Department, Shanghai Public Health Clinical Center, Fudan University, No. 2901 Caolang Rd, Jinshan District, Shanghai, China; email: ywhu0117@126.com

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Technical Appendix

Samples and Sample Collection

A total of 11 paired nasopharyngeal swab specimens and sputum specimens were collected from 9 patients who were given a diagnosis of infection with influenza A(H7N9) virus at the Shanghai Public Health Clinical Centre, Shanghai, China, during April 4–27, 2013. Sputum samples treated with 4% sodium hydroxide solution and nasopharyngeal swab specimens were placed in virus transport medium (minimum essential medium containing 2% fetal bovine serum, 5% penicillin/streptomycin, and amphotericin B) immediately after collection and subsequently stored at −80°C. All the samples were detected by using a hemagglutinin 7–specific real-time reverse transcription PCR (RT-PCR) to determine viral load, as described by Hu et al. (*1*).

Plasmid Construction

Template RNA was extracted from nasopharyngeal swab specimens of patients infected with influenza A(H7N9) virus by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's instructions. Extracted RNAs were amplified by using primers N9-NA346F and N9-NA925R, which are specific for the neuraminidase (NA) gene of influenza A(H7N9) virus (online Technical Appendix Table 1). The PCR products were inserted into the pMD18-T vector (TaKaRa, Dalian, China), and clones were sequenced by using primers N9-NA697F and N9-NA943R (online Technical Appendix Table 1). Clones containing specific fragments with NA R292 or292

K (N2 numbering) were used to generate plasmid standards, which were serially diluted 10-fold (10^1–10^{11} copies) in sterile water and used in assay validation.

Single-Nucleotide Polymorphism Real-Time RT-PCR

Sequences of influenza A(H7N9) viruses from the Global Initiative on Sharing Avian Influenza Data database were aligned by using PrimerExpress software version 3.0 (Applied Biosystems, Foster City, CA, USA). One pair of primers and 2 minor groove–binding probes were designed to discriminate the codon for lysine (AAG) at position 292 in the NA gene for the mutant virus strain and the codon for arginine (AGG) at position 292 in the NA gene for the wild-type virus strain (online Technical Appendix Table 1). The assay was composed of 2 reactions: 1 contained the FAM-labeled probe specific for the 292K mutant strain and 1 contained the VIC-labeled probe specific for the R292 wild-type strain.

Viral RNAs were extracted by using QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacture's instructions. The reaction was performed by using the One-Step Fluorescent Quantitative RT-PCR Kit (TaKaRA). Real-time RT-PCRs were performed in a 25-μL reaction volume containing 12.5 μL of reaction buffer, 5 μL of viral RNA, 400 nmol/L of each primer, and 200 nmol/L of probe. The reaction program was 42°C for 10 min; 95°C for 30 s; 5 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 30 s; and 40 cycles at 95°C for 10 s and 60°C for 40 s. The assay was performed by using the ABI StepOne Plus System (Applied Biosystems), and data were processed by using StepOne software version 2.2 (Applied Biosystems).

Results were considered positive if the cycle threshold (C_t) values were <35 cycles and the ΔRn values (representing changes in the linear amplification plot of the fluorescent signal) were >30,000 for the FAM-labeled probe and 11,000 for the VIC-labeled probe. The baseline and threshold values were set by default. Cross-hybridization was not

observed between the 292R probe and the 292K plasmid or the 292K probe and the 292R plasmid.

Detection of Mixed Population by Using Quantified Plasmid Mixtures

To test the ability of the assay to detect the 292K mutant when both variants are present at various ratios and different viral loads, we prepared mixtures containing the 292K plasmid and the R292 plasmid, respectively, at copy numbers of 10^4 at the following ratios: 2:98, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:5 and 98:2. All mutant:wild-type mixtures were analyzed, and results are shown in online Technical Appendix Table 2. The $\Delta C_t K - R$ value for the mixture at a ratio of 50:50 was used as the assay-specific normalization value in determining the percentage of 292K mutant in mixed populations as dscribed by Liu et al. (*2*). The percentage of the 292K population in the sample was calculated by using the formula of Liu et al. (*2*): $\Delta C_t K - R = C_t K - C_t R - \Delta C_t k50$, where $C_t K$ is the threshold cycle for 292K detection, $C_t R$ is the threshold cycle for R292 detection, and $\Delta C_t k50$ is the $\Delta C_t K - R$ value of the R:K mixture with 10^4 copies at a ratio of 50:50, which was used for normalization. The percentage of 292K on the population was $1/(2^{\Delta C_t k-R} + 1) \times 100$.

Patients 15 and 17

Patient 15 was a 58-year-old man who was admitted to the Shanghai Public Health Clinical Centre on April 7, 2013, because of fever and cough with white sputum for 10 days and polypnea for 5 days. At admission, he had a temperature of 39°C, a pulse rate of 96 beats/min, and a respiration rate of 26 breaths/min. He had hypertension as an underlying condition. Influenza A(H7N9) virus was detected in throat swab specimens on the day of admission. The patient had received oseltamivir, 75 mg twice a day, on April 5. He was then given oseltamivir, 150 mg twice a day, on April 13–15, and peramivir on April 16, He was also given corticosteroids during April 5–26. Extracorporeal membrane

oxygenation was used on June 1 just before the patient died of multiple organ dysfunction. Influenza A (H7N9) virus was weakly detected by quantitative PCR in a throat swab specimen obtained 48 days after admission of the patient.

Patient 17 was a 67-year-old man who was admitted to the Shanghai Public Health Clinical Centre on April 21, 2013, because he had exhibited cough and fever for 8 days. At admission, he had a temperature of 38.5°C, a pulse rate of 76 beats/min, and a respiration rate of 26 breaths/min. Laboratory test showed that he was infected with influenza A(H7N9) virus 1 week before admission. The patient had received oseltamivir, 75 mg twice a day, since April 17. He was then given a double dose of oseltamivir, 150 mg twice a day, during April 21–27. Influenza A(H7N9) virus was not detected in throat swab samples obtained after 15 days of treatment with oseltamivir.. He was considered cured and discharged on May 10.

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Technical Appendix Table 1. Primer and probe sequences designed for detection of the NA R292K mutation and clone construction for influenza A(H7N9) virus*

Procedure	Primer or probe	Sequence, 5→3′	Nucleotide location†
NA R292K assay	N9-NA827F	CATGTTACGGGGAACGAACAGG	827–848
	N9-NA894R	TGGTCTATTTGAGCCCTGCCA	874–894
	N9-K	(FAM)-CACATGC**AAG**GACAA-(MGB)	858–872
	N9-R	(VIC)-CACATGC**AGG**GACAA-(MGB)	858–872
Sanger sequencing	N9-NA697F	TGTGTATGCCACAACGGYGTATGCC	697–703
Clone construction	N9-NA346F	GTCACAAGRGARCCTTATGT	346–366
	N9-NA925R	GTGTCATTGCYACTGGRTCTATC	903–925

*NA, neuraminidase. The single-nucleotide polymorphism position is indicated in in **boldface**.

†Numbering is according to the reference sequence A/Shanghai/4664T/2013(H7N9) (GenInfo accession no. KC853231).

Technical Appendix Table 2. Proportion estimation of neuraminidase 292K mutant in influenza A(H7N9) virus mixtures prepared by using quantified R292 and 292K plasmids at copy numbers of 10^4 per reaction*

			% of 292K mutant†	
292K:R292 ratio	C_tK value, mean (SD)	C_tR value, mean (SD)	Mean (SD)	95% CI
2:98	30.4 (1.2)	22.3 (0.1)	0.9 (0.6)	−0.7 to 2.4
5:95	26.8 (0.9)	19.1 (1.5)	0.9 (0.3)	0.1–1.7
10:90	25.4 (0.1)	21.7 (0.4)	12.1 (1.8)	7.6–16.5
20:80	24.7 (0.1)	21.8 (0.2)	20.2 (2.1)	15.0–25.4
30:70	24.6 (0.2)	22.5 (0.2)	29.9 (2.6)	23.6–36.2
40:60	24.1 (0.2)	22.7 (0.1)	40.4 (3.9)	30.9–50.0
50:50	23.7 (0.2)	23.1 (0.1)	55.2 (0.8)	53.3–57.0
60:40	23.9 (0.2)	24.0 (0.2)	67.9 (1.4)	64.4–71.4
70:30	23.6 (0.4)	24.3 (0.3)	74.2 (3.3)	65.9–82.5
80:20	24.0 (0.3)	25.2 (0.3)	80.7 (2.8)	73.7–80.7
90:10	28.7 (0.5)	31.4 (1.3)	91.9 (4.0)	82.0–101.8
95:5	23.6 (0.4)	26.3 (0.7)	92.3 (2.5)	86.3–98.4
98:2	23.4 (0.1)	28.5 (0.1)	98.4 (0.1)	97.3–99.6

*C_tK, cycle threshold for 292K detection; C_tR, cycle threshold for R292 detection.

†Percentage indicates calculated ratio of mutant strain or wild-type strain in mixture population. The method of calculation is as described by Liu et al. (*2*).