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BRIEF COMMUNICATION

Detection of low copies of drug-resistant influenza viral gene by a single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe

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Influenza virus infection causes endemics almost yearly and pandemics occasionally. Although antivirals are available for the clinical treatment of influenza virus infection, the emergence of a drug-resistant virus has reduced the effectiveness of therapy and prophylaxis. Therefore, the timely detection of drug-resistant influenza viruses is important. A single-tube reaction using peptide nucleic acid (PNA) as both a polymerase chain reaction (PCR) clamp and a sensor probe was established to detect the low numbers of copies of viral genes that carry the resistant marker. Influenza A H1N1 viruses resistant to a clinically used antiviral, amantadine, are selected for the experimental design. The PNA-mediated reverse transcription-PCR detected 10 copies/μL of RNA from the resistant strain among 2×10^4 copies/μL of RNA from the sensitive strain. A rapid and sensitive method was established for detecting low numbers of drug-resistant genes of the influenza virus. The assay would help to monitorthe emergence of adrug-resistant influenza virus.

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Influenza virus infection causes high mortality and morbidity in humans and animals. This virus belongs to orthomyxoviridae, a family of RNA viruses. Like other RNA viruses, there is no proofreading activity of viral RNA polymerase so that mutant viruses are easily generated and form a quasispecies in virus population. A rare amount of mutant viruses is generally undetectable by reverse transcription-polymerase chain reaction (RT-PCR) because the reaction would amplify the major form of RNA that derived from wild-type viruses. However, the undetectable mutant viruses would sometimes cause severe clinical impacts. Therefore, it is important to detect the low copies of drug-resistant mutant RNA in clinical specimens to avoid the emergence of drug-resistant mutant viruses.

Peptide nucleic acid (PNA) is a synthetic DNA analog in which the phosphodiester backbone is replaced with an *N*-(2-amonoethyl)glycine chain so that it is resistant to nuclease cleavage. However, nucleobases of PNA are complementary to its target DNA in the normal A—T and G—C geometry and form a PNA—DNA complex that is more stable than a DNA—DNA duplex.[1–3] When using a PNA probe designed to cover the variable region and having a sequence complementary to the wild type in a PCR, the PNA probe binds tightly to perfectly matched wild-type DNA template but not to the mismatched mutant DNA sequences, which specifically inhibits the PCR amplification of wild-type DNA without interfering in the amplification of mutant DNA. It is the so called "PNA-mediated PCR clamping".[4] This design has been applied to detect oncogenic K-*ras* and mitochondrial mutations[5–7] as well as some virulent or drug-resistant mutations in microorganisms, such as the *uid*A gene mutation of *Escherichia coli* and DNA polymerase gene mutation of hepatitis B virus.[8–10]

Dual hybridzation probes and melting curve analysis were also applied in this assay. The dual hybridization probes comprise a pair of probes labeled with different fluorescent dyes (a donor and an acceptor), between which fluorescence resonance energy transfer occurs when the two probes hybridize to approximate regions of complementary templates.[11] Monitoring the acceptor emission along with temperature change generates a melting curve of the probes. To detect DNA variants, the shorter of the two probes is designed to be positioned over the variable region. Hence, the shorter probe serves as a sensor because any change in the DNA sequence in this region results in a shift in its melting temperature and a change in the melting curve profile. In the current study, a design combining PNA-mediated PCR clamp and dual hybridization probes was applied for detection of drug (amantadine)-resistant influenza A H1N1 viruses.

To achieve efficient clamping of wild-type amplification, the probe was designed following a guideline described in our previous report.[12] Briefly, an 18-mer PNA tagged with fluorescein on its N-terminal (Fluorescein-OO-CCCAATTA TA**C**TTGCGGC) was used as both PCR clamp and sensor probe. The PNA probe covers the mutation site (underlined; that cause S to N change at position 31 of M2 protein) of M2 gene and has a distance of 126 nucleotides from the primer annealing another strand. Note that the distance may affect clamping efficiency.[13] A DNA probe tagged with LC-Red640 on its 3′ end (5′-GCTTTTGGAAAAAAGCCGATCAATAATCCACAAT ATCAGGTGCACA-Red640-3′) serves as the anchor probe. RT-PCR of M2 gene was performed using Qiagen One-Step RT-PCR Kit (Qiagen, Hilden, Germany). The 20-μL reaction mixture contained 1× reaction buffer and enzymes provided by the kit, 0.8 mM of each dNTP, 4.5 mM $MgCl_2$, 0.6 μM forward (5′-ATGGTGCAGGCAATGAGA-3′) and reverse (5′-TACTCC TTCCGTAGAAGG-3′) primers, 0.25 μM PNA probe, and 5 μL viral RNA. The reaction conditions consisted of a 30-minute incubation at 50 °C for RT, 15 minutes at 95 °C for activation of the hot-start *Taq* polymerase, followed by 45 cycles of 95 °C, 30 seconds for denaturation; 52 °C, 30 seconds for annealing, and 60 °C (clamping condition) or 72 °C (non-clamping condition), 1 minute for extension. After RT-PCR, a 10-μL aliquot of the product was mixed with 1 μL of 10 μM anchor probe and transferred into a capillary tube. The melting curve was analyzed on a LightCycler v1.0 (Roche Applied Science) with the following program: 95 °C for 60 seconds, 40 °C for 30 seconds, slowly increase the temperature (0.7 °C/s) to 90 °C and read the fluorescent signal at the step mode.

One of the advantages of applying PNA-mediated PCR for monitoring the drug-resistant virus is to detect the mutants in the existence of wild-type in specimens. Therefore, different copies of RNA extracted from amantadine-sensitive strain (A/Taiwan/72458/2007, A/Somomon_Islands/3e/2006-like) and resistant strain (A/Taiwan/10067/2007, A/Malaysia/862/2007-like) were mixed together and subjected to the PNA-mediated PCR amplification. The RNA copies were measured by real-time RT-PCR using ABI 7900 machine. The copy number of sensitive strain was fixed at 2×10^4 copies/μL, and the copy number of the resistant strain was varied from 2×10^4 to 10 copies/μL. Therefore, the ratio of sensitive to resistant viruses was from 1:1 to 1:2000. Fig. 1 shows the melting curve of the reaction, and the results demonstrated that the sensitivity of the detection of amantadine-resistant virus (Malaysia strain) by PNA-mediated PCR amplification is 1:2000, in which there were 2×10^4 copies/μL of RNA from the sensitive strain and 10 copies/μL of RNA from the resistant strain.

To understand whether the clamping of target DNA derived from the sensitive strain (wild-type) by PNA increases the detection sensitivity of the resistant strain (mutant), the same ratio of RNAs were subjected to the

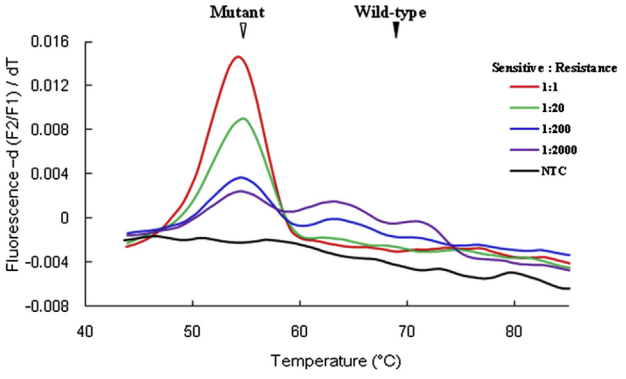

Figure 1. Sensitivity of detecting the amantadine-resistant H1N1 virus by PNA-mediated PCR amplification (clamping). Filled and open arrowheads indicate the melting peaks of wild-type and mutant, respectively.

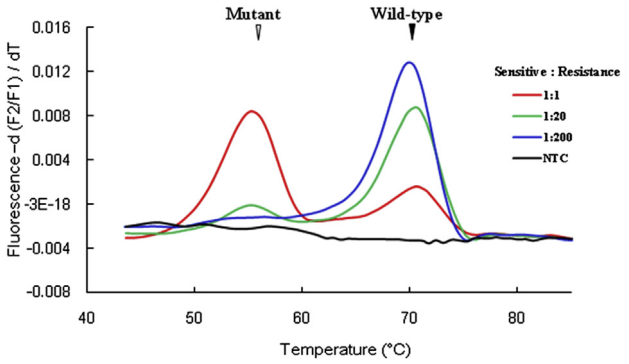

Figure 2. Sensitivity of detecting amantadine-resistant H1N1 virus by PNA-mediated PCR amplification (non-clamping). Filled and open arrowheads indicate the melting peaks of wild-type and mutant, respectively.

same real-time RT-PCR without "clamping". The detection limitation became 1:20, in which there are 1000 copies/μL of resistant RNA among 2×10^4 copies/μL of RNA in the reaction (Fig. 2).

To assess the efficacy of the novel method for detection of the amantadine-resistant influenza A H1N1 viruses, 116 clinical specimens collected from 2001 to 2008 were selected for the evaluation. All of them have been identified as influenza A H1N1 positive using viral culture, immunofluorescent assay (typing) and sequencing (subtyping). PNA-mediated RT-PCR detected 22 specimens containing amantadine-resistant viruses. The sequence analysis of M gene of these isolates revealed that those 22 viruses have N at position 31 of M2 protein (amantadine-resistant marker).

According to the different melting curve from that of amantadine-resistant gene, the PNA-mediated RT-PCR can also detect the amantadine-sensitive gene. Indeed, 90 specimens were detected as the amantadine-sensitive influenza A H1N1 virus. Together with the 22 amantadine-resistant ones, 112 clinical specimens were positive for influenza A virus. However, 35 out of 116 were positive in the use of traditional real-time RT-PCR (based on C_t value), and 55 out of 116 were positive in the use of melting curve typing method (based on T_m value). The difference in sensitivity may be attributed to the primers targeting different viral gene sor the same gene but different regions. The sensitivity of PNA-mediated RT-PCR targeting M gene is greater than that of the real-time RT-PCR based on detection of HA gene which is commonly used is many clinical virology laboratories.

Most laboratories monitor the drug-resistant viruses by sequencing the resistant markers from the viral isolate. The whole procedure takes several days. This study proposes a novel strategy, PNA-mediated RT-PCR, to monitor the drug-resistant influenza virus directly from clinical specimens, which only takes a few hours. The method can be applied to monitor the emergence of drug-resistant viruses during the treatment course of an influenza virus-infected patient.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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