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Development of Multiplex RT-PCR Assays for Rapid Detection and Subtyping of Influenza Type A Viruses from Clinical Specimens

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We developed multiplex RT-PCR assays that can detect and identify 12 hemagglutinin (H1–H12) and 9 neuraminidase (N1–N9) subtypes that are commonly isolated from avian, swine, and human influenza A viruses. RT-PCR products with unique sizes characteristic of each subtype were amplified by multiplex RT-PCRs, and sequence analysis of each amplicon was demonstrated to be specific for each subtype with 24 reference viruses. The specificity was demonstrated further with DNA or cDNA templates from 7 viruses, 5 bacteria, and 50 influenza A virus–negative specimens. Furthermore, the assays could detect and subtype up to 10^5 dilution of each of the reference viruses that had an original infectivity titer of 10^6 EID_{50}/ml. Of 188 virus isolates, the multiplex RT-PCR results agreed completely with individual RT-PCR subtyping results and with results obtained from virus isolations. Furthermore, the multiplex RT-PCR methods efficiently detected mixed infections with at least two different subtypes of influenza viruses in one host. Therefore, these methods could facilitate rapid and accurate subtyping of influenza A viruses directly from field specimens.

Keywords: Influenza A virus, multiplex RT-PCR, subtyping, clinical specimens

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus, which is an enveloped, single-stranded, negative RNA virus of the *Orthomyxoviridae* family. Influenza A virus frequently causes widespread and fatal disease in birds as well as mammals, including humans. Influenza A viruses can be classified into various subtypes on the basis of antigenic differences between the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA); there are 16 subtypes of HA (H1-16) and 9 subtypes of NA (N1-9) [7]. Amino acid sequence identity among subtypes of HA and NA ranges from 25-80% and 42–57%, respectively [6]. All influenza A virus subtypes have been found in aquatic and domesticated birds, and a few subtypes have been recovered from mammals. Influenza A viruses that have infected humans during the past 90 years have been limited to the H1, H2, and H3 subtypes. However, human infections with several AI subtypes such as H5N1 [4, 15, 21, 22], H7N7 [11], and H9N2 [3, 8, 16] have occurred, thus demonstrating direct crossing of the species barrier. Therefore, there is a great need for more rapid and precise methods to detect HA and NA subtypes irrespective of the species (avian, swine, and human).

Methods for detecting and subtyping influenza viruses utilize the propagation of virus in tissue culture or embryonated eggs before subtyping by hemagglutination inhibition (HI) [13] and neuraminidase inhibition (NI) tests, which use a monospecific antiserum to each subtype [2]. Although virus propagation in tissue culture or embryonated eggs is sensitive and accurate, it requires several days for a viable virus to cause observable cytopathic effects; thus, such assays are time-consuming and laborious. Other diagnostic tests have also been used, such as immunofluorescence staining and enzyme-linked immunosorbent assay (ELISA) based on the detection of nucleoprotein antigen [20]. ELISAs can rapidly detect viruses, but the sensitivity is comparatively poor. Molecular techniques such as PCR-based methods, however, have enabled major advances in the speed and sensitivity of the laboratory diagnosis of viral infections. Specifically, these methods have higher sensitivity (93%) for influenza A viruses than cell culture methods (80%) and ELISA (62%) [19]. Among the 16 HA subtypes, only H5 and H7 are highly virulent in poultry,

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and the rest of the viruses cause a much milder, primary respiratory disease known as low-pathogenic avian influenza [1]. However, no influenza virus subtype can be ruled out as a candidate for a potential pandemic, because all three pandemic influenza viruses in the 20th century originated directly or indirectly from avian influenza viruses.

We have monitored influenza viruses from domesticated and migratory birds in Korea since 2004. Of the 144 subtypes, the H1 to H12 subtypes of the HA gene and N1 to N9 subtypes of the NA gene have been commonly detected from migratory birds and domesticated animals in Korea (unpublished data). To facilitate more rapid detection of these subtypes, we have developed multiplex RT-PCR assays that use appropriate primer mixtures specifically designed to detect and identify 12 HA (H1-H12) and 9 NA (N1-N9) subtypes of avian, swine, and human influenza viruses. These multiplex RT-PCR assays were used to investigate the prevalence of each avian influenza virus subtype and to rapidly detect highly pathogenic avian influenza (HPAI) from clinical specimens and viral isolates.

MATERIALS AND METHODS

Isolation of Viruses

Influenza virus strains were isolated in embryonated eggs and MDCK cells from tracheal swabs and fecal samples of animals during 2004–2007. Tracheal swabs and fecal samples were collected into tissue culture medium containing penicillin G (2×10^6 U/l), polymyxin B (2×10^6 U/l), gentamicin (250 mg/l), nystatin (0.5×10^6 U/l), ofloxacin HCl (60 mg/l), and sulfamethoxazole (0.2 g/l). Specimens were inoculated into MDCK and embryonated eggs and influenza viruses purified as previously described [10, 14]. Virus isolates were subtyped with a panel of reference antisera recommended by the World Health Organization (http://www.who.int/csr/resources/publications/en/#influenza). One hundred eighty-eight type A influenza virus isolates, cultured from cells or embryonated eggs, 85 virus-positive fecal specimens from birds, and 40 lung samples from swine were selected from archived isolates/samples at Chungbuk National University. Thirty-five influenza virus-negative samples (20 from fecal specimens of birds, and 15 swine lung tissues) were also obtained. These samples were collected from swine and domestic poultry between 2004 and 2007. Approximately 1 g of fecal specimens was placed in a tube with 3–5 ml of Eagle's minimum essential medium (MEM) and vortexed vigorously. Approximately 10% suspension of each lung homogenate was also prepared in Eagle's MEM. The suspensions of fecal specimens or lung homogenates were centrifuged at 800 ×*g* for 20 min, and 200 µl of the supernatants was used for virus isolation and RNA extraction. Virus-positive allantoic or cell culture fluids were harvested and stored as stocks at −80°C until used.

Extraction of RNA and Synthesis of cDNA

Viral RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Briefly, 200 µl of the specimens was mixed with 550 µl of RLT buffer and incubated for at least 5 min at room temperature. After the addition of 550 µl of absolute ethanol, the mixture was vortexed and applied to a spin-column. After the washing and drying steps, RNA was eluted in 40 µl of RNase-free water. Reverse transcription was carried out under standard conditions by using influenza-specific primers [9].

Multiplex PCR Reaction

The PCR reaction in a 50 µl volume contained 5 U of TaKaRa EX *Taq* (Takara Bio Inc., Shiga, Japan), 6 µl of 20 mM Mg^{2+}, and 3 µl of 2.5 mM of each dNTP, appropriate concentrations of template cDNA, and 1 µl of 10 pM primer mixture (Table 2). Each PCR product was amplified by the following conditions: denaturation step for 5 min at 94°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 10 min. The amplified products were analyzed by 0.8% agarose gel electrophoresis.

The multiplex RT-PCR assays were also tested for specificity using five bacteria (10^4 CFU of each bacterium) and seven viruses (10^4 PFU of each virus) that commonly infect swine. Bacterial pathogens tested were *Mycoplasma hyopneumoniae* (ATCC 27719) and *M. hyorhinis* (ATCC 23839), and field isolates of *Streptococcus suis*, *Hemophilus parasuis*, and *Bordetella bronchiseptica*. Viruses tested were porcine reproductive and respiratory syndrome virus (ATCC VR-2332), transmissible gastroenteritis virus (ATCC VR-743), encephalomyocarditis virus (EMCV-CBNU) [18], porcine parvovirus (NADC-8), pseudorabies virus (Shope strain), avian pneumovirus (CNV-PL1), and avian New Castle disease viruses (clone 4 vaccine strain and several field isolates).

Sequence Analysis

The DNA fragments were extracted and purified with a QIAquick gel extraction kit, and sequencing of the amplified DNA was performed at Macrogen (Seoul, S. Korea) with an ABI 373 XL DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). DNA sequences were compiled and edited using the Lasergene sequence analysis software package (DNASTAR, Madison, WI, U.S.A.).

RESULTS

Design of HA and NA Primers

At least 20 sequences encompassing human, avian, and swine HA and NA sequences of each subtype were selected from the Flu Database (http://www.flu.lanl.gov/) and aligned to determine common regions. According to the similarity and length of the nucleotide sequences, we designed primer sets for 12 HAs (H1–H12) and 9 NAs (N1–N9) (Table 1). Initially, we attempted to amplify 12 HA and 9 NA genes using all 12 HA and 9 NA primer sets in one tube with known reference viruses. However, the amplification was relatively poor compared with the efficiencies attained using individual primer sets. Moreover, because of nonspecific bands and overlapping DNA fragments, precise subtyping was not possible. Therefore, we optimized the number of primers per mixture based on the criteria of amplification efficiency, specificity, and the ability to

Table 1. The multiplex PCR primers used to amplify avian influenza viruses.

Name	Sequence of oligonucleotide	Name	Sequence of oligonucleotide
HA 1F	5'-AGC AAA AGC AGG GGA-3'	N1 1F	5'-AGC AAA AGC AGG AGT-3'
HA 5F	5'-AGC AAA AGC AGG GGT-3'	N3 1F	5'-AGC AAA AGC AGG TGC-3'
H1 1077R	5'-TAC CAY CCA TCT ATC ATK CCT G-3'	N6 1F	5'-AGC AAA AGC AGG GTG AAA ATG-3'
H2 1325R	5'-TCA AGT GTC CTC TCA TTT TCC AT-3'	N7 1F	5'-AGC AAA AGC AGG GTG ATT GAG AAT-3'
H3 267R	5'-RTA GRG CAT CTA TYA RTG TRC A-3'	N9 1F	5'-AGC AAA AGC AGG GTC-3'
H4 342R	5'-GYA CAT CAA ATG GRT ARC AAG T-3'	N1 546R	5'-GCA CTT GCY GAC CAA GCA ACW GA-3'
H5 545R	5'-ARC TYC TCT TTA TTG TTG GGT A-3'	N2 734R	5'-GCR CTY CCA TCA GTC ATT ACT ACT-3'
H6 669R	5'-AAT TCA TRC TTT CAG TTC CCA T-3'	N3 233R	5'-ATT ATT GTT GTT GTT ATG TTG TT-3'
H7 1155R	5'-GCW GCA GTT CCY TCY CCT TGT-3'	N4 644R	5'-TCT GTT ATT ATA CCA TTR TAT TT-3'
H8 848R	5'-TTT TGA ATT ATT CTG CCA TGG CT-3'	N5 726R	5'-GGA CCA TCY GTC ATT ACC CAA TAA-3'
H9 796R	5'-GRG CRA TTA RAT TCC CAT TGG A-3'	N6 1219R	5'-CCT GAG TAY CCY GAC CAG TTT TG-3'
H10 1342R	5'-TCA ATT GTG TGC TGA TTT TCC ATT-3'	N7 1378R	5'-CCG GAC CCA ACT GGG AMT GGG C-3'
H11 794R	5'-GCA CCA TTW GAC TCA AAT GTT ATT-3'	N8 1342R	5'-CTC CAC ACA TCA CAA TGG AGC T-3'
H12 429R	5'-CAG TGT ATG TGA CAT TCC ATT TGG-3'	N9 451R	5'-TCG TGT ATT GTT CCA TTT GAG TGT-3'

*A+G: R C+T: Y A+C: M G+T: K G+C: S A+T: W A+T+C: H G+A+T: D G+T+C: B G+A+C: V A+G+C+T: N

distinguish products by size. Five to six primers were selected to comprise three HA and NA primer mixtures. The three groups (I, II, and III) of HA and NA genes containing 5–6 primers demonstrated the best conditions for sensitivity and specificity for subtyping of HA and NA genes (Table 2). Each multiplex HA primer could detect the following subtypes of HA genes: group I (H3, H5, H7, and H9), group II (H1, H2, H4, and H8), and group III (H6, H10, H11, and H12). Similarly, each multiplex NA primer could amplify the following subtypes of NA genes: group I (N3, N4, and N6), group II (N5, N8, and N9), and group III (N1, N2, and N7) (Table 2).

Detection and Subtyping of Influenza A Virus

To evaluate our multiplex RT-PCR methods, we tested our multiplex RT-PCR with 24 reference influenza type A viruses isolated from swine, and wild and domesticated poultry in Korea (Table 3). Twelve HA and nine NA subtypes of the influenza viruses could be clearly differentiated by the size of the amplified DNAs (Fig. 1). Qualitative comparison between the multiplex amplified bands and individually amplified bands shown in Fig. 2 demonstrated that the multiplex primers were capable of amplifying the appropriate PCR amplicons with the same efficiency.

The specificity of RT-PCR products was confirmed by sequencing (data not shown). The specificity was demonstrated further by testing with DNA or cDNA templates from seven viruses and five bacteria (listed in Materials and Methods) or mock-infected MDCK cells. None of the multiplex RT-PCR reactions was positive when the primers were tested with non-influenza A virus specimens. To evaluate the sensitivity of the multiplex RT-PCR, the primer sets were tested with 10-fold serially diluted RNAs extracted from 200 μl of each virus stock that was adjusted to contain 10^6 EID_{50}/ml of 24 reference influenza A viruses. Amplification could be visualized with a 10^5 dilution of each of the reference viruses (data not shown).

Evaluation of the Multiplex RT-PCR Assay with Field Specimens

To evaluate the multiplex RT-PCR using field specimens, we tested 188 influenza A virus isolates, 40 lung homogenates, and 85 fecal or tracheal specimens from swine, and wild or domesticated poultry in Korea (Table 4). The results for detection and subtyping of influenza A viruses from field cases using multiplex RT-PCR were in complete agreement with those for virus isolation in MDCK cells or chicken embryonated eggs and with amplification *via* RT-PCR with individual primers. Six virus isolates and 11 fecal/tracheal swabs were diagnosed as a mixture of at least two different subtypes of influenza viruses (Fig. 2). In addition, no positive amplification was observed when the

Table 2. Components of HA and NA primer mixtures of groups I, II, and III.

HA primer mixtures			NA primer mixtures		
I	II	III	I	II	III
H3 (267 bp)	H1 (1,077 bp)	H6 (669 bp)	N3 (233 bp)	N5 (726 bp)	N1 (546 bp)
H5 (545 bp)	H2 (1,325 bp)	H10 (1,342 bp)	N4 (644 bp)	N8 (1,342 bp)	N2 (734 bp)
H7 (1,155 bp)	H4 (342 bp)	H11 (794 bp)	N6 (1,219 bp)	N9 (451 bp)	N7 (1,378 bp)
H9 (796 bp)	H8 (848 bp)	H12 (429 bp)			

Table 3. The reference influenza A viruses tested for multiplex RT-PCR.

No.	Isolates	HA type	NA type	Host
1	A/PR/8/34	H1	N1	Human
2	A/SW/Kor/CAN/04	H1	N1	Swine
3	A/SW/Kor/JL1/05	H1	N2	Swine
4	A/WB/Kor/W145/06	H1	N9	Avian
5	A/ WB/Kor/W180/07	H2	N4	Avian
6	A/SW/Kor/CA04/05	H3	N2	Swine
7	A/ WB/Kor/W128/06	H3	N8	Avian
8	A/ WB/Kor/L81/06	H3	N2	Avian
9	A/WB/Kor/W20/05	H4	N6	Avian
10	A/WB/Kor/W150/06	H5	N1	Avian
11	A/WB/Kor/W120/06	H5	N2	Avian
12	A/WB/ Kor/W16/05	H5	N3	Avian
13	A/WB/ Kor/W69/05	H6	N5	Avian
14	A/WB/ Kor/W133/06	H7	N7	Avian
15	A/WB/ Kor/W152/06	H7	N9	Avian
16	A/WB/ Kor/W141/06	H8	N4	Avian
17	A/WB/ Kor/W71/05	H9	N1	Avian
18	A/WB/ Kor/L77/06	H9	N2	Avian
19	A/WB/ Kor/L87/06	H9	N2	Avian
20	A/WB/ Kor/W182/07	H10	N6	Avian
21	A/WB/ Kor/W158/07	H10	N7	Avian
22	A/WB/ Kor/W157/07	H11	N2	Avian
23	A/WB/ Kor/W160/07	H11	N9	Avian
24	A/WB/ Kor/W134/06	H12	N5	Avian

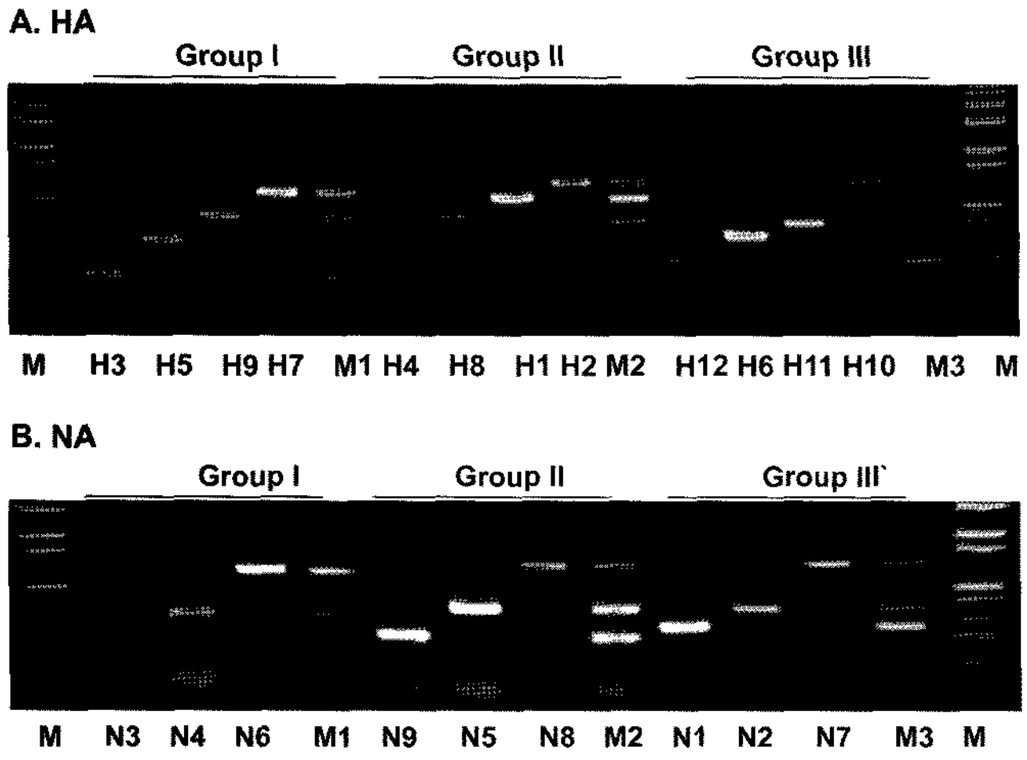

Fig. 1. Detection and subtyping of type A influenza virus by multiplex RT-PCR assay.
The virus strains representing the 12 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes used for RT-PCR are shown in Table 3. **A**. PCR reactions for detection and subtyping of H1 to H12 subtypes using a specified primer mixture; group I (H3, H5, H8, and H9), group II (H1, H2H4, and, H8), and group III (H6, H10, H11, and H12). **B**. PCR reactions for detection and subtyping of N1 to N9 subtyping using a designated primer mixture; group I (N3, N4, and N6), group II (N5, N8, and N9), and group III (N1, N2, and H7). The M1, M2, and M3 lanes represent the PCR reaction with all groups I, II, and III virus mixtures in one tube, respectively.

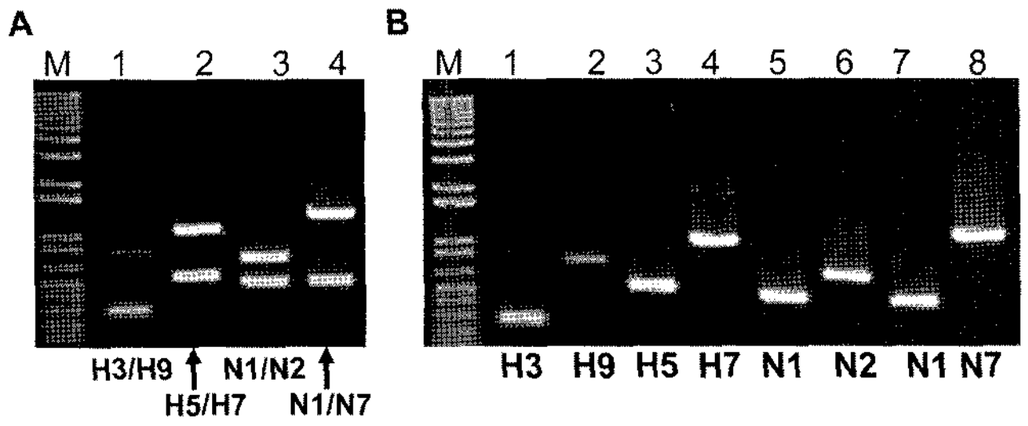

Fig. 2. Detection and subtyping of dual infections by multiplex RT-PCR (**A**) and single PCR (**B**) assays of avian influenza A viruses from fecal specimens of wild ducks.
A. PCR reaction with HA group I primer mixture (lanes 1–2) and NA group III primer mixture (lanes 3–4). Lane M, Hi-Low size DNA marker; lane 1, dual positive of H3 and H9; lane 2, dual positive of H5 and H7; lane 3, dual positive of N1 and N2; lane 4, dual positive of N1 and N7. **B**. Single PCR assay using individual primer set of each HA and NA subtype with the same cDNA template used in multiplex RT-PCR assay. The result of the single PCR reaction with individual primer set completely agreed with the multiplex RT-PCR assay.

multiplex RT-PCR assays were performed on 50 influenza A virus-negative specimens (30 fecal/tracheal swabs and 20 lung tissues) (Table 4).

To further confirm whether the PCR products amplified using multiplex primers were the actual HA or NA subtype of the influenza virus, we sequenced the purified genomic DNA. All amplified DNAs had the correct sequence for the corresponding subtype (data not shown).

DISCUSSION

To date, influenza A viruses comprise 16 HA and 9 NA subtypes, which implies a total of 144 possible combined subtypes. Currently, human infections of highly pathogenic avian H5N1 and H7N7 subtypes are being reported, and certain avian influenza A viruses seem to cross the species barrier to infect other species that originally were not known as the natural host [23]. Given this scenario, we need a simple, rapid, and accurate method to detect the various subtypes of influenza A viruses regardless of species origin. Many multiplex PCR methods have been developed and applied to various viral agents to satisfy the need for rapid and economical detection and diagnosis of viral infections. However, most of the methods have focused only on highly pathogenic or commonly found subtypes, such as H1, H3, H5, H7, and H9 influenza viruses [5, 12, 17, 24]. Furthermore, we acknowledge the fact that no influenza virus subtype can be ruled out as a candidate for a potential next pandemic outbreak. Therefore, we devised a simple and rapid multiplex RT-PCR method to identify 12 HA subtypes (H1–H12) and 9 NA subtypes (N1–N9), which were commonly isolated in South Korea. After various attempts to select the appropriate number of primers to be used in one mixture, four HA subtypes and

Table 4. Detection and subtyping of type A influenza virus isolates, virus-positive/negative fecal/tracheal swabs or lung samples from poultry and pigs by multiplex RT-PCR assays.

Specimen collected	No. of specimens	No. of positive specimens	No. matched with individual PCR (%)	No. of multiple infections
		Virus-positive by virus isolation or HA tests		
Virus isolates	188	188	188 (100%)	6
Fecal/tracheal swabs[a]	35/50	35/50	35/50 (100%)	4/7
Lung samples[b]	40	40	40 (100%)	0
		Virus-negative by virus isolation or HA tests		
Fecal/tracheal swabs	15/15	0	0 (100%)	0
Lung samples	20	0	0 (100%)	0

[a]Poultry specimens.
[b]Pig specimens.

three NA subtypes were chosen as the optimal number of subtypes to be detected in one tube. Comparison of the visual images of amplification utilizing individual primer sets and multiplex primer sets revealed that multiplex PCR methods were as specific and efficient as individual PCR methods (Fig. 1). In addition, our method has the advantages of being able to conserve time and reagents, considering that 3 to 4 subtypes could be distinguishably identified in a single amplification run for each of the NA and the HA genes, respectively. The cost-effective feature, however, does not necessarily affect or compromise the proven specificity and sensitivity of the procedure. The multiplex RT-PCR assays developed in the present study could differentiate 12 HA and all 9 NA subtypes of influenza A viruses from cultured virus isolates and, more importantly, field specimens (Table 4). As such, these assays may facilitate influenza virus diagnosis with easier identification and more rapid subtyping compared with other methods. It is important to note that multiple infections have been detected in virus isolates and clinical field samples, indicating the possibility of concurrent or sequential infection in host animals and the occurrence of more complex influenza virus subtypes or genotypes (Fig. 2). Given the multiple and complex infections caused by many of the known influenza viruses, the application of our multiplex PCR assays will also contribute to the rapid detection and diagnosis of recent reassortment events among avian, swine, and human viruses, and hopefully prevent the further spread of these viruses.

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References

1. Alexander, D. J. 2000. A review of avian influenza in different bird species. *Vet. Microbiol.* **74:** 3–13.
2. Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenza virus neuraminidase and neuraminidase-inhibition test procedures. *Bull. World Health Organ.* **48:** 199–202.
3. Butt, K. M., G. J. Smith, H. Chen, L. J. Zhang, Y. H. Leung, K. M. Xu, *et al.* 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J. Clin. Microbiol.* **43:** 5760–5767.
4. Chan, P. K. 2002. Outbreak of avian influenza A (H5N1) virus infection in Hong Kong in 1997. *Clin. Infect. Dis.* **34(Suppl 2):** S58–S64.
5. Choi, Y. K., S. M. Goyal, S. W. Kang, M. W. Farnham, and H. S. Joo. 2002. Detection and subtyping of swine influenza H1N1, H1N2, and H3N2 viruses in clinical samples using two multiplex RT-PCR assays. *J. Virol. Methods* **102:** 53–59.
6. Colman, P. M., J. N. Varghese, and W. G. Laver. 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature* **303:** 41–44.
7. Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus. 2005. Characterization of a novel influenza a virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79:** 2814–2822.
8. Guo, Y. J., J. W. Li, I. Cheung, M. Wang, Y. Zhou, and X. H. Li. 1999. Discovery of human infected by avian influenza A (H9N2) virus. *Chin. J. Exp. Clin. Virol.* **15:** 105–108.
9. Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* **146:** 2275–2289.
10. Jo, S. K., H. S. Kim, S. W. Cho, and S. H. Seo. 2007. Genetic and antigenic characterization of swine H1N2 influenza viruses isolated from Korean pigs. *J. Microbiol. Biotechnol.* **17:** 868–872.

11. Koopmans, M., B. Wilbrink, M. Conyn, G. Natrop, H. van der Nat, H. Vennema, *et al.* 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in The Netherlands. *Lancet* **363:** 587–593.
12. Ong, W. T., A. R. Omar, A. Ideris, and S. S. Hassan. 2007. Development of a multiplex real-time PCR assay using SYBR Green 1 chemistry for simultaneous detection and subtyping of H9N2 influenza virus type A. *J. Virol. Methods* **144:** 57–64.
13. Palmer, D. F., W. R. Dowdle, M. T. Coleman, and G. C. Schild. 1975. Advanced laboratory techniques for influenza diagnosis. U.S. Department of Health, Education and Welfare Immunology Series. U.S. Department of Health, Education and Welfare, Washington, D.C.
14. Park, K. J. and H. H. Lee. 2005. *In vitro* antiviral activity of aqueous extracts from Korean medicinal plants against influenza virus type A. *J. Microbiol. Biotechnol.* **15:** 924–929.
15. Peiris, J. S., W. C. Yu, C. W. Leung, C. Y. Cheung, W. F. Ng, J. M. Nicholls, *et al.* 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* **363:** 617–619.
16. Peiris, M., K. Y. Yuen, C. W. Leung, K. H. Chan, P. L. Ip, R. W. Lai, W. K. Orr, and K. F. Shortridge. 1999. Human infection with influenza H9N2. *Lancet* **354:** 916–917.
17. Poddar, S. K., R. Espina, and D. P. Schnurr. 2002. Evaluation of a single-step multiplex RT-PCR for influenza virus type and subtype detection in respiratory samples. *J. Clin. Lab. Anal.* **16:** 163–166.
18. Song, M. S., Y. H. Joo, E. H. Lee, J. Y. Shin, C. J. Kim, K. S. Shin, M. H. Sung, and Y. K. Choi. 2006. Genetic characterization of encephalomyocarditis virus isolated from aborted swine fetus in Korea. *J. Microbiol. Biotechnol.* **16:** 1570–1576.
19. Steininger, C., M. Kundi, S. W. Aberle, J. H. Aberle, and T. Popow-Kraupp. 2002. Effectiveness of reverse transcription-PCR, virus isolation, and enzyme-linked immunosorbent assay for diagnosis of influenza A virus infection in different age groups. *J. Clin. Microbiol.* **40:** 2051–2056.
20. Takimoto, S., M. Grandien, M. A. Ishida, M. S. Pereira, T. M. Paiva, T. Ishimaru, E. M. Makita, and C. H. Martinez. 1991. Comparison of enzyme-linked immunosorbent assay, indirect immunofluorescence assay, and virus isolation for detection of respiratory viruses in nasopharyngeal secretions. *J. Clin. Microbiol.* **29:** 470–474.
21. Tam, J. S. 2002. Influenza A (H5N1) in Hong Kong: An overview. *Vaccine* **20(Suppl 2):** S77–S81.
22. Tran, T. H., T. L. Nguyen, T. D. Nguyen, T. S. Luong, P. M. Pham, V. C. Nguyen, *et al.* 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* **350:** 1179–1188.
23. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56:** 152–179.
24. Xie, Z., Y. S. Pang, J. Liu, X. Deng, X. Tang, J. Sun, and M. I. Khan. 2006. A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7, and H9 hemagglutinin subtypes. *Mol. Cell. Probes* **20:** 245–249.