

Use of Reverse Transcriptase PCR To Subtype N1 to N9 Neuraminidase Genes of Avian Influenza Viruses[∇]

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Reverse transcriptase PCR designed to amplify the N1 to N9 neuraminidase (NA) genes of avian influenza viruses detected 118 of the 119 NA genes tested (99.2%) in a subtype-specific manner. This technique successfully subtyped all 167 recent avian influenza viruses isolated from birds. Subtype specificity was confirmed by sequence analyses of all 285 PCR products.

The spread of the H5N1 highly pathogenic avian influenza (AI) virus in Asia, Europe, and Africa raises concerns that a pandemic will occur (1, 15). Migratory ducks that are more resistant to the H5N1 virus can carry the virus long distances, while more-susceptible swans and geese serve as indicators of the infections in wild aquatic bird populations (3, 4). Active surveillance programs have been instituted worldwide to determine the current H5N1 prevalence among wild aquatic birds (1, 14). The AI virus has two glycoprotein spikes, hemagglutinin (HA) and neuraminidase (NA). NA comprises a mushroom-shaped tetramer and is divided serologically into nine subtypes by using an NA inhibition (NI) test (8, 12). NA subtyping using PCR is used for AI global surveillance, since this test can be performed easily in general diagnostic laboratories. In contrast, the NI test can be performed only in reference laboratories.

Comparison of 263 complete nucleotide sequences of NA genes downloaded from the Influenza Sequence Database (<http://www.flu.lanl.gov/>) (10) revealed that the virus membrane anchor and stalk region have a great deal of nucleotide sequence variability (8). This variability can be exploited to distinguish the different subtypes. For each NA subtype, we thus designed forward primers that were complementary to the membrane anchor (nucleotides ~53 to 115) and reverse primers complementary to the region carrying the stalk to the 5' end of the head region (nucleotides ~209 to 365) (Table 1). These subtype-specific primers were screened against previously and recently isolated AI viruses and further modified using sequence data for 41 NA genes that were not detected initially by PCR.

Viral RNA was extracted from allantoic fluid by using Trizol solution (Invitrogen, Carlsbad, CA) and used for cDNA synthesis with random primers (6-mers) and PrimeScript reverse transcriptase (Takara, Shiga, Japan) (13). The PCR amplifica-

tion was performed using the GeneAmp PCR System 9700 (ABI, Foster City, CA) and Ex *Taq* polymerase (Takara, Shiga, Japan) (13). PCR products were sequenced directly using forward and reverse primers (ABI-3100 sequencer; PE ABI, Foster City, CA) (13).

The detection rate of NA genes by the NA subtyping primer sets was determined using previously isolated AI viruses ($n = 119$; isolated between 1946 and 2006). These 119 viruses consisted of 83 duck strains, 16 chicken strains, 7 turkey strains, and 13 strains from other birds. They included 77 strains from Japan and 42 strains from Korea, Thailand, Italy, The Netherlands, the United States, or Chile; alternatively, the viruses can be viewed as 92 Eurasian and 27 American lineages. The primer sets amplified 118 NA genes: 12 N1, 32 N2, 20 N3, 6 N4, 12 N5, 17 N6, 3 N7, 5 N8, and 11 N9 genes. The NA subtype specificity was confirmed by sequence analyses of all 118 PCR products by using the same primers. Only one gene, an N2 gene, was not amplified by PCR. Full genome analysis showed that the unamplified N2 gene belonged to the American lineage. Serial 10-fold dilutions of allantoic fluids containing nine AI viruses (one strain for each of subtypes N1 to N9) were used to compare PCR sensitivity to infectivity, expressed as 50% egg-infective dose. The detection limits of the PCRs were 10^{2-5} to 10^{4-5} 50% egg-infective doses.

The cross-reactivity of the primer sets was tested with 45 AI viruses (5 viruses per subtype for nine NA subtypes). Homologous NA genes were amplified efficiently; faint, smeared, or size-different PCR products that were distinguishable from specific products by their size or intensity on agarose gels were occasionally detected. Sequence analyses showed that some faint bands were confirmed as representing cross-reactivity or nonspecific products, while two clear NA genes in a single sample were indicative of the presence of two AI viruses. Such faint bands might be inevitable in our NA subtyping PCR. The NA subtyping PCR could detect a deletion in the NA stalk region which was present in some AI viruses, such as A/chicken/Korea/ES/2004 (H5N1) and A/turkey/Italy/4580/99 (H7N1). It is surmised that the deletion might be associated with the adaptation of AI virus strains to terrestrial birds, such as chickens or turkeys.

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TABLE 1. Primers for subtyping NA genes of AI viruses

NA subtype	Primer no.	Sequence position	GenBank accession no. for reference sequence	Product size (bp)	Primer sequence (5' → 3') ^a	No. of mixed nucleotides
N1	685	N1-54F	CY003931	245	TCARTCTGYATGRYAAAYTGG GGRCARAGAGAKGAATTGCC	5
	469	N1-298R				3
N2	470	N2-59F	CY005306	278	TYTCTMTAACYATTGCRWCARTATG GARTT GTCYT TRGAR AAVGG	6
	695	N2-336R				5
N3	687	N3-79F	AY650272	287	GCCCTTCTYATYGGRRRTKGGRAA ACTATDRCRTCYTTGTTYTC	6
	688	N3-365R				5
N4	483	N4-55F	CY003986	236	AGTGYKAGYATTRTAYTRAC ARGTCTYTYCCACTRGARTA	6
	484	N4-290R				5
N5	509	N5-115F	CY005693	178	GARTA ATATCAGYRACVAAAAG GATACATYRCAGAGAGGTTTC	4
	87	N5-279R				2
N6	88	N6-57F	CY005464	264	AGGAATGACACTATCSGTAGTAAG GAYAGRATRTGCCATGAGTTYAC	1
	89	N6-307R				4
N7	472	N7-53F	CY004347	261	TCWGGAGTGGCMATAGCACT CACKACCCAYCCTTCAACWTTG	2
	473	N7-313R				3
N8	107	N8-93F	CY004056	137	CATRTVGTBAGYATYAYARTAAC ACAYTRGYATTGTRCCATTG	7
	690	N8-209R				4
N9	691	N9-64F	CY004701	227	GTAATAGGCACRATYGCAGT CCTTRGTYARRTTATTGAA	2
	692	N9-290R				4

^a Codes for mixed-base positions: B, C/G/T; D, A/G/T; K, G/T; M, A/C; R, A/G; V, A/C/G; W, A/T; Y, C/T.

The usefulness of the PCR for subtyping NA genes was assessed using 167 AI viruses: 163 low-pathogenicity (LP) AI viruses isolated from wintering migratory ducks in Japan in 2006/2007 and 2007/2008 (K. Tsukamoto, T. Ashizawa, K. Nakanishi, N. Kaji, R. Kakizawa, M. Shishido, K. Suzuki, T. Tada, and M. Mase, unpublished data) and 4 H5N1 highly pathogenic AI viruses isolated from chickens in Japan in 2007 (M.

Mase, Y. Yamamoto, M. Okamatsu, Y. Uchida, T. Saito, K. Nakamura, K. Tsukamoto, and S. Yamaguchi, unpublished data). All 167 AI viruses were successfully subtyped, and nucleotide sequence analysis of the PCR products confirmed the NA subtype specificity. PCRs that determined the NA subtypes of nine AI viruses (one strain per NA subtype) are shown in Fig. 1. PCR was used successfully to subtype 18 N1, 40 N2, 24

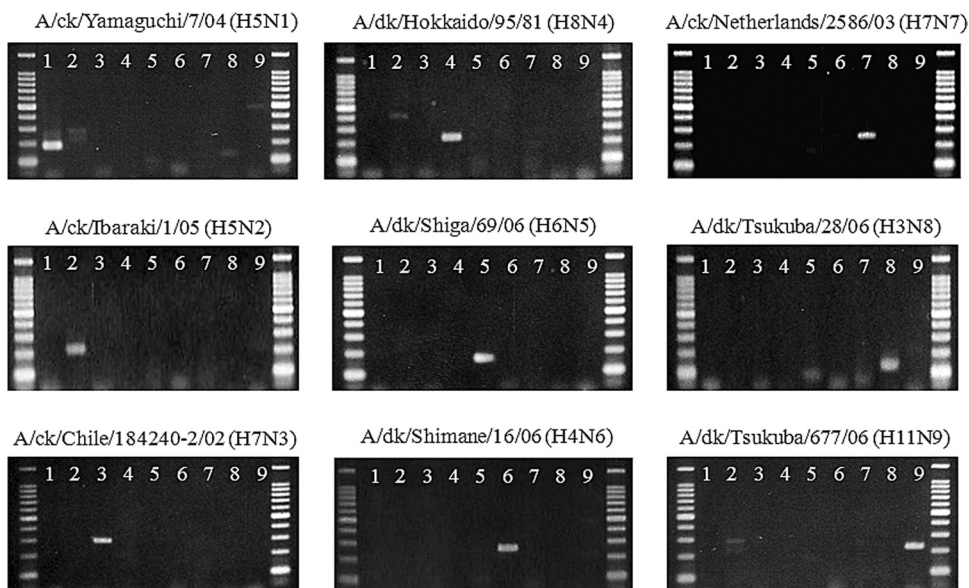


FIG. 1. Subtyping PCR for NA genes of nine AI viruses.

N3, 9 N4, 10 N5, 15 N6, 7 N7, 23 N8, and 21 N9 genes. The total rate of detection of NA genes by PCR was 99.7% (285 genes/286 genes).

To summarize, NA-subtyping PCR for the N1 to N9 genes of AI viruses is both sensitive (99.7%; 285/296) and highly specific (100%; 285/285) and useful for amplifying widely diverse NA genes from 59 reference strains and 227 wild-duck strains. The strains from wild ducks may be representative of Eurasian strains since they were isolated in five prefectures over a 3-year period in Japan; these strains comprise a variety of subtypes and represent molecularly diverse viruses. Alvarez et al. (2) developed a universal primer set for detecting all nine NA subtypes with 97% (32/33) sensitivity; however, sequencing of the PCR products was essential for NA subtyping (2). Qiu et al. (11) utilized nine primer sets for NA subtyping using 101 reference strains or isolates; the reported sensitivity was 97.3%, and the specificity was 91.1% (11). Fereidouni et al. (5) developed an NA subtyping PCR to analyze a total of 162 NA genes from 43 reference strains and 119 diagnostic samples (5). We designed the PCR to be subtype specific by targeting the most subtype-specific regions of NA genes. The subtype specificity was confirmed by using 45 isolates and sequence analysis of all 285 PCR products. Although direct detection of NA genes in diagnostic samples was not performed in this study, PCR is a viable option if the cDNA is prepared appropriately. Our NA subtyping PCR could be useful in determining the adaptation of AI viruses to terrestrial birds, such as chickens or turkeys. It can also be used to detect mixed viruses (data not shown) and is rapid (~4 h) compared to the conventional serological NI test (2 days). In contrast to the use of microarray technology, PCR does not require expensive equipment or training (6, 7, 9). Our NA-subtyping PCR, together with HA-subtyping PCR (13), holds great potential for use in general laboratories for simultaneous subtyping of HA and NA genes of AI viruses.

Nucleotide sequence accession numbers. The sequences of the 41 NA genes were registered in GenBank under accession numbers AB472013 to AB472040 and AB472051 to AB472063.

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