Ecological and Epidemiological Studies on Avian Influenza Virus in Wild Birds

(野鳥における鳥インフルエンザウイルスの生態学的および疫学的研究)

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CONTENTS

GENERAL INTRODUCTION

Influenza viruses are belonging to *Orthomyxoviridae* family [39] and are calssified as A, B and C based on antigenic differences in their nucleoprotein (NP) and matrix (M1) protein [19, 68]. Influenza A viruses have been isolated from variety of animal species, including humans, pigs, horses, mink, marine mammals and a wide range of domesticated and wild birds [19, 68]. Whereas, influenza B and C viruses are predominantly human pathogens that have been occasionally isolated from seal and pigs, respectively [15, 42]. Phylogenetic studies of these viruses have revealed that aquatic birds are the source of all influenza viruses in other animal species and they have achieved an optimal level of adaptation in this natural reservoir [19]. While influenza A viruses are generally non-pathogenic in wild aquatic birds, especially of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (shorebirds, gulls, terns and auks), the viruses sometimes cause significant morbidity and mortality upon transmission to other species, including domestic birds and mammals [68]. Influenza A virus has two types of surface glycoprotein, hemagglutinin (HA) and neuraminidase (NA) and a total of 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been identified based on their antigenic

specificity [13, 68]. To date, all HA and NA subtypes of influenza A virus have been found in avian viruses, in contrast, viral subtypes of mammalian viruses are limited [19].

Influenza A virus genomes comprise eight negative-sense, single-stranded viral RNA segments. Hence, it is possible to generate progeny viruses possessing the random combination of viral genes derived from parent viruses, if co-infection with more than two different viruses into a host cell. As described above, wild aquatic birds are the primordial reservoir of all influenza viruses, and the vast influenza genes pool exists in those species [68]. Although the probability of an avian influenza virus entering the human population has been extremely rare, a novel lineage of human influenza virus resulted from the reassortment between avian and human influenza viruses has the potential to become the new pandemic strain. For example, analysis of nucleotide sequence data has demonstrated that the 1957 (H2N2) and 1968 (H3N2) human pandemic influenza viruses resulted from the reassortment of three (HA, NA and PB1) and two (HA and PB1) genes of avian influenza viruses with five and six human influenza viral genes, respectively [19, 24, 68]. Therefore, avian influenza viruses have gained an ecological niche presenting in a newly pandemic threat to humans.

Influenza A viruses do not replicate indiscriminately across animal species but rather show a clear pattern of host range restriction, despite their common origin. For

example, in experimental infection of non-human primates, avian influenza viruses do not replicate efficiently, while human influenza viruses replicate well [38]. The molecular basis for host range restriction is not well understood; however, HA plays a key role in the restriction of interspecies transmission [40]. The HA protein mediates virus binding to *N*-acetylneuraminic (sialic) acid containing host cell surface molecules. By contrast, the sialidase activity of the NA protein removes sialic acid to liberate newly synthesized viruses from infected cells. Thus, efficient virus replication requires the balanced actions of HA receptor binding activity and NA sialidase activity [36, 67]. Furthermore, the receptor specificity of the HA differs: most avian influenza viruses preferentially bind to the sialic acid-α2,3-galactose (SAα2,3Gal) linkage, while human influenza viruses favor the SAα2,6Gal linkage on cell surface sialyloligosaccharides [23]. It was shown that the presence of SAα2,6Gal but not of SAα2,3Gal sialyloligosaccharides on the surface of epithelial cells from human trachea, by contrast, the epithelial cells of duck intestine contain SAα2,3Gal but not SAα2,6Gal sialyloligosaccharides [11, 21]. Thus, the host range of influenza A virus may also correspond to the presence or absence of certain sialic acid-galactose linkages in host animals. Recently, a number of reports that viral proteins other than HA protein may be responsible for the overcoming the interspecies barrier has been increasing [17, 34, 50]. These findings indicate that the host range restriction of influenza A

virus is reflected by multiple factors and a further investigating is essential to understand the detail mechanisms.

Avian influenza viruses can be classified into two distinct groups, low pathogenic avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) viruses by their pathogenicity in poultry. LPAI viruses mainly cause respiratory illnesses in the birds and generally low mortality. By contrast, HPAI viruses cause systemic diseases that affect most organ systems including the nervous and cardiovascular systems, often resulting in high mortality in turkeys and chickens. H5 or H7 subtype of avian influenza virus can be either low pathogenic or highly pathogenic, all other known HA subtypes have only low pathogenic forms [58]. In wild aquatic birds and domestic ducks, both of LPAI and most HPAI viruses usually have caused no disease with the exception of high mortality in common terns of South Africa during 1961 (A/turn/South Africa/61) [5] and infections and mortality in a variety of wild birds with H5N1 HPAI viruses that originated from Asia in 1996 (A/Goose/Guangdong/1/96) [72].

However, in 2005, more than 6000 wild aquatic birds including mainly bar-headed geese, great black-headed gulls, brown-headed gulls, ruddy shelducks and great cormorants died from infection with H5N1 HPAI viruses at Qinghai Lake in western China [9, 32]. Following this outbreak, Qinghai-like H5N1 viruses have been repeatedly isolated from

dead goose and swans in many Asian and European countries [1]. At present, whether aquatic birds can continue to migrate long distances after being infected with recent H5N1 viruses remains controversial. However, these findings may suggest that the migration of affected aquatic birds is thought to be one of varied possible reasons why the virus has been rapidly distributed and transmitted in wide area. Thus, role of free-flying aquatic birds in the spread of HPAI viruses cannot be ruled out. These findings indicate necessitate continuing virological surveillance of avian influenza in the natural hosts to provide available information on the invasion of HPAI viruses for the outbreak prevention measures.

In January 2007, there were outbreaks of HPAI caused by avian influenza viruses of H5N1 subtypes among chicken farms at Miyazaki prefecture and Okayama prefecture, Japan. In addition, before the Japanese outbreaks, the H5N1 virus was isolated from a female adult mountain-hawk eagle found at the roadside in Kumamoto prefecture, which is 75 km away from the site of the first outbreak in Miyazaki [20, 54]. Phylogenetic analysis showing all of these isolates were closely related and belonged to the A/bar-headed goose/Qinghai/5/2005-like lineage, thus suggesting that the Qinghai Lake strains might have entered Japan before 2007 [20, 30]. Although there is no direct evidence, such as isolation of virus from migratory birds, one possible hypothesis is that the H5N1 viruses might have introduced into Japan from foreign country by such migratory birds infected

with the virus and those wild birds and/or animals brought them onto the farms. Wild aquatic birds obviously favor rivers, ponds and lakes; however, such sites did not exist around the farms in Japan affected by HPAI in 2007. Therefore, it is thought that terrestrial birds, rather than waterfowl, were involved in viral dissemination and introduction into the farms. However, whereas there have been many reports about the susceptibility to HPAI viruses infection and the potential to transmit the viruses in migratory aquatic birds [31, 72], only limited information has been available in terrestrial wild birds.

This thesis provides the information on ecological roles for the distribution of avian influenza virus in wild birds and biological and genetic basis for the host range restriction of the virus. In chapter 1, the author reports on current epidemiological data on the survey for avian myxoviruses in migratory aquatic birds during 2001 to 2008 in western Japan. In chapter 2, the author reports on the result of experimental infection with H5N1 HPAI virus using terrestrial wild birds and assesses the possibility that those birds may act as carriers for the virus. In chapter 3, the author reports on the contribution of NA protein for viral replication in duck intestine, which is involved in the host range restriction of influenza virus.

CHAPTER 1

Avian Influenza Virus and Paramyxovirus Isolation from Migratory Waterfowl and Shorebirds in San-in District of Western Japan from 2001 to 2008

Introduction

Avian influenza virus (AIV) belongs to the family *Orthomyxoviridae* and has two types of surface glycoprotein, HA and NA, which are divided into H1 to H16 and N1 to N9 subtypes based on their antigenic specificity [13, 68]. If poultry, such as chickens and turkeys, are infected with AIVs, they exhibit various disease symptoms, ranging from mild respiratory signs caused by LPAI viruses to high mortality induced by HPAI viruses, which are restricted to H5 and H7 subtypes; therefore, AIVs are considered to be the most important diseases with regard to economic damage to the poultry industry.

In addition to AIV, avian paramyxovirus (APMV) is a virus that causes an important disease impacting international trade in poultry and poultry products. APMV belongs to the family *Paramyxoviridae* and has nine antigenic serotypes (APMV-1 to -9) based on their hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assay results [2]. Very little is known about the molecular and biological characteristics and pathogenicity of APMV serotypes 2–9, while APMV-1, known as Newcastle disease virus (NDV), has been studied extensively. NDVs are divided into three major pathotypes based on their pathogenicity in chickens: lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence) [2]; therefore, outbreaks of Newcastle disease,

similarly to avian influenza, have resulted in severe economic losses in the poultry industry.

Wild birds, particularly waterfowl, are a reservoir of all known AIV subtypes other than H13 and H16 strains, which are found exclusively in shorebirds, such as gulls [13, 68], and APMV-1, -4, -6, -8 and -9 serotypes [2]. Wild birds, including waterfowl and shorebirds, are thus considered to be important carriers for the transmission of AIVs and APMVs. In Japan, waterfowl such as geese, swans and ducks are well-known wintering migratory birds. These birds fly to Japan from Alaska, the Russian Far East, eastern Siberia, eastern Mongolia, and northeastern China. Since December 1979, Otsuki *et al.* have conducted continued surveillance of AIVs in migratory Anseriformes (e.g., ducks, geese and swans) and Charadriformes (e.g., gulls) in western Japan [43-46, 52, 63]. No highly pathogenic AIVs have been isolated, but many subtypes of viruses from various species of wild birds have been detected. In a previous study, Ito *et al.* experimentally demonstrated that non-pathogenic H5N3 AIV and APMV-1 (NDV) isolated from wild waterfowl became highly pathogenic after several cycles of infection in chickens [22, 51], thus suggesting that wild birds are able to transmit and spread viruses that are potential precursors for highly pathogenic derivatives in poultry birds. Therefore, continuous surveillance of AIVs and APMVs in wild birds, particularly waterfowl, is important for providing information on the prevalent subtypes of the viruses circulating in the field and emerging highly pathogenic

viruses.

This paper described the survey results of AIVs and APMVs in wild waterfowl and shorebirds in winter during their migratory seasons from 2001 to 2008 in the San-in district of western Japan. In addition, the results of phylogenetic analysis on two strains of the AIV isolates identified as H5 subtype was also reported.

Materials and Methods

Collection of fecal samples

Fresh fecal samples were collected from whistling swans (*Cygnus columbianus jankowskii*), mallards (*Anas platyrhynchos*), common teals (*Anas crecca*), Eurasian wigeons (*Anas penelope*), northern pintails (*Anas acuta*), gadwalls (*Anas strepera*), white-fronted geese (*Anser albifrons frontalis*), black-tailed gulls (*Larus crassirostris*) and duck spp. (*Anas* spp.) during winter (November–March) of fiscal years 2001 to 2008 (fiscal year 2001, for example, refers to the period from November 2001 through March 2002). Samples were collected from eight different sites: Lake Koyama, Pond Nikko, Lake Togo, Tenjin River, Hino River, Ito Coast, the Yonago waterbird sanctuary and rice fields of the

suburbs of Yasugi City in the San-in district (Tottori and Shimane Prefectures) of western Japan. Fecal samples were collected individually into screw-capped tubes and stored at -80°C until assayed.

Virus isolation

Virus isolation was performed as described elsewhere [52] with slight modification. Fecal samples were suspended at a concentration of approximately 30% in phosphate-buffered saline (pH 7.2) containing penicillin at 10,000 units per ml and streptomycin at 10 mg per ml. The suspension was centrifuged at 1,000 × g for 10 min. Two hundred microliters of supernatant was inoculated into the allantoic cavities of two 9- to 11-day-old embryonated chicken eggs and were incubated at 37°C for 3 days unless death of the embryo was detected. After the inoculated eggs were chilled at 4°C, their allantoic fluids were tested for hemagglutination activity.

All hemagglutinin agents were tested by ESPLINE® INFLUENZA A&B-N (FUJIREBIO INC., Tokyo, Japan) which is a highly sensitive kit for avian influenza virus [4]. All positive agents were identified by HI and NI tests with specific antisera against influenza A virus strains as described elsewhere [52, 63]. Samples that were negative for

the influenza virus by the detection kit were further tested by HI using specific antisera against APMV strains APMV-1/goose/Alaska/415/91, APMV-2/chicken/California/Yucaipa/56 and APMV-4/duck/Mississippi/320/75. Tested samples that were positive for any of the above antisera were identified as APMV. Methods for HI and NI tests were based on established procedures [71].

Genomic sequencing

Viral RNA was isolated from infected allantoic fluid using a QIAamp Viral RNA Mini Kit (QIAGEN, CA, USA). HA gene segments coding full-length ORFs were amplified using PrimeScript™ Reverse Transcriptase (TaKaRa, Shiga, Japan) for RT and KOD Dash (TOYOBO, Osaka, Japan) for PCR. After extraction from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), viral cDNA was sequenced using a BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, CA, USA) on a 3130xl Genetic Analyzer (Applied Biosystems). The set of HA gene-specific primers and conditions used for RT, PCR and sequencing are available upon request. DNA sequence data were edited and aligned with BioEdit version 7.0.8.0. software [16].

Phylogenetic analysis

Phylogenetic analysis of HA gene segments was performed by applying the clustal W method using MEGA 4 software [61]. The phylogenetic tree was constructed with the Kimura two-parameter nucleotide model. The robustness of the grouping in neighbor joining analysis was assessed using 1,000 bootstrap resampling.

Results and Discussion

A total of 4,335 fecal samples were collected from whistling swans (1,890 samples), mallards (471 samples), white-fronted geese (189 samples), common teals (139 samples), Eurasian wigeons (106 samples), northern pintails (36 samples), black-tailed gulls (26 samples), gadwalls (2 samples) and duck spp. (1,476 samples) in the San-in district of western Japan. From these samples, 41 strains of AIV (28 from whistling swans, 3 from common teals, 2 from mallards and 8 from duck spp.) and 13 strains of APMV (4 from whistling swans, 2 from white-fronted geese, 1 each from mallards, Eurasian wigeons and gadwalls and 4 from duck spp.) were isolated (Table 1). From 2001 to 2008, the overall isolation rate of AIV was 0.95% and that for APMV was 0.30%. This rate for AIVs was

similar to that in our previous surveillance in the same district since 1979 (1.9%, 129 isolates/6,801 fecal samples). Subtypes of the 41 strains of AIV isolated were characterized by HI and NI tests and were classified into 12 different subtypes: H1N1, H3N9, H4N6, H5N3, H6N1, H6N2, H6N5, H6N8, H9N2, H9N6, H10N4 and H11N9 (Table 2). Predominant combinations of HA and NA subtypes were H4N6 (14 isolates) and H6N8 (7 isolates). Isolation of H3N9 and H9N6 subtypes, which were isolated in 2001 and 2008, respectively, has not been reported in the field in Japan.

A large number of H5N1 HPAI viruses have been isolated from swan species in European countries and Mongolia since 2005, and HPAI outbreaks in poultry caused by H5N1 viruses have been reported after isolation from swans [1]. These incidents indicate that swans play a key role in the spread of HPAI viruses in Europe. In addition, in April and May 2008, H5N1 HPAI viruses were isolated from dead or moribund whooper swans (*Cygnus cygnus*) in Aomori, Akita and Hokkaido Prefectures in northern Japan [64, 65]. The present study showed that whistling swans had the largest number of AIVs among all tested species (Table 2). Our findings suggest that HPAI viruses may have been introduced into the San-in district via the migration of wild swans.

Western Japanese poultry farms experienced two outbreaks of HPAI viruses caused by H5N1 subtypes in 2004 and 2007 [20]. Before the outbreaks, we isolated two H5

subtypes of AIV, designated A/teal/Tottori/150/2002 (H5N3) and A/whistling swan/Shimane/580/2002 (H5N3) (GeneBank Accession numbers of HA gene: AB535130 and AB535131, respectively) in the field specimens. Sequencing analysis of HA genes revealed that the deduced amino acid sequence at the cleavage site of both isolates showed typical avirulent motifs (RETR/G), indicating that they were presumably low or non-pathogenic AIVs. Phylogenetic analysis based on nucleotide sequences of the HA gene segment showed that A/teal/Tottori/150/2002 and A/whistling swan/Shimane/580/2002 belonged to Eurasian lineages and clustered in a different branch with the H5N1 HPAI viruses that were isolated in Japan in 2004, 2007 and 2008 (Figure 1). Both isolates were also separate from the H5N2 subtype of Japanese LPAI viruses isolated from chicken farms in 2005. Ito *et al.* [22] demonstrated that a non-pathogenic AIV strain, A/whistling swan/Shimane/499/83 (H5N3), developed into a highly pathogenic virus showing 100% mortality in chickens after several rounds of infection in chickens. This suggests that circulation of non-pathogenic field isolates in chickens can lead to the development of HPAI variants. In fact, H5N2 LPAI viruses isolated in the Unites States in 1983 or Mexico in 1993 to 1995 increased in pathogenicity and emerged in poultry as HPAI viruses [14, 25]; however, which H5 low pathogenic isolates from wild birds adapt and evolve to become highly pathogenic to chickens is still unclear. Therefore, in order to understand this

issue, experimental investigations using both H5 isolates in the present study is considered to be carried out.

In the present study, 13 APMVs were also isolated from wild birds (Table 1). To determine the APMV serotypes, a set of antisera against APMV-1 to -9 is essential. Because we did not have access to antisera for all serotypes, the isolated APMVs could not be serotyped. The HI test using anti-APMV-1, -2 and -4, as described above, confirmed that 5 of 13 isolates were positive only for APMV-1 antiserum, indicating that these isolates may be NDV strains. On the other hand, of the remaining 8 isolates, four isolates were positive only for APMV-2 and the remainders were positive only for APMV-4 antiserum. In Japan, from 2001 to 2007, NDVs, including virulent pathotypes for chicken, were sporadically found in wild birds and poultry [33]. Among these, several Japanese isolates were genetically similar to Korean isolates, suggesting that they were derived from an immediate common ancestor. As a possible explanation for viral introduction into Japan, it was considered that the movement of wild birds may be related to viral dissemination [33]; therefore, to better understand the epidemiology of NDVs in wild birds, further genetic and serological studies of the APMV isolates in this study are necessary.

These data indicate that various subtypes of AIV and APMV are prevalent in numerous species of migratory wild birds flying into the San-in district of western Japan.

Continued surveillance over multiple years will allow for a better understanding of the role of wild birds in the introduction and dissemination of AIVs and APMVs in the field. Therefore, it is necessary to continue the surveillance of these serious poultry pathogens in migratory waterfowl and shorebirds.

Table 1. Isolation of AIVs and APMVs from fecal samples from waterfowl and shorebirds in the San-in district of Western Japan from 2001 to 2008

Species	AIV and APMV isolation in each fiscal year								Total
	2001	2002	2003	2004	2005	2006	2007	2008	
Whistling swan	10/3/291[a)]	3/1/363	5/0/414	0/0/216	8/0/353	0/0/17	0/0/135	2/0/101	28/4/1890
Duck spp.	5/0/212	0/0/43	1/0/119	1/1/630	0/0/140	1/1/127	0/0/89	0/2/116	8/4/1476
Mallard	0/0/12	0/0/116					0/1/75	2/0/268	2/1/471
White-fronted goose	0/0/11	0/2/129					0/0/49		0/2/189
Common teal	3/0/75					0/0/6		0/0/58	3/0/139
Eurasian wigeon							0/1/106		0/1/106
Northern pintail	0/0/31	0/0/5							0/0/36
Black-tailed gull	0/0/26								0/0/26
Gadwall						0/1/2			0/1/2
Total	18/3/658	3/3/656	6/0/533	1/1/846	8/0/493	1/2/152	0/2/454	4/2/543	41/13/4335
Isolation rate (%)	2.74/0.46[b)]	0.46/0.46	1.13/0	0.12/0.12	1.62/0	0.66/1.32	0/0.44	0.74/0.37	0.95/0.30

a) Number of AIV isolates/APMV isolates/total tested samples.
b) Isolation rate of AIVs/APMVs

Table 2. Antigenic characterization of AIV isolates from fecal samples from waterfowl and shorebirds in the San-in district of Western Japan from 2001 to 2008.

Species	Total no.	Subtypes of AIV isolates in each fiscal year							
		2001	2002	2003	2004	2005	2006	2007	2008
Whistling swan	28	H3N9 (2)[a)] H6N8 (6) H9N11 (2)	H1N1 (2) H5N3 (1)	H1N1 (1) H6N5 (3) H6N8 (1)	NI	H4N6 (6) H6N1 (1) H11N9 (1)	NI	NI	H6N1 (1) H9N2 (1)
Mallard	2	NI[b)]	NI	NI	NI	NI	NI	NI	H9N6 (1) H11N9 (1)
Common teal	3	H4N6 (1) H5N3 (1) H10N4 (1)	NI	NI	NI	NI	NI	NI	NI
Duck spp.	8	H4N6 (5)	NI	H4N6 (1)	H4N6 (1)	NI	H6N2 (1)	NI	NI

a) Number of isolates of each antigenic subtype is shown in parentheses.
b) No viruses were isolated.

Figure 1


A/chicken/Kyoto/3/2004
A/crow/Kyoto/53/2004
A/chicken/Yamaguchi/7/2004
A/crow/Osaka/102/2004
A/chicken/Oita/8/2004
A/chicken/Korea/ES/2003
clade 2.5
A/mountain hawk-eagle/Kumamoto/1/2007
A/duck/Korea/Asan5/2006
A/chicken/Korea/IS/2006
A/black-headed goose/Qinghai/1/2005
clade 2.2
A/Indonesia/CDC1046/2007
A/Indonesia/5/2005
clade 2.1.3
A/Indonesia/CDC594/2006
A/Indonesia/CDC596/2006
clade 2.1.2
A/chicken/Indonesia/11/2003
A/chicken/Indonesia/7/2003
clade 2.1.1
A/chicken/Yunnan/447/2005
A/duck/Guangxi/13/2004
clade 2.4
A/muscovy duck/Vietnam/39/2007
A/duck/Laos/3295/2006
clade 2.3.4
A/chicken/Guiyang/3055/2005
A/goose/Guiyang/3422/2005
clade 2.3.3
A/whooper swan/Aomori/1/2008
A/whooper swan/Akita/1/2008
A/whooper swan/Aomori/2/2008
A/whooper swan/Hokkaido/1/2008
A/chicken/Primorje/1/2008
A/whooper swan/Hokkaido/2/2008
A/duck/Vietnam/568/2005
A/goose/Guangxi/345/2005
clade 2.3.2
A/duck/Hunan/139/2005
A/duck/Hunan/127/2005
clade 2.3.1
A/Hong Kong/213/2003
clade 1
A/chicken/Hong Kong/YU777/2002
clade 8
A/duck/Fujian/897/2005
clade 9
A/duck/Hubei/wg/2002
clade 6
A/chicken/Hebei/326/2005
clade 7
A/goose/Guiyang/337/2006
clade 4
A/chicken/Hong Kong/YU562/2001
clade 3
A/goose/Guangxi/914/2004
clade 5
A/goose/Guangdong/1/1996
clade 0
A/whistling swan/Shimane/580/2002
A/duck/Yunnan/435/2002
A/teal/Tottori/150/2002
A/duck/Mongolia/54/2001
Eurasian Avian
A/mallard/Maryland/302/2001
A/chicken/Ibaraki/1/2005
A/chicken/Mexico/435/2005
American Avian
0.02

Figure legend

Figure 1. Phylogenetic tree for HA gene in recent H5 isolates. The phylogenetic tree was generated using the neighbor-joining method and bootstrap testing (n = 1,000) in MEGA (4.0.2). Analysis was based on nucleotides 105–1659 (1555 bp) of the HA gene. ● represents isolates in Japan in 2004, ■ in 2005, ◆ in 2007 and ▲ in 2008. H5 isolates in this study are underlined.

CHAPTER 2

Susceptibility of Two Species of Wild Terrestrial Birds to Infection with a Highly Pathogenic Avian Influenza Virus of H5N1 Subtype

Introduction

Avian influenza viruses are divided into subtypes based on virion surface proteins, hemagglutinin (H1 to H16) and neuraminidase (N1 to N9). All virus subtypes, other than strains found exclusively in shorebirds such as gulls (H13 and H16), have been isolated from waterfowl [13, 68]. Therefore, wild waterfowl are thought to be a natural reservoir that plays a role in the prevalence of viruses [68]. If poultry such as chicken and turkey are infected with avian influenza viruses, they exhibit various disease signs ranging from mild respiratory signs caused by LPAI viruses to high mortality induced by HPAI viruses. On the other hand, in infected wild waterfowl, clinical signs are rarely seen, although the viruses replicate efficiently in their bodies [68]; an exception was the recent fatal H5N1 viruses that caused systemic infection, neurological dysfunction and death in ducks [8, 56].

Since 2003, HPAI outbreaks in Southeast Asia have continued to smolder and spread, and H5N1 virus has been seen in many parts of Asia, Europe, Africa and the Middle East. Some migratory waterfowl appear to carry and disseminate the currently circulating highly pathogenic H5N1 strains. Whether these birds can migrate long distances after being infected, however, remains controversial [62].

In the beginning of 2007, H5N1 HPAI outbreaks occurred at three chicken farms in Miyazaki Prefecture and one chicken farm in Okayama Prefecture, Japan. Genetically, the causative viruses were closely related to viruses isolated in Korea at the end of 2006 and their hemagglutinin genes were classified into clade 2.2, represented by viruses isolated from dead wild birds at Lake Qinghai, China, 2005 [20, 35]. In addition, the results of epidemiological investigations conducted in Japan suggested that H5N1 HPAI viruses had not persisted in Japan until the 2007 outbreaks; therefore, the 2007 isolates are thought to be newly introduced from elsewhere.

One possible explanation is that the H5N1 viruses might have introduced through imported poultry, birds, eggs and meat products or illegally imported exotic birds. However, according to the report on epidemiological surveillance and quarantine measures in outbreak countries, direct evidences of connection between the outbreak farms and foreign countries, for example farm-related persons who had a history of contact to the infected sources or travel to outbreak countries or import of feed and medicines from such countries, have not been reported [35]. In addition to this, other conceivable explanation is that the H5N1 viruses might have been carried by infected wild birds, as many species of migratory bird travel from Korea to Japan in winter from October to March. The reports of HPAI viruses being isolated from live wild birds support this hypothesis [29, 31, 72].

Wild waterfowl obviously favor rivers, ponds and lakes; however, such sites did not exist around the farms in Japan affected by HPAI in 2007. Therefore, it is possible that terrestrial birds, rather than waterfowl, were involved in viral dissemination and introduction into the farms. However, there have been few reports on the susceptibility of terrestrial birds to H5N1 HPAI viruses. In this study, in order to obtain more information, the author performed experimental infection of terrestrial birds that migrate among HPAI-prevalent countries with the recent H5N1 virus, and discuss the possibility that they may act as carriers for the virus.

Materials and methods

Virus

A HPAI virus, A/mountain hawk-eagle/Kumamoto/1/07 (MHE/Kumamoto/07; H5N1), was the first isolated from a wild terrestrial bird in Japan. Genetically, all Japanese isolates in 2007 containing MHE/Kumamoto/07 were closely related and classified as clade 2.2 Qinghai Lake-related strains [35]. MHE/Kumamoto/07 was confirmed the highly pathogenic phenotype by experimental infection of chickens, which led up 100% mortality

within 24 to 45 hours postinoculation. The stock virus used in this study was deposited at the Avian Zoonosis Research Centre, Faculty of Agriculture, Tottori University.

Animals

Pale thrushes (*Turdus pallidus*) and great reed warblers (*Acrocephalus arundinaceus*) used for experimental infection were harvested using mist nets inside Tottori Prefecture with permission from the Japanese Ministry of the Environment. Birds were quarantined for 3 to 5 days before the experiment.

Experimental infection

Ten pale thrushes and eight great reed warblers were intranasally inoculated with 50 μl containing $10^{6.3}$ EID_{50} and 30 μl containing $10^{6.4}$ EID_{50} of the stock virus, respectively. Infected birds were observed twice a day for clinical signs and mortality for an 8-day period. At 5 days postinoculation (d.p.i.), two randomly selected pale thrushes were killed humanely in order to determine virus titers in each organ, as described below. Control groups consisted of seven pale thrushes and six great reed warblers, which were

intranasally given phosphate buffer saline (PBS), were observed for the same period as inoculated birds of each species. Control and infected birds were housed separately in negative-pressure isolator cages.

In order to determine the virus titer in organs, the brain, trachea, lung, heart, liver, colon and kidney were collected from two great reed warblers (selected randomly among the dead birds at 3 d.p.i.), two sacrificed pale thrushes without any clinical signs at 5 d.p.i. and one pale thrush that died at 8 d.p.i.. Collected organs were homogenized into 10% w/v homogenates with PBS and were centrifuged (2,200 × g, 5 min). One-hundred microliters of serial 10-fold dilutions of supernatants were inoculated into the allantoic cavities of three 10-day-old embryonated hens' eggs [26]. Virus titers were calculated by the Reed and Muench method [49] and were expressed as EID_{50} per gram of tissue.

When the inoculated great reed warblers died, tracheal and cloacal swabs were collected for virus recovery. In the case of pale thrushes, because it was difficult to obtain the swabs from surviving birds without use of anesthesia, collection was not conducted. Control birds were swabbed after death or were killed humanely. These experiments were performed in a biosafety level 3 facility at Tottori University Avian Zoonosis Research Center, Japan.

Histopathological and immunohistological tests

Tissues including the brain, trachea, lung, heart, liver, colon and kidney were taken from one bird showing typical symptoms in each group (i.e. dead great reed warbler at 2 d.p.i. and sacrificed pale thrush at 8 d.p.i.). Tissues from control birds were also collected on the same days as inoculated birds of each species. Collected tissues were fixed in 10% formalin in PBS and then dehydrated, embedded in paraffin and cut into 5-μm sections that were stained with standard hematoxylin and eosin (HE). Immunohistological analysis for viral antigen detection was also performed as described [53] using anti-A/Vietnam/1203/04 (H5N1) prepared in rabbits as a primary antibody.

Results

In order to investigate the susceptibility of terrestrial wild birds to H5N1 HPAI virus, two bird species (great reed warbler and pale thrush) were inoculated with A/mountain hawk-eagle/Kumamoto/1/07 (MHE/Kumamoto/07) virus. Within 3 d.p.i., all 8 inoculated great reed warblers had died after exhibiting depression (Table 3). In contrast, only 3 of the 8 pale thrushes died (one died suddenly at 2 d.p.i. and two died suddenly at 8

d.p.i.), while the others showed no clinical signs during the observation period. In the case of control birds, all great reed warblers and pale thrushes survived. These results indicate that pale thrushes infected with the H5N1 virus are capable of survival for a longer period than great reed warblers. These findings suggest that there is a significant difference in the mortality rate of H5N1 viral infection among wild terrestrial bird species.

Virus titers in the brain, trachea, lung, heart, liver, colon and kidney of infected birds revealed that the virus was able to replicate efficiently in all of the tested organs from great reed warblers (Table 4). The virus titers at 3 d.p.i. in different organs were ranging from $10^{3.7}$ to $10^{9.5}$ EID_{50}/g. Virus titers in pale thrushes at 5 d.p.i. were below the detection limit (< $10^{1.5}$ EID_{50}/g) in the brain and $10^{1.5}$ to $10^{5.5}$ EID_{50}/g in other organs. Virus titers in pale thrushes that died at 8 d.p.i. were only obtained from the brain and trachea ($10^{5.6}$ and $10^{1.7}$ EID_{50}/g, respectively).

The results of virus recovery from swabs of great reed warblers showed that 7 of 8 tracheal samples and 4 of 8 cloacal samples were positive for the virus. The virus recovery rate from tracheal swabs was higher than from cloacal swabs, thus suggesting that this species may shed larger amounts of virus in the trachea than the cloaca. No virus was isolated from swabs of any control birds.

On histological examination of infected birds, noteworthy alterations were not seen in any tissues from the great reed warbler. In contrast, the brain of pale thrush showed apparent perivascular inflammation of mononuclear cells and nonsuppurative meningitis (Figure 2a). In control birds, no histopathological changes were observed in any organs (Figure 2b).

Discussion

In the present study, the author investigated the susceptibility of two species of wild terrestrial bird (great reed warbler and pale thrush) belonging to the order *Passeriformes* by performing experimental infections using a recently isolated H5N1 HPAI virus. The tested species are widely distributed in Asian countries where HPAI outbreaks have frequently occurred. Every spring, the great reed warbler migrates from Southeast Asia into Japan. On the other hand, in autumn, the pale thrush arrives from Northeast Asia through the Korean Peninsula. In addition, both species were seen around the farms affected in Japan by the HPAI in 2007. Furthermore in order to discuss the possibility that these terrestrial birds may act as carriers for HPAI viruses, MHE/Kumamoto/07, which was isolated from wild birds and showed highly pathogenicity for chicken, was used in this study. The present

results showed that both of two species have high susceptibility to infection with the H5N1 virus and have the potential to become carriers for the virus.

Boon *et al.* [6] demonstrated that the mortality rate of house sparrows was 66-100%, but no deaths were seen in starlings infected with H5N1 HPAI viruses, which were classified as clade 2.3.4 and isolated from dead wild terrestrial birds. In addition, Perkins and Swayne [48] reported that zebra finches and house finches inoculated with H5N1 HPAI virus, A/chicken/Hong Kong/220/97, showed high mortality and morbidity rates, while all of the inoculated sparrows and starlings survived, and only some of the sparrows experienced mild disease signs. In the present study, the great reed warbler showed high fatality rates within 3 d.p.i., while many of the inoculated pale thrushes survived for 8 days, despite showing high virus titres at 5 d.p.i. (Table 3 and 4). All of these birds are classified into the order *Passeriformes*. These results indicate that there are different survival rates among terrestrial wild bird species following H5N1 viral infection, even within the same order.

As shown by the virus recovery from swab samples, more virus appear to be excreted from the upper respiratory tract than from the intestinal tract of great reed warblers infected with the recent H5N1 virus. Similar findings were observed in waterfowl [7, 56]. It is important for more efficient viral detection to collect specimen materials likely to contain

larger amounts of virus. Therefore, virus isolation from both tracheal and cloacal swabs is necessary for surveillance in wild terrestrial birds.

In the present study, the author demonstrated that two species of migratory wild terrestrial birds have high susceptibility to H5N1 infection and these birds have the potential to carry H5N1 HPAI viruses. It has been considered that the 2007 HPAI isolates entered Japan and were disseminated over large areas by infected wild birds. In addition, the habitats of terrestrial birds, such as those used in this study, are in close proximity to those of poultry and humans. Therefore, the possibility that these terrestrial birds can contribute to the spread of H5N1 HPAI viruses cannot be ignored, although direct evidence of viral dissemination is lacking [20, 35]. Hence, to establish an early warning system for HPAI outbreaks, it is vitally important to perform surveillance of both wild waterfowl and terrestrial birds.

Table 3. Mortality of wild terrestrial birds infected with H5N1 HPAI virus, A/mountain hawk-eagle/Kumamoto/1/07

Species	Mortality rate (No. of dead/total)	No. of death on day postinfection							
		1	2	3	4	5	6	7	8
Great reed warbler	8/8	0	4	4	—	—	—	—	—
Pale thrush	3/8	0	1	0	0	0	0	0	2

—: Not applicable.
In case of control birds, all the 7 pale thrushes and 6 great reed warblers survived.

Table 4. Virus titers in organs of wild terrestrial birds infected with H5N1 HPAI virus, A/mountain hawk-eagle/Kumamoto/1/07

Species	No. of tested birds	Virus titer (log EID_{50}/g)						
		Brain	Trachea	Lung	Heart	Liver	Colon	Kidney
Great reed warbler	2 (both died at 3 d.p.i.)[a]	9.5	7.5	7.5	7.5	6.5	3.7	5.2
Pale thrush	2 (both sacrificed at 5 d.p.i.)[a]	—[b]	2.8	5.5	3.2	2.5	1.5	3.5
Pale thrush	1 (died at 8 d.p.i.)[a]	5.6	1.7	—	—	—	—	—

[a]Collected organs were pooled and virus titers were then determined using embryonated chicken eggs.

[b] <1.5 log EID_{50}/g .

Figure 2

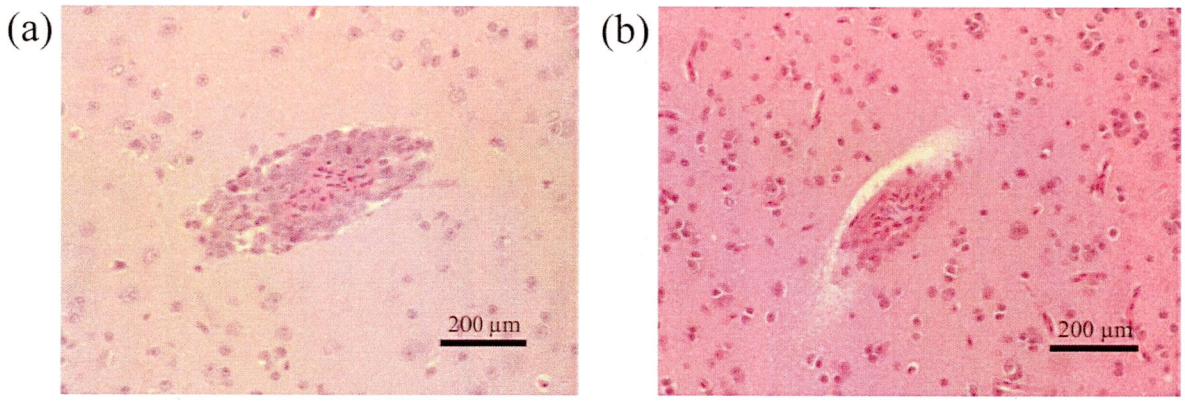

Figure legend

Figure 2. Histological examination of tissues from wild terrestrial birds experimentally infected with H5N1 HPAI virus, A/mountain hawk-eagle/Kumamoto/1/07 (a) and control birds given PBS (b). Perivascular inflammation with mononuclear cells were observed in brain sections of infected pale thrush sacrificed at 8 d.p.i.. HE stain (a). No inflammation was observed in brain sections of control pale thrush sacrificed at 8 d.p.i.. HE stain (b).

CHAPTER 3

Low-pH Stability of Neuraminidase Associated with Intestinal Replication of Influenza A Virus in Duck

Introduction

Influenza A viruses possess two glycoprotein spikes on the virion surface, corresponding to HA and NA which are divided into 16 HA and 9 NA subtypes on the basis of the antigenic property [13, 19, 68]. HA binds to oligosaccharide receptors containing terminal sialic acids on the target cell surface. In addition, after virus entry into an endosome through receptor-mediated endocytosis, HA mediates the fusion of the viral envelope with the endosomal membrane to release the viral genome into the infected cells [55, 70]. On the other hand, NA removes sialic acids from progeny viral particles and cell surface, which results in preventing viral particles from self-aggregation or reattachment to previously infected-cells at the final infectious stage [47]. Recently, Ohuchi et al. [41] suggested that at the initial infectious stage, if virus attach to unsuitable sites for the entry into the target cells, NA facilitates viral transfer from one site to another site so that the efficacy of viral infection is enhanced.

Genomic genes of influenza A virus are consisted of eight unique RNA segments. It is possible that co-infection with more than two different viruses into a host cell can allow the generation of progeny viruses containing various and novel combination of genes (i.e., genetic reassortant) [68]. Indeed, the emerging of the 1957 Asian H2N2 strain (Asian

influenza) were caused by the replacing HA, NA and PB1 genes of previous circulating human H1N1 virus with those genes of avian H2N2 virus [19, 24]. This event evidenced that the genetic reassortant between a prevalent human virus and an avian virus which have been maintained exclusively in wild bird's population, causing the sudden appearance of new types of influenza virus that can become pandemic strains.

Host range of influenza A viruses have been known to show a wide distribution in different mammalian species including humans, pigs, horses, mink, marine mammals and avian species including domestic and wild birds. In particular, wild birds, predominantly ducks, geese and shorebirds, are proposed as the reservoir of influenza A viruses in nature [19, 37, 68]. Avian influenza viruses replicate both in the respiratory and the intestinal tract (predominant site) of ducks and the virus is shed in the feces [26, 69], whereas human influenza A virus limited replicate in the respiratory tract and did not recover from intestine of duck [69]. Hinshaw et al. [18] reported that a reassortant virus, containing only HA and NA genes from a human virus, A/Udorn/307/72 (H3N2), and the remaining genes from an avian virus, A/mallard/NY/6750/78 (H2N2), did not replicate in ducks. This observation indicated that the biological property of HA and NA significantly contribute to the viral growth in infected animals. However, in contrast to the well-characterized role of HA on

the tissue tropism or the host range restriction of influenza A viruses, at present, there are limited information about the role of NA.

The N2 subtype of NA, originally derived from an avian virus, first appeared in human viruses in the 1957 outbreak of the H2N2 virus. Kobasa et al. [28] generated a panel of reassortant viruses, containing the N2NA gene from human viruses isolated between 1957 and 1968 and all other genes from avian virus A/duck/Hong Kong/278/78 (H2N9; Dk78). The reassortant virus containing the NA gene from A/Singapore/1/57 (H2N2), Dk78/Sing57N2 (H2N2), grew well in duck intestine via oral-route infection, whiles the reassortant virus containing the NA gene from A/England/12/62 (H2N2), Dk78/Eng62N2 (H2N2), did not grow to detectable levels [28]. These findings suggested that the later human virus N2 had lost the ability to support viral growth in ducks, which might be caused by the reduction of sialidase activity, compared to that of avian virus N2, to maintain an optimal balance between NA activity and the lower receptor binding affinity of human virus HA [28]. However, another report showed that the low pH sensitivity of influenza A virus N2NA was responsible for the determination of viral growth capacity in duck intestine [60]. Like these, there are several hypotheses regarding the contribution of N2NA to influenza A virus replication in duck.

Here, in order to better understand the role of NA on the viral replication and enterotropism in ducks, a variant virus enabling to grow in the intestine was generated from Dk78/Eng62N2 by passage in duck and then, the characteristics of its NA and the molecular mechanisms which are responsible for the viral replication was demonstrated by *in vitro* analyses.

Materials and Methods

Viruses and Cells

Three human influenza viruses: A/Singapore/1/57 (H2N2), A/Udorn/307/72 (H3N2) and A/Tottori/872/94 (H3N2), two avian influenza viruses: A/mallard/New York/6750/78 (H2N2) and A/duck/Hokkaido/5/77 (H3N2) and a reassortant virus, Dk78/Eng62N2 (H2N2), were used in this study. The detail methods of generation of Dk78/Eng62N2 (H2N2), whose NA genes were derived from human virus A/England/12/62 (H2N2) and all remaining genes were derived from avian virus A/duck/Hong Kong/278/78 (H2N9), was referred in other report [28]. These viruses were propagated in allantoic cavities of 10-day-

old embryonated chicken eggs at 37°C for two days. The infectious allantoic fluids were harvested and stored at -80°C as a stock virus.

293 cells were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% heat-inactivated fetal bovine serum and were maintained at 37°C in 5% CO_2.

In vivo selection of variant virus, generated from Dk78/Eng62N2, that developed the ability of replication in duck intestine

At first, concentrated viruses of Dk78/Eng62N2 as inoculums into ducks were prepared. Aliquots (0.2 ml) of Dk78/Eng62N2 suspension were injected into the cavities of approximately one hundred 10-day-old embryonated chicken eggs and after 48 hours incubation at 35°C, the allantoic fluid of individual eggs was collected and pooled. Then, pooled infectious allantoic fluid was clarified particular host debris by centrifugation at 4,000 × g for 30 minutes at 4°C and was subsequently precipitated by ultracentrifugation at 55,000 × g for 2 hours at 4°C. The pellet of sediment was resuspended in 3 ml of PBS (pH 7.2) and was subjected to animal experiment as described follows.

Mallard ducks (*Anas platyrhynchos platyrhynchos*) were purchased from Tsumura Corporation (Osaka, Japan) and used 4-6 weeks of age. Initially, five ducks were inoculated rectally with 0.5 ml of the concentrated Dk78/Eng62N2. After 3 d.p.i., the ducks were sacrificed humanly to collect aseptically the colon, and then the colons were individually homogenized into 10% w/v homogenates with PBS (containing 10,000 units/*ml* penicillin and 10 mg/ml streptomycin) as described by Kida et al. [26]. After centrifuged at 2,200 × g for 5 min, the supernatant was inoculated into 10-day-old embryonated chicken eggs to confirm the viral recovery. One milliliter of each allantoic fluid confirmed viral propagation was then orally inoculated into a new duck. At 3 d.p.i., the virus recovery from colons of the inoculated ducks was performed in the same way as described above. The recovered virus was cloned by triplicate plaque method using MDCK cells, resulted that a cloned recovered virus was collected.

Measurement of the enzymatic activity of viral NA after low-pH treatment

For the preparation of virus whose NA enzymatic activity was adjusted to a fixed level, the activity of each virus was determined by neuraminidase assay according to standard procedures recommended by WHO [3]. Briefly, 25 μl of a serial two-fold diluted

virus was incubated with 25 μl of PBS and 50 μl of fetuin (SIGMA, St. Louis, USA) solution prepared as 12.5 mg/ml in phosphate buffer (pH 5.9) at 37 °C for 18 hours. Following that, the reaction reagent was incubated with 50 μl of periodate reagent at room temperature for 20 min, after then 500 μl of arsenite reagent was added and vortexed until the color disappeared. Soon after the disappearance of the color, 1.25 ml of thiobarbituric acid reagent was added and heated in boiling water for 15 min. Then, the sample was cooled down at room temperature for 10 min. The colored chromosphere was extracted into 2 ml of *n*-butanol-5% HCl (Vol/Vol) and the absorbance was measured at 549 nm in Ultrospec 1100 *pro* UV/Vis spectrophotometer (Amersham Biosciences, Uppsala, Sweden). A diluted virus that had a value of Optical Density 1.0 unit for NA activity was used in following experiment.

The pH treatment for virus was performed as following methods. Two milliliter of the prepared virus suspension was adjusted to various pH (pH7, 5, 4 or 3) by 0.1 M citric acid and was added each pH of 0.01 M citric acid-sodium citrate buffer solutions up to 3 ml, then was incubated at 4°C for 60 min (low-pH treatment). After that, the pH was returned pH7 by 1.0 M NaOH and added distilled water up to 4 ml. Subsequently, the enzymatic activity of 50 μl of the aliquot virus after the low-pH treatment was measured as described above. The residual NA activity in various low-pH treatments were shown in the ration of

the activity after each low-pH treatment versus after pH 7 treatment. The experiment was performed independently for three times and the average value is reported in results.

Sequence analysis

In order to compare the amino acid sequence of viruses, the consensus sequence of full-length viral genes were determined. Viral RNA was isolated from infectious allantoic fluid using a QIAamp Viral RNA Mini Kit (QIAGEN, CA, USA). Gene segments were amplified using PrimeScript™ Reverse Transcriptase (TaKaRa, Shiga, Japan) for RT and KOD Dash (TOYOBO, Osaka, Japan) for PCR. After electrophoresis and extraction from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), viral cDNA was sequenced with the use of BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, CA, USA) on a 3130xl Genetic Analyzer (Applied Biosystems). The set of gene specific primers and temperature conditions applied in RT, PCR and sequencing are available upon request.

Construction of plasmid expression NA protein

Full-length cDNAs corresponding to ORFs of the NA genes of Dk78/Eng62N2 and Dk-rec6 were amplified by PCR using a KOD Plus (TOYOBO) with oligonucleotides, N2-Start(*Eco*RI); 5'-CGgaattcACCATGAATCCAAATCAAAAGATAATAAC-3' (small letters represent *Eco*RI site) and N2-Stop(*Xho*I); 5'-CCGctcgagTTATATAGGCATGAAATTGATGTTCGCC-3' (small letters represent *Xho*I site). The PCR products were individually cloned into pT7Blue vector by using pT7Blue Perfectly Blunt Cloning Kit (Novagen, Darmstadt, Germany) according to the provided instructions and designed pTEng62NA and pTDk-rec6NA, respectively. These constructed plasmids were confirmed to introduce each desired NA gene by sequence analysis of the entire region generated by PCR.

Subsequently, six kinds of pT7 plasmids contained NA genes, which possess various amino acid residues differed between Dk78/Eng62N2 and Dk-rec6 (at position 165, 356 and 431), were constructed by QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according to the provided instructions. Primer sets; NA-I165V-F (5'-GAATGAGTTGGGTGTTCCATTTCATTTAGG-3' [altered nucleotide are underlined]) and NA-I165V-R (5'-CCTAAATGAAATGGAACACCCAACTCATTC-3') which change isoleucine to valine at position 165 , NA-D356N-F (5'-GCTGGGCCTTTGACAATGGAGATGACG-3') and NA-D356N-R (5'-

CGTCATCTCCATTGTCAAAGGCCCAGC-3') which change aspartic acid to asparagine at position 356, NA-P431Q-F (5'-GATAAGGGGAAGGCAACAGGAGACTAGAG-3') and NA-P431Q-R (5'-CTCTAGTCTCCTGTTGCCTTCCCCTTATC-3') which change proline to glutamine at position 431 were utilized. Ile165→Val, Asp356→Asn and Pro431→Gln mutations were introduced into pTDk-rec6NA as a temperate and were produced pTEng62NA-N356D/Q431P, pTEng62NA-V165I/Q431P and pTDK-rec1NA, respectively. Then, Pro431→Gln mutation was introduced into pTEng62NA-V165I/Q431P as a temperate, resulted that pTEng62NA-V165I was generated. pTEng62NA-Q431P and pTEng62NA-N356D were generated by introduction Asp356→Asn and Pro431→Gln, respectively, into pTEng62NA-N356D/Q431P. Introduction of only the desired mutation in each constructed plasmid was confirmed by sequence analysis.

Each NA fragment digested by *Eco*RI and *Xho*I from the constructed plasmid was subcloned into the expression vector pCAGGS/MCS by using DNA ligation Kit Ver.2.1 (TaKaRa). Generated NA protein expression plasmids were designated pCEng62NA, pCDk-rec1NA, pCDk-rec6NA, pCEng62NA-V165I, pCEng62NA-N356D, pCEng62NA-Q431P, pCEng62NA-V165I/Q431P and pCEng62NA-N356D/Q431P. Plasmid DNA was purified using Plasmid Midi Kit (QIAGEN). Expression of NA proteins on 293 cells derived from these plasmids were confirmed by fluorescent antibody test using anti-

A/Singapore/1/57 (H2N2) chicken serum as a primary antibody and FITC conjugated anti-chicken/turkey IgG rabbit serum (Zymed Laboratories, CA, USA) as a secondary antibody.

Enzymatic activity of cell-expressed NA proteins

293 cells at 80 % confluency of 12-well plates were transfected with 0.4 μg of each NA proteins expressed by the plasmid using TransIT-LT1 transfection reagent (Mirus, WI, USA) according to the procedure of the manufacture. After incubation at 37°C for 48 hours, the cells were washed three times with PBS containing $CaCl_2$ and $MgCl_2$ [PBS (+)]. Following that, 500 μl of fetuin solution (25 mg/ml) was added and incubated for 1 hour at 37°C. The content was collected and centrifuged at 10,000 × g for 1 min, and 40 μl of the supernatant was used for measurement of the NA enzymatic activity. The following operation was obeyed by the described method in section of measurement of the enzymatic activity of viral NA, supplied with 20 μl of periodate reagent, 200 μl of arsenite reagent, 500 μl of thiobarbituric acid reagent and 1 ml of *n*-butanol-5% HCl.

To correct the level of cell-expressed NA proteins, enzyme-linked immunosorbent assay (ELISA) was performed. The 293 cells at 48 hours post-transfection as mentioned above were fixed with formalin at 4°C over night, then washed three times with PBS.

Following, PBS containing 1% bovine serum albumin were covered at room temperature for 3 hours. Subsequently, antigen-antibody complex reaction was carried out using anti-A/Singapore/1/57 (H2N2) prepared in chicken as a primary antibody and anti-chicken/turkey IgG labeled horseradish peroxidase rabbit serum (Zymed Laboratories) as a secondary antibody at room temperature for 1 hour per each reaction. After rinsed unreacted antiserum with PBS, the bound horseradish peroxidase activity was detected by 150 μl of ABTS solution (Roche Diagnostics, Basel, Switzerland) at room temperature for 5 min and then, the content was collected and centrifuged at 10,000 × g for 1 min. After that, 100 μl of the supernatant was dropped in 96-well plate and was measured the optimal density by model 3550 microplate reader (BIO-RAD, CA, USA) at wavelength of 450 nm. The determined NA enzymatic activity of cell-expressed proteins was then normalized to the relative level of each protein expression for respective experiments. We performed independently the measurement for three times. The enzymatic activity is shown in relation as compared to that of expressed NA proteins of Dk78/Eng62 as 100% and the average value is reported in Results.

Measurement the enzymatic activity of cell-expressed NA proteins after Low-pH treatment

The 293 cells were transfected by NA expression plasmids on 12-well plate as described above. After 48 hours post-transfection, the cells were washed twice with PBS (+) and covered with 500μl of PBS adjusted different pH (pH 6, 5, 4 and 3) by 0.1 M citric acid and incubated at 37°C for 5 min. After washing the cells with PBS (+) for three times, the NA enzymatic activity was determined as mentioned above. The residual enzymatic activity after respective low-pH treatments are shown in ratio to that of each NA protein at pH 6 treatment as 100%. The values are reported the means for triplicate experiments in Result.

Results

In vivo, selection of variant virus that enables to grow in duck intestine via orally inoculation

It have been previous reported that a reassortant virus, Dk78/Eng62N2, could not be recovered from duck intestine via orally inoculation [28]. Hence, as an initial step towards to recover a variant virus possessing the ability to replicate in lower intestine, five mallard

ducks were inoculated rectally with the concentrated Dk78/Eng62N2 prepared as described in materials and methods. The result showed that viruses were recovered from intestine of four of the five inoculated ducks at 3 d.p.i.. Additionally, there was not any difference in clinical signs among all inoculated ducks. Subsequently, to investigate whether four recovered viruses could establish the natural route of infection in duck or not, the viruses were orally inoculated into an individual new ducks. At 3 d.p.i., among four inoculated ducks, only one duck, which was inoculated with Dk-rec1, exhibited slight lethargy and a virus was recovered from its rectum. Then, the recovered virus was subjected to the plaque cloning on MDCK cells and a obtained cloned virus (Dk-rec6), which was confirmed the productive replication in duck intestine by orally experimental inoculation, was used for the following experiments.

The full-length nucleotide sequences of Dk78/Eng62N2, Dk-rec1 and Dk-rec6 were determined to compare the deduced amino acid residues (Table 5). Dk-rec1 had amino acid substitutions of valine to isoleucine at position 165 and asparagine to aspartic acid at position 356 in NA protein. In addition, Dk-rec6 had changing of glutamine to proline at position 431 in NA. Our findings indicated that some of these amino acid substitutions enabled Dk-rec6 to support viral growth in duck.

Residual NA activity of recovered viruses after low-pH treatment

One of the most notable differences in infective forms between human and avian viruses is that the former transmit via airborne route, whereas the later take via oral-fecal route. Therefore, avian viruses are supposed to pass through glandular stomach containing digestive fluid at low-pH before reaching the intestinal tract. Wherein, the residual NA enzymatic activity after low-pH treatment between Dk78/Eng62N2, Dk-rec1 and Dk-rec6 were compared. As shown in Table 5, NA activities of all viruses were gradually decreased until treatment at pH 5. However, the activity of Dk78/Eng62N2 and Dk-rec1 were sharply decreased at pH4 (46% and 40%, respectively as shown to relative ratio of the active of each virus at pH 7) and almost disappeared at pH 3 (4% and 6%, respectively). In contrast, Dk-rec6 conserved the activity was 72% at pH4 and 35% at pH3. These results implies that Dk-rec6 NA were higher resistant to low-pH than Dk78/Eng62N2 and Dk-rec1, which may lead to the difference in the viral replication in lower intestine of orally inoculated duck.

The mechanism of low-pH resistance of Dk-rec6 NA

Based on the results of highly residual NA activity of Dk-rec6 after low-pH treatment, it was hypothesized that two mechanisms might be given for the explanation. One possible explanation is that NA of Dk-rec6 is more stable than Dk78/Eng62N2 under low-pH conditions, although the original NA activities are equally under ordinary condition. On the contrary, the other is that NA stability under low-pH conditions are similar level (i.e., NA activities were equal reduction rate.), but Dk-rec6 has intrinsically higher NA activity than Dk78/Eng62N2 under normal condition. In either case, the NA of Dk-rec6 appears to possess higher resistance to low-pH conditions than that of Dk78/Eng62N2.

Therefore, in order to confirm the mechanism contributing the low-pH resistance of Dk-rec6 NA, plasmids which individually express NA protein of Dk78/Eng62N2 (corresponding to NA of A/England/12/62), Dk-rec1 and Dk-rec6 were constructed. Then, the enzymatic activity and the residual activity of the cell-expressed NAs after low-pH treatment were measured. When compared with the enzymatic activity of Dk78/Eng62N2 NA as 100%, the relative activity of Dk-rec1 NA and Dk-rec6 NA were 94% and 134%, respectively, which demonstrated that there was no significant difference in the enzymatic activity among these viruses. On the other hand, under low-pH conditions (Figure 3), the residual enzymatic activities of all NA proteins at pH 5 or 4 were ranged from 80% to 100%, indicated that there was not notable difference. However, after pH 3 treatment, the

enzymatic activity of Dk78/Eng62N2 NA and Dk-rec1 NA were sharply decreased to 9% and 13% respectively, whereas that of Dk-rec6 NA retained 59%. This considerable difference in this result suggested that Dk-rec6 NA were higher resistant to low-pH than Dk78/Eng62N2 NA and Dk-rec1 NA. Based on these results, we conclude that the mechanism of the low-pH resistance of Dk-rec6 NA was responsible for the difference in NA stability at low-pH conditions rather than in the original enzymatic activity under ordinary condition.

Amino acid residue associated with low-pH stability of NA

In order to ascertain amino acid residues contributing to low-pH stability of Dk-rec6 NA, the five kinds of plasmids, which were replaced various combinations of amino acid residue at 165, 356 and 431 in Dk78/Eng62N2 NA with identified residues in Dk-rec6 NA, were constructed (Table 6). The NA proteins derived from the constructed plasmids were expressed on 293 cells and were measured the residual enzymatic activity after low-pH treatments. The result was shown in Figure 3. All mutant NA maintained similar level of the NA activity ranging between almost 80% and 100% after pH5 or pH4 treatment compared with the individual proteins at pH6 treatment. However, at pH3 treatment, the

enzymatic activities of Eng62NA-V165I and Eng62NA-N356D (glutamine at position 431 in both proteins) caused an acute decrease to 7% and 18%, respectively. In contrast, mutant NAs: Eng62NA-Q431P, Eng62NA-V165I/Q431P and Eng62NA-N356D/Q431P whose residues at position 431 were proline remained relatively high NA activity of 54%, 44% and 62%, respectively, which were compatible with Dk-rec6 NA (59%). These results demonstrated that proline at position 431 obviously contributed to the low-pH stability of Dk-rec6 NA.

The relation of N2NA amino acid residue at position 431 with the low-pH stability in field isolated viruses

To obtain further information about the low-pH stability of N2NA, the residual NA activity of field isolated viruses after low-pH treatment were determined. As shown in Table 5, after pH3 treatment, both of duck isolates, A/duck/Hokkaido/5/77 (H3N2) and A/mallard/New York/6750/78 (H2N2), retained more than 40% of the activity compared with each virus at pH7 treatment. In contrast, with the exception of A/Singapore/1/57 (H2N2), two human viruses, A/Udorn/307/73 (H3N2) and A/Tottori/872/94 (H3N2), considerably lost the NA activity to less than 10%. A/Singapore/1/57 retained about 20% of

the NA activity at pH3 treatment, indicated that NA of this strain had slightly higher the low-pH resistance than other human viruses. The comparison of N2NA amino acid at position 165, 356 and 431 in these viruses revealed that all viruses whose NA displayed the low-pH resistance (A/duck/Hokkaido/5/77, A/mallard/New York/6750/78 and A/Singapore/1/57) were observed proline at position 431 as same as Dk-rec6 (Table 5). Interestingly, Kobasa et al. [27, 28] reported that all avian and early human N2NA proteins before 1960 conserved proline at position 431, whereas human N2NA after 1962 did not. These findings may also support that residue at position 431 contributes to the low-pH resistance of N2NA and the viral replication in duck intestine.

Discussion

Because influenza A viruses primarily replicate in the intestine of duck via oral-route (natural-route) infection and high concentration of the viruses are excreted in the feces, the virus isolated from the intestine of naturally infected duck are supposed to pass through the digestive tract including gizzard. Thereby, it has been considered that the maintenance of biological property of the virus is needed to undergo growth in duck after the exposure to low-pH environment [69]. Function of NA is an important role for

influenza virus to replicate efficiently [41, 47]. Previous reports [12, 60] suggested NAs of avian influenza viruses maintained relatively higher enzymatic activity at low-pH than that of mammalian virus. In this study, the author compared the residual NA activity after low-pH treatment between Dk78/Eng62N2 that could not replicate in duck and its variant (Dk-rec6) that was obtained by passage in duck. The result showed that NA of Dk-rec6 had remarkably higher resistance to low-pH than Dk78/Eng62N2 (Table 5). Taken together, these findings suggest that the low-pH resistance of NA protein is essential characteristics for influenza A virus to replicate in duck via natural infection. The author conceived that this study is the first evidence that high NA resistance to low-pH condition is one of the most important functions to determine the faculty of the replication in duck intestine by *in vivo* experiments.

Compared with Dk78/Eng62N2, there were three amino acid substitutions at position 165, 356 and 431 in NA protein of Dk-rec6. Among these amino acid residues, introduction of proline 431 into Dk-rec6 was corresponded to the same passage step as its NA acquired the low-pH resistance. Analysis of mutant NA proteins expressed on 293 cells revealed that proline 431 was critical residue to contribute to the low-pH stability of Dk-rec6 NA (Figure 3). In addition, field isolates whose NAs displayed low-pH resistant were also found proline at position 431 (Table 5). Taken together, these results indicate that

amino acid residue at position 431 play an important role in the low-pH stability of N2 NA. Kobasa et al. reported that residue 431 is located near the edge of the sialic acid-binding pocket of the enzymatic active site [27] and that N2NA of all avian isolates and early human isolates before 1960 harbored proline 431, while that of human isolates after 1962 did not [28]. These findings also suggested that residue 431 was responsible for an important property of NA that associated with host range. Other research group reported that 1968 human pandemic strains (H3N2 subtype) also possess sialidase activity under low-pH conditions and some of them do not have proline, but lysine at position 431 [57, 60]. Furthermore, they demonstrated that two amino acid residues, arginine at position 344 and phenylalanine at position 466 were responsible for the low-pH stable A/Hong Kong/1/68 NA [59]. It has been known that both of the amino acid residues were located near calcium ion binding site and in addition, residue at 466 was also located near the subunit interface [10, 59, 66]. Therefore, these findings indicated that multiple mechanisms responsible for the low-pH stability of NA enzymatic activity of influenza A virus might be present. By contrast, with regard to the residue at position 165 and 356, these positions are unidentified in that contributing to some functions of NA as described elsewhere [10, 66]. Moreover, there were not notable correlations between amino acid substitutions and host species or isolation year of field viruses (data not shown).

Kobasa et al. [28] showed that reassortant viruses containing N2NA genes derived from human strains in the genetic background of a duck isolate, A/duck/Hong Kong/278/78, were orally inoculated into ducks, resulting in well viral growth in intestine of all birds inoculated with Dk78/Sing57N2 (containing NA gene of A/Singapore/1/57), while inadequate growth in all birds inoculated with Dk78/Eng62N2 (containing NA gene of A/England/12/62). They proposed that the different results were caused by the distinguishable enzymatic activities; NA of A/Singapore/1/57 had higher level of activity as compared with that of A/England/12/62. In the present study, however, significant differences between NA activities of Dk78/Eng62N2 and Dk-rec6 were not observed, although Dk-rec6 displayed slightly increase activity by 1.3-fold compared with Dk78/Eng62N2. This result can explain that Dk-rec6 may be present in an intermediate stage of the adaptive change to ducks. It was also reported [28] that A/England/12/62 NA, which was substituted together with all amino acid residues at position 331, 339, 367, 370 and 431 with corresponding residue of A/Singapore/1/57, dramatically raised the enzymatic activity to the similar level as A/Singapore/1/57. Residue 331, 339, 367 and 370 (with the exception of residue 431) in Dk-rec6 NA identified with Dk78/Eng62N2 (A/England/12/62) NA, thereby this might be one of the account for unremarkable differences in the enzymatic activities between those viruses. In any way, the author

suggested that the high NA activity was an additive factor, but not critical factor, for influenza virus to grow in duck intestine.

On Asian flu of 1957 and Hong Kong flu of 1968, several genes from avian influenza gene pool in wild ducks were introduced into human virus, which have been considered conclusive event to break out the pandemic strains [19, 68]. However, the detail mechanism of adaptation of influenza A viruses to a new host species and emerging of new human pandemic strain remains undefined. The role of NA glycoprotein on the enterotropism in duck was focused in this study and the author demonstrated that low-pH resistance of NA contributes to the biological property of influenza virus and its molecular mechanism. The findings in the present study suggested that only a few molecular changes are possible to break the barrier of interspecies transmission. Thereby, further studying the precise molecular changes to cross the host species barriers is essential to develop an effective means of prediction of influenza virus epidemics.

Table 5. Comparison of amino acid residues at position 165, 356 and 431 in N2NA of influenza A virus and residual enzymatic activity of viral NAs after low-pH treatment

Virus strain	Amino acid at position			NA activity (%)[a]		
	165	356	431	pH5	pH4	pH3
Dk78/Eng62N2	Val	Asn	Gln	71	46	4
Dk-rec1	Ile	Asp	Gln	62	40	6
Dk-rec6	Ile	Asp	Pro	78	72	35
A/Singapore/1/57	Val	Asn	Pro	87	64	20
A/Udorn/307/72	Val	Asp	Gln	72	18	3
A/Tottori/872/94	Val	Asp	Lys	80	46	10
A/duck/Hokkaido/5/77	Val	Asn	Pro	88	88	48
A/mallard/NY/6750/78	Val	Asn	Pro	86	85	43

[a] The residual NA activity in various low-pH treatments were shown in the ration of the activity after each low-pH treatment versus after pH 7 treatment.

Table 6. The schematic diagram of amino acid residues in NA proteins expressed on 293 cells surface from constructed plasmids

Cell expressed NA protein	Amino acid position[a]		
	165	356	431
Dk78/Eng62N2	V	N	Q
Dk-rec1	I	D	
Dk-rec6	I	D	P
Eng62NA-V165I	I		
Eng62NA-N356D		D	
Eng62NA-Q431P			P
Eng62NA-V165I/Q431P	I		P
Eng62NA-N356D/Q431P		D	P

[a] Only the residues that differ from Dk78/Eng62N2 NA are shown.

Figure 3

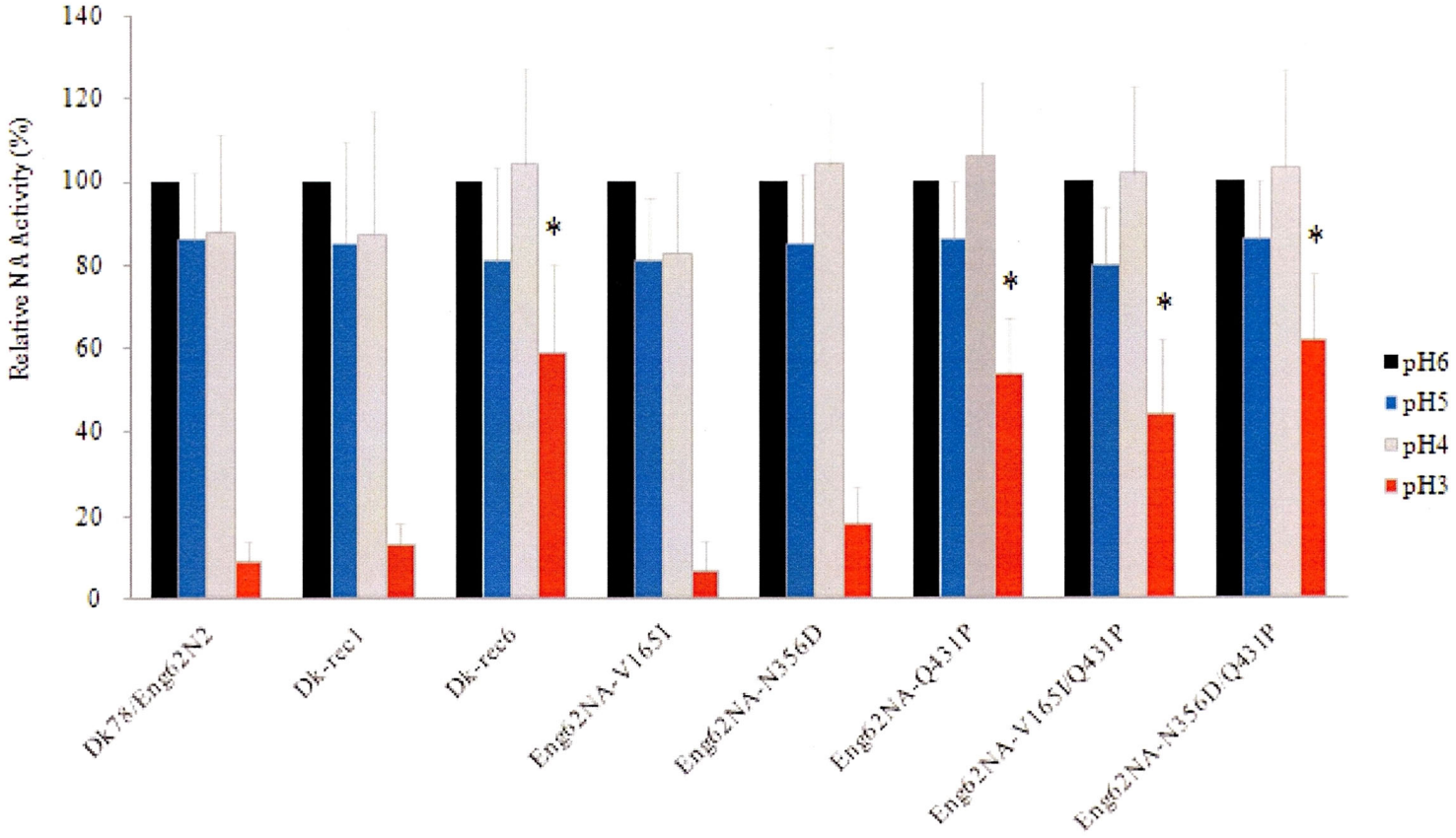
Relative NA Activity (%)
140
120
100
80
60
40
20
0
*
*
*
*
pH6
pH5
pH4
pH3
DK78/Eng62N2
Dk-rec1
Dk-rec6
Eng62NA-V165I
Eng62NA-N356D
Eng62NA-Q431P
Eng62NA-V165IQ431P
Eng62NA-N356D Q431P

Figure legend

Figure 3. Residual enzymatic activity of cell-expressed NAs under low-pH conditions. 293 cells were transfected with each constructed plasmid expression NA protein and after 48h post-transfection, the enzymatic NA activity after low-pH treatment were measured as described in Materials and Methods section. The relative NA activities after pH treatment at pH6 (black bars), pH5 (blue bars), pH4 (gray bars) and pH3 (red bars) were determined. The NA activity at each pH is expressed as a percentage relative to that of individual NAs at pH6. Error bars indicate the standard deviations of the mean of three independent experiments. An asterisk (*) indicates significant differences ($P<0.01$) compared with the Dk78/Eng62N2 at each pH by ANOVA.

GENERAL CONCLUSION

In this thesis, the author described the epidemiological and ecological researches on avian influenza virus in wild birds, including the biological and genetic studies for the host range restriction of the virus. In the first study, migratory waterfowl and shorebirds of several species staying in San-in district, western Japan were surveyed for avian myxoviruses between 2001 and 2008. A total of 41 avian influenza viruses of 12 different HA and NA combinations including two H5N3 strains and 13 of avian paramyxoviruses was isolated from 4,335 fecal samples of wild aquatic birds. During 2001-2008, three outbreaks caused by avian influenza viruses of H5 subtypes were occurred in 2004, 2005 and 2007, and H5N1 HPAI viruses were isolated from wild birds in 2008 in Japan. Phylogenetic analysis of HA genes revealed that both of the H5N3 isolates in the present study clustered in a different branch with all the recent Japanese isolates of H5 subtype. However, these results suggested that possible precursor viruses for HPAI viruses are still brought into Japan by numerous species of migratory waterfowls. The results also support the contention that continued surveillance of wild waterfowl population should be an integral part of control policies for these serious poultry diseases.

To obtain information on the susceptibility of terrestrial wild birds to H5N1 HPAI viruses and assess the possibility that they may act as carriers for the virus, the author

performed experimental infections of two species of terrestrial wild birds (great reed warbler and pale thrush) with a recent H5N1 HPAI virus, A/mountain hawk-eagle/Kumamoto/1/07. The results showed that both species were highly susceptible to the virus. The great reed warbler showed fatal infection with 100% mortality, but the pale thrush survived for longer periods with viral shedding. These findings suggest that there is variation in clinical appearance after infection among the wild terrestrial birds, and that some bird species may become carriers of H5N1 viruses.

Finally, in order to determine the role of NA in host range restriction of influenza virus, the author studied a reassortant virus that replicated with poor efficiency in ducks. The reassortant virus (Dk78/Eng62N2), in which NA of an avian virus, A/duck/Hong Kong/278/78 (H2N9), was replaced with that from a human virus, A/England/12/62 (H2N2), was rectally inoculated into ducks. The viruses recovered were then orally inoculated into new ducks and a variant (Dk-rec6) that replicated efficiently in the intestinal tract was isolated. Dk-rec6 was supposed to pass through the digestive tract (acidic environment) before attachment to the intestine, therefore the residual NA activity of the viruses after low-pH treatment was examined. The results demonstrated that the NA activity of Dk78/Eng62N2 almost disappeared after low pH treatment, while that of Dk-rec6 was conserved under the same conditions. Sequence analysis showed that Dk-rec6 NA

contained three amino acid substitutions at positions 165, 356 and 431. Among these residues, the amino acid at position 431 was found to determine the low-pH stability of NA by the experiments using cell-expressed NAs which were introduced various combinations of those amino acid residues into Dk78/Eng62N2 NA. These findings indicate that amino acid residue at position 431 on the NA molecule contribute to low-pH stability on viral NA protein and this characteristic play an important factor for influenza A virus to determine the ability of intestinal replication in duck.

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学位論文要旨

カモなどの野生水禽を自然宿主とする A 型インフルエンザウイルスは、鳥類から哺乳動物にまで感染する幅広い宿主域をもつ病原体である。2005 年以降、世界的大流行を引き起こし、今尚、各国の養鶏産業界に甚大な被害を与え続けている H5N1 亜型の高病原性鳥インフルエンザウイルスは鶏、七面鳥およびアヒルなどの家禽以外にも多くの鳥類が感受性を有することから、特に野生鳥類が本ウイルスの伝播、流行拡大に重要な役割を果たしている可能性が示唆されている。一方、ヒトのアジア風邪ウイルスや香港風邪ウイルスはそれまで人に流行していたウイルスと野鳥の集団内で長期間保存されていたウイルス間での遺伝子再集合により出現したパンデミックウイルスであると考えられている。このように野鳥は地球上に存在する全てのインフルエンザウイルスの流行において重要な役割を担っていると考えられる。そこで本研究では、１）野鳥における鳥インフルエンザウイルスの保有状況を明らかにすること２）野鳥による鳥インフルエンザ流行拡大（ウイルス伝播）の可能性を評価すること、また３）水禽類における鳥インフルエンザウイルスの腸管増殖（組織向性）のメカニズムを明らかにすることを目的として、以下の調査・実験を行った。

第 1 章では、2001 年から 2008 年度の冬季に山陰地方に飛来した野生水禽における鳥類ミクソウイルスの疫学調査を実施した。合計 4,335 個の水禽由来糞便試料か

ら 41 株の鳥インフルエンザウイルスおよび 13 株の鳥パラミクソウイルスが分離された。分離された鳥インフルエンザウイルスは、12 種類の HA および NA 血清亜型の組み合わせに分類され、その中には 2 株の H5N3 亜型のウイルスが含まれていた。H5 亜型分離株の HA 遺伝子における系統学的解析の結果、2004、2005 及び 2007 年に国内で発生した H5 亜型鳥インフルエンザの原因ウイルスや 2008 年に死亡したオオハクチョウから分離された H5N1 亜型高病原性鳥インフルエンザウイルスとは異なる系統に属していることが明らかとなった。しかし、高病原性鳥インフルエンザウイルスに変化する可能性を有する前駆ウイルスが野生水禽により国内に持ち込まれている可能性は否定できない。従って、今後も野生水禽を対象にした調査を実施し、家禽に重要な感染症の制圧対策として渡り鳥の監視を続けることが重要であると考えられた。

第 2 章では、陸生野鳥の高病原性鳥インフルエンザウイルスに対する感受性を明らかにし、それら野鳥が本ウイルスの保有動物になる可能性を考察するため、感染実験を実施した。H5N1 亜型高病原性鳥インフルエンザウイルス、A/mountain hawk-eagle/Kumamoto/1/07 を 2 種類の陸生野鳥であるオオヨシキリおよびシロハラに経鼻接種した結果、接種後 3 日以内に全てのオオヨシキリが死亡した一方、シロハラでは実験期間（8 日間）を通して 8 羽中 3 羽のみ死亡が確認された。また、臓器ウイルス力価ではオオヨシキリおよびシロハラともに高いウイルス力価が測

定された。以上の成績からシロハラはオオヨシキリと比較して本ウイルス感染後に比較的長期間生存することが示唆されたことから、陸生野鳥の中には本ウイルスの伝播動物となる可能性を有する野鳥が存在すると考えられた。

第 3 章では、インフルエンザウイルスのカモの腸管増殖能に関わる NA 蛋白の役割を明らかにする目的で、A/duck/Hong Kong/278/78 (H2N9)の NA 蛋白をヒト由来ウイルス，A/England/12/62 (H2N2)で置換したリアソータントウイルス Dk78/Eng62N2 を用いて実験を行った。Dk78/Eng62N2 は、経口接種したアヒルの腸管で増殖しない。まず始めに、Dk78/Eng62N2 をアヒルの結直腸に接種し、回収したウイルスをさらに経口接種することにより、腸管増殖能獲得変異株(Dk-rec6)を得ることに成功した。Dk-rec6 は増殖場所であるアヒルの腸管に到達する前に、酸性環境である消化管を通過する必要があることから、両ウイルスの low-pH 処理後における NA 活性の比較を行った。その結果、pH3 処理後においてDk78/Eng62N2 は NA 活性が消失するのに対し、Dk-rec6 では比較的高い NA 活性が残存した。推定アミノ酸配列を比較した結果、Dk-rec6 の NA 蛋白には 165 位、356 位および 431 位の 3 か所にアミノ酸置換が認められた。そこで、これらの変異を種々の組合せで持つ細胞発現 NA 蛋白を用いて low-pH 処理後における NA 活性を比較した結果、431 位のアミノ酸にプロリンを有する発現蛋白において low-pH 抵抗性が認められた。以上の成績から、431 位のアミノ酸が NA 蛋白に low-pH 抵抗

性を付与し、この性質がインフルエンザウイルスのカモにおける腸管増殖能を決定する重要な因子であると考えられた。

以上のように本研究において、鳥インフルエンザウイルスの野鳥における生態学的特性並びに本ウイルスの野生水禽類に対する組織向性の分子基盤が明らかとなった。今後も出現が予想される鳥類由来インフルエンザウイルスの防遏対策確立のためには、本研究で得られた情報を基礎とし、さらなる組織的且つ広範な疫学調査を継続実施することが重要であると考えられる。