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Duck and Human Pandemic Influenza A Viruses Retain Sialidase Activity under Low pH Conditions[1]

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The majority of influenza A viruses isolated from wild birds, but not humans, can replicate in the duck intestinal tract. Here we demonstrate that all duck isolates tested universally retain sialidase activities under low pH conditions independent of their neuraminidase (NA) subtypes. In contrast, the sialidase activities of most isolates from humans and pigs practically disappear below pH 4.5, with the exception of four human pandemic viruses isolated in 1957 and 1968. Sequence comparisons among duck, human, and swine N2 NA subtypes indicate that amino acids at positions 153, 253, 307, 329, 344, 347, 356, 368, 390, and 431 may be associated with the low pH stability of duck and human pandemic N2 NAs. This finding suggests that the low pH stability of duck influenza A virus NA may be a critical factor for replication in the intestinal tract through the digestive tract of ducks, and that the properties of NAs are important for understanding the epidemiology of the influenza virus.

Key words: influenza virus, neuraminidase, pH stability, sialidase.

Influenza A viruses have been isolated from many animal species including humans, pigs, horses, and birds (*1*). All of the subtypes (H1 to H15 and N1 to N9) have been isolated from wild aquatic birds, which appear to play an important role as the reservoir for the viruses transmit to other animals (*2*). Experimental infection of influenza A viruses across animal species shows a distinct restriction of host range on replication (*3–5*). Most avian influenza virus isolates replicate in the intestinal tract through the digestive tract of ducks, which remain completely asymptomatic when infected (*6*). However, human influenza A viruses do not replicate in ducks (*6*, *7*) In a previous study, the replication of human-avian reassortant influenza A viruses in ducks showed that reassortant viruses containing either the hemagglutinin (HA) or neuraminidase (NA) gene from a human strain failed to infect the intestinal tracts of ducks (*8*) These observations suggest that the biological properties of avian virus HA and NA contribute to the host range restriction.

The HA receptor specificity of influenza viruses differs according to the animal species of origin (*9–12*). Recently, the importance of the recognition of *N*-glycolylneuraminic acid (Neu5Gc) linked to galactose (Gal) by the α2,3 linkage on the replication of avian influenza viruses in the duck intestine was demonstrated by a comparison of receptor specificity among the viruses and immunohistochemical analysis of the sialoglycoconjugates in duck colon (*13*).

Avian, but not human or swine, H1N1 influenza A viruses retain their infectivity and sialidase activities after low pH treatment (*14*). However, the conservation of low pH stability among various NA subtypes of avian virus isolates and the molecular mechanism of their stability remain unknown. In the present study, we report that avian influenza virus isolates universally retain sialidase activities under low pH conditions independent of their NA subtypes, and that N2 NAs of four human pandemic viruses isolates in 1957 and 1968 also exhibit avian-like properties, but not other human isolates.

MATERIALS AND METHODS

Viruses—The influenza A viruses tested were propagated in the allantoic sac of 10-day-old embryonated eggs and were purified by sucrose-density-gradient centrifugation as described previously (*15*).

Sialidase Assay—Five microliters of each influenza A virus suspension (500 ng protein) in various pH buffers were stored at 37°C for 30 min and then incubated with 5 μl of 4 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MUα-Neu5Ac) (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 100 mM carbonate buffer (pH 10.7). The fluorescence of the released 4-methylumbelliferone was measured with a fluorescence spectrophotometer (F-4010;

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Abbreviations HA, hemagglutinin, Gal, galactose, 4-MUα-Neu5Ac, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, NA, neuraminidase, Neu5Gc, *N*-glycolylneuraminic acid

Hitachi, Tokyo) with excitation at 355 nm and emission at 460 nm. NA enzymatic activity is expressed as milliunits (mU) per mg of each viral protein. One unit of enzyme activity is defined as the amount that releases 1 μmol of sialic acid from 4-MUα-Neu5Ac per minute at 37°C. The values are the means for duplicate experiments.

NA Amino Acid Sequence Analysis—Each virion RNA was extracted with TRIzol reagent (Life Technologies, Rockville, MD, USA). cDNAs of the NA genes from influenza viruses were synthesized using AMV reverse transcriptase and amplified by PCR with forward primer oligonucleotides (5′-TCTCTTCGAGCAAAAGCAAAAGCAGGAGTGAAGATG-3′, 5′-AGAACCTTATGTGTCATGC-3′, 5′-CAGTATTGGTTCATGGTC-3′, and 5′-GCAGGAGTGAAGATGAATCCA-3′) and reverse primer oligonucleotides (5′-ATTAACCCTCACTAAAAGTAGAAACAAGGAGTTTTTTTC-3′, 5′-CCCTCTTTAATGAATAGTATTC-3′, 5′-CATTTCCATTGTCAAAGGC-3′, and 5′-TAAAATTGCGAAACGTTATATAGGCAT-3′) and *Pfu* (Stratagene, La Jolla, CA) or *Taq* (Takara Shuzo, Tokyo) DNA polymerase. The PCR-derived dsDNA was used as a template for automated sequencing on an Applied Biosystem 373A automated sequencer (Perkin-Elmer, Foster City, CA). Six internal forward primers (5′-CATAACAGAGATAGTGT-3′, 5′-ATGCAACTGCTAGCTTCA-3′, 5′-AGTGAAAGGCTGGGCCTTT-3′, 5′-TGAAAGGCTGGGCCTTT-3′, 5′-TTATGTGTGCTCAGGGC-3′, and 5′-GGCAAAAGCTGCATCATCAAT-3′) and four reverse primers (5′-ATGACACATAAGGTTCTC-3′, 5′-CATCAGTCATTACTACTG-3′, 5′-TTAGGTGTGGACCAACC-3′, and 5′-CAATACTGTTTGAGGTCC-3′) were used to sequence the coding region of the NA gene. The amino acid sequences of the NAs were compared among viruses in the regions of

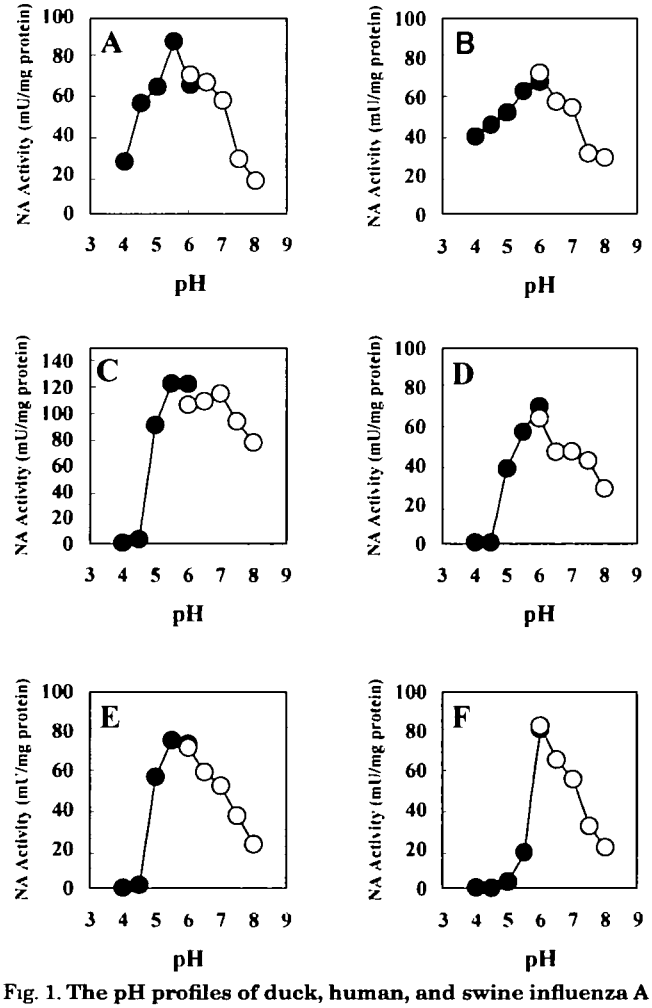

Fig. 1. **The pH profiles of duck, human, and swine influenza A virus NAs.** Five microliter samples of each influenza A virus suspension (500 ng protein) in various pH buffers (20 mM acetate buffer, ●, 20 mM phosphate buffer, ○) were stored at 37°C for 30 min and then incubated with 5 μl of 4-MUα-Neu5Ac at 37°C for 30 min. The fluorescence of the released 4-methylumbelliferone was measured as described in "MATERIALS AND METHODS." NA activity is expressed as mU per mg of each viral protein. The values are the means of duplicate experiments. A, A/duck/849/3/80 (H4N1); B, A/duck/276/2/78 (H2N2); C, A/Texas/91 (H1N1); D, A/Kumamoto/55/76 (H3N2), E, A/swine/Hong Kong/168/93 (H1N1); F, A/swine/Italy/526/85 (H3N2)

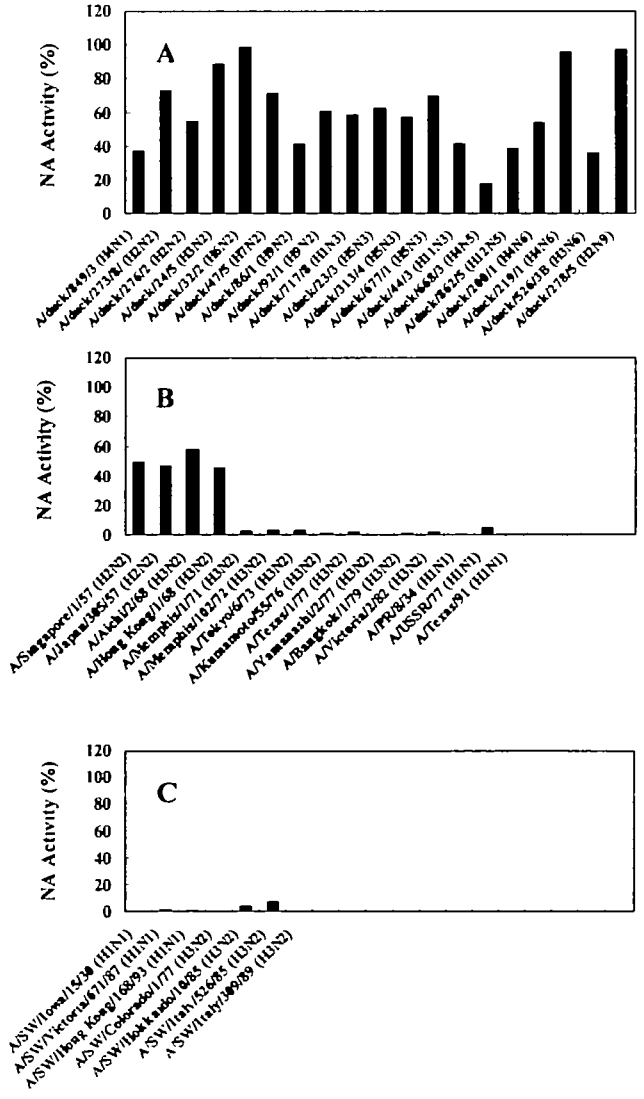

Fig. 2 **Activities of duck, human, and swine influenza A virus NAs under low pH conditions.** The fluorescent substrate was incubated at 37°C for 30 min at pH 4.0 and pH 5.0 with each influenza virus as described in the legend to Fig. 1. The NA activity at pH 4.0 is expressed as a percentage relative to the activity of each virus at pH 5.0. The values are the means of duplicate experiments. A, duck isolates, B, human isolates; C, swine isolates

residues 78 to 469.

Phylogenetic Tree of Influenza Virus N2 NA Subtypes—The NA amino acid sequences (amino acid positions 78 to 469 of NA) were analyzed using the Clustal W 1.7.4. computer program (*16*). The tree was constructed with TREEVIEW 1.5.2 (*17*). The scale shows the mutational distances between the viruses.

RESULTS AND DISCUSSION

Comparison of the Low pH Stability of Various NA Subtypes of Avian, Human, and Swine Influenza A Virus Isolates—To compare the pH profiles of NA among different species, we examined the NA activities of N1 and N2 subtypes from duck, human, and swine virus isolates by the fluorometric assay using 4-MUα-Neu5Ac (Fig 1). All influenza viruses tested showed a very similar optimal pH (pH 5.5–6.0). In contrast, the NA activities of the viruses under low pH conditions showed distinct differences among animal species The NA activities of human and swine influenza viruses completely disappeared at pH values below pH 4.5; however, the duck isolates retained about 70% of their optimal activities under similar conditions. In order to determine the pH stability of the duck NAs, the sialidase activities of the duck, human, and swine isolates were assayed at optimal pH after 30 min exposure to pH 4.0. The enzymatic activities of the duck isolates retained about 40% of their original activity, while the sialidase activities of the isolates from humans and pigs nearly disappeared under the same conditions (data not shown) We, therefore, examined whether duck viruses generally possess sialidase activities under low pH conditions. The NA activities of nineteen duck, fifteen human, and seven swine isolates were analyzed at pH 4.0 and compared as percentage activity relative to the activity at pH 5.0 (Fig. 2). All duck NAs (N1, N2, N3, N5, N6, and N9 NA subtypes) exhibited high activities at pH 4.0 independent of their subtype. The N1 and N2 NAs of human and swine isolates exhibited high activities at pH 5.0, however, their activities almost disappeared at pH 4.0, with the exception of four human viruses isolated in 1957 and 1968.

NA pH Profiles of Pandemic Human N2 Influenza Viruses—We examined the pH profiles of A/Singapore/1/57 (H2N2) and A/Japan/305/57 (H2N2) NAs that showed activities at pH 4.0 as high as those of duck viruses. The pH profiles of both A/Singapore/1/57 (H2N2) and A/Japan/305/57 (H2N2) were different from the profile of A/Kumamoto/55/76 (H3N2) used as a human control (Fig. 3), but were very similar to the profiles of duck NAs as shown in Fig. 1 In addition, the profiles of A/Aichi/2/68 (H3N2) and A/Hong Kong/1/68 (H3N2) were also similar to those of duck NAs (data not shown). The substrate specificities of early human N2 NAs of viruses isolated between 1957 and 1962, was similar to that of avian viruses (*18*). The present findings, therefore, suggest that earlier human N2 viruses retain the properties of avian NAs in pH profile in addition to their substrate specificities.

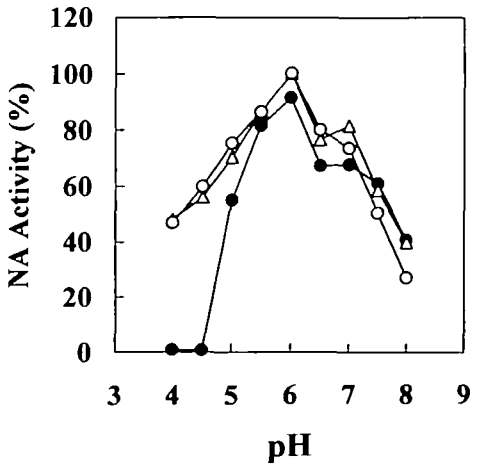

Fig 3. **The pH profiles of A/Singapore/1/57, A/Japan/305/57, and A/Kumamoto/55/76 N2 NAs.** The NA activity of each virus at varying pH was measured as described in the legend to Fig. 1. NA activity is expressed as a percentage relative to the activity of each virus at optimal pH. The values are the means of duplicate experiments. A/Singapore/1/57 (open triangles), A/Japan/305/57 (open circles), A/Kumamoto/55/76 (closed circles).

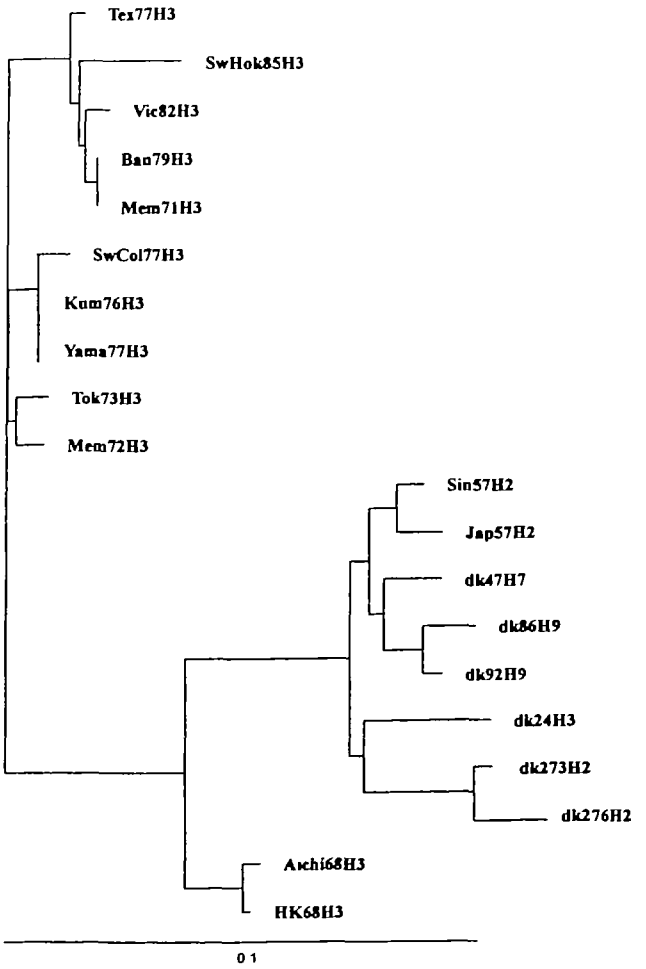

Fig 4. **Phylogenetic tree of influenza virus N2 from different hosts.** The nucleotide sequences of influenza virus N2 NAs tested, except A/Bangkok/1/79, A/swine/Italy/526/85, and A/swine/Italy/309/89, were determined as described in "MATERIALS AND METHODS." The NA amino acid sequences (amino acid positions 78 to 469 of NA) were analyzed using the Clustal W 1.7 4. computer program (*16*) The tree was constructed with TREEVIEW 1.5.2 (*17*) The scale shows the mutational distances between the viruses

TABLE I **Comparison of amino acid residues in the N2 NA of duck, human, and swine influenza viruses.**

Virus strains	Amino acid position									
	153	253	307	329	344	347	356	368	390	431
Duck										
A/duck/24/5/76	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/duck/47/5/76	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/duck/86/1/76	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/duck/92/1/76	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/duck/273/8/78	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/duck/276/2/78	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
Human										
A/Singapore/1/57	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/Japan/305/57	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	**Pro**	**Asn**	**Lys**	**Ser**	**Pro**
A/Aichi/2/68	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	Gln	**Asn**	**Lys**	**Ser**	Lys
A/Hong Kong/1/68	**Ile**	**Arg**	**Met**	**Asp**	**Arg**	Gln	**Asn**	**Lys**	**Ser**	Lys
A/Memphis/1/71	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Memphis/102/72	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Tokyo/6/73	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Kumamoto/55/76	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Texas/1/77	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Yamanashi/2/77	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Bangkok/1/79	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/Victoria/2/82	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
Swine										
A/sw/Colorado/1/77	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu
A/sw/Hokkaido/10/85	Thr	Lys	Val	Asn	Lys	His	Asp	Glu	Leu	Glu

Boldface lettering indicates consensus amino acid residues in duck and pandemic human virus N2 NAs that may be associated with the stability of NA at low pH Underlining indicates the amino acid residues are only present in the NA of A/Aichi/2/68 and A/Hong Kong/1/68

Comparison of Amino Acid Residues in Duck, Human, and Swine N2 NAs—We assumed that the amino acid residues of the duck virus N2 NA glycoprotein confer confirm stability at low pH, and so determined the NA nucleotide sequences of all tested N2 viruses, including duck viruses that were not available in the GenBank database except A/Bangkok/1/79 (H3N2), A/swine/Italy/526/85 (H3N2), and A/swine/Italy/309/89 (H3N2). A phylogenetic analysis of the N2 NA of duck, human, and swine viruses was performed using of the Clustal W 1.7.4 (*16*) and TREEVIEW 1.5.2 (*17*) computer programs. The NA genes of earlier human viruses and all later human viruses belong to distinct phylogenetic lineages (Fig. 4). In addition, the earlier human viruses had NA genes located on the same branch represented by the duck viruses. The duck and pandemic human virus N2 NA consensus sequences were compared with the consensus sequences of swine and all later human viruses. Nucleotide sequence analysis suggested that amino acids at positions 153, 253, 307, 329, 344, 347, 356, 368, 390, and 431 may be associated with the properties of duck N2 NAs under low pH conditions (Table I). In particular, residues 347 and 431, which are located beside the catalytic site, may contribute to the low pH stability. A further direct approach by comparing the sialidase activities of mutant N2 NAs remains to verify this hypothesis.

Wild aquatic birds appear to play an important role as the reservoir for the origin viruses transmitted to other animals (*2*). Most avian influenza virus isolates can replicate in the duck intestinal tract, however, the molecular mechanism of their enterotropism remains unknown. Ito *et al.* (*13*) showed that the recognition of the Neu5Gcα2,3Gal linkage on the receptor specificity of the viral HA was associated with intestinal replication of influenza viruses in ducks. In the present study, we found that the NAs of duck influenza A viruses show distinct differences in their low pH profiles compared with human and swine viruses, independent of their NA subtype. An earlier study showed that duck viruses exhibit infectivity in embryonated eggs or MDCK cells after exposure to acidic pH, whereas human viruses do not (*7*, *14*). The present findings, therefore, suggest that the nature of duck and earlier human NAs are suitable for passing through the digestive tract of the duck in comparison with the NAs of all later human and swine isolates, and that their NAs play a critical role in the process of viral infection to the intestine after passing through the digestive tract of the duck. Further studies are needed to confirm the critical role of NA by experimental infection of ducks with mutant viruses

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