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Multiplex real-time reverse transcription polymerase chain reaction for differential detection of H5, N1, and N8 genes of highly pathogenic avian influenza viruses

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ABSTRACT: Rapid and differential diagnosis of highly pathogenic avian influenza virus (HPAIV) subtype H5 is essential for the effective prevention and control of outbreaks caused by this pathogen. In this study, we describe a one-step multiplex real-time reverse transcription polymerase chain reaction (mRRT-PCR), using H5-, N1-, and N8-specific primers and probes, for differential detection of two HPAIVs (H5N1 and H5N8) and other H5-subtype AIVs. Using the mRRT-PCR assay, we were able to detect H5N1, H5N8, and other H5-subtype AIVs in a one-tube reaction, with high specificity; furthermore, using an *in silico* PCR program, we confirmed that this assay can detect nearly all H5, N1, and N8 genes of AIVs currently available in the Influenza Sequence Database. The limit of detection of the assay was determined to be as low as 100 copies/reaction for each target gene, and was comparable to limits of detection of previously reported mRRT-PCR assays. Thus, the mRRT-PCR assay described here can serve as a rapid and reliable differential diagnostic tool for the monitoring and surveillance of H5N1, H5N8, and other H5-subtype AIVs in countries where these pathogens are problematic.

Keywords: highly pathogenic avian influenza virus; real-time RT-PCR; H5N1; H5N8

Highly pathogenic avian influenza viruses (HPAIVs) of subtype H5 continue to circulate in poultry and wild birds, and pose a threat to animal and human health worldwide. Furthermore, these viruses are continually evolving – because of extensive genetic diversity, and because of reassortment with other subtypes of influenza viruses (WHO 2015). Recently, several H5-lineage AIVs have acquired neuraminidase genes from unrelated AIVs via reassortment, and novel H5N2, H5N3, H5N5, H5N6, and H5N8 reassortant subtype viruses were reported in Asia, Europe, and North America (Smith et al. 2015). In the Republic of Korea, outbreaks of H5N1 HPAI occurred four times between 2003 and 2011, causing devastating economic losses in the poultry industry, as well as serious public health concerns (Kim et al. 2010; Kim et al. 2012). Subsequently, the first outbreak of H5N8 occurred in Jeonbuk province in the southern part of the Republic of Korea in January of 2014 (Lee et al. 2014). H5N8 HPAIVs isolated from infected birds belonged to two distinct genetic groups, with one of these genetic groups predominant in the Republic of Korea (Lee et al. 2014). Despite extensive national control strategies, H5N8 HPAIVs were sporadically detected in domestic poultry and wild birds in 2015 and 2016. Unlike

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H5N1 HPAIVs, the predominant H5N8 HPAIVs did not cause a dramatic increase in mortality and infection in commercial chickens. Nevertheless, H5N1 and H5N8 HPAIVs manifest similar clinical signs at an early stage of infection in poultry and wild birds (Jeong et al. 2014; Lee et al. 2014).

Rapid and differential diagnoses of these HPAIVs are essential for effective prevention and control of outbreaks caused by these pathogens. The real-time reverse transcription polymerase chain reaction (RRT-PCR) is a powerful tool for the sensitive and specific detection of viral nucleic acids in clinical samples, and it is faster and more specific than conventional RT-PCR. Therefore, the RRT-PCR assay has been widely used, and is recommended by the WHO for rapid HPAIV detection (WHO 2014). Moreover, multiplex RRT-PCR (mRRT-PCR) assays allow the measurement of several fluorophores for simultaneous detection of different target sequences in a single reaction – saving time, labour, and costs in clinical diagnostic laboratories. Several mRRT-PCR assays have been successfully developed for the detection of H5 and H7 genes (Spackman et al. 2003), H5 and H9 genes (Li et al. 2008), and H5 and N1 genes (Payungporn et al. 2006; Wu et al. 2008), in a single reaction. However, no mRRT-PCR assay has been described for the differential detection of H5, N1, and N8 genes of HPAIVs.

In our previous report, we described a single-step RRT-PCR for the detection of AIV matrix, H5, N1, and N8 genes using individual, gene-specific primers and probe sets (Kim and Park 2015). Although the assay could be useful for the rapid and sensitive detection of H5N1, H5N8, and other subtypes of AIVs, there were some limitations in its application to large-scale surveillance of HPAIVs. Namely, the assay was carried out in a uniplex RRT-PCR (uRRT-PCR) format using four separate reaction tubes, each containing different target gene-specific primers and probe sets. Therefore, in this study, we developed a one-step mRRT-PCR assay for the simultaneous detection of HPAIV H5, N1, and N8 genes, which could be applicable for rapid and differential diagnoses of H5N8, H5N1, and other H5-subtype AIVs.

MATERIAL AND METHODS

Viruses. AIV reference strains with different haemagglutinin and neuraminidase subtypes, Korean-representative HPAIV strains, and other avian pathogens, including vaccine strains of Newcastle disease virus (La Sota strain), infectious bronchitis virus (AVR1 strain), and the infectious bursal disease virus (W2512 strain), were used to evaluate the mRRT-PCR assay (Table 1). The Animal and Plant Quarantine Agency of the Republic of Korea kindly provided inactivated AIVs, including different subtypes (H1–16) of reference AIVs, four H5N1 HPAIVs isolated from previous Korean outbreaks of HPAI (2003–2010), and five H5N8 HPAIVs isolated from recent outbreaks of HPAI (2014–2015) in the Republic of Korea.

RNA extraction. Viral RNAs were extracted from stock viruses and field samples using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Viral RNA extractions were conducted at Biosafety Level 3 (BSL-3) facilities in the Avian Disease Division of Animal and Plant Quarantine Agency. Extracted nucleic acids were stored at –20 °C until needed.

Primers and probes for mRRT-PCR. For the simultaneous detection of H5N1, H5N8, and other H5-subtype HPAIVs, three sets of primers and probes (H5- and N1-specific) described in previous reports (Kim et al. 2013; Kim and Park 2015), were used. For accurate differential detection of HPAIVs using mRRT-PCR, it was essential that sequence-specific probes were labelled with reporter dyes whose fluorescence spectra were distinct, or exhibited only minimal overlap (Navarro et al. 2015). In this study, for the simultaneous and differential detection of H5, N1, and N8 genes in a single reaction, the probes for these genes were labelled differently at the 5' and 3' ends: the 6-carboxyfluorescein (FAM) reporter dye and black hole quencher 1 for the H5 probe, the hexachlorofluorescein (HEX) reporter dye and black hole quencher 1 for the N1 probe, and the cyanine 5 (CY5) reporter dye and black hole quencher 2 for the N8 probe. All reporters and dyes were used according to the manufacturer's guidelines (Bioneer, Korea; Table 2).

mRRT-PCR conditions. A number of commercial kits are currently available for multiplex real-time assays without optimisation. In this study, we pre-tested five commercial kits to verify that they were suitable for the creation of the mRRT-PCR assay; we then selected the kit that worked best for mRRT-PCR, and conducted subsequent experiments using this kit. The mRRT-PCR assay with H5, N1, and N8 primers and probe sets was performed

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Table 1. Avian influenza viruses and other avian viruses used to evaluate the multiplex real-time reverse transcription polymerase chain reaction (mRRT-PCR) assay

Virus (clade)	Subtype	Detection of the AIV gene[a]		
		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/West Australia/2576/79	H15N9	–	–	–
A/gull/Denmark/68110	H16N3	–	–	–
HPAI isolates from Korean HPAI outbreaks				
A/chicken/Korea/ES/2003 (2.5)	H5N1	+	+	–
A/chicken/Korea/IS/2006 (2.2)	H5N1	+	+	–
A/chicken/Korea/Gimje/2008 (2.3.2.1)	H5N1	+	+	–
A/duck/Korea/Cheonan/2010 (2.3.2.1)	H5N1	+	+	–
A/breeder duck/Korea/Gochang1/2014 (2.3.4.4)[b]	H5N8	+	–	+
A/broiler duck/Korea/Buan2/2014 (2.3.4.4)[b]	H5N8	+	–	+
A/broiler duck/Korea/H1731/2014 (2.3.4.4)[b]	H5N8	+	–	+
A/domestic mallard duck/Korea/H1924/2015 (2.3.4.4)[b]	H5N8	+	–	+
A/mallard duck/Korea/H2102/2015 (2.3.4.4)[b]	H5N8	+	–	+
Other avian pathogens				
Newcastle disease virus, La Sota strain		–	–	–
Infectious bronchitis virus, AVR1 strain		–	–	–
Infectious bursal disease virus, W2512 strain		–	–	–

+ = mRRT-PCR positive, – = mRRT-PCR negative

[a]Viral RNA amplification by mRRT-PCR was evaluated using H5, N1, and N8 gene-specific primer and probe sets

[b]This group of HA gene segments was re-designated clade 2.3.4.4, from the original clade 2.3.4.6, according to a recommendation made by the WHO/OIE/FAO H5 Working Group

using a THUNDERBIRD Probe One-step qRT-PCR Kit (Toyobo, Japan) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). A total reaction volume of 20 µl, containing the following components, was prepared according to the manufacturer's instructions: 10 µl of 2 × reaction buffer, 0.5 µl RT enzyme mix, 0.5 µl DNA polymerase, 0.5µM of each primer, 0.25µM of each probe, and 5 µl of viral RNA template (extracted from strains H5N1 [A/chicken/Korea/Gimje/2008], H5N8 [A/broiler duck/Korea/Buan2/2014], or H5N3 [A/duck/Hong Kong/820/80]). The reaction program consisted of 10 min at 50 °C for reverse transcription, 1 min at 95 °C for the initial denaturation, and 40 cycles of 95 °C for 15 s, and 58 °C for 45 s, for amplification. FAM, HEX, and CY5 fluorescence signals were obtained at the end of each annealing step. For the interpretation of the mRRT-PCR

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Table 2. Primers and probes for the proposed multiplex real-time reverse transcription polymerase chain reaction used in this study

Targetgene	Primer or probe	Sequence (5'-3')	Genome position[a]	T_m (°C)	Product size (bp)
H5	H5F	TGACTACCCGCAGTATTCAG	1506–1525	55.8	147
	H5R	AGACCAGCTAYCATGATTGC	1633–1652	56.7	
	H5P	FAM-TCAACAGTGGCRAGTTCCYTAGCA-BHQ1	1606–1629	63.3	
N1	N1F	GATTGGTCAGGATATAGCG	1132–1150	51.5	97
	N1R	GAATGGCAACTCAGCACCG	1317–1335	58.6	
	N1P	HEX-TTGTCCAGCATCCRGAACT-BHQ1	1157–1175	55.3	
N8	N8F	CCAGTGACACTCCAAGAGG	964–982	56.1	253
	N8R	CCACTGTATCCCGACCAAT	1198–1216	53.0	
	N8P	CY5-CCATGGGAAATCAGGGATATGG-BHQ2	1018–1039	57.3	

BHQ = black hole quencher, FAM = 6-carboxyfluorescein, HEX = hexachlorofluorescein, CY5 = cyanine 5

[a]The position of the primer- or probe-binding sequence is marked according to the genetic sequence of the H5 and N1 genes of the Korean-representative H5N1 HPAIV (A/duck/Korea/Cheonan/2010 (H5N1), GenBank accession numbers JN807950 and JN808043) and the N8 gene of H5N8 HPAIV (A/broiler duck/Korea/Buan2/2014 (H5N8), GenBank accession number KJ413844.1)

results, samples producing cycle threshold (Ct) values less than 37 were considered positive, and those with high Ct values (greater than 37) were considered negative (Kim and Park 2015).

mRRT-PCR specificity. The specificity of the mRRT-PCR assay was evaluated using viral RNA samples extracted from 16 reference AIV strains, nine Korean-representative H5N1 and H5N8 strains, and other avian respiratory viral pathogens, including Newcastle disease virus, infectious bronchitis virus, and infectious bursal disease virus vaccine strains (Table 1). In addition, we further evaluated the efficacy of mRRT-PCR with each primer set using the FastPCR software package, version 5.4 (PrimerDigital Ltd., Finland); these *in silico* evaluations were based on all available, complete sequences of H5, N1, and N8 genes obtained from the Influenza Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) between 1996 and 2016, as previously described (Kalendar et al. 2011; Kim and Park 2015).

mRRT-PCR sensitivity. The sensitivities of all assays (the mRRT-PCR assay and its corresponding monoplex assays for H5N1 and H5N8 HPAIVs) were evaluated with the specific target H5, N1, and N8 gene segments of two representative Korean H5N1 (A/chicken/Korea/Gimje/2008 [H5N1]) and H5N8 (A/broiler duck/Korea/Buan2/2014 [H5N8]) HPAIVs (Kim et al. 2010; Lee et al. 2014). The H5, N1, and N8 genes were amplified from H5N1 and H5N8 viral RNAs using previously described RT-PCR methods (Hoffmann et al. 2001), and were cloned into the pTOP TA V2 vector (Enzynomics, Korea) according to the instructions accompanying the TOPcloner TA core Kit (Enzynomics). The concentrations of each of the positive plasmid DNAs were determined by measuring absorbance at 260 nm using a NanoDrop Lite (Thermo Scientific, USA). Copy numbers of each of the cloned genes were quantified using the following formula from Parida et al. (2011):

$$\text{copies}/\mu l = \text{concentration of plasmid } (g/\mu l)/[(\text{plasmid length} \times 660) \times (6.022 \times 10^{23})]$$

Each mRRT-PCR assay was carried out three times, with 10-fold serially diluted DNAs (from 10^7 to 10^0 copies/µl) for each target. This was done to assess the average limit of detection of the mRRT-PCR assay, and to compare it with the results of its corresponding uRRT-PCR assay.

Clinical evaluation of mRRT-PCR vs M-RRT-PCR. To evaluate the mRRT-PCR assay on clinical samples, we selected 67 tissue samples from stored specimens (collected during the 2010–2015 Korean HPAI outbreaks) in our laboratory (Table 3). Prior to application of mRRT-PCR, AIV matrix gene-specific RRT-PCR (M-RRT-PCR) assays were performed on the samples for the detection of the matrix (M) gene of AIVs, using our previously described method (Kim and Park 2015). Those results were compared with the results of the mRRT-PCR assays that were

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Table 3. Diagnostic results from clinical samples assayed with the matrix gene-specific RRT-PCR (M-RRT-PCR) and the newly-developed multiplex RRT-PCR (mRRT-PCR)

M-RRT-PCR	mRRT-PCR			Subtype assayed by mRRT-PCR (*n*)
	positive	negative	subtotal	
Positive	4	1[a]	5	H5 (1), H5N1 (1), H5N8 (2)
Negative	0	62	62	
Subtotal	4	63	67	

[a]One sample that was M-RRT-PCR-positive and mRRT-PCR-negative was confirmed as an H9-subtype avian influenza virus

performed on the same RNAs extracted from the samples. To further evaluate virus-positive samples, the haemagglutinin protein was then subtyped as previously described (Kim et al. 2012).

RESULTS

Interpretation of mRRT-PCR results

The primers and probes summarised in Table 2 were selected to facilitate mRRT-PCR detection, in multiplex format, of FAM, HEX, and CY5 fluorescent signals, which corresponded to the H5, N1, and N8 genes of H5-subtype AIVs, respectively. All primers and probes were designed to work under identical PCR conditions in the multiplex format. The results showed that double-fluorescent signals of FAM and HEX or FAM and CY5 could be detected by the mRRT-PCR system for the H5N1 (Figure 1A) or H5N8 (Figure 1B) HPAIVs. Triple-fluorescent signals of FAM, HEX, and CY5, for the viral mixture of H5N1 and H5N8 (Figure 1C), could also be detected. In addition, a FAM signal was detected by the system for other NA subtypes of H5 AIV, such as H5N3 (Figure 1D). These results demonstrate that mRRT-PCR successfully amplified three specific target genes, H5, N1, and N8, without spurious amplifications or significant cross-communications among the three fluorescent reporter dyes.

mRRT-qPCR specificity

The mRRT-PCR assay successfully amplified (1) H5 genes from all tested H5-subtype AIVs, including the H5N3, H5N1, and H5N8 strains; (2) N1 genes from all tested N1-subtype AIVs, including the H1N1 and H5N1 strains; and (3) N8 genes from all tested N8-subtype AIVs, including the H3N8 and H5N8 strains. However, the mRRT-PCR assay did not amplify genes from other AIV subtypes or other avian pathogens. Furthermore, as expected, the mRRT-PCR assay successfully co-amplified the H5 and N1, and H5 and N8 genes from H5N1 and H5N8 HPAIVs, respectively (Table 1). The results indicate that the mRRT-PCR assay is a precise and reliable diagnostic tool for the H5 subtype of influenza virus.

Table 4. In silico evaluation of the H5, N1, and N8 primer and probe sets for the multiplex real-time reverse transcription polymerase reaction assay

Target gene	Subtype	Results of *in silico* PCR analysis[a]		
		tested (*n*)	successes (*n*)	success rate (%)
H5	H5N1	2866	2861	99.8
	H5N8	157	157	100.0
	All	4006	3813	95.6
N1	H5N1	2304	2299	99.8
	H1N1	364	362	99.5
	All	3372	3365	99.8
N8	H5N8	157	157	100.0
	H3N8	788	787	99.9
	All	1598	1591	99.6

[a]Each gene-specific primer and probe set was evaluated using corresponding avian influenza virus (AIV) gene sequences deposited in the Influenza Sequence Database between 1996 and 2016. The H5 primer and probe sets were evaluated using H5 gene sequences of all H5 AIVs, including H5N1 and H5N8; the N1 primer and probe sets were evaluated using N1 gene sequences of all N1 AIVs, including H5N1 and H1N1; and the N8 primer and probe sets were evaluated using N8 gene sequences of all N8 AIVs, including H5N8 and H3N8

Evaluation of the mRRT-PCR using *in silico* PCR (Table 4)

The predictive success rate (PSR) of the mRRT-PCR with each primer set was assessed as follows: the H5 primer and probe set showed a PSR of 99.8% (2861/2866), 100.0% (157/157) and 95.6% (3813/4006) for H5 gene sequences from H5N1, H5N8 and all neuraminidase subtypes of H5 AIVs, respectively; the N1 primer and probe set showed

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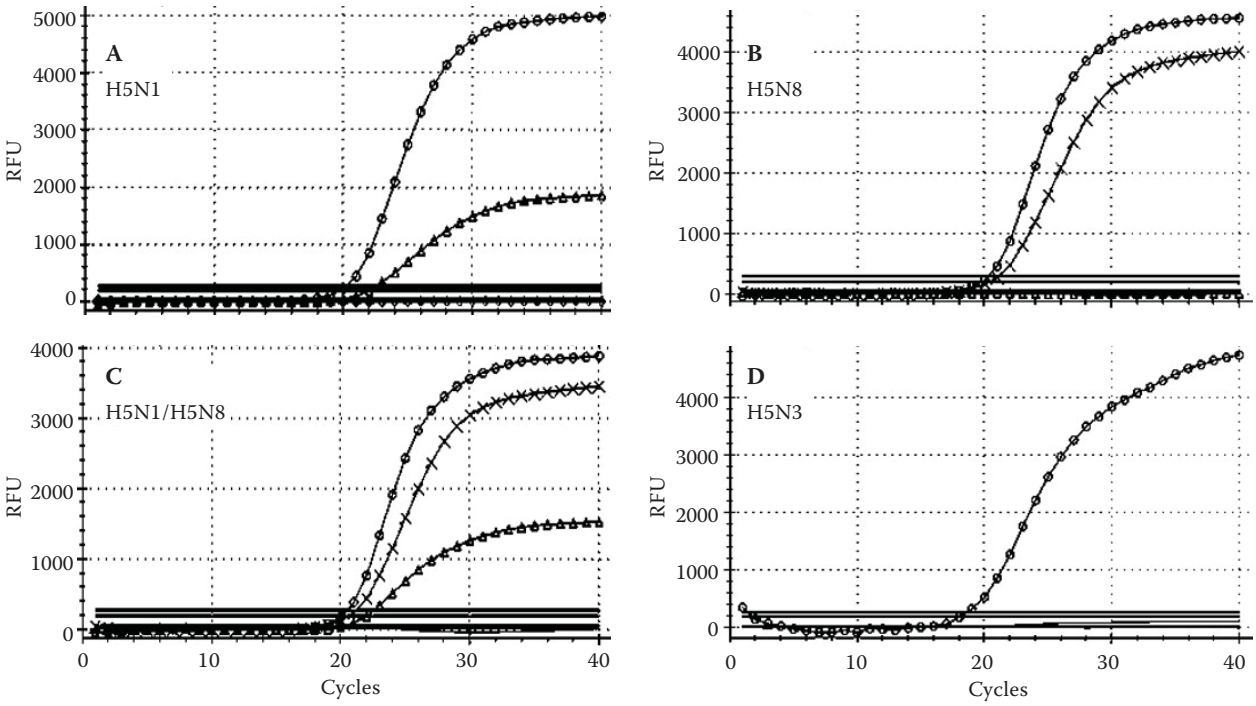

Figure 1. Interpretation of H5-subtype HPAIV detection in the multiplex real-time reverse transcription polymerase chain reaction (○ = H5 gene, △ = N1 gene, × = H8 gene)

a PSR of 99.8% (2299/2304), 99.50% (362/364) and 99.8% (3365/3372) for N1 gene sequences from H5N1, H1N1 and all haemagglutinin subtypes of N1 AIVs, respectively; The N8 primer and probe set showed a PSR of 100.0% (157/157), 99.9% (787/788) and 99.6% (1591/1598) for N8 gene sequences from H5N8, H3N8 and all haemagglutinin subtypes of N8 AIVs, respectively. Based on the above *in silico* PCR results, each primer and probe set for the mRRT-PCR was proven to have high specificity for the current circulating H5 subtype of AIVs.

Sensitivity of the mRRT-PCR

To determine the limit of detection (LOD) of H5, N1 and N8 by mRRT-PCR (or its corresponding uRRT-PCR), standard curves for target genes were generated by plotting their Ct numbers versus their dilution factors (Figure 2). High correlation values ($R^2 > 0.99$) between Ct values and dilution factors were obtained for the results of the mRRT-PCR and uRRT-PCR assays. The LOD values for H5 by mRRT-PCR were 4.5 and 99.9 copies for H5N1 and H5N8 HPAIV, respectively; for H5N1, this was similar to the corresponding uRRT-PCR LOD (4.4 copies), whereas for H5N8, this was higher than the corresponding uRRT-PCR LOD (36.3 copies). For N1, the mRRT-PCR LOD was 8.7 copies – a little higher than the corresponding uRRT-PCR LOD (3.1 copies). Lastly, for N8, the mRRT-PCR LOD was 4.4 copies, significantly lower than the corresponding uRRT-PCR LOD (15.9 copies; Figure 2).

Clinical evaluation of mRRT-PCR vs M-RRT-PCR

The M-RRT-PCR assay identified five out of 67 clinical samples as AIV-positive, whereas the mRRT-PCR assay identified four samples as positive: H5 (n = 1), H5N1 (n = 1), and H5N8 (n = 2; Table 3). The one AIV sample that was M-RRT-PCR-positive and mRRT-PCR-negative was subsequently confirmed as an H9 subtype, using the previously described method (Kim et al. 2012). The 62 samples that were identified as negative by the M-RRT-PCR were all confirmed as negative by the newly-developed mRRT-PCR assay. These results showed that mRRT-PCR could be used for clinical diagnoses by detecting H5N1 and H5N8 HPAIVs, and with the same sensitivity and specificity as the previously described M-RRT-PCR assay; however, this was not the case for non-H5 subtypes of AIVs.

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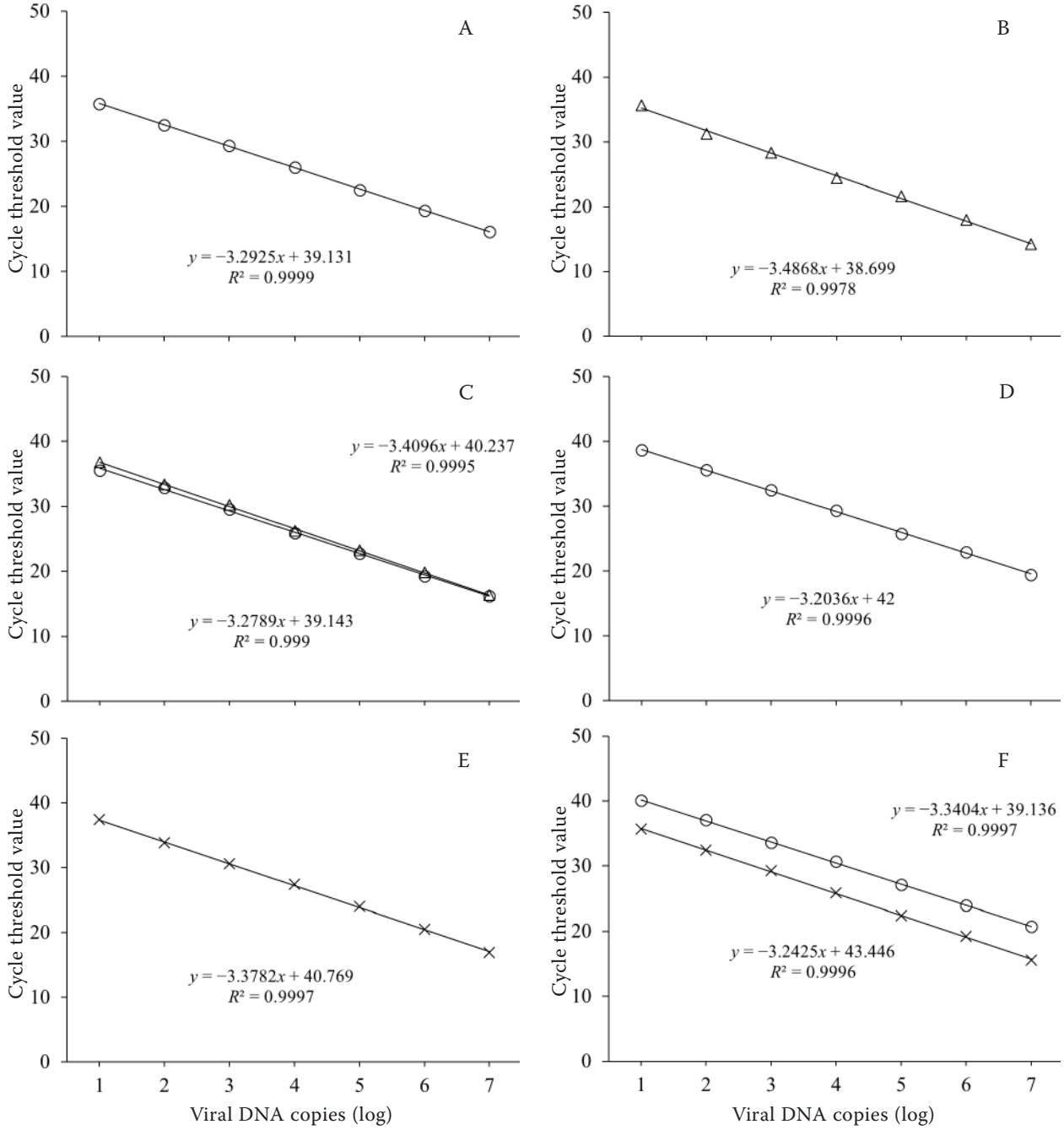

Figure 2. Limit of detection values of the uniplex or multiplex real-time reverse transcription polymerase chain reaction assays for the H5N1 and H5N8 highly pathogenic avian influenza viruses (HPAIVs)

A = Monoplex H5 (FAM), B = Monoplex N1 (HEX), C = Multiplex H5 and N1, D = Monoplex H5 (FAM), E = Monoplex N8 (CY5), F = Multiplex H5 and N8

DISCUSSION

In recent times, the increasingly broad geographic distributions of H5-subtype HPAIVs in domestic and wild birds have raised serious concerns regarding the control of these viruses (Smith et al. 2015; WHO 2015). In parts of Asia, including Korea, H5-subtype AIVs – including H5N1, H5N8, and recently H5N6 – have been continuously detected in poultry and wild bird populations, suggesting that there is a potential risk for endemic HPAI in these countries (Jeong et al. 2014). For the effective prevention and control of these potential HPAI outbreaks, a rapid and sensitive mRRT-PCR assay

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that allows differential detection of HPAIV subtypes H5N1, H5N8, and other H5-subtype AIVs, is required. Until now, many uRRT-PCR assays have been reported for the detection of M, H5, H7, H9, N1, or N8 genes (Spackman et al. 2002; Lee and Suarez 2004; Monne et al. 2008; Sidoti et al. 2010; Tsukamoto et al. 2010; Kim et al. 2013; Kim and Park 2015). Also, some mRRT-PCR assays with multiple gene-specific primer and probe sets have been reported for the simultaneous detection and/or subtype discrimination of different haemagglutinin and neuraminidase subtypes of influenza A or B viruses (Payungporn et al. 2006; Li et al. 2008; Suwannakarn et al. 2008; Wu et al. 2008; Choi et al. 2013). However, to the best of our knowledge, no protocol for mRRT-PCR has been reported for the simultaneous detection of H5, H1, and N8 genes that can discriminate between H5N1 and H5N8 HPAIVs. In this study, we developed and evaluated an mRRT-PCR assay that can differentially detect the H5, N1, and N8 genes of H5N1, H5N8, and other H5-subtype AIVs

Due to the high variation in the nucleotide sequences of the haemagglutinin and neuraminidase genes between different AIV isolates, it is difficult to select completely conserved sites against which to design primers and probes that match, with 100% accuracy, the targeted AIV isolates of any given subtype. Therefore, we used three primer and probe sets with diagnostic efficacies that had already been confirmed through previous studies (Kim et al. 2013; Kim and Park 2015). We further evaluated the capability of each primer and probe set to detect the target genes of AIVs using a web-based *in silico* PCR software application (FastPCR). The FastPCR application can help in estimating the efficiency of a designed primer set for PCR, and will indicate the likelihood of PCR success with each primer set against given target sequences (Kalendar et al. 2011). The PSRs of mRRT-PCR assays using H5, N1, and N8 primers and probe sets were 95.6%, 99.8%, and 99.6% (respectively) for all available H5, N1, and N8 AIV gene sequences. The PSRs for target genes of the H5N1 and H5N8 HPAIVs were confirmed to be nearly 100% (Table 2); this suggests that almost all H5N1 and H5N8 HPAIVs, and other H5-subtype AIVs, can be specifically detected in the mRRT-PCR assay. The analytical sensitivity of the mRRT-PCR assay was at least 100-fold higher than that of the method previously reported by Spackman et al. (2003), and was comparable to that of the method reported by Li et al. (2008) and Wu et al. (2008). Considering these results, the sensitivity of the mRRT-PCR assay in this study should be sufficient for the monitoring and surveillance of H5-subtype HPAIVs.

Several factors in this new assay need to be optimised, including the concentrations of reagents (primers and probes, Mg^{2+}, *Taq* DNA polymerase, and dNTPs) and the composition of the PCR buffer, to ensure that all multiplex reactions occur with efficiencies equal to their corresponding uniplex reactions. However, the process of optimising multiplex real-time assay parameters can be tedious and time-consuming (Henegariu et al. 1997; Markoulatos et al. 2002). A number of commercial kits are currently available for multiplex real-time assays that do not require optimisation. However, despite the manufacturers' claims, using these kits does not guarantee successful multiplex reactions in all cases. In this study, we pre-tested five commercial kits to verify that they were suitable for the establishment of the mRRT-PCR assay, and we selected the kit that worked best (with minor optimisation steps for annealing temperatures; data not shown). Therefore, researchers who want to develop multiplex RT-PCR assays can save the time and effort required for assay optimization by conducting preliminary experiments with various commercial kits.

In addition to factors that that may affect the sensitivity of the RRT-PCR assay (such as the RNA extraction procedure used, efficiency of Taq polymerase used, and choice of fluorescent reporter dyes), two additional factors may cause decreases in the sensitivity of multiplex reactions: (1) cross-interaction between primer-pairs, probes, and amplicons can inhibit subsequent PCR reaction cycles; and (2) the efficiency of reverse transcription can be affected by competition between amplicons of different sizes (Makoulatos et al. 2002; Wu et al. 2008; Kalendar et al. 2009). In this study, the LOD of the mRRT-PCR assay using three primer-probe sets was a little higher than that of each of the three individual corresponding uRRT-PCR assays (Figure 2). This outcome probably reflects the influence of interaction between primers, probes, and amplicons in the multiplex environment (Makoulatos et al. 2002; Kalendar et al. 2009). The mRRT-PCR LOD values for H5 and N8 were rather higher than that for N1, probably because of slightly larger amplicons for H5 and N8 than for N1 (Spackman et al. 2003; Wu et al. 2008).

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Although the proposed mRRT-PCR in this study simultaneously detected H5, N1, and N8 genes from tested viruses and clinical samples, the assay is limited by the fact that it cannot detect the non-H5-subtype AIVs that are circulating in bird populations (Table 3). To address this, one diagnostic strategy would be to combine the proposed mRRT-PCR assay and the previously described AIV M-gene-specific RRT-PCR (Kim et al. 2013; Kim and Park 2015), to enable the simultaneous detection of all AIV subtypes while maintaining the ability to discriminate between the H5N1 and H5N8 HPAIVs. More preferably, a multiplex RRT-PCR needs to be developed for the simultaneous detection of the M, H5, N1, and N8 genes of AIVs. Based on these results, the mRRT-PCR assay developed in this study showed good sensitivity and specificity, and will be useful for laboratory surveillance and rapid diagnosis of H5N1, H5N8, and other N-subtype H5 HPAIVs in countries where various H5-subtype HPAIVs are circulating in avian populations. However, although the primers and probe sets were carefully designed to hybridise with conserved regions of the target genes (based on most AIV sequences available from the Influenza Sequence Database at the time of this study), the emergence of novel H5-subtype HPAIVs that are not detectable by this assay can be expected, due to the mutation-prone nature of AIVs. Therefore, continuous evaluation with new influenza isolates and clinical samples is required to confirm the suitability of the primers and probe sets used in this mRRT-PCR assay.

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