Surveillance for Reassortant Virus by Multiplex Reverse Transcription-PCR Specific for Eight Genomic Segments of Avian Influenza A H5N1 Viruses†[▽]

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Avian influenza H5N1 virus is a global threat. An emergence of a reassortant virus with a pandemic potential is a major concern. Here we describe a multiplex reverse transcription-PCR assay that is specific for the eight genomic segments of the currently circulating H5N1 viruses to facilitate surveillance for a virus resulting from reassortment between human influenza virus and the H5N1 virus.

The genome of influenza A viruses is composed of eight gene segments, which encode polymerase (PB1, PB2, and PA), nucleocapsid (NP), hemagglutinin (HA), neuraminidase (NA), matrix (M) protein, and nonstructural (NS) protein. Since influenza viruses have segmented genomes, they are able to evolve through reassortment (1). Both the 1957 and 1968 pandemic strains are believed to be reassortants in which HA and PB1 genes, with or without the NA gene, were replaced by the corresponding genomic segments of avian influenza virus strains (5). The reassortments provided the viruses with new HA and NA antigenic profiles and the ability to spread efficiently from human to human. The ongoing outbreak of the highly virulent avian influenza virus H5N1 is concerning because of the possibility of a reassortant virus emerging with pandemic potential (3). Monitoring for a reassortant with mixed genomic segments from human and avian viruses is therefore very important. However, detection of a reassortant requires sequencing of all eight genomic segments, a process which is laborious and time consuming. Therefore, we developed a simple and high-throughput screening method using multiplex reverse transcription-PCR (RT-PCR) specific for eight genomic segments of recent H5N1 viruses to detect the probable reassortant viruses worthy of further characterization.

For the primer design, 100 full-length sequences arbitrarily selected from the Influenza Virus Resource Database (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/genomes/FLU/>) were used to search for the sequence(s) that was conserved in H5N1 and that differed from

those of other subtypes. The viruses used in this study comprised 28 human influenza virus isolates and 103 H5N1 isolates from various animal species (94 isolates from poultry and other birds, 6 isolates from tigers, 2 isolates from leopards, and 1 isolate from a cat). These viruses have been collected since the beginning of the epidemic in the country. In 2003, 2004, 2005, and 2006, 7, 34, 60, and 2 animal H5N1 virus isolates were collected, respectively. The human viruses comprised 6 H5N1, 3 H1N1, 8 H3N2, and 11 influenza B viruses. These viruses were propagated in MDCK cells or embryonated eggs. The H5N1 viruses were originally subtyped by RT-PCR. The subtyping was confirmed by sequencing for all human H5 and some animal H5 viruses (2). Available accession numbers of some of the H5N1 virus isolates used in this study are shown in Table S1 in the supplemental material and also in a report by Puthavathana et al. (4). The human H1, H3, and influenza B viruses were characterized and confirmed with respect to their subtypes by hemagglutination inhibition assay at the WHO Reference Laboratories, Melbourne, Australia, or the Centers for Disease Control and Prevention of the United States via the National Reference Laboratory on Influenza, NIH, Thailand.

RNA was extracted by using a QIAamp viral RNA Mini kit (QIAGEN, Valencia, CA). A 140- μ l volume of virus suspension from the infected culture or embryonated egg yielded a volume of 60 μ l of extracted RNA. Multiplex RT-PCR was carried out in three reaction tubes with combinations of the following primer sets: NA plus PB1 plus NP, HA plus PA plus PB2, or NS plus M. Sequences of these primers are shown in Table 1. The thermocycling was performed in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer) using a QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA) with standard reaction conditions (a 50 μ l volume containing 1 $\times$ PCR buffer, 5 to 10 μ l of extracted RNA, 400 μ M (each) deoxynucleoside triphosphate, 2.5 mM MgCl₂, 2 μ l enzyme mix, and 2 U RNase inhibitor). The thermocycling profile was 50°C for 30 min, 95°C for 15 min, 35 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C

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TABLE 1. Sequences, concentrations used in the reaction, nucleotide positions, and product sizes of PCR primers for the eight gene segments

Gene	Primer name	Sequence	Concn in PCR (μ M)	Nucleotide positions	Product size (bp)
PB2	SPB2F	GAA TGA ACA AGG GCA GAC G	0.3	237–255	117
	SPB2R	ACT GCA CTT GTC GCC GGC	0.3	336–353	
PB1	SPB1F	AAT GAG AAT CAG AAT CCT AGG	0.4	952–972	377
	SPB1R	GTG GTT TTG GTG TAC CTT TTC	0.4	1308–1328	
PA	SPAF	AAT TGA AAG CAT GAT TGA AGC C	0.3	1797–1818	167
	SPAR	TGA ACA CAG ACT TCG CCA GC	0.3	1944–1963	
HA	HA-H5f	ACT CCA ATG GGG GCG ATA AA	0.6	897–916	352
	HA-H5r	CAA CGG CCT CAA ACT GAG TGT	0.6	1228–1248	
NP	SNPF	GAC AGC TAT CCA CCA GAG GG	0.3	1112–1131	174
	SNPR	CCG AGA AAG TGG GCT GAA CG	0.3	1266–1285	
NA	NA-N1f	CTC ATG CTC CCA CTT GGA	0.6	332–349	515
	TNAr5	CCC TGC ACA CAC ATG TG	0.6	830–846	
M	SMF	TCG CAC TCA GCT ACT CAA CC	0.3	369–388	142
	SMR	TGA GAC CGA TGC TGT GAA TCT	0.3	490–510	
NS	SNSF	GAT AAG GCA CTT AAA ATG CCG	0.4	250–270	253
	SNSR	ACG GTG AGA TTT CTC CCA CG	0.4	483–502	

for 45 s, and a final extension of 72°C for 10 min. The PCR products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel.

In our multiplex RT-PCR assay, potential reassortants would show the lack of one or more of the eight amplicons in the case of H5N1 viruses and the presence of one or more amplicons in the case of H1N1 and H3N2 viruses. With the designed primer set, we could initially amplify all eight genomic segments from 105 out of 109 H5N1 viruses. Four viruses initially gave negative amplification results for NA. However, these four viruses yielded positive NA band results

in separated single-gene amplifications, and multiplex reamplification using 1.5- to 2-fold-higher RNA input gave positive amplification results for all genes. Representative results obtained with the multiplex RT-PCR products are shown in Fig. 1. None of the human H3N2, H1N1, or influenza B virus isolates yielded any amplification products by this method (data not shown). In order to verify the lack of reassortment, all human H5N1 viruses and a subset of animal viruses (62 out of 103) were subjected to a full-genome sequencing protocol as previously described (4). Phylogenetic analyses of the sequence data revealed that all viruses contained their respective genomic segments and that no evidence of reassortment was found (data not shown). In addition to detecting human-avian virus reassortants, our assay may also be able to detect some reassortants that acquire genomic segments from some other unrelated avian viruses. However, the assay was not designed to detect reassortment among avian viruses, which are unlikely to play a role in viral adaptation to the human host. The absence of a band in the amplification of H5N1 viruses is not always due to reassortment. Full-genome sequencing is needed for result confirmation. Because the performance of this assay is directly affected by variation in the sequences of circulating H5N1 strains, the primer sequences should be frequently re-evaluated and updated accordingly.

Our multiplex RT-PCR was designed for egg- or MDCK-propagated viruses. Although the assay may be able to detect some of the genes in some clinical specimens, the level of sensitivity is probably not sufficient to reliably detect all the genomic segments simultaneously. This makes our assay less suitable for direct screening using clinical specimens. Nevertheless, the possibility of using a more sensitive version of this assay with clinical specimens should be further explored.

FIG. 1. A representative photograph of multiplex RT-PCR products from an H5N1 virus after electrophoresis in 2% agarose and ethidium bromide staining. Each lane shows products from each tube. The first lane is a 100-bp DNA ladder, and the target gene of each amplification product is labeled on the right.

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REFERENCES

1. Guan, Y., J. S. Peiris, A. S. Lipatov, T. M. Ellis, K. C. Dyrting, S. Krauss, L. J. Zhang, R. G. Webster, and K. F. Shortridge. 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc. Natl. Acad. Sci. USA* **99**:8950–8955.
2. Hoffmann, E., J. Stech, Y. Guan, and R. G. Webster. 2001. Universal primer set for the full-length amplification of all influenza viruses. *Arch. Virol.* **146**: 2275–2289.
3. Li, K. S., Y. Guan, J. Wang, G. J. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**:209–213.
4. Puthavathana, P., P. Auewarakul, P. C. Charoenying, K. Sangsiriwut, P. Pooruk, K. Boonnak, R. Khanyok, P. Thawachsupha, R. Kijphati, and P. Sawanpanyalert. 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J. Gen. Virol.* **86**:423–433.
5. 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.