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SHORT COMMUNICATION

Pyrosequencing reveals an oseltamivir-resistant marker in the quasispecies of avian influenza A (H7N9) virus

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Prompt diagnosis of an oseltamivir-resistant marker is important for patient management, in particular to prevent the spread of resistant strains in the recent human H7N9 outbreak. We tailored a pyrosequencing assay to reveal neuraminidase R292K, a resistant marker found in one isolate from China, and demonstrated its performance in both sensitivity and specificity. In addition, a semi-nested polymerase chain reaction was applied, which enhanced the detection rate by at least 10-fold. We validated this assay by examining the marker in Taiwan's first imported human case and found R and K in quasispecies.

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Introduction

The emerging avian influenza A (H7N9) virus has been reported to infect humans in China since February 2013.[1] Taiwan is located in proximity to mainland China, and is closely connected in business; therefore, Taiwan is highly alerted to any reported cases of this disease. The Taiwan Centers for Disease Control (CDC) and its collaborating laboratories of virology began a real-time polymerase chain reaction (PCR)-based influenza A H7N9 virus surveillance for H7N9 probables from pneumonia cases with unspecified pathogens. The laboratories examined clinical samples including throat swabs, sputum, and tracheal aspirates. On April 24, 2013, a sputum specimen collected from a patient in Taipei City, Taiwan tested positive for H7N9.[2,3]

Several studies reported that E119V, I222V, R224K, R292K, and N294S are markers for N2 in reducing oseltamivir susceptibility.[4–6] N9 is known to be genetically connect to N2.[7] Although most H7N9 human isolates thus far do not contain any of these markers, one isolate from Shanghai, China showed 292K in its neuraminidase (NA) gene. We further examined this marker by NA pyrosequencing of the first Taiwanese sputum specimen and also located R292K in the quasispecies of the virus population.

Materials and methods

Clinical specimen and RNA extraction

The sputum specimen submitted to the Taiwan CDC, which was the first laboratory-confirmed case of avian influenza A (H7N9) virus infection in Taiwan, was used for this study. The extraction of the viral RNA was conducted by the Taiwan CDC with the MagNA Pure LC automated nucleic acid extraction system (Roche Diagnostics, Mannheim, Germany).

Primer design

Human, avian, and environment H7N9 NA sequences reported in China[8] and 21 NA sequences of Eurasian lineage of avian influenza virus[9] were retrieved from the Global Initiative on Sharing Avian Influenza Database and aligned by BioEdit v. 7.0.4.[10] The consensus regions that covered N9 were chosen for primer design. The consensus-degenerated primers were designed to target an NA fragment 488 nucleotides long, encompassing the R292K mutation site (Fig. 1A).

Reverse transcription—polymerase chain reaction

SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used to perform cDNA synthesis according to the manufacturer protocol, with a 1 μL RNA template and a HuN9NA-F460-biot primer (Table 1). The PCR was executed using a proofreading KOD-Plus DNA polymerase (1 U) in a 50 μL reaction containing dNTPs (0.2 mM), $MgSO_4$ (1 mM), two primers (HuN9NA-F460-biot and HuN9NA-R947, 0.3 μM each), and 2 μL of cDNA in a PCR buffer under the following cycling conditions: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final elongation step of 68°C for 7 minutes. The PCR condition was the same for the semi-nested PCR with the HuN9NA-F460-biot and HuN9NA-R919 primers (Table 1). The PCR products were analyzed by gel electrophoresis on a 1% agarose gel containing 2 μg/mL ethidium bromide. For Sanger sequencing, the PCR products were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA).

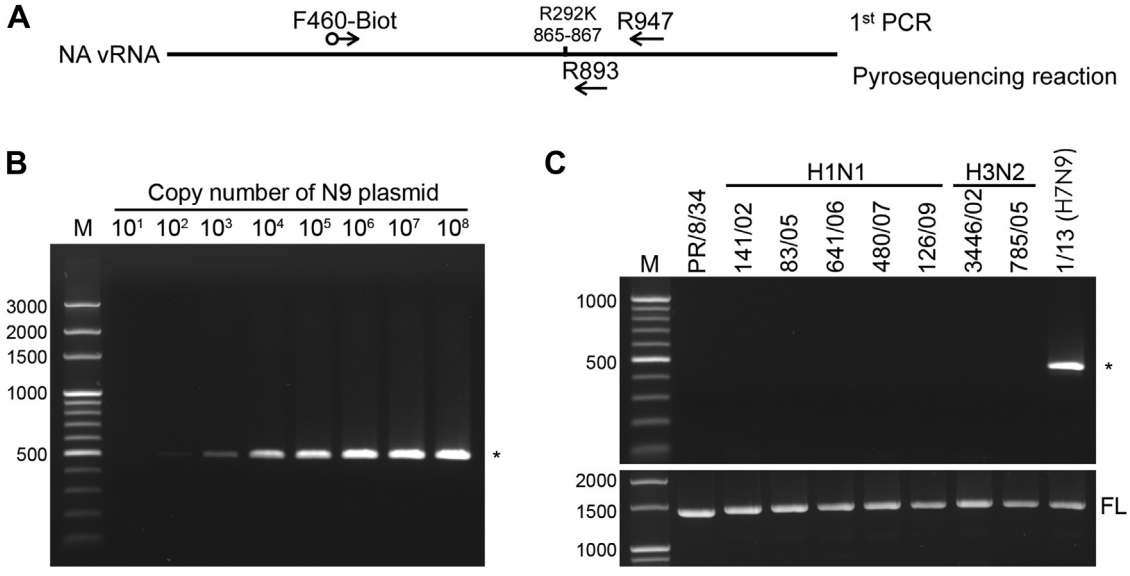

Figure 1. Sensitivity and specificity of reverse transcription polymerase chain reaction (PCR). (A) The reverse transcription-PCR primers were designed for detecting an emerging H7N9 virus NA gene. (B and C) A serially diluted N9 plasmid, PR8 plasmid, and selected Taiwanese seasonal influenza A neuraminidase (NA) plasmids (including one H1N1pdm - A/Taiwan/126/2009 labeled as 126/09) were amplified and analyzed in 1% agarose gel. Each lane was loaded with 5 μL of a PCR product, and the expected PCR product is indicated by an asterisk. A full-length (FL) NA product was obtained from each strain tested as a loading control.

Table 1 RT-PCR and pyrosequencing primers for avian influenza A (H7N9) virus targeting the neuraminidase (NA) gene

Primer	Sequence (5′ to 3′)	Nt position[a]	DNA size
Reverse transcription			
HuN9NA-F460-biot	CTGATAAGCTGGCCACTATCATCAC	460—484	
PCR			
HuN9NA-F460-biot	CTGATAAGCTGGCCACTATCATCAC	460—484	488 bp
HuN9NA-R947	CTGCATATRTAYTGACTAGTRTGTGTCA	947—920	
Semi-nested PCR			
HuN9NA-F460-biot	CTGATAAGCTGGCCACTATCATCAC	460—484	460 bp
HuH7NA-R919	TTGCTACTGGRTCTATCTGAAYCACT	919—894	
Pyrosequencing			
HuN9NA-292&294-R893-seq	GGTCTATTTGAGCCCTGCCA	893—874	

[a] Nt position is numbered by A/Taiwan/1/2013(H7N9) NA cds, reference no. EPI ISL140356 in GISAID.

The nucleotide sequence of the purified fragments was determined using an automated ABI3730 DNA sequencer (PE-Applied Biosystems, Foster City, CA, USA). The full-length cDNA of the H7N9 NA gene was amplified with universal primers[11] and cloned using the pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). A plasmid carrying the R292K mutation was used to validate the sensitivity of the PCR and pyrosequencing assay. The NA plasmids from pandemic H1N1 (A/Taiwan/126/2009), and seasonal H1N1 (A/Taiwan/141/2002), and H3N2 (A/Taiwan/3446/2002) viruses were used for evaluating the specificity of the PCR primers.

Pyrosequencing assay

The pyrosequencing assay was performed according to the manufacturer instructions by using the PyroMark Q24 system (Qiagen). In brief, 10 μL of a biotinylated PCR product was bound to 2 μL of streptavidin-coated Sepharose beads by agitating at room temperature for 10 minutes. Single-stranded DNA was purified by washing the DNA with a 70% EtOH, a denaturation solution, and a washing buffer by using a PyroMark Q24 vacuum workstation. The single-stranded DNA was then incubated with 0.3 μM sequencing primer (HuN9NA-292&294-R893-seq) in 25 μL of a PyroMark annealing buffer at 80°C for 2 minutes, and then cooled to room temperature for at least 5 minutes. Pyrosequencing reactions were performed on the PyroMark Q24 instrument by using the allele quantification mode of PyroMark version 2.0.6 software (Qiagen).

Results

Sensitivity and specificity of reverse transcription-PCR in detecting NA of H7N9 virus

The NA gene of this emerging H7N9 virus is known to be derived from the Eurasian lineage of the avian influenza virus,[8,9] and the primers for reverse transcription (RT)-PCR were therefore designed to target to the conserved region among the N9 subtypes of the Eurasian lineage (Fig. 1A, Table 1). A 488-nucleotide DNA was amplified that contained the 292nd codon. A serial dilution of N9 plasmid was made to validate the RT-PCR sensitivity, in which as few as 1000 copies of target DNA were detected in the agarose gel stained by ethidium bromide (Fig. 1B). To test the specificity of our proposed RT-PCR primers, we used full-length NA plasmids derived from each of the seasonal H1N1, H3N2, pandemic H1N1 2009, and PR8 strain of influenza A viruses as DNA templates for RT-PCR. All the NA plasmids were amplified by the full length NA primers[11] for complete genome amplification, by contrast, only the N9NA plasmid was specifically amplified by our primers without a cross-reaction to other subtypes (Fig. 1C).

Pyrosequencing for detection of the R292K mutation

The NA mutation R292K (AGG to AAG) was found in A/Shanghai/1/2013. In all other H7N9 strains found in humans, however, this residue remained R (AGG). To examine whether R or K existed in the first imported case in Taiwan, the N9NA detection based on the RT-PCR was performed directly on the sputum, and a specific amplified DNA was successfully observed in the specimen as well as the plasmid control (an NA clone derived from the H7N9 virus), but not in the reagent control (Fig. 2A). The PCR product from the sputum was then analyzed using a pyrosequencing assay. Conventional Sanger sequencing was also performed for comparison. The reverse primer (HuN9NA-292&294-R893-seq) was used in the pyrosequencing assay. The sequences of the pyrosequencing read out, therefore, were reverse complements of the original sequences. As shown in Fig. 2B, mixed nucleotides of C and T were detected at the corresponding triplets of the residue for 292 at a ratio of 44.08% to 55.92%, in which C resulted in a sensitive marker (C<u>C</u>T was complementary/reversed to A<u>G</u>G at codon 292; R292), and T resulted in a resistant marker (C<u>T</u>T was complementary/reversed to A<u>A</u>G at codon 292; R292K). The NA plasmid control that contained an R292K mutation, however, showed a pure T at the corresponding nucleotide position (Fig. 2C). This suggests that a mixed population of different viruses was present in the sputum specimen. The mixed nucleotide sequences were displayed with mixed peaks by Sanger sequencing (in the right panel of Fig. 2B), and the dominant peak (T, leading to R292K) was automatically used in the output sequence. Similarly to Sanger sequencing, the use of the standard nucleotide

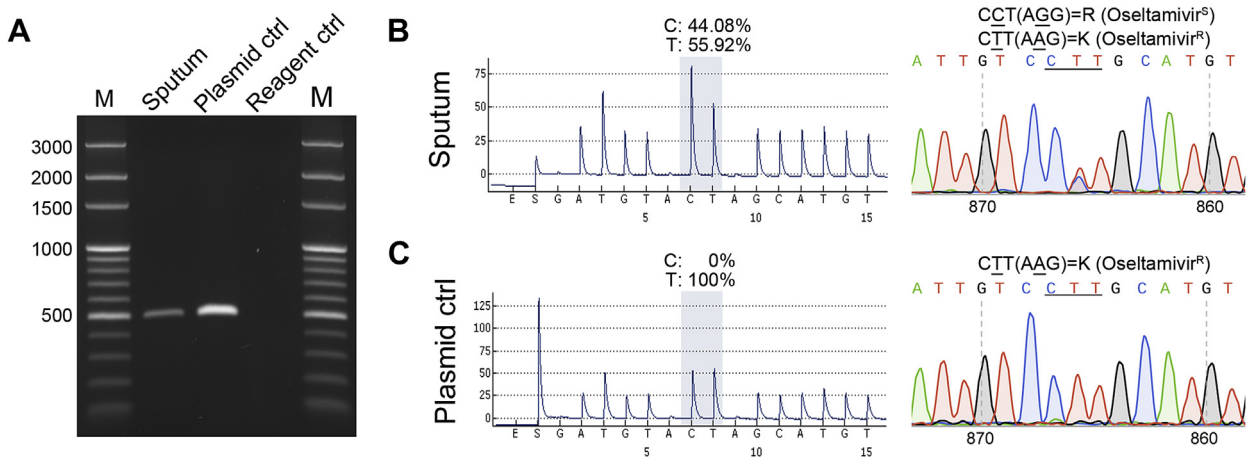

Figure 2. Pyrosequencing for detecting R292K mutation. (A) The presence of neuraminidase (NA) in the sputum specimen collected from the first imported Taiwanese patient was detected by reverse transcription polymerase chain reaction and analyzed with an electrophoresis gel. (B) The polymerase chain reaction products (200 ng) amplified from the sputum and N9 plasmid were analyzed using pyrosequencing (left subpanels) and Sanger's sequencing method (right subpanels) by employing a reverse primer (HuN9NA-292&294-R893-seq). The Sanger sequence readouts were in a negative sense. The positive-sensed sequence of R292 appeared as AGG and the oseltamivir-resistant marker R292 K as AAG (shown in parentheses).

dispensation protocol for the pyrosequencing assay also only displayed the major T nucleotide and failed to detect the underlying mixed variants. We implemented a customized nucleotide dispensation order (as shown in the chromatogram in Fig. 2B) that specifically read out the 292 position signals in the N9NA pyrosequencing assay; this yielded a successful detection of the resistant variants in the H7N9 virus.

Semi-nested PCR improved sensitivity for the detection of the H7N9 virus

We understand that the low sensitivity of a detection method may yield a false-negative result. We used a semi-nested PCR based on a nested reverse PCR primer (HuN9NA-R919) and, after the first round of PCR, the semi-nested PCR detected the virus cDNA in solutions that were at least

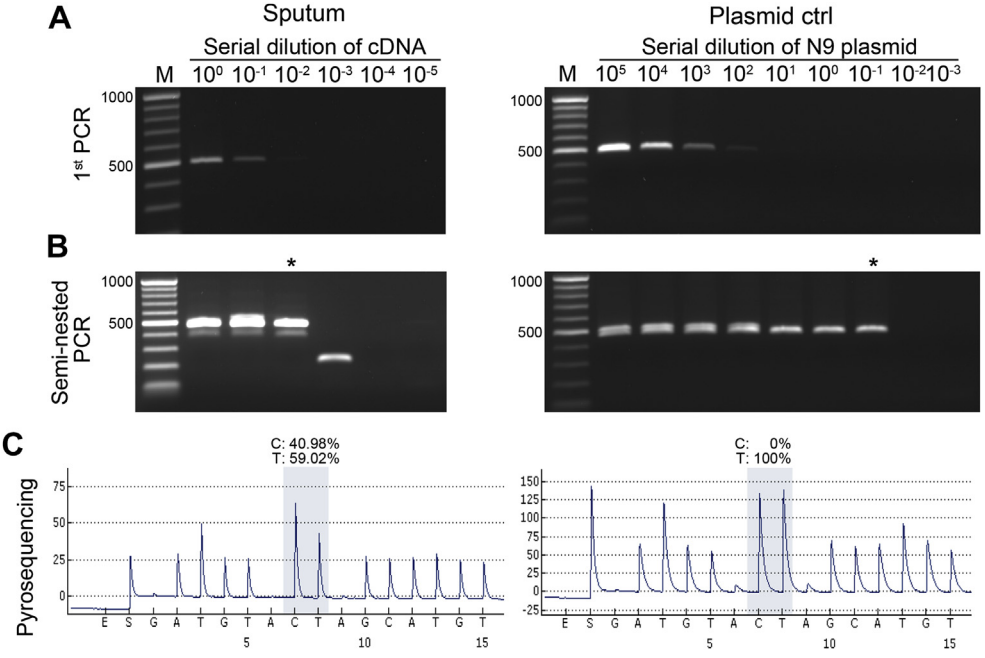

Figure 3. The semi-nested polymerase chain reaction (PCR) improved the sensitivity of detecting avian influenza A (H7N9) virus. (A) The serially diluted cDNA from the sputum and the N9 plasmid (0.44 pg, approximately 10^5 copies of DNA) were used to indicate the sensitivity of the reverse transcription-PCR method. (B) After 1 μL of the first PCR product was amplified in a semi-nested PCR, the detection rate was enhanced 10-fold for the sputum and 10^4-fold for the N9 plasmid control. (C) Analyzing the PCR products (the lowest detected samples labeled with an asterisk in panel B) by using pyrosequencing showed similar results as in Fig. 2B and C.

10 times more diluted than the original solution. In addition, the sensitivity of the semi-nested PCR showed a 10^4-fold increase in the N9 plasmid control; the plasmid is a highly pure preparation and is more stable than the specimen isolation (Fig. 3A and B). The semi-nested PCR products from first-round diluted samples were subsequently analyzed using a pyrosequencing assay, and a similar ratio of mixed nucleotides was also obtained in the specimen shown in Fig. 2B and C.

Discussion

Oseltamivir is an effective and easy-to-use (by oral administration) clinical drug for treating influenza. However, increasingly more reports are indicating that drug-resistant viruses have been found in patients with or without oseltamivir treatment. Therefore, it is critical to detect oseltamivir-resistant markers promptly and accurately to provide guidelines for clinical treatment. Many medical virological laboratories often perform virus isolation and then Sanger sequencing, which usually takes several days. Pyrosequencing analysis reduces this process to several hours because of its higher sensitivity for revealing nucleotide sequence information directly from specimens. Comparatively, several approaches have been well-established for detecting the NA drug resistance marker, such as the rare-variant-sensitive high-resolution melting-curve analysis established by Chen et al,[12] which also detects with exceptional sensitivity. However, this technique was specifically designed to detect H275Y in 2009 N1NA; its design cannot be changed arbitrarily and it is limited to detecting only certain circulating NA subtypes. The analysis should be specifically designed for this emerging avian influenza A (H7N9) virus because the various subtypes of an influenza virus can have varied NA sequences. Furthermore, because pyrosequencing and Sanger sequencing are both primer-based design analysis methods, both possess easily designed sets of primers with which to establish the detection purpose.

We implemented a highly specific and sensitive pyrosequencing assay to detect the oseltamivir-resistant markers in the sputum specimen collected from the first imported human H7N9 case in Taiwan. We located R292K in the form of a viral quasispecies. In this case, other NA inhibitors, such as zanamivir and peramivir, should be considered alternative drugs for patient treatment.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in the manuscript.

Acknowledgments

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