Detection of molecular markers of antiviral resistance in influenza A (H5N1) viruses
using pyrosequencing method

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Abstract

Resistance to antiviral drugs in influenza viruses can emerge following medication or may result from natural variation. Two classes of anti-influenza drugs targeting either the M2 protein (amantadine and rimantadine) or neuraminidase (NA) (oseltamivir and zanamivir), are currently licensed. These drugs are expected to be important in controlling early stages of a potential pandemic. In the present study, we describe how a pyrosequencing method can be used to rapidly detect established molecular markers of resistance to M2 blockers and to NA inhibitors in A(H5N1) viruses. The residues L26, V27, A30, S31, and G34 in the M2 were targeted for pyrosequencing. The NA residues for pyrosequencing analysis included the established markers of drug-resistance, (H274 and N294) as well as residues of less certain relevance (V116, I117, Q136, K150, and I222). A single pair of pyro RT-PCR primers was designed to allow amplification of an approximately 600nt-long amplicon of the NA gene of H5N1 viruses from various clades/subclades associated with infections in humans. The sensitivity of the assay was demonstrated by the successful pyrosequencing of RNA extracted from samples of serially diluted (10^-5 to 10^-7) virus stocks with initial concentrations ranging from 10^5 to 10^9 pfu/ml. The markers of resistance were detected in samples with Ct values ranging from 32 to 37 determined by real-time RT-PCR. The pyosequencing approach may provide a valuable tool in rapid detection of markers of drug resistance in H5N1 viruses and facilitate the elucidation of the role of such changes in natural and acquired drug resistance.
Introduction

Avian influenza A(H5N1) viruses that first emerged in 1997, have become endemic in Asia and pose a serious threat to agriculture and human health (12, 14, 42). Since their re-emergence in 2003, influenza A(H5N1) viruses continue to infect humans with high mortality rate (~63%) (46). Although vaccines are the primary line of defense for the prophylactic control of influenza infections, effective antiviral drugs provide a valuable option in the early containment of highly virulent and/or novel strains. Presently, there are two classes of anti-influenza drugs licensed by the U.S. Food and Drug Administration (FDA) that are effective against influenza A viruses, including H5N1 viruses. These drugs are M2 channel blockers, also known as adamantanes (amantadine and rimantadine), and NA inhibitors (NAIs) (zanamivir and oseltamivir) (20, 34). Molecular markers of resistance to the older class of drugs, adamantanes, are well established and comprise changes at residues L26, V27, A30, S31, and G34 within the transmembrane domain of the M2 protein (6, 11, 22). Since 2005, a dramatic rise in resistance to adamantanes in seasonal influenza A viruses has led to changes in the CDC recommendations for the use of adamantanes in the control of influenza infections (8). In response to a need for rapid monitoring of the susceptibility of seasonal influenza A viruses to adamantanes, the pyrosequencing assay was developed (7). This assay was subsequently adopted by various virus surveillance laboratories for high throughput screening for adamantane resistance in seasonal influenza A viruses and proved its usefulness in the timely detection of the emergence and spread of adamantane-resistant H3N2 and H1N1 viruses (3, 4, 9, 17, 39, 40). More recently, pyrosequencing was used to detect quasispecies of influenza virus in clinical material as well as after virus isolation in
cell culture (16, 18, 27). In recent years, a significant increase of adamantane resistance was also reported among some groups of influenza A(H5N1) viruses (11, 24, 25). This necessitates a close monitoring of adamantane resistance among diverse groups of rapidly evolving influenza A(H5N1) viruses, including viruses circulating in avian populations.

Emergence and spread of adamantane resistant virus variants in three antigenic subtypes H3N2, H1N1, and H5N1 of influenza A viruses influenced the decision to stockpile NAIs to be used for mitigation of pandemic influenza. Emergence of oseltamivir-resistant viruses in treated individuals have been seen at a low frequency (~1%) in the adult population (44) and more often (up to 30%) in young children (26) and immunocompromised patients. Nevertheless, until recently, resistance to this class of drugs has been very low (<0.5%) in community isolates (23, 25, 32, 33, 36). During the season 2007-2008, however, a significant rise in resistance to oseltamivir was detected among seasonal influenza A(H1N1) viruses recovered from untreated individuals of different ages and in distant geographic locations (28, 41). The emerged oseltamivir-resistant H1N1 viruses retain sensitivity to zanamivir. These recent findings emphasized the need for close monitoring of NAI resistance among seasonal influenza viruses as well as among highly virulent H5N1 viruses. In contrast to adamantanes, molecular markers of resistance to NAIs are not yet well characterized. Moreover, resistance to NAIs is drug-specific and NA type/subtype-specific, reviewed by (19). Of note, H5N1 viruses of different genetic groups (clades/subclades) exhibit substantial variance in their NA sequences and susceptibility to NAIs (10, 32, 47). For these reasons, the NA activity inhibition assay is currently used as the primary assay for monitoring resistance to NAIs (21, 35, 41, 43, 45). This method requires virus propagation in cell culture prior to testing.
and needs to be complemented by sequence analysis of the NA to confirm known or to
detect novel markers of NAI-resistance. Oseltamivir-resistant H5N1 viruses with amino
acid changes at two residues H274Y and N294S have emerged in humans during
oseltamivir treatment (15, 29). The impact of natural drift mutations in the NA on the
drug susceptibility of H5N1 viruses has been reported (13, 32, 37) but is not readily
predicted. Moreover, when existing NAIs were designed, crystal structures of the
influenza N1 subtype enzyme, including that of H5N1 viruses were not available. Recent
crystal structure data and conformational studies of influenza N1 enzyme showed that
replacements at residues outside of the conserved active site (e.g. residues Q136 and
K150 in the 150-loop of the NA molecule) could affect binding of the existing drugs to
the NA by interfering with their induced fit into the active site (10, 30, 38, 47). Although
the exact mechanisms by which some changes (e.g., residues V116, I117, Q136, K150,
and I222) in the N1 enzyme affect susceptibility to a particular NAI are not well
understood, there is a need to monitor their presence in multiple and evolving clades of
H5N1 viruses.

The ability to rapidly detect markers of antiviral drug resistance is a valuable tool
in the view of influenza pandemic preparedness. Especially desirable are rapid, high-
throughput methods which are sensitive and minimize handling of live viruses.
Pyrosequencing has been proven to provide such an option for the detection of resistance
to adamantanes in seasonal influenza A viruses and more recently this method was
expanded for detection of resistance to NAIs in seasonal and H5N1 viruses (16, 18, 27).
In the present study, we enhanced the existing pyrosequencing approach by expanding the number of targeted markers (from 2 to 7) in the NA of H5N1 viruses and also provided validation of pyrosequencing assays of both M2 and N1NA by testing H5N1 viruses from a variety of genetic groups (clades/subclades). Based on results of the enzyme inhibition assays (2, 5, 25, 27, 32), replacements at some of the targeted residues in the NA (e.g., V116, I117, Q136, K150, and I222) have previously been linked to reduced drug-susceptibility in avian and human viruses carrying N1. However, some of them may yet be proven to have little relevance for clinical resistance. Nevertheless, the pyrosequencing assay developed here provides a robust tool for detection of influenza virus variants including those directly from clinical specimens and thus would be instrumental in identifying new mechanisms of resistance to the newer NAI class of anti-influenza drugs.

**Material and Methods**

*Viruses.* Influenza A H5N1 viruses isolated from humans and birds between 1997 and 2008 and submitted to the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the CDC, Atlanta, GA, were propagated in allantoic cavities of 9-11 day-old embryonated chicken eggs and harvested 24-48 h post-inoculation. Viruses were handled in the biosafety level 3 containment, with enhancement required by the USDA and select agents program. Viruses representing different genetic groups (clades and subclades) and associated with confirmed human infections (1) as well as viruses carrying known markers of resistance to adamantanes and NAIs were chosen for the study. Those viruses included but were not limited to: clade 0 - A/Hong Kong/482/1997 (I222T); clade 1 - A/Vietnam/1203/2004 and
A/duck/Vietnam/NCVD-19/2007; clade 2.3.4 - A/duck/Vietnam/NCVD100/2007 (I117V) and A/chicken/Laos/NCVD-38/2007 (K150N, I222L); clade 3 - A/duck/Hong Kong/380.5/2001 (N294S); and clade 7 - A/chicken/Vietnam/NCVD-93/2008 (Table 1). Throughout the manuscript, amino acid residues in the N1 NA are shown according to the universal N2 subtype numbering.

**Pyrosequencing primer design.** Full length NA sequences obtained from the CDC sequence database and the Los Alamos Influenza sequence database (31) from 1391 human and avian H5N1 viruses collected between 1997 and 2008 were aligned. Using BioEdit software (version 5.0.6, North Carolina State University), the N1 consensus sequence was generated. Similarly, a consensus sequence was generated based on alignment of their M gene sequences. These consensus sequences were used to design the pyro RT-PCR and sequencing primers using Pyrosequencing Assay Design software (Biotage). In several instances, primers were modified to accommodate degenerate nucleotides. The primers were synthesized at the CDC Biotechnology Core facility (Table 2).

Resistance to adamantanes is associated with mutations occurring at one or more of the residues 26, 27, 30, 31 and 34 (7, 11) in the M2 protein. The RT-PCR primers were designed to amplify a region encompassing these amino acids (from nt 739 to nt 1027).

**RT-PCR and pyrosequencing.** Viral RNAs were extracted from viral supernatants in lysis buffer. RT-PCR amplifications were performed using one-step RT-PCR system (Qiagen, Valencia, CA). Primers were used at 20µM in a standard 25µl reaction mixture and amplification for 45 cycles. Biotinylated amplicons were purified and the pyrosequencing reactions carried out as previously described in (7) using the PSQ
MA 96 platform pyrosequencer. Amplification products were washed in a series of buffers and single-strand, biotinylated DNA products were hybridized to mutation-specific sequencing primers in a 96 well plate used at 0.45µM final concentrations in 40µl of annealing buffer. Pyrosequencing was performed using Pyrogold reagents according to Biotage recommendations. The accuracy of sequences obtained using pyrosequencing was confirmed by comparison to the results of conventional sequencing.

**Assessment of pyrosequencing assay sensitivity.** To determine the sensitivity of the designed pyrosequencing assay in terms of the amount of RNA needed for detection of markers of resistance in the NA of H5N1 viruses, we performed the following experiments. The H5N1 viruses grown in eggs had initial concentrations of $10^5$ to $10^8$ pfu/ml. Viral RNA was extracted from the initial stocks and then serially 10-fold diluted in water ($10^{-2}$ to $10^{-8}$). The RNA dilutions were used for amplification of the NA gene fragment using the RT-PCR primers designed for pyrosequencing and were also subjected to conventional real-time RT-PCR analysis (the conditions of the rRT-PCR are consistent with the CDC Diagnostic assay for detection and subtyping of influenza viruses currently in use). Primers and probes used in this study are available upon request through a material transfer agreement with the US CDC.

In addition, the sensitivity of the pyrosequencing assay was assessed using RNA extracted from serially 10-fold diluted virus stocks. One hundred microliters from each virus dilution was added to equal volume of lysis buffer in BSL3+ containment. RNAs were used to perform the RT-PCR amplifications as described above and pyrosequencing was done using the primer F-796A to analyze codon 274 for the entire panel of viruses ($n=12$). This approach allowed the determination of the highest dilution of virus stock
that could be used to generate pyrograms of high-quality resolution using the designed pyrosequencing primers. The RNAs from the highest dilution of the virus stock were also subjected to real-time RT-PCR analysis.
Results

Detection of molecular markers of A(H5N1) virus resistance to M2 blockers.

Because of the conserved nature of influenza A M2 sequences, it was possible to use the same set of primers to detect adamantane resistance-conferring mutations in two antigenic subtypes of seasonal influenza, H1N1 and H3N2 (7). Here, we assessed the ability of the same primers to detect adamantane resistance in H5N1 subtype. We found that overall the same assay protocol was suitable for the majority of the H5N1 viruses. However, when the modified forward primer (Table 2) was used, it improved the quality of pyrograms for some H5N1 viruses. A single pair of primers (a modified forward and the previously described reverse primer (7) allowed amplification of all H5N1 viruses tested in the study and the same forward primer was used to generate sequences from these viruses. The pyrogram (Figure 1A) for the A/Hong Hong/482/97 virus shows no markers of resistance to adamantanes, whereas pyrogram (Figure 1B) for A/chicken/Vietnam/NCVD-93/2008 clearly indicates the mutation AGT to AAT (serine to asparagine) at residue 31. The A/Vietnam/1203/2004 virus has mutations CTT to ATT (leucine to isoleucine) at position 26 and AGT to AAT (serine to asparagine) at position 31 (Figure 1C). This combination of amino acid changes was previously reported in clade 1 H5N1 viruses (11). The M2 protein of the A/duck/Vietnam/NCVD-19/2007 virus contains 3 mutations: V27A (GTT to GCT) at residue 27, in addition to L26I and S31N (Figure 1D).
Detection of molecular markers of A(H5N1) virus resistance to NAIs.

The sequence variance of NA of avian H5N1 viruses presents a challenge for designing a universal set of primers for pyrosequencing. The codons of interest included H274, N294, I222, Q136, K150, V116, and I117 and thus requires a RT-PCR amplicon to encompass all seven codons. Therefore, primers were designed to allow amplification of the portion of the NA gene from nt 322 to nt 924 (Methods and Table 2). Because the codons are distant from each other and because a single pyrosequencing primer can provide reliable sequences up to 60 nts, an individual primer for sequence reading at each codon was designed and tested (Table 2). The results of pyrosequencing analysis for the representative H5N1 viruses that belong to five different clades/subclades are shown below. It is worth noting that the designed assay was also successfully used to analyze sequences at these residues of interest in H5N1 viruses from other subclades (2.1, 2.2, and clade 4) (results not shown).

Codon 274: Amino acid change histidine to tyrosine at residue 274 (CAT to TAT) in the NA of H5N1 viruses has been reported to confer resistance to oseltamivir (15, 29). As anticipated, only wild type sequence with CAC (histidine) at codon 274 was detected among the viruses tested. According to the pyrograms and conventional sequencing, A/chicken/Laos/NCVD-38/2007 and A/chicken/Vietnam/NCVD-093/2008 viruses from clades 2.3.4 and 7, respectively, shared identical sequences in the region analyzed (not shown), whereas sequences of two viruses from clade 1- (A/Vietnam/1203/2004), and from clade 2.3.4- (A/duck/Vietnam/NCVD100/2007), differed by a single nucleotide (Figure 2 A-B).
**Codon 294:** The effect of N294S change on NAI-susceptibility of H5N1 viruses in vitro has previously been reported (29, 48). Pyrograms confirmed the presence of wild type NA sequence in A/Hong Kong/482/97 (Figure 2C) and AAT to AGC mutation (asparagine to serine) at residue 294 in the A/duck/Hong Kong/380.5/2001 virus from clade 3 (Figure 2D); the latter virus is oseltamivir-resistant based on NAI assays (data not shown).

**Codon 222:** Substitutions at a conserved residue I222 have been shown to alter susceptibility to NAIs (2, 19, 41). As expected, wild type sequence ATA (isoleucine) at codon 222 was detected in the A/Vietnam/1203/2004 virus (Figure 3A), while pyrograms show mutations at codon 222 in A/Hong Kong/482/1997 virus (threonine, ACA) and in A/chicken/Laos/NCVD-38/2007 virus (leucine, TTA) (Figure 3B-C).

**Codons 136 and 150:** The analysis of crystal structures of N1NA from H1N1 and H5N1 viruses (10, 38) revealed the importance of the residues forming the 150-loop and glutamine at residue 136 for the drug binding to the NA. Moreover, Q136K mutation was reported to cause zanamivir-resistance in seasonal influenza viruses carrying the enzyme of N1 subtype (Hurt AC, personal communication; CDC unpublished data). Results (Figure 3D-E) show the presence of either CAA or CAG at codon 136, which corresponds to the wild type Q136.

The ability of the assay to detect changes at codon 150 was tested using viruses from three clades: 1, 2.3.4, and clade 7. These viruses: A/Vietnam/1203/2004, A/duck/Vietnam/NCVD100/2007 (clade 2.3.4), and A/chicken/Vietnam/NCVD-93/2008 (clade 7) viruses shared identical wild type sequence AAA (lysine) at position 150.
In contrast, the pyrogram from A/chicken/Laos/NCVD-38/2007 (clade 2.3.4) virus showed the presence of drug resistance conferring mutation AAA to AAC (lysine to asparagine) (Figure 3G).

**Codons 116 and 117:** Amino acid residue V116 appears to be conserved among NA types and subtypes and replacement of valine with alanine at this position in H5N1 viruses resulted in reduced susceptibility to NAIs (25). Furthermore, mutation I117V was reported to be associated with reduced susceptibility to NAIs in both H5N1 (25, 32) and seasonal H1N1 (27) viruses. In our tests, A/Hong Kong/482/1997, A/Vietnam/1203/2004, and A/duck/Hong Kong/380.5/2001, from clades 0, 1, and 3, respectively, shared the identical target sequences with GTT (valine) at position 116 and ATA (isoleucine) at position 117 (Figure 3 H, for simplicity only one pyrogram is shown which corresponds to the wild type at both residues). In accordance with conventional sequence data, pyrograms (Figure 3 I) showed the presence of wild type sequence GTT (valine) at position 116, and mutant sequence GTT (valine) at residue 117 of the NA of the A/duck/Vietnam/NCVD100/2007 virus from clade 2.3.4.

To evaluate the sensitivity of the pyrosequencing assay, real-time RT-PCR analysis was conducted on RNAs dilutions ranging from $10^{-5}$ to $10^{-7}$ which yielded quality pyrograms following pyrosequencing (see Methods). Results from the real-time RT-PCR amplification analysis confirmed that the pyrosequencing assay was successful in reliably identifying each of the targeted NA mutations at all seven codons in samples with Ct values ranging from 31 to 35 (Figure 4). In addition, the sensitivity of the pyrosequencing assay was tested by analyzing the pyrograms obtained directly from the
highest dilutions of the H5N1 virus stocks. Similarly to the RNAs dilutions, the sensitivity of the pyrosequencing assay was found to range from $10^{-5}$- to $10^{-7}$ virus dilutions of the initial virus stocks ($10^5$ to $10^8$ pfu/ml). Real-time-RT-PCR on these highest virus dilutions showed Ct value ranging from 32 and 37. This sensitivity was within the range of detection using the established CDC influenza diagnostic real-time RT-PCR assay.

**Discussion**

Monitoring of drug resistance among H5N1 viruses is a challenging task due to their rapid evolution and the need for BSL-3-enhanced containment for work with live viruses. In this study, we demonstrate the development of a rapid and high-throughput pyrosequencing method that could be utilized for the reliable detection of known molecular markers of influenza viruses resistant to neuraminidase inhibitors (NAIs) or adamantanes from a variety of influenza A H5N1 viruses. It is worth noting that because our assay was designed to encompass all 7 currently known markers associated with drug resistance in the influenza NA, it differs markedly from other described methods (18, 27), which were designed to only target 1 or 2 markers. Moreover, recent work elucidating N1 NA structure have shown that previous work on the design of neuraminidase inhibitors may not have been optimal for targeting the N1 subtype of the NA and natural resistance to such designed drugs can be conferred by changes in residues outside of the immediate N1 active site (10, 30, 38, 47, 49). Indeed, drug susceptibility testing using the NA inhibition assay showed that naturally occurring amino acid replacements outside of the NA active site (e.g. V116, I117) can alter the susceptibility of avian H5N1 viruses to oseltamivir and/or zanamivir (25, 32). Although evaluation of
clinical resistance to NAIs associated with each of these genetic markers has not been established conclusively, it is prudent to have reliable tools for rapid and high-throughput detection of such variants in event of an epidemic or pandemic outbreak of influenza. It is noteworthy that the pyrosequencing assay developed and reported here can be easily performed with small amounts of available RNA such as might be limiting in clinical specimens and generates short gene sequences making the product less affected by the RNA quality in virus samples. For seasonal influenza viruses, we and others have demonstrated a reproducible ability to detect molecular markers associated with drug resistance from a variety of clinical specimens (16, 18, 27).

In the present study, we conducted assessment of the pyrosequencing assay using two approaches. The viral RNA was extracted from the virus stocks and then serially diluted RNA preparations were subjected to RT-PCR and pyrosequencing. Alternatively, the serial dilutions were made from the virus stocks and then viral RNA was extracted and used for RT-PCR amplifications followed by pyrosequencing. The second approach may provide a better estimation of the assay sensitivity. In both instances, RNAs were used to determine Ct values of the highest dilutions that generated good quality pyrograms (sequences) in real-time RT-PCR assay. The Ct values (ranging from 31-35 vs. 32-37) obtained with either approach yielded results indicative of high sensitivity of the pyrosequencing assay designed in the present study. Noteworthy, the latter approach reflects more accurately the content of viral RNA available in a specimen.

Unlike NAIs, resistance to amantadine and rimantadine is relatively high among clades 1 and 2.1 H5N1 viruses (11, 24, 25, 49). Using pyrosequencing methodology, we were able to analyze the presence of the 5 molecular markers of resistance to M2
blockers in a wide variety of H5N1 viruses. We have shown here only examples of
detection of mutations I26L (CTT to ATT), V27A (GTT to GCT), and S31N (AGT to
AAT) in H5N1 viruses from clades 0, 1, and 7, however, it is important to emphasize that
the assay also successfully allowed testing viruses from subclades 2.1, 2.2, and clade 4
(data not shown). In conclusion, the sensitivity of the pyrosequencing assay was
optimized to allow analysis of viral samples with small quantities of RNA. This could
permit detection of drug resistant H5N1 viruses in clinical specimens thus reducing time
needed to complete the antiviral susceptibility testing.

The assay developed in this study lends itself to be used in a rapid and reliable
detection of known molecular markers of resistance to anti-influenza drugs. The results
obtained can be utilized to guide decisions regarding antiviral use in prophylaxis and
treatment in the event of local outbreaks or potential pandemic.
Acknowledgements

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The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. We declare that we have no conflict of interest.
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   adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide.

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FIGURE LEGENDS

**Figure 1.** Detection of established molecular markers of resistance to M2 blockers in H5N1 viruses using pyrosequencing. Sequences generated encompass the region of the M gene which contains the 5 amino acid residues of M2 protein associated with resistance. (A)-sensitive A/Hong Kong/482/1997 virus has no change at any of the 5 codons (in blue); (B)- resistant A/chicken/Vietnam/NCVD-093/2008 virus has S31N (AGT to AAT) mutation. (C)-the resistant A/Vietnam/1203/2004 virus contains two mutations L26I (CTT to ATT) and S31N (AGT to AAT).(D)- resistant A/chicken/Vietnam/NCVD-093/2008, with 3 mutations L26I (CTT to ATT), V27A (GTT to GCT), and S31N (AGT to AAT).

**Figure 2.** Detection of the most common oseltamivir-resistance conferring mutations in H5N1 using pyrosequencing. One pair of primers (F322C/R924B-biot) was used to generate biotinylated PCR products from the set of H5N1 viruses used in the study. The pyrograms show the NA sequences at codon 274: Panels A-B, wild type viruses A/Vietnam/1203/2004 and A/duck/Vietnam/NCVD100/2007 with CAC (H274), from clades 1 and 2.3.4, respectively. Panels C-D: oseltamivir-sensitive A/Hong Kong/482/1997 with AAT (N294) and oseltamivir-resistant A/duck/Hong Kong/380.5/2001, with AGC (N294S).

**Figure 3.** Pyrosequencing analysis of codons 222, 136, 150, and 117 in neuraminidase of H5N1 viruses. Viruses from clades 0, 1, and 2.3.4 were tested. Panel A shows wild type sequence ATA (I222) in A/Vietnam/1203/2004, panels B-C show mutants ACA (I222T) and TTA (I222L) for viruses A/Hong Kong/482/1997 and A/chicken/Laos/38/2007, respectively.
Panels D-E represent wild type viruses A/Hong Kong/482/1997 and A/duck/Vietnam/NCVD100/2007 with CAA and CAG at residue 136, respectively.

Panels F-G, wild type A/chicken/Vietnam/NCVD-093/2008, contains AAA (lysine) at position 150 and mutant A/chicken Laos/NCVD-38/2007 with AAC (asparagine) at position 150.

Panel H: wild type A/Vietnam/1203/2004 with ATA (I117), and variant A/duck/Vietnam/NCVD100/2207, from clade 2.3.4, had GTT, corresponding to mutant I117V, (I)

**Figure 4.** Evaluation of the sensitivity of the pyrosequencing assay designed for detection of markers of resistance to NAIs in H5N1 viruses. RNAs were prepared from the 7 viruses shown in table 1 and from 5 additional viruses that belong to clades/subclades 1, 2.1, and 2.2. Viral RNA was extracted and ten fold ($10^{-2}$ to $10^{-8}$) dilutions of viral RNA were prepared for each virus. Pyrosequencing analysis was performed on each RNA dilution. The highest dilution, at which sequences were obtained, was used in a conventional real-time RT-PCR amplification to determine the Ct (threshold cycle) values. Figure 4 shows amplification curves from the 12 respective RNA samples. The Ct values ranged from 31 to 35.
Table 1. The H5N1 viruses recovered from humans and birds and tested in the present study:

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<th>Virus</th>
<th>Clade</th>
<th>N1 numbering</th>
<th>N2 numbering</th>
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<td>0</td>
<td>I223T</td>
<td>I222T</td>
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<td>L26I, S31N</td>
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<td></td>
<td>L26I, V27A, S31N</td>
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<td></td>
<td>S31N</td>
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</table>

‡The GenBank accession numbers of the NA sequences of the viruses in the order they are listed in the table are: AF084272, EF541467, CY030377, FJ538947, CY030487, AY075034, FJ538949; their M gene sequence accession numbers are: AF084282, EF541453, CY030378, FJ538948, CY030488, AY075035, FJ538950.
**Table 2:** The designed RT-PCR and sequencing primers to perform pyrosequencing analysis

<table>
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<th>RT-PCR primers</th>
<th>Sequence</th>
<th>Target gene</th>
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<td>H5N1-M-R1027-biot</td>
<td>5’-AGTAGAAACAAGGTAGTTTTTACTCTC-3’</td>
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<tr>
<td>H5N1-NA-F322C</td>
<td>5’-ATTGGTTTCCARGGGGGATG-3’</td>
<td>H5N1-NA</td>
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<tr>
<td>H5N1-NA-R924B-biot</td>
<td>5’-TTGATTGAAARGAYACCATG-3’</td>
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<tr>
<th>Pyrosequencing primers</th>
<th>Sequence</th>
<th>NA target residue</th>
</tr>
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<tbody>
<tr>
<td>H5N1-NA-F321</td>
<td>5’-GATTGGTTTCAAGGGGGATGTG-3’</td>
<td>I117</td>
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<tr>
<td>H5N1-NA-F374</td>
<td>5’-CCC ACY TGG AAT GCA GAA C -3’</td>
<td>Q136</td>
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<td>H5N1-NA-F421</td>
<td>5’-AATGACAAGCACTCCAAYGGGAC-3’</td>
<td>K150N</td>
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<td>H5N1-NA-F640</td>
<td>5’-GACACYATCAAGAGTYTGGGA-3’</td>
<td>I222</td>
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<td>H5N1-NA-F796A</td>
<td>5’-TCAGTCGAATTTGAATGCT-3’</td>
<td>H274</td>
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<tr>
<td>H5N1-NA-F851</td>
<td>5’-ATGCCGYYGAAATCACRTGTGT-3’</td>
<td>N294</td>
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</table>

* Same primer was used at 100 µM for pyrosequencing the region encompassing residues 26, 27, 30, 31, and 34 of the M2 protein.
Figure 1

(A) Clade 0: A/Hong Kong/482/97 (clade 0): sensitive
Readout: ATCCTCTGTTGTCAGCAAGTATCATTGGGA

(B) A/chicken/Vietnam/NCVD-093/2008 (clade 7): 31 (AAT) = resistant S31N
Pyrosequencing readout: ATCCTCTGTTGTCAGCAAGTATCATTGGGA
Clade 1: A/Vietnam/1203/2004 (clade 1): 26 (ATT) and 31 (AAT) = resistant L26I + S31N
Readout: ATCCTATTGTGTGGCCGCAATATCATTGGGA

Clade 1: A/duck/Vietnam/NCVD-19/2007: 26 (ATT), 27 (GCT), and 31 (AAT) = resistant L26I + V27A + S31N
Readout: ATCCTATTGCTGTGGCCGCAATATCATTGGGA
Figure 2

Clade 1: A/Vietnam/1203/2004
Readout: CCTAATTATCACT

Clade 2.3.4: A/duck/Vietnam/NCVD100/2007
Readout: CCTAATTACCACT

Clade 0: A/Hong Kong/482/97
Readout: GTGCAGGGGATATTGG

Clade 3: A/duck/Hong Kong/380.5/2001
Readout: GTGCAGGGGATAGCT
Figure 3

Clade 1: A/Vietnam/1203/2004
Readout: GGAACAACATAC

Clade 0: A/Hong Kong/482/97
Readout: GGAACAACACAC

Clade 2.3.4: A/chicken/Laos/NCVD-38/2007
Readout: GGAACAACTTAC
Clade 0: A/Hong Kong/482/1997
Readout: CTTTTTCTTG ACCAAAG

Clade 2.3.4: A/duck/Vietnam/NCVD100/2007
Readout: TTTTTTTTTT TGACTCAGG

Clade 2.3.4: A/chicken/Laos/NCVD-38/2007
Readout: TGTCAACGA

Clade 7: A/chicken/Vietnam/NCVD-093/2008
Readout: TGTCAAAGA

CAA = wild type Q136

AAA = wild type K150

AAC = mutant K150N
Clade 1: A/Vietnam/1203/2004
Readout: TTTGTTATAA

Clade 2.3.4: A/duck/Vietnam/NCVD100/2007
Readout: TTTGTTGTTA

GTT = wild type V116
ATA = wild type I117

GTT = wild type V116
GTT = mutant I117V
Figure 4

Fluorescence (dRn) vs. Cycles

Ct value: 31, 35