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# Single-Step Real-Time Reverse Transcription-Polymerase Chain Reaction for Simultaneous Detection of H5N1 and H5N8 Highly Pathogenic Avian Influenza Viruses

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**Abstract:** For efficient monitoring of Highly Pathogenic Avian Influenza Viruses (HPAIVs) in countries where various subtype H5 HPAIVs co-circulate in avian populations, a Simultaneous Differential Detection Method for major and minor subtypes of HPAIVs is needed. In this study, we developed a single-step Real-time Reverse Transcription-Polymerase Chain Reaction (sRRT-PCR) assay for the simultaneous detection of *HPAIVM*, *H5*, *N1* and *N8* genes. The *M* gene primer set in the assay detected all subtypes of AIV including representative H5N1 and H5N8 strains from Korea as reported in earlier studies and the *H5*, *N1* and *N8* gene primer sets were able to specifically detect H5, N1 and N8 subtypes of HPAIV, respectively. The detection limits for the *M*, *H5*, and *N1* or *N8* genes were 0.6, 0.7 and 0.9 $EID_{50}$ for H5N1 HPAIV and 1.0, 0.8 and 1.3 $EID_{50}$ for H5N8 HPAIV, respectively. The assay was sufficiently sensitive for monitoring and surveillance of HPAIVs and can be used in the differential diagnosis of H5N1, H5N8 and other subtypes of AIV in countries where subtype H5 HPAIV outbreaks are occurring.

**Key words:** Highly pathogenic avian influenza virus, real-time RT-PCR, H5N1, H5N8, detection

## INTRODUCTION

Highly Pathogenic Avian Influenza Viruses (HPAIVs) have caused considerable economic losses in the poultry industry and pose potential threats to animal and human health worldwide. Furthermore, subtype H5 HPAIVs are continually evolving because of extensive genetic diversity and reassortment with other subtypes of influenza viruses (OIE Method, 2008; Donis and Smith, 2015). In early 2014, a distinct group of HPAI H5 reassortant viruses (H5N8 subclade 2.3.4.4) caused outbreaks in poultry in the Republic of Korea (ROK) and by late 2014, this subclade had spread to Japan, the Russian Federation and Europe (OIE Method, 2008).

In ROK, outbreaks of H5N1 HPAI occurred four times from 2003-2011, causing devastating economic losses in the poultry industry (Kim *et al*., 2012; Lee *et al*., 2008; Wee *et al*., 2006). On 16 January, 2014, the fifth HPAI outbreak and the first outbreak involving H5N8 occurred in Jeonbuk Province in the Southern part of ROK. The H5N8 viruses isolated from wild birds and domestic ducks and chickens were divided into two distinct genetic groups with one of these genetic groups predominant in ROK (Lee *et al*., 2014). Unlike the H5N1 HPAI outbreaks that had occurred previously, the predominant H5N8 viruses did not cause a dramatic increase in mortality and infection in chicken farms. Nevertheless, H5N1 and H5N8 HPAI manifest similar clinical signs at an early stage of infection in poultry and wild birds (Jeong *et al*., 2014). Although, the number of outbreak cases has declined significantly, H5N8 HPAIVs have been detected in domestic poultry and wild birds sporadically in 2015.

Early detection and differential diagnosis of these HPAIVs are essential for effective prevention and control of these outbreaks. Real-time Reverse Transcription-Polymerase Chain Reaction (RRT-PCR) is a powerful method for the sensitive and specific detection of viral nucleic acids in clinical samples (Mackay *et al*., 2002). RRT-PCR is time-saving and specific relative toconventional RT-PCR. Therefore, the RRT-PCR assay has been widely used and is recommended by WHO for rapid HPAIV detection (Suarez *et al*., 2007). Moreover, multiplex RRT-PCR assays allow the measurement of several fluorophores in one well for simultaneous detection of different target sequences (Wittwer *et al*., 2001). Since, the outbreak of H5N1 HPAIV, many RRT-PCR assays have been developed to detect the virus (Aguero *et al*., 2007; Chen *et al*., 2007;

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Payungporn *et al*., 2006). Some multiplex RRT-PCR assays for simultaneous typing (A/B) and subtyping of H1, H2, H3, H5, H7 and H9 influenza A viruses have also been reported (Kang *et al*., 2010; Kim *et al*., 2013; Li *et al*., 2008; Monne *et al*., 2008; Spackman *et al*., 2002; Suwannakarn *et al*., 2008; Wu *et al*., 2008). However, to our knowledge, no protocol has been reported for simultaneous differential diagnosis of the novel H5N8 and H5N1 viruses, along with other subtype H5 HPAIVs.

In this study, we developed a single-step RRT-PCR (sRRT-PCR) assay for simultaneous detection of *HPAIV M*, *H5*, *N1* and *N8* genes which could be useful for rapid differential diagnosis of H5N1, H5N8 and other influenza A viruses.

## MATERIALS AND METHODS

**Viruses:** AIV reference strains with different H and N subtypes, representative HPAIV isolates from Korea and other avian pathogens including vaccine strains of Newcastle Disease Virus (NDV), Infectious Bronchitis Virus (IBV) and the Infectious Bursal Disease Virus (IBDV) were used to evaluate the sRRT-PCR assay (Table 1). The Animal and Plant Quarantine Agency (QIA), ROK, provided inactivated AIVs including different subtypes (H1-16) of reference AIVs, four H5N1 HPAIVs isolated from previous Korean outbreaks of HPAI (2013-2010) and five H5N8 HPAIVs isolated from a 2014-2015 outbreak in ROK.

Table 1: Specificity of the single-step Real time Reverse-Transcription Polymerase Reaction (sRRT-PCR) assay for the detection of Avian Influenza Viruses (AIV) with *Matrix*, *H5*, *N1* and *N8* gene-specific primers and probe sets

| | | Detection of *AIV* gene[a] | | | |
|---|---|---|---|---|---|
| Viruses | Subtypes | *Matrix* | *H5* | *N1* | *N8* |
| **Reference strains of different subtypes** | | | | | |
| A/PR/8/34 | H1N1 | + | - | - | - |
| A/Singapore/1/57 | H2N2 | + | - | - | - |
| A/duck/Ukraine/1/63 | H3N8 | + | - | - | - |
| A/duck/Czechoslovakia/56 | H4N6 | + | - | - | - |
| A/duck/Hong Kong/820/80 | H5N3 | + | + | - | - |
| A/shearwater/Australia/1/72 | H6N5 | + | - | - | - |
| A/wild duck/Kr/CSM42-34/11 | H7N9 | + | - | - | - |
| A/turkey/Ontario/6118/68 | H8N4 | + | - | - | - |
| A/turkey/Wisconsin/1/66 | H9N2 | + | - | - | - |
| A/wild duck/Kr/CSM42-9/11 | H10N7 | + | - | - | - |
| A/duck/Memphis/546/74 | H11N9 | + | - | - | - |
| A/duck/Alberta/60/76 | H12N5 | + | - | - | - |
| A/wild duck/Kr/SH38-45 | H13N2 | + | - | - | - |
| A/mallard/Gurjer/263/82 | H14N5 | + | - | - | - |
| A/shearwater/WestAustralia/2576/79 | H15N9 | + | - | - | - |
| A/gull/Denmark/68110 | H16N3 | + | - | - | - |
| HPAI isolate from HPAI epidemic | | | | | |
| A/chicken/Korea/Gimje/2008 | H5N1 | + | + | + | - |
| A/broiler duck/Korea/Buan2/2014 | H5N8 | + | + | - | + |
| **Other avian pathogens** | | | | | |
| Newcastle disease virus (vaccine strain) | | - | - | - | - |
| Infectious bronchitis virus (vaccine strain) | | - | - | - | - |
| Infectious bursal disease virus (vaccine strain) | | - | - | - | - |

[a]Viral RNA amplification was evaluated by each sRRT-PCR using *Matrix*, *H5*, *N1* or *N8* gene-specific primer and probe sets (+: RRT-PCR-positive; -: RRT-PCR-negative)

**RNA extraction:** Viral RNA was extracted from stock viruses and field samples using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Viral RNA extractions were conducted in Biosafety Level 3 (BSL-3) facilities at the Avian Disease Division, QIA. Extracted nucleic acids were stored at -20°C until further use.

**Primers and probes for sRRT-PCR:** A primer and probe set for the detection of the Matrix (*M*) gene inall subtypes of AIVs was taken from a previous report (Kim *et al*., 2013) and an additional three sets of primers and probes for the *H5*, *N1* and *N8* genes were designed based on sequences of *H5*, *N1* and *N8* genes of subtype H5 AIV including H5N1 and H5N8 HPAIV sequences that were deposited in the Influenza Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) between 2010 and 2015. Multiple sequence alignments of the *H5*, *N1* and *N8* genes were performed using *DNAStar* Laser gene (DNAStar Inc., Madison, WI, USA). The H5 primer and probe set was designed based on an alignment of over 1,000 *H5* gene sequences of representative AIV strains from Asia, North America, Europe and Africa. The N1 primer and probe set was designed based on an alignment of over 550 *N1* gene sequences of H5N1 AIV and the N8 primer and probe set was designed based on 72 *N8* gene sequences of H5N8 AIV from Korea, China, USA and else where. All the primer and probe sets were designed using Primer Express 3.0 Software (Applied Biosystems, Foster City, CA, USA) and synthesized by Bioneer (Daejeon, Korea). All probes were labeled with 6-carboxyfluorescein (FAM) (reporter dye) at the 5' end and with 6-carboxytetramethylrhodamine (TAMRA) (quencher dye) at the 3' end. The primers and probes used in this study are listed in Table 2.

**sRRT-PCR conditions:** The sRRT-PCR amplification was carried out using RealMOD Probe HiSenScriptqRT-PCR Mix (Intron Biotechnology, Seongnam, Korea) and a real-time PCR instrument (Applied Biosystems, Waltham, MA, USA). A total reaction volume of 20 μL, containing 10 μL of 2×qRT-PCR reaction solution, 1 μL 20×qRT-PCR enzyme mix, 0.4 μM of each primer and probe and 5 μL RNA template was prepared according to the manufacturer's instruction. The sRRT-PCR program consisted of 10 min at 45°C for reverse transcription and 30 sec at 95°C for activation of Taq enzyme followed by 40 cycles of 95°C for 15 sec and 55°C for 30 sec for

Table 2: Primer and probe sets for detecting the *M*, *H5*, *N1* and *N8* genes of avian influenza viruses

| Genes | Primer and probe | Sequence (5'-3') | Product size (bp) | References |
|---|---|---|---|---|
| *Matrix* | MF (forward) | AAGACCAATCCTGTCACCTCTGA | 104 | Kim *et al.* (2013) |
| | MR (reverse) | CAAAGCGTCTACGCTGCAGTCC | | |
| | MP (probe) | FAM-TTTGTNTTYACGCTCACCGTGCC-TAMRA | | |
| *H5* | H5F (forward) | GCTCCDGAATATGCVTACAAAATTGTC | 355 | This study |
| | H5R (reverse) | TGTCTGCAGCGTAYCCAC | | |
| | H5P (probe) | FAM-ATAGCAGGNTTTATAGARGGAGGDTGGCA-TAMRA | | |
| *N1* | N1F (forward) | GATTGGTCAGGATATAGCG | 97 | This study |
| | N1R (reverse) | GAATGGCAACTCAGCACCG | | |
| | N1P (probe) | FAM-TTGTCCAGCATCCRGAACT-TAMRA | | |
| *N8* | N8F (forward) | CCAGTGACACTCCAAGAGG | 253 | This study |
| | N8R (reverse) | CCACTGTATCCCGACCAAT | | |
| | N8P (probe) | FAM-CCATGGGAAATCAGGGATATGG-TAMRA | | |

Table 3: Evaluation of the matrix, H5, N1 and N8 primer and probe set for the single-step real time reverse-transcription polymerase reaction assay by *in silico* PCR program

| | | Results of the *in silico* PCR analysis[a] | | |
|---|---|---|---|---|
| Target genes | Subtypes | No. of tested | No. of successes | Success rate (%) |
| *Matrix* | All | 10,979 | 10,978 | 99.9 |
| *H5* | H5 | 3,489 | 3,475 | 99.6 |
| | H5N1 | 2,629 | 2,623 | 99.8 |
| | H5N8 | 75 | 75 | 100.0 |
| *N1* | N1 | 3,054 | 3,043 | 99.6 |
| | H5N1 | 2,092 | 2,092 | 100.0 |
| *N8* | N8 | 1,297 | 1,274 | 98.2 |
| | H5N8 | 75 | 75 | 100.0 |

[a]Each gene specific primer and probe set was evaluated with the corresponding *AIV* gene sequences deposited in the influenza sequence database between 1996 and 2015. The matrix primer and probe set was evaluated with the *Matrix* gene sequences of all subtypes of AIVs; the H5 primer and probe set was evaluated with *H5* gene sequences of H5 AIV, H5N1 and H5N8 HPAIV and the N1 and N8 primer and probe sets were evaluated with *N1* gene sequences of N1 AIV and H5N1 HPAIV and *N8* gene sequences of N8 AIV and H5N8 HPAIV, respectively

Table 4: Evaluation of the single-step Real time Reverse-Transcription Polymerase Reaction (sRRT-PCR) assayfor the detection of H5N1 and H5N8 HPAIV isolates from chickens and mallard ducks in Korean HPAI epidemics from 2003-2015

| | | Detection of HPAIV gene[a] | | | |
|---|---|---|---|---|---|
| Viruses | Clade | *Matrix* | *H5* | *N1* | *N8* |
| **H5N1 strain** | | | | | |
| A/chicken/Korea/ES/2003 | 2.5 | + | + | + | - |
| A/chicken/Korea/IS/2006 | 2.2 | + | + | + | - |
| A/chicken/Korea/Gimje/2008 | 2.3.2.1 | + | + | + | - |
| A/duck/Korea/Cheonan/2010 | 2.3.2.1 | + | + | + | - |
| **H5N8 strain[b]** | | | | | |
| A/breeder duck/Korea/Gochang1/2014 | 2.3.4.4 | + | + | - | + |
| A/broiler duck/Korea/Buan2/2014 | 2.3.4.4 | + | + | - | + |
| A/broiler duck/Korea/H1731/2014 | 2.3.4.4 | + | + | - | + |
| A/domestic mallard duck/Korea/H1924/2014 | 2.3.4.4 | + | + | - | + |
| A/mallard duck/Korea/H2102/2015 | 2.3.4.4 | + | + | - | + |

[a]Viral RNA amplification was evaluated by each sRRT-PCR using *Matrix*, *H5*, *N1* or *N8* gene-specific primer and probe sets (+: RRT-PCR-positive; -: RRT-PCR-negative). [b]This group of *HA* gene segments was redesignated as clade 2.3.4.4 from theoriginal clade of 2.3.4.6 according to a recommendation of the WHO/OIE/FAO H5 working group

amplification. For interpretation of the sRRT-PCR results, samples producing a Cycle threshold (Ct) of <37 were considered positive and a high Ct-value (>37) were considered negative.

**Specificity of the sRRT-PCR:** The specificity of the sRRT-PCR assay was evaluated using viral RNA samples extracted from 16 reference AIV strains, two Korean representative H5N1 [A/chicken/Korea/Gimje/2008 (H5N1)] and H5N8 [A/broiler duck/Korea/Buan2/2014 (H5N8)] strains and other avian respiratory viral pathogens including NDV, IBV and IBDV vaccine strain (Table 1). In addition, we evaluated the efficacy of the sRRT-PCR assay with each primer set *in silico* using FastPCR software, version 5.4 (PrimerDigital Ltd., Helsinki, Finland) based on all available complete *M*, *H5*, *H1* and *N8* gene sequences of obtained from the Influenza Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) between 1996 and 2015 (Table 3) (Kalendar *et al*., 2011).

**Sensitivity of the sRRT-PCR:** The sensitivity of the sRRT-PCR assay for H5N1 and H5N8 HPAIV was evaluated with two Korean representative HPAIV H5N1 [A/chicken/Korea/Gimje/2008(H5N1)] and H5N8 [A/broiler duck/Korea/Buan2/2014(H5N8)]. The viruses were propagated in embryonated Specific Pathogen-Free (SPF) Chicken Eggs (ECEs) and 50% egg infectious dose ($EID_{50}$) titers were determined in 11 day old SPF ECEs using the Reed and Muench Method (Reed and Muench, 1938). To calculate the limit of detection of the sRRT-PCR assay, viral RNAs were extracted from viruses with titers of 5.5 log $EID_{50}$/0.1 mL, serially diluted 10 fold in molecular biology grade water and tested with Table 2 primer sets.

**Evaluation for Korean H5N1 and H5N8 isolates:** The usefulness of the proposed sRRT-PCR was evaluated with representative H5N1 and H5N8 strains isolated from chickens and ducks in Korean HPAI epidemics from 2003 to 2015 (Table 4). Four H5N1 strains isolated in 2003, 2006, 2008 and 2010 HPAI epidemics and five H5N8 strains isolated in a 2014-2015 epidemic were propagated in 11 days old SPF ECEs and the viral RNAs extracted from the allantoic fluids were used for sRRT-PCR with *M*, *H5*, *N1* or *N8* gene-specific primer and probe sets (Table 2).

## RESULTS AND DISCUSSION

Recently, the increasing geographic distribution of H5N1, H5N8 and other subtype HPAIVs as well as the co-circulation of HPAIVs in domestic and wild birds have raised serious concerns regarding the control of these viruses (OIE Method, 2008). In ROK, three different clades of the *HPAIV H5* gene were detected during four different H5N1 HPAI outbreaks, i.e., A/chicken/Korea/ES/2003 (clade 2.5), A/chicken/Korea/IS/2006 (clade 2.2), A/chicken/Korea/Gimje/2008 and A/duck/Korea/Cheonan/2010 (clade 2.3.2.1) (Jeong *et al*., 2014). During the 2014 outbreak in ROK, the *HA* gene of the H5N8 HPAIVs belonged phylogenetically to the proposed clade 2.3.4.6 (Lee *et al*., 2014). Now, these H5N8 HPAIVs are classified in a new clade, clade 2.3.4.4 on the recommendation of the WHO-OIE-FAO H5 evolution working group (WHO, 2015). The outbreaks of H5N1 and H5N8 HPAI that have periodically occurred in ROK and the various subtypes of AIV that have been detected in wild bird populations suggest a potential risk for endemic HPAI in the country. For effective prevention and control of these HPAI outbreaks, a rapid and sensitive RRT-PCR assay that allows simultaneous detection of subtype H5 HPAIVs and other subtype AIVs is required. However to our knowledge, no protocol has been reported for simultaneous differential diagnosis of the novel H5N8 and H5N1 viruses as well as other subtype H5 HPAI viruses. In this study, we developed and evaluated a sRRT-PCR assay with M, H5, N1 and N8-specific primer and probe sets for simultaneous detection and differentiation of HPAIV H5N1 and H5N8 subtypes from other subtypes of AIV.

For the simultaneous detection of H5N1, H5N8 and other subtype HPAIVs, we used four sets of primers and probes (M, H5, N1 and N8-specific) in our sRRT-PCR assay (Table 2). We included a previously reported *M* gene-specific primer and probe set to detect all subtypes of AIVs (Kim *et al*., 2013). The *H5*, *N1* and *N8* gene-specific primer and probe sets were newly designed in this study. The *M* gene primer set was able to detect all subtypes of AIV including representative H5N1 and H5N8 strains from Korea as expected based on earlier studies and the *H5*, *N1* or *N8* gene primer sets was able to specifically detect H5, N1 and N8 subtypes of HPAIV, respectively (Table 1). However, the assay failed to detect the *N1* gene of the A/PR/8/34(H1N1) strain and the *N8* gene of the A/duck/Ukraine/1/63(H3N8) strain, even though one or two mismatches should be tolerated (Table 1). The reason for this failure in unclear but it may be due to mismatches of the reverse primer or the probe with both viruses (Kim *et al*., 2013, 2006).

To expand our evaluation of sRRT-PCR specificity which was originally performed with a small number of reference AIVs and field isolates, we further evaluated each primer and probe set for the detection of each subtype of AIV using a web-based *in silico* PCR program (FastPCR Software, Version 5.4, PrimerDigital Ltd., Helsinki, Finland) following the developer's instructions. FastPCR can help to estimate the efficiency of a designed primer set for PCR and indicate the likelihood of PCR success with each primer set against target sequences (Kalendar *et al*., 2011). Table 3 shows the results of the *in silico* PCR with the proposed primer and probe sets targeting complete sequences of the genes of different AIV subtypes, representing isolates from 1996-2015 available in the influenza sequence database. The Predictive Success Rate (PSR) of the sRRT-PCR with each primer set was assessed as follows: the M primer and probe showed a PSR of 99.9% (10,978/10,979) for *M* gene sequences of all subtypes of AIVs; the H5 primer and probe set showed a PSR of 99.6% (3475/3489), 99.8% (2623/2629) and 100.0% (75/75) for *H5* gene sequences from H5 AIVs with different N subtypes, H5N1 and H5N8 HPAIVs, respectively the N1 primer and probe set showed a PSR of 99.6% (3043/3054) and 100.0% (2092/2092) for *N1* gene sequences from subtype N1 AIVs with different H subtypes and H5N1 HPAIVs, respectively and the N8 primer and probe set showed a PSR of 98.2% (1274/1297) and 100% (75/75) for the *N8* gene sequences from subtype N8 AIVs with different H subtypes and H5N8 HPAIVs, respectively. Based on the above *in silico* PCR results, each primer and probe set in the sRRT-PCR was found to be highly specific for each subtype of AIV. The assay could detect almost all H5, N1, and N8 subtypes of HPAIVs prevalent in ROK and other countries, suggesting that this assay can be very useful for the differential diagnosis of H5N1, H5N8 and other subtypes of AIV in countries where subtype H5 HPAIV outbreaks are occurring.

The detection thresholds of the sRRT-PCR assay for the *M*, *H5* and *N1* or *N8* gene were 0.6, 0.7 and 0.9 $EID_{50}$ for H5N1 HPAIVs and 1.0, 0.8 and 1.3 $EID_{50}$ for H5N8 HPAIVs, respectively (Fig. 1). Previous studies have shown that the sensitivity of RRT-PCR assays ranges from $10^{-1}$ to $10^{2.8}$ $EID_{50}$ (Kim *et al*., 2013; Monne *et al*., 2008; Spackman *et al*., 2002). Considering these earlier results, the sensitivity of the proposed sRRT-PCR assay should be considered sufficient for the monitoring and surveillance of HPAIVs.

The AIV strains tested in this study were limited to a small number of reference strains and field samples. Therefore, validation using additional influenza isolates and clinical samples is required to determine the utility of this assay. Additionally, continuous surveillance and

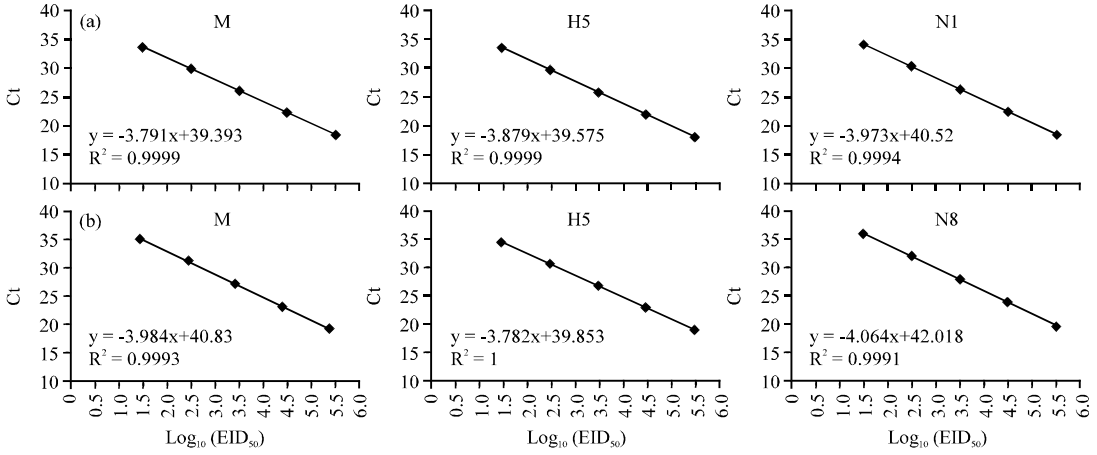


Fig. 1: Sensitivity and dynamic range of the single-step real-time RT-PCR assay for detection of H5N1 and H5N8 viral RNA. Serial 10 fold dilutions of viral RNA standard (from $10^{5.5}$ to $10^{0.5}$ $EID_{50}$/0.1 mL) were plotted against the threshold Cycle (Ct): a) A minimum of 0.6, 0.7 and 0.9 $EID_{50}$ of H5N1 virus or b) 1.0, 0.8 and 1.3 $EID_{50}$ of H5N8 virus per reaction could be detected using M, H5, N1 and N8-specific primer and probe sets, respectively. The coefficient of determination ($R^2$) and a regression curve (y) was calculated

genetic characterization of animal AIVs will be required to confirm the value of the primer and probe sets in this sRRT-PCR assay. However, to address this limitation of the study, we assessed, *in silico*, the sensitivity of the sRRT-PCR assay using PCR Software and almost all AIV sequences available in the influenza sequence database. The results showed that the sRRT-PCR assay can serve as a rapid differential diagnostic tool for currently prevalent HPAIVs in ROK and other countries where various subtypes of HPAIVs are circulating in avian populations.

## CONCLUSION

In this study, we developed an sRRT-PCR assay for simultaneous detection of the AIV *matrix*, *H5*, *N1* and *N8* genes. This sRRT-PCR assay can be used for the differential diagnosis of H5N1, H5N8 and other subtypes of AIV, especially in countries where H5 subtype HPAIV outbreaks are occurring.

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