HOKKAIDO UNIVERSITY

Title	Studies on the Diagnosis and Molecular Epidemiology of Avian Influenza
Author(s)	Manzoor, Rashid
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第8826号
Issue Date	2008-09-25
DOI	https://doi.org/10.14943/doctoral.k8826
Doc URL	https://hdl.handle.net/2115/34933
Type	doctoral thesis
File Information	Manzoor.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Studies on the Diagnosis and Molecular Epidemiology of Avian Influenza

（鳥インフルエンザの診断ならびに分子疫学に関する研究）

Rashid Manzoor

Contents

Preface ---------- 1

Chapter I

Development of a Pen-site Test Kit for the Rapid Diagnosis of H7 Highly Pathogenic Avian Influenza

Introduction ---------- 4

Materials and Methods ---------- 8

Results

Selection of monoclonal antibodies for the kit ---------- 13

Specificity and sensitivity of the kit ----------13

Detection of H7 HA antigen from swabs and tissues of experimentally infected chickens ---------- 15

Discussion ---------- 18

Brief summary ---------- 21

Chapter II

Phylogenic Analysis of the M Genes of Influenza Viruses Isolated from Free-Flying Water Birds from their Northern Territory to Hokkaido, Japan

Introduction ---------- 22

Materials and Methods ----------25

Results

Isolation of Influenza A viruses from fecal samples of free-flying water birds ---------- 28

Phylogenic analysis of the M genes of influenza virus isolates ----------28

Discussion ---------- 41

Brief summary ---------- 44

Chapter III

PB2 gene is responsible for restriction in replication of influenza A virus strain A/chicken/Yamaguchi/7/2004 (H5N1) in pigs

Introduction ---------- 45

Materials and Methods ---------- 48

Results

Selection of *in vivo*-generated reassortant viruses capable of replication in pigs ---------- 52

Virus clones recovered by *in vivo* selection in pigs are capable of replication in pigs ---------- 52

Potential of replication of viruses produced by reverse genetics is similar to that of parent viruses ---------- 55

Amino acid substitutions found in the PB2 protein of rg-H1N1-PB2H5N1 after a single passage in pigs ---------- 55

Discussion ---------- 59

Brief summary ---------- 63

Conclusion ---------- 65

Acknowledgement ---------- 67

References ---------- 69

和文要旨 ---------- 86

Preface

Avian influenza is caused by type A influenza viruses belonging to *Orthomyxoviridae* family. The virions are spherical to filamentous in shape and surface is covered with two types of spikes, the hemagglutinin (HA) and neuraminidase (NA). These spikes are projecting from the lipid envelope enclosing the genome of the virus. The genome comprises of eight segments of single-stranded, negative sense RNA in the form of ribonucleoprotein complex. Each gene segment carries its own polymerase complex consisting of polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA) which along with nucleoprotein (NP), matrix protein 1 (M1) and non-structural protein 2 (NS2) is involved in virus replication [66]. The surface glycoproteins, HA and NA, are divided antigenically into H1-H16 and N1-N9 subtypes, respectively [27, 117]. The type A influenza viruses are serologically classified on the basis of the combination of HA and NA subtypes. On the basis of their pathogenicity for chicken, they are divided into apathogenic avian influenza viruses (APAIV), low pathogenic avian influenza viruses (LPAIV) or highly pathogenic avian influenza viruses (HPAIV) [2, 59, 62].

Free-flying water birds are the natural reservoir of influenza viruses. All of the HA (H1-H16) and NA (N1-N9) subtypes of influenza A viruses in various combinations have been isolated from this natural reservoir [2, 27]. APAIV or LPAIV belong to all of the H1-H16 HA subtypes while HPAIV have been restricted to H5 and H7 HA subtypes, although not all viruses of these subtypes are highly pathogenic for chickens. Influenza viruses of H5 and H7 subtypes exist as APAIV in the free-flying water birds. The available evidence indicates that these APAIV strains are first transmitted to domestic water fowls, quails or turkeys, and then in live bird markets to chickens. During multiple cycles of infection in

chicken population, they may acquire pathogenicity for this species to become HPAIV [2, 107]. The HPAIV of H5 and H7 subtypes have inflicted colossal economic losses to the poultry industry worldwide. There are reports of occasional bird-to-human and rare human-to-human transmissions of these HPAIV. Since 2003, 385 laboratory-confirmed human cases of H5 highly pathogenic avian influenza (HPAI) virus infection have been reported from 15 countries of the world [119]. In addition, H7 influenza viruses are responsible for many laboratory confirmed human cases since 2002. These include >80 cases of human infection with one fatality in 2003 in Netherlands caused by HPAI H7N7 virus, two cases of conjunctivitis in Canada in 2004 caused by HPAI H7N3 virus and multiple cases of conjunctivitis and influenza like-illness in United kingdom in 2007 caused by LPAI H7N2 virus [1, 28, 40, 63, 113]. These LPAI and HPAI viruses were transmitted to humans from poultry; therefore it is of utmost importance to control influenza virus infections in poultry if human infections are to be prevented or controlled. For efficient control, early diagnosis is a pre-requisite. Although virus isolation is the golden standard for the diagnosis of avian influenza, its large scale applicability is hampered by the requirement of bio-safety level-3 facility. To adjunct this method many rapid diagnostic methods have been developed which either detect viral nucleic acids or antigens. A rapid diagnosis test kit based on immunochromatography has been descried earlier for the detection of H5 HA antigen [112]. In chapter 1, we describe development of a rapid diagnosis kit for the detection of H7 HA antigen.

In 2005, a large outbreak of H5N1 infection occurred in wild bird population at Qinghai Lake in China, a major congregating site for free-flying water birds. Later, viruses like those isolated at Lake Qinghai were isolated in many countries of Asia, Europe and Africa suggesting probable role of free-flying water birds in the spread of HP H5N1 viruses globally [3, 116]. If the H5 HPAI viruses would start perpetuating in the wild birds, there

would be a catastrophe world wide. Therefore there is a dire need for continuing surveillance of avian influenza in the natural hosts. The genetic and antigenic analysis has already proved the role of free-flying water birds in the generation of human pandemic virus A/Hong Kong/1968 (H3N2) [59, 60, 122]; therefore surveillance and genetic analysis of influenza viruses will enable us to better prepare for future pandemics. The matrix protein (M) encoding gene of avian influenza viruses is highly conserved [67]. Its genetic analysis would help to determine the relationship between influenza viruses isolated form natural hosts and those from outbreaks. In chapter II, the findings of phylogenetic analysis of M gene of influenza viruses isolated form the free-flying water birds arriving in Hokkaido have been described.

Pigs act as "mixing vessels" in the generation of influenza viruses of new genotypes [31, 93]. Genetic analysis indicated their role as intermediate host in the generation of pandemic influenza virus strain in 1968 [60]. Their role as mixing vessels is further supported by the presence of avian type (α2-3 Gal) and human type (α2-6 Gal) receptors for influenza viruses [48]; therefore, they should support the growth of human and avian influenza viruses. Kida *et al.* [58] showed that some influenza viruses of duck origin did not replicate in pigs. Recently a highly pathogenic H5N1 influenza virus, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04 (H5N1)), was also shown not to replicate in pigs [47]. Influenza viruses have been found circulating in the pigs in China and Europe [16, 34]. Kida *et al.* [58] showed that swine isolate contributed NP, NA, M or NS genes to the non-replicable duck isolate and enabled it to replicate in pigs. It is possible that viruses circulating in pig population can contribute genes to viruses like Ck/Yamaguchi/2004 (H5N1) and enable them to cross host-species barrier. Therefore in chapter III, genetic basis of host-range restriction of Ck/Yamaguchi/04 (H5N1) was determined.

Chapter 1

Development of a Pen-site Test Kit for the Rapid Diagnosis of H7 Highly Pathogenic Avian Influenza

Introduction

Avian influenza viruses belong to genus Influenza A virus of family *Orthomyxoviridae*. Influenza A viruses are divided into 16 HA and 9 neuraminidase (NA) subtypes on the basis of their antigenic specificity [27]. In addition, they are grouped on the basis of their pathogenicity for chicken into HPAIV, in which mortality may be as high as 100%, and low pathogenic avian influenza viruses (LPAIV) causing much milder respiratory disease [2]. Since they are antigenically and genetically stasis in their natural hosts, feral water birds that do not show clinical signs, they can be designated as apathogenic avian influenza viruses (APAIV) [59, 62]. HPAIV have been restricted to H5 and H7 HA subtypes, although not all viruses of these subtypes are highly pathogenic for chickens. Feral water birds are endemically infected with the APAIV strains of H5 and H7 HA subtypes. The available evidence indicates that these APAIV strains are first transmitted to domestic water fowls, quails or turkeys, and then in live bird markets to chickens. During multiple infections in chicken population, they may acquire pathogenicity for this species to become HPAIV. Consequently outbreaks of HPAI in poultry are difficult to prevent [2, 107]. Besides causing havoc in poultry, infrequent

chicken-to-human and human-to-human transmissions of H7 HPAIV have been reported [23, 28]. To-date, outbreaks caused by H7 subtype influenza viruses have been reported through out the globe (Fig. 1).

Rapid diagnosis of HPAI on the outbreak is critical for its control. WHO, OIE and FAO have also emphasized the need for early detection, reporting and introduction of measures to control this menace. Although virus isolation is the standard test for diagnosis of avian influenza [83], it requires time and bio-safety level 3 (BSL-3) facilities [105]. To supplement this method other rapid diagnostic tests have been developed which detect either the viral nucleic acids or viral antigens. Nucleic acid based molecular diagnostic techniques share the principle of amplifying the nucleic acid to detection levels. Most common techniques are reverse transcriptase polymerase chain reaction (RT-PCR) [70], RT-PCR with enzyme linked immunosorbant assay (ELISA) [25], real-time reverse transcriptase polymerase chain reaction (RRT-PCR) [104], nucleic acid sequence-based amplification (NASBA) [18, 19] and loop mediated isothermal amplification (LAMP) [45, 46]. Some of the common techniques used for the detection of viral antigens are indirect fluorescence antibody test (IFA) and ELISA [37]. Although these techniques provide results in shorter time than the classic method of virus isolation, they require extensive laboratory set up, special equipment and skilled personnel. To overcome these defects, an antigen capture method based on immunochromatography was used in the present study. The main advantage of using this test is that no extensive laboratory set up, special equipment or skilled personnel are required and the result can be obtained within 15-30 minutes [6, 7, 13, 15, 112].

Most of the commercially available rapid diagnosis kits detect the nucleoprotein (NP) antigen, whose antigenicity is common among type A influenza viruses [6, 7, 13]. Therefore, availability of a rapid diagnosis kit for the HA antigen subtype determination particularly of HPAIV will be a useful adjunct to the rapid diagnosis of HPAI and consequent implementation of rapid control measures. Rapid diagnosis kit for the HPAI caused by H5 avian influenza viruses has been described [112]. Here we report an immunochromatography test kit for the rapid diagnosis of H7 avian influenza virus infection.

Materials and Methods

Viruses and bacteria

In this study 33 strains of influenza A viruses were used (Table 1). These viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for 48 hours at 35^{o}C. The infectious allantoic fluid was harvested and was used as virus strain stock. Other viruses and bacteria used included avian paramyxovirus (AMPV) strains APMV-1/ruddy-shelduck/Mongolia/19/2006, APMV-4/duck/Hokkaido/W337/2006, APMV-7/pintail/Hokkaido/U6/2006 from our repository and infectious bronchitis virus strain B42, infectious laryngotracheitis virus strain NS175, fowl pox virus strain Sishui, avian adenovirus-7, *Staphylococcus aureus* strain Hyogo, *Escherichia coli* and *Mycoplasma gallisepticum* strain C5PT purchased from the Japanese Association of Veterinary Biologics (Tokyo, Japan).

Development of the kit for rapid detection of H7 HA antigen

Hybridoma cell lines producing monoclonal antibodies (MAbs) against the H7 HA antigen of A/duck/Hokkaido/Vac-2/2004 (H7N7) were established and examined for reactivity with 21 different strains of influenza viruses of H7 HA by immunofluorescent antibody test [91]. The immunochromatography kit was manufactured under the contract with BL Corporation Ltd. (Shizuoka, Japan). Details in the preparation of the kit are proprietary. In brief, the kit consists of a plastic support on which a nitrocellulose membrane is mounted. Anti-mouse immunoglobulin antibodies and anti-H7 HA MAbs were immobilized onto nitrocellulose membrane as capture antibodies in the control and test judgment regions, respectively. A reagent pad containing colloidal-gold-labeled anti

H7 HA MAbs was mounted on the nitrocellulose membrane between test judgment region and sample port (sample-dropping region) (Fig. 2).

Test procedure

The efficacy of the present kit was compared with that of an NP antigen detection kit, Capilia® Flu A+B (BL Co., Ltd., Japan). The samples were prepared in the extraction buffer (BL Co., Ltd., Japan). The immunochromatography test was performed by adding 100 μl of test solution in the sample port of each kit. Results were read visually after 15 minutes of incubation at room temperature. A single colored line appearing in the control judgment region indicates the absence of H7 HA antigen. The concurrent presence of colored lines in both control and test judgment areas indicate the presence of H7 HA antigen in the samples.

Specificity and sensitivity of the kit

Specificity of the kit was determined by using reference influenza virus strains of 16 HA subtypes (H1-H16), 14 additional strains of H7 influenza viruses, 3 strains of highly pathogenic avian H5N1 influenza viruses and other avian bacterial and viral pathogens mentioned earlier.

Sensitivity of the kit was assessed and compared with that of Capilia® Flu A+B kit using A/chicken/Netherlands/2586/2003 (H7N7), A/duck/Hokkaido/Vac-2/2004 (H7N7), A/seal/Massacheussettes/1/1981 (H7N7) and A/chicken/Pakistan/447/1995 (H7N3) influenza viruses. Serial four-fold dilutions of each virus were tested. In parallel, these viruses were inoculated into embryonated chicken eggs to determine the

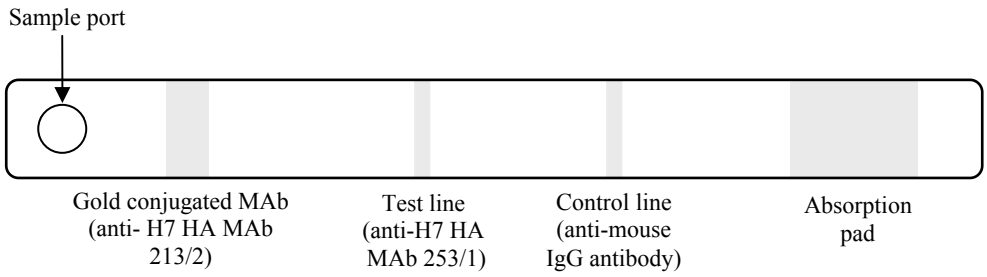

Fig. 2 Schematic diagram of H7 HA antigen detection kit showing different components. Anti-mouse immunoglobulin antibodies and anti-H7 HA MAb 253/1 are striped on the nitrocellulose membrane in the control and test judgment regions as capture antibodies. A reagent pad containing anti-H7 HA MAb 213/2, conjugated to colloid gold particles is placed between sample port and test judgment regions. An adsorption pad is placed next to the control region which absorbs the extra sample after it has migrated through the membrane. When test sample containing antigen is dropped in the sample port, it migrates on the nitrocellulose membrane. The H7 HA antigen in the sample binds to the colloidal-gold-labeled MAbs and this complex is arrested by the immobilized MAbs in the test judgment region producing a colored line. Some of the MAbs are arrested by the anti-mouse Immunoglobulin G (IgG) thus producing a colored line in the control judgment region.

50% egg infectious dose (EID_{50}). The sensitivity was the lowest virus titer detectable by the kit and was expressed as $\log_{10}$ EID_{50}/test.

Experimental infection of chickens with H7 HPAIV

Four-week-old chickens (Boris-brown) were purchased from Hokuren Central Breeding Farm (Hokkaido, Japan). The chickens were infected intranasally with 100µl of infectious allantoic fluid containing 100 CLD_{50} of A/chicken/Netherlands/2586/2003 (H7N7). The chickens, in this study, were housed in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Japan) in a BSL-3 facility at Graduate School of Veterinary Medicine, Hokkaido University, Japan. This study was conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Tracheal and cloacal swabs were collected daily for 6 days post-inoculation (p.i.) and were either placed in the extraction buffer or virus transport medium (Minimum essential medium containing 10,000U/ml Penicillin G, 10mg/ml Streptomycin, 0.3 mg/ml Gentamicin, 250 U/ml Nystatin and 0.5% bovine serum albumin) for titration of infectivity. The swab samples collected in the extraction buffer were then treated with an enzyme pad (BL Co., Ltd., Japan) for 15 minutes at room temperature. Then immunochromatography test was performed using the present kit and Capilia® Flu A+B kit as explained earlier. In addition to swab samples, trachea, kidney and colon were also collected daily for 6 days p.i. from dead or sacrificed chickens. Ten percent tissue homogenates were prepared in transport medium as test samples for titration of infectivity. The test samples were diluted 5-fold with extraction buffer, treated with enzyme pad as for swabs, and applied to the

sample port of the kit. The results were recorded after 15 minutes of incubation at room temperature.

The infectivity titers of the virus in the swabs and supernatants of the tissue homogenates were calculated by fifty percent end point method [88] and expressed as EID_{50}/ml of swab or EID_{50}/ g of tissue.

Results

Selection of monoclonal antibodies for the kit

To develop an immunochromatography kit for rapid diagnosis of avian influenza caused by influenza viruses of H7 subtypes, MAbs were produced against the H7 HA of A/duck/Hokkaido/Vac-2/2004 (H7N7) [91]. Two MAbs, 213/2 and 253/1 (Fig. 2) were selected on the basis of sensitivity in immunochromatography assay. These MAbs were tested as both conjugate and capture antibodies. In the present kit, MAb 213/2 is conjugated to gold-colloid particles and MAb 253/1 is immobilized to the nitrocellulose membrane, as the capture antibody, by overnight drying at room temperature.

Specificity and sensitivity of the kit

The specificity of the kit was evaluated by using reference influenza virus strains of H1-H16 HA subtypes and other avian viruses and bacteria. The kit was also evaluated using 14 strains of H7 subtype influenza viruses. The kit detected each of the H7 influenza viruses and did not show any non-specific reaction with the influenza viruses of other HA subtypes (Table 1), other avian viruses and bacteria (data not shown).

The sensitivity of the kit was, then, evaluated using four H7 influenza virus strains. Four-fold serial dilutions of infectious allantoic fluid were prepared and applied to the sample port of the present kit and Capilia® Flu A+B kit. The detection limit of the present kit ranged $10^{3.7}$-$10^{5.3}$ EID_{50} / test while detection limit for Capilia® Flu A+B kit ranged $10^{3.1}$-$10^{4.4}$ EID_{50} / test (Table 2).

Table 1. Specificity of the kit for the detection of the H7 HA antigen

Viruses	Subtypes	Results
A/duck/Tottori/723/1980	H1N1	–
A/duck/Hokkaido/17/2001	H2N3	–
A/duck/Mongolia/4/2003	H3N8	–
A/duck/Czechoslovakia/1956	H4N6	–
A/duck/Pennsylvania/10218/1984	H5N2	–
A/chicken/Yamaguchi/7/2004	H5N1	–
A/whooper swan/Mongolia/2/2006	H5N1	–
A/duck/Yokohama/aq-10/2003	H5N1	–
A/turkey/Massachusetts/3740/1965	H6N2	–
A/seal/Massachusetts/1/1980	H7N7	+
A/chicken/Netherlands/2586/2003	H7N7	+
A/duck/Hokkaido/Vac-2/2004	H7N7	+
A/turkey/England/1963	H7N3	+
A/turkey/Italy/4580/1999	H7N1	+
A/chicken/Pakistan/447/1995	H7N3	+
A/duck/Mongolia/47/2001	H7N1	+
A/duck/Hokkaido/143/2003	H7N7	+
A/gull/Shimane/91/1988	H7N8	+
A/duck/Taiwan/4201/1999	H7N7	+
A/swan/Shimane/42/1999	H7N8	+
A/duck/Hong Kong/47/1976	H7N2	+
A/turkey/Tennessee/1/1979	H7N3	+
A/duck/Hokkaido/W128/2004	H7N7	+
A/duck/Hokkaido/W182/2004	H7N7	+
A/turkey/Ontario/6118/1968	H8N4	–
A/turkey/Wisconsin/1/1966	H9N2	–
A/chicken/Germany/N/1949	H10N7	–
A/duck/England/1/1956	H11N6	–
A/duck/Alberta/60/1976	H12N5	–
A/gull/Maryland/704/1977	H13N6	–
A/mallard/Astrakhan/263/1982	H14N5	–
A/duck/Australia/341/1983	H15N8	–
A/black-headed gull/Sweden/5/1999	H16N3	–

Detection of H7 HA antigen from swabs and tissues of experimentally infected chickens

The applicability of the present kit for the diagnosis of H7 influenza virus infection was evaluated for the chickens infected with A/chicken/Netherlands/2586/2003 (H7N7). The infected chickens started showing respiratory signs on day 4 p.i. Later, some chickens developed paralysis and two died. The viruses were first recovered from the respiratory and intestinal tracts on day 1 p.i. and later from the kidneys on day 2 p.i. (Table 3). During first 3 days p.i., overt clinical signs were not observed and the virus titers were low in the swab and organ samples. Therefore, during this phase of infection, H7 HA antigen was not detected by the kit. In comparison, Capilia® Flu A+B detected NP antigen in some samples (Table 3). From day 4-6 p.i., chickens showed clinical signs and the present kit detected H7 HA antigen from both swab samples and tissue homogenates (Table 3). It is apparent from the results that H7 rapid diagnosis kit detected antigen from the clinically sick chickens thus demonstrating its usefulness for pen-site diagnosis.

Discussion

Most of the commercially available diagnosis kits are to diagnose influenza A or B virus infection by detecting NP antigen in the samples, hence do not identify the HA subtype of the influenza A viruses [6, 7, 13, 15]. In the present study, a rapid diagnosis kit based on immunochromatography was developed and evaluated for the diagnosis of H7 influenza virus infection. Epitope mapping of the MAbs, used in the present kit, was done by analysing escape mutants selected in the presence of MAbs. Escape mutants produced in the presence of MAb 213/2 had amino acid substitutions at S145N and S152Y while those produced in the presence of MAb 253/1 had amino acid substitution at A169D (Amino acid numbering started from start codon). The binding sites for these MAbs are located in the globular head of the HA molecule. These MAbs bound each H7 HA antigen of the tested influenza virus strains currently circulating in Eurasia and North America [91]. The kit detected specifically the H7 HA antigen and did not react with any of the tested influenza viruses of the other HA subtypes including recently isolated H5N1 subtype HPAIV (Table 1) neither with other viruses nor bacteria. Since HPAIV are restricted to H5 and H7 HA of influenza A viruses, the kit developed for the detection of H5 HA antigen [112] and the present kit for the detection of H7 HA antigen must be useful for the control of HPAI.

The sensitivity of the present kit was determined and compared with that of Capilia® Flu A+B kit using both HPAIV and LPAIV (Table 2). The detection limit of the present kit ranged from $10^{3.7}$ to $10^{5.3}$ EID_{50}/test while detection limit for Capilia® Flu A+B kit ranged from $10^{3.1}$ to $10^{4.4}$ EID_{50}/test. Tsuda *et al.* [112] reported the detection limit of the H5 rapid diagnosis kit ranging from $10^{3.5}$ to $10^{5.5}$/test. Similarly Chua *et al.* [15]

reported the detection limits of $10^{3.8}$/0.1 ml and $10^{4.8}$/0.1 ml for NP antigen detecting immunochromatography kits. The detection limit of the present kit is, thus, comparable to the commercially available kits.

The field application for the present kit was evaluated by determining the capability of the present kit to detect the H7 HA antigen in the samples collected from the chickens inoculated intranasally with A/chicken/Netherlands/2586/2003 (H7N7). As shown in Table 3, virus titers were higher in the tissue homogenates ($10^{1.5}$-$10^{7.8}$ EID_{50}/g) than in the swab samples ($10^{1.5}$-$10^{5.8}$ EID_{50}/ml) and virus titers were higher in tracheal swabs ($10^{1.5}$-$10^{5.8}$ EID_{50}/ml) than the cloacal swabs ($10^{1.5}$-$10^{3.5}$ EID_{50}/ml). Bai *et al.* [7] and Tsuda *et al.* [112] also reported that HPAIV cause systemic infection and organs like kidney and colon have higher virus titers than swab samples. On day 5 p.i., H7 HA antigen was detected by the present kit, but virus was not isolated from this sample. Furthermore, NP antigen was also not detected by the Capilia® Flu A+B kit. These results suggest that it was a false positive reaction. This false positive result could be due to the presence of sticky materials like mucus or other fecal materials in the cloacal swab samples. Briefly, the present kit detected H7 HA antigen in both of swab samples and tissue homogenates of at least $10^{4.5}$ EID_{50}/ml or g infectivity titer.

The sensitivity of the present kit is lower than that of Capilia® Flu A+B kit, however, samples positive by the Capilia® Flu A+B can be immediately sub-typed using present kit at pen-site. It is assumed that even the detection of a small number of positive influenza samples should be enough for the diagnosis of H7 influenza virus infection in birds as a flock. Other rapid subtype determination techniques like RT-PCR [70] may be available. Such facilities are, however, not always available particularly in remote areas

and other developing countries. Therefore, under such circumstances, the present kit would be a proper adjunct for pen-site diagnosis.

The present results indicate that the kit developed should be a useful tool for the diagnosis and control of HPAI caused by H7 subtype influenza viruses.

Brief summary

As well as H5 HPAIV, H7 HPAIV strains have caused serious damages in poultry industries worldwide. Cases of bird-to-human transmission of H7 HPAIV have also been reported [28]. On the outbreak of avian influenza, rapid diagnosis is critical not only for the control of HPAI but also for human health. In the present study, a rapid diagnosis kit based on immunochromatography for the detection of H7 hemagglutinin (HA) antigen of influenza A virus was developed using 2 monoclonal antibodies that recognize different epitopes on the H7 HAs. The kit detected each of the tested 15 H7 influenza virus strains and did not react with influenza A viruses of the other subtypes than H7 or other avian viral and bacterial pathogens. The kit detected H7 HA antigen in the swabs and tissue homogenates of the chickens experimentally infected with HPAIV strain A/chicken/Netherlands/2586/2003 (H7N7). The results indicate that the present kit is specific and sensitive enough for the diagnosis of HPAI caused by H7 viruses, thus, recommended for the field application as a pen-site test kit.

Chapter II

Phylogenic Analysis of the M Genes of Influenza Viruses Isolated from Free-Flying Water Birds from their Northern Territory to Hokkaido, Japan

Introduction

Each of the known influenza virus subtypes of 16 hemagglutinin (HA) and 9 neuraminidase (NA) in various combinations, is maintained in free-flying water birds belonging to Anseriformes (particularly ducks, geese and swans) and Charadriiformes (particularly gulls, terns and waders) [27, 117]; with relatively higher isolation rate of wide variety of influenza virus subtypes from the Anseriformes particularly from ducks than from members of Charadriiformes [53, 78, 85]. Many avian species are known to perform regular short or long distance migrations. The most common being the seasonal migration from the northern breeding areas to the southern wintering lands starting in autumn and then back to the northern breeding areas in spring [10]. Influenza viruses replicate in the epithelial cells forming crypts in the colon and are shed in the fecal materials therefore transmission among birds is mainly by fecal-oral route [62, 117]. During their migration to the south and at the time of over-wintering they distribute influenza viruses [50, 84, 85].

Influenza A viruses are in evolutionary stasis in their natural hosts [33] that do not show clinical signs [62]. Influenza virus prevalence in the natural hosts depends on

species, time of sampling and geographical area [78]. Immunologically naïve juvenile birds have been shown to have higher prevalence of influenza A viruses particularly before southward migration. It was found that the prevalence fell from ~60% in ducks, sampled at marshalling sites close to Canadian breeding areas, to 0.4% to 2% at the wintering grounds in southern USA [39, 85]. The role of influenza virus gene pool in the emergence of strains of disease potential for both humans and animals has been documented on many occasions. Genetic and antigenic analysis of H3 influenza viruses have shown that H3 HA of the human pandemic strain, A/Hong Kong/1968 (H3N2) was of migratory duck origin [59, 60, 122]. H5N1 viruses genetically closely related to those isolated at lake Qinghai have been isolated in many countries of Asia, Europe and Africa, suggesting the role of migratory birds in the spread of these viruses [3, 116]. Thus, role of free-flying water birds in the spread of highly pathogenic avian influenza (HPAI) viruses cannot be ruled out. These findings necessitate continuing virological surveillance of avian influenza in the natural hosts to provide information on the emerging HPAI viruses and human pandemic strains.

Avian influenza viruses are divided into Eurasian and American lineages probably as a result of long-term circulation in different host bird populations. The genome of influenza virus comprises of 8 single-stranded negative sense RNA segments [65]. The genetic analyses of influenza virus HA and NA genes in natural hosts have been done extensively [8, 11, 27, 51, 59, 115]. However, information on genes encoding the internal proteins of influenza virus in natural hosts is poor. The available evidences indicate that M genes are highly conserved [67] and have evolved into different host-specific lineages [49]. It encodes two partially overlapping M1 (252 amino acid) and M2 (97 amino acid) proteins.

The information on how the M gene has evolved in the natural hosts and its relationship to the HPAI viruses is poor. Therefore, M genes of influenza viruses isolated from free-flying water during 2000-2007 have been phylogenetically analysed in the present study.

Materials and Methods

Sample collection

A total of 3,718 fecal samples were collected during October-November 2000-2007 from free-flying water birds at Ohnuma Lake, Wakkanai and Ohno-ike pond of Hokkaido University, Sapporo, Japan. The fecal samples were collected in sterile containers, chilled, transported to our laboratory.

Virus isolation

Virus isolation from fecal samples was carried out in 9-10 day-old embryonated eggs as previously described [61] with a slight modification. The fecal samples were resuspended in virus transport medium [73], centrifuged at 2,000 rpm for 5 minutes and then inoculated. After 48 hours of incubation at 35^{o}C, the inoculated eggs were chilled overnight at 4^{o}C and then their allantoic fluids were tested for hemagglutination activity. Later, all hemagglutinating agents were identified by hemagglutination inhibition [96] and neuraminidase inhibition [5] tests using panel of specific anti-sera against reference strains of influenza viruses [61].

RNA extraction, RT-PCR and nucleotide sequencing

One virus of each of the combination of HA and NA subtypes was selected by year of isolation (Table 1) and propagated in embryonated chicken eggs as mentioned above. Viral RNA was extracted using TRI reagent LS (Sigma, USA) from the harvested allantoic fluid according to manufacturer instructions, reverse transcribed using Uni 12 primer and full length M gene was amplified using M-gene specific primer set (Bm-M-1 and Bm-M-1027R) [43]. The PCR products were separated by 1% agarose gel electrophoresis and

purified using the MiniElute™ Gel Extraction Kit (Qiagen, USA) according to manufacturer instructions. The purified products were used as templates in sequencing reactions using GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, USA) according to manufacturer instructions and analysed with CEQ™ 2000XL sequencer (Beckman Coulter, USA). The primers used for sequencing were same as used for amplification. The PA, HA, NP, NA and NS genes of A/duck/Hokkaido/W95/2006 (H10N8) were amplified using gene-specific primer pairs [43] while PB2 and PB1 genes were amplified using PB2-807F (5'-AGC AAC AGT ATC AGC AGA CC-3') – Ba-PB2-2341R [43] and PB1-1240F (5'-GGA ATG ATG ATG GGC ATG TT-3') – Bm-PB1-2341R [43] primer sets, respectively. The primers used for partial sequencing of PB2, PB1 and PA genes were PB2-807F, PB1-1240F , Bm-PB1-2341R [43] and PA-1074F (5'-GAA CAT GAA GAA AAC AAG CC-3'). The HA, NP, NA and NS genes were partially sequenced by using Bm-NS-890R [43], Bm-NP-1565R [43], Ba-NA-1413R [43] and Bm-NS-1 [43] primers respectively.

The accession numbers of M genes sequenced in this study are available from DDBJ/EMBL/GenBank under accession numbers given in Table 2.

Phylogenetic analysis

In addition to the M genes of selected isolates, BLAST homology searches (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) were used to retrieve sequences for tree construction. Phylogenetic analysis was based on partial sequence of M genes (position 85-904 bp) of influenza viruses. The nucleotide sequences were analysed using GENETYX (Genetyx Corp., Japan) for Windows ver. 7 software. The nucleotide sequences were aligned by Clustal W [110]. and evolutionary distances were calculated by

Maximum Composite Likelihood method [109]. Phylogenetic dendrograms were constructed using Neighbor Joining (NJ) method and bootstrap analysis (1,000 replicates) and bootstrap interior branch test [22] for phylogeny was conducted using Mega 4.0 software [108]. Mega 4.0 software [108] was used for the construction of phylogenetic dendrograms of 7 genes (PB2, PB1, PA, HA, NP, NA and NS) of A/duck/Hokkaido/W95/2006 (H10N8) using NJ method (1,000 replicates). Evolutionary distances were calculated using Maximum Composite Likelihood method [109].

Results

Isolation of Influenza A viruses from fecal samples of free-flying water birds

During 2000-2007, total of 3,718 fecal samples (1,626 from Sapporo and 2,092 from Wakkanai) were collected. From these, 218 influenza A viruses (104 from Sapporo and 114 from Wakkanai) were isolated. Predominant combinations of HA and NA subtypes of isolates were H3N8, H7N7, H6N2, H11N9, H4N6 and H10N4 (Table 1).

Phylogenic analysis of the M genes of influenza virus isolates

Total of 151 M gene sequences (67 in present study shown in Table 2 and the others from gene bank) were analysed and results are presented in Fig.1. The phylogenetic analysis showed that the M genes from 65 isolates belonged to Eurasian non-gull-avian, one to Eurasian-gull and the remaining one gene to North American non-gull-avian lineages. The M genes, within Eurasian non-gull-avian lineage, grouped into three apparent sublineages. The assortment of genes into sublineages was not related to isolation time or location such as A/mallard/Astrakhan/263/1982 (H14N5) had 98.4% and 98.2% nucleotide identity with A/quail/Nanchang/7-026/2000 (H3N6) and A/duck/Hokkaido/1019/2001 (H4N6) respectively, although these viruses were isolated 18-19 years apart at different locations.

Sublineages I and III (Fig. 1 B, C) in Eurasian non-gull-avian lineage comprised of viruses isolated from migratory ducks, domestic ducks, chickens, quails, pheasants and humans in Japan, China, Taiwan, Mongolia and Europe. In sublineage II, four influenza viruses isolated in Hokkaido from migratory ducks clustered with H6N1

Fig. 1 Phylogenetic tree for influenza A virus M genes. The phylogenetic tree was constructed using NJ method (1000 replicates) and M gene of A/equine/Prague/1/1956 (H7N7) was included in the analysis as an outgroup sequence. For construction of this tree, 151 M gene sequences each comprising of 820 nucleotides (position 85-904) were analysed. (A), Complete, simplified phylogram showing avian influenza virus lineages. (B), Part of phylogram from A showing sublineages I and II of Eurasian non-gull-avian lineage. (C), Part of phylogram showing sublineage III of Eurasian non-gull-avian lineage and Eurasian-gull, North American non-gull-avian and North American-gull lineages. Numbers at each branch point indicates boot strap values of ≥ 50%/ bootstrap interior branch test value ≥ 75 % in boldface. The strains sequenced in this study are indicated in boldface. The scale shows number of nucleotide substitutions per site. Vertical lines are used for spacing the branches and their lengths are not significant. Abbreviations: BH-gull, black-headed gull; BW-tl, blue-winged teal; Ck, chicken; Dk, duck; Eq, equine; Gf, guinea fowl; Goo, goosander; Gs, goose; H-gull, herring gull; L-gull, laughing gull; Mal, mallard; Mig-dk, migratory duck; Par, partridge; Phe, pheasant; Pin, pintail; Qa, quail; Sb, shorebird; ST-sandpiper, sharp-tailed sandpiper; Sw, swan; Tk, turkey; Tl, teal; H?N?, HA and NA subtypes not given in GenBank.

Fig. 2 Phylogenetic trees of seven gene segments of A/duck/Hokkaido/W95/2006 (H10N6). Nucleotides 844-1,385 (542bp) of PB2, 1,279-2,294 (1,016bp) of PB1, 1,110-1,612 (503) of PA, 1,200-1683 (484bp) of HA, 962-1,497 (536bp) of NP, 893-1,419 (527bp) of NA and 4-547 (544bp) of NS genes were used for the phylogenetic analysis. The phylogenetic trees were constructed using NJ method (1,000 replicates). The scale shows number of nucleotide substitutions per site. The influenza virus strain sequenced is indicated in bold face. Number at each branch point indicates boost strap values of ≥50%. Abbreviations: BW-tl, blue-winged teal; Chu, chukar; Ck, chicken; Com-scot, common scoter; Dom-dk, domestic duck; Dk, duck; Gar, garganay; Gf, guinea fowl; Gs, goose; GW-tl, green-winged teal; H-gull, herring gull; L-gull, laughing gull; Long-dk, longtail duck; Mal, mallard; Mig-dk, migratory duck; Mk, mink; Ost, ostrich; Phe, pheasant; Pin, pintail; Qa, quail; RN-stint, red-necked stint; Rt, ruddy turnstone; Sb, shorebird; ST-sandpiper, sharp-tailed sandpiper; Sw, swan; Tk, turkey; Tl, teal; Tn, tern; WB-feces, wild bird feces N?, NA subtypes not given in GenBank.

viruses isolated from Taiwan [69] and A/goose/Guandong/1/1996 (H5N1) (Gs/Gd/96) like viruses [24]. Both, BLAST homology and BLAST tree searches for each of the M genes from Hokkaido isolates showed high nucleotide identity with viruses isolated from Taiwan. A/duck/Hokkaido/95/2001 (H2N2) showed 97.9% and 96.8% nucleotide homology with A/chicken/Taiwan/0824/1997 (H6N1) and Gs/Gd/96 respectively.

The M gene of A/duck/Hokkaido/W189/2006 (H13N6) grouped with those from H13 and H16 subtype viruses, distinctively from Eurasian non-gull-avian lineage viruses (Fig. 1C). It is noteworthy that H13 subtype viruses have been rarely isolated from species other than gulls [54, 85]. In this study we report two H13 subtype viruses isolated in our laboratory from ducks in Hokkaido (AB428712) and Siberia (AB284991). The M genes from these isolates clustered with those from gull isolates indicating their probable origin in gulls.

Since, the M gene of A/duck/Hokkaido/W95/2006 (H10N8) phylogenetically grouped with North American non-gull-avian lineage viruses (Fig. 1C); the remaining 7 genes were sequenced to find out their lineage. Phylogenetic analysis of remaining 7 genes revealed that NA gene also belonged to North American non-gull-avian lineage while remaining 6 genes belonged to Eurasian non-gull-avian lineage (Fig. 2, Table 3), suggesting that inter-lineage reassortment occurs in the lakes where free-flying water birds congregate during migration to the south.

Discussion

Despite the important role of the influenza virus gene pool in natural hosts in the emergence of HPAI and human viruses, few genes particularly those coding for internal proteins from this gene pool have been sequenced and analysed. In the present study, we sequenced the M gene of influenza viruses isolated from free-flying water birds arriving in Hokkaido, Japan.

Influenza viruses of twenty eight different combinations of HA and NA subtypes were isolated from fecal samples of free-flying water birds arriving in Hokkaido. This diversity of HA and NA combinations could be due to reassortment occurring in the lakes where free-flying water birds congregate [84]. The predominant and frequent isolation of H3, H4 and H6 subtype influenza viruses is in consistence with early reports, both from Eurasia and North America [53, 78]. These findings suggest that influenza viruses of these HA subtypes might be well adapted to their reservoir hosts [98]. Some influenza virus like H1N1, H5N3 and H11N9 were intermittently isolated. Ito *et al.,* [50] proposed that intermittent isolation of influenza viruses could be due to co-circulation of different influenza A virus subtypes in duck population in their breeding areas.

The results in this study have revealed that evolution of influenza virus M gene in natural hosts has resulted in the distinguishable sublineages in Eurasian non-gull-avian lineage, indicating that these sublineages are independently circulating in the bird populations. However, the pattern or number of sublineages may vary if more extensive data are analysed. The assortment of M genes into sublineages does not seem to be related to location of isolation or isolation year. Similar findings were shown by Widjaja *et al.* [120]. In sublineages I and III, clustering of M genes sequenced in the present study with

those of from many Asian and European countries suggests that mixing of genes, not only along but also across the migratory routes due to their overlapping nature [26], has occurred. This finding indicates the dynamic nature of the influenza virus gene pool in this region.

Previous surveillance studies done in our laboratory indicated that influenza viruses perpetuating in ducks nesting in Siberia should have provided genes for the emergence of H5N1 viruses in Hong Kong in 1997 [84]. Duan *et al.* [24] found that A/duck/Hokkaido/120/2001 (H6N2) had the close phylogenetic relationship with Gs/Gd/96 and its variants. They proposed that the precursor gene of Gs/Gd/96 like viruses might have been in migratory birds along the western Pacific flyway. Lee *et al.* [69] revealed that a unique lineage of H6N1 influenza viruses has established in Taiwan which are genetically and antigenically distinct from H6N1 viruses circulating in southern China and Hong Kong. The results in this study are not only in agreement with proposition of Duan *et al.* [24] but also suggest that precursor genes of H6N1 viruses in Taiwan should have come from viruses in the free-flying water birds flying along the Pacific flyway.

The predominant isolation of H13 and H16 subtype influenza viruses from gull species confirms the notion that these viruses belong to influenza virus "gull lineage" thus constituting a distinct gene pool [54, 85]. Although H13 subtype viruses were isolated from ducks in present study, their close phylogenetic relationship with H13 subtype viruses isolated from gulls indicates that the ducks might have acquired these viruses from the shorebirds. It is also evident that M genes from gulls, like NP genes [32] were divided into Eurasian-gull and North American-gull lineages, distinct from Eurasian non-gull-avian or North American non-gull-avian lineages.

Genetic reassortment between avian influenza viruses [35, 72] or between human influenza viruses [95] has been demonstrated earlier. In the present study, one virus isolate was found to be a reassortant and it is difficult to predict how and where this reassortment occurred. It is noteworthy that isolation of one reassortant virus, in this study, does not represent the actual reassortment frequency because all genes of only A/duck/Hokkaido/W95/2006 (H10N8), whose M and NA genes belonged to North American lineage, were sequenced. More reassortant viruses could be found if other gene segments were sequenced. In northern hemisphere, East Asia/Australia and Pacific Americas flyways show some overlapping [26] and some shorebirds or ducks may migrate across these flyways at Bering Strait. Furthermore, boundaries between avifauna of Eurasia and North America are not well demarcated. Some ducks such as Northern pintail (*Anas acuta*) are common in both regions and they may serve as potential intercontinental carriers of influenza viruses [85].

The present study enhances our understanding on how the influenza virus M gene has evolved into distinct sublineages in Eurasia. The present results also help us to understand the relationship between influenza viruses in natural hosts and those causing outbreaks in animals. Virological surveillance of avian and animal influenza, thus, must be continued globally for the preparedness of future pandemics.

Brief summary

During 2000-2007, 218 influenza viruses of 28 different combinations of HA (H1-H13) and NA (N1-N9) subtypes were isolated from fecal samples of free-flying water birds at two distant lakes in Hokkaido, Japan. Phylogenic analysis of the matrix (M) genes of 67 strains, selected on the basis of their subtype combinations, revealed that A/duck/Hokkaido/W95/2006 (H10N8) was a reassortant whose M and NA genes belonged to North American non-gull-avian and the other 6 genes to Eurasian non-gull-avian lineages. The M genes of other 65 strains belonged to Eurasian non-gull-avian and the one to Eurasian-gull lineages. The M genes of 65 strains were grouped into 3 different sublineages, indicating that influenza viruses circulating in different populations of free-flying water birds have evolved independently in nature.

Chapter III

PB2 gene is responsible for restriction in replication of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) in pigs

Introduction

Influenza A viruses have been isolated from a variety of species, including humans, birds, pigs, horses, minks, seals, whales, cats, dogs and tigers [56, 102, 103, 117]. Indeed, influenza A viruses exhibit a restricted host range with efficient replication in their natural hosts and poor or no replication in other host species. The studies have shown that avian influenza viruses do not replicate efficiently in humans [9] and other primates [79]. Similarly, human influenza viruses do not replicate efficiently or show restricted replication in avian species [36, 38]; however, influenza viruses may cross this species barrier. Inter-species transfer of human, swine and avian influenza viruses has been documented on several occasions [12, 16, 80, 114]. In both Asian (1957) and Hong Kong (1968) pandemics, the causative viruses were reassortants which acquired three genes (PB1, HA and NA) in the former pandemic and two genes (PB1 and HA) in the latter from avian influenza viruses [55, 60, 94, 118, 122]. The role of pigs in the generation of new influenza viruses is well documented [58]. It was shown that the H3 HA gene of Hong

Kong pandemic strain A/Hong Kong/1968 (H3N2) was of migratory duck origin and was acquired as a result of reassortment with the precedent human H2N2 influenza virus in pigs [60, 122]. Furthermore, avian-human reassortant viruses were isolated from Italian pigs [12] and those isolated from children in Netherlands in 1993 were found to be avian-human reassortants circulating in pigs in Europe [16]. These findings suggest that pigs should support the growth of both avian and human influenza viruses and are therefore termed as "mixing vessels" [31, 93]. Nevertheless, not all influenza viruses replicate in pigs. Kida *et al*. [58] demonstrated this by studying the growth potential of 38 different H1-H13 subtypes of avian influenza viruses in pigs.

The genetic basis of influenza virus host-range restriction and adaptation to a new host species are poorly understood. The first host-range barrier is offered at the cell surface where receptor-mediated entry into cells starts. Avian and human viruses differ in the receptor specificity of their surface glycoprotein; hemagglutinin. Human viruses bind preferentially to sialic acid-α2,6-galactose whereas avian viruses bind preferentially to sialic acid-α2,3-galactose linkages on cell surface sialyloligosaccharides [20, 89, 90]. After cell entry a second level of host-range barrier is offered when interaction between viral and cellular proteins takes place. In addition to surface glycoproteins, influenza virus internal proteins also harbor determinants for host range and virulence [17, 71, 111]. Among these internal proteins, polymerase basic protein 2 (PB2) is well documented, and is a component of the viral polymerase complex required for virus replication. The PB2 protein has been shown to be involved in host-range restriction and pathogenicity [4, 106].

In late December 2003, there was an outbreak in a layer chicken farm in Yamaguchi Prefecture, Japan. The causative agent was identified as highly pathogenic avian influenza

virus, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04 (H5N1)) [75]. This virus was shown to be highly pathogenic to chickens, quails, budgerigars and ducklings, while mice showed low mortality rate; miniature pigs were resistant to the infection [47]. This virus offers a good model to study the mechanism underlying interspecies transmission/adaptation to a new host. The classical swine influenza viruses or avian-human reassortant viruses have been reported to be circulating in pigs in Europe and Asia [16, 34, 92, 99]. These viruses can contribute genes to viruses like Ck/Yamaguchi/04 (H5N1) and help them to cross the species barrier. Therefore, the present study was conducted to address the genetic basis of such an interspecies adaptive process.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were maintained in Minimum essential medium (MEM) (Nissui, Japan) supplemented with 5% calf serum. Human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10 % fetal calf serum. Both cell lines were maintained at 37^{o}C in 5% CO_2 atmosphere. Two viruses (designated as parent viruses) were used in this study; Sw/Hokkaido/81 (H1N1) was obtained from the virus repository of our laboratory while Ck/Yamaguchi/04 (H5N1) was provided by the National Institute of Animal Health, Ibaraki, Japan [75].

The viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. The eggs were incubated at 35^{o}C for maximum of 48 hours. Before harvesting the infectious allantoic fluid, the eggs were chilled at 4^{o}C overnight and the harvested allantoic fluid was stored at -80^{o}C until use.

Experimental infection of pigs

Three- to four-week-old, landrace, specific pathogen-free (SPF) pigs, free of antibodies against influenza A viruses (Takikawa swine station, Hokkaido, Japan) were housed in the Biosafety level-3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

The viruses used in the present study were inoculated intranasally with 500 µl of infectious allantoic fluid containing $10^{7.0} \sim 10^{7.5}$ 50 % egg infectious dose (EID_{50}) of virus except Ck/Yamaguchi/04 (H5N1). The Ck/Yamaguchi/04 (H5N1) was inoculated

intranasally with 500 µl of infectious allantoic fluid containing $10^{8.4}$ EID_{50} of virus. The nasal swabs were collected either for 7 days post-inoculation (p.i.) from pigs inoculated with infectious allantoic fluid prepared from co-inoculated eggs or for 10 days p.i. from pigs inoculated with other viruses used in this study. The nasal swabs were collected in 1 ml of virus transport medium. Pre and day 14 p.i. blood samples for serum were collected and antibody titers were determined using ELISA [57]. The infectivity titers of the different viruses in the nasal swabs of pigs were calculated in chicken embryonated eggs by 50% end point method [88] and were expressed as EID_{50}/ml of swab.

All animal experiments were conducted in accordance with the guide-lines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

In vivo selection of H5N1 reassortant viruses between Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) capable of replication in pigs

The virus inoculum containing reassortant viruses was produced by co-inoculation of 10-day-old embryonated chicken eggs with 100 µl of inoculum containing Ck/Yamaguchi/04 (H5N1) ($10^{7.4}$ EID_{50}/50 µl) and Sw/Hokkaido/81 (H1N1) viruses ($10^{6.0}$ EID_{50}/50 µl) at a ratio of 1:10,000, respectively. The eggs were incubated at 35°C for 48 hours and chilled at 4°C overnight. The infectious allantoic fluid was used as inoculum for pigs to select the H5N1 reassortant viruses capable of replication in the pigs. The inoculum contained parental H5N1, H1N1 and reassortant viruses. The nasal swabs were collected for 7 days p.i. and were used for selecting virus clones by plaque cloning.

Virus clones were selected from nasal swabs by plaque cloning on MDCK cells as described by Kida *et al.* [58]. Individual virus clones were selected and propagated in 10-

day-old embryonated chicken eggs for maximum of 48 hours at 35^{o}C. The eggs were chilled at 4^{o}C overnight and allantoic fluid was harvested. The HA subtype of virus clones was determined by hemagglutination inhibition assay (HI) [96].

Evaluation of replicative potential of H5N1 subtype virus clones recovered from pigs

Eleven H5N1 subtype virus clones were isolated by plaque cloning. All gene segments of these clones were amplified and partially sequenced. It was found that two clones were single gene reassortants while the remaining nine clones derived all genes from Ck/Yamaguchi/04 (H5N1). To determine their replicative potential, two single gene reassortant clones and two clones of Ck/Yamaguchi/04 (H5N1) like gene constellation were re-inoculated in pigs. Nasal swabs were collected for 10 days p.i. and infectivity titers were measured as described earlier.

Generation of viruses by reverse genetics

Eight genes from each of Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) viruses were cloned to produce viruses by reverse genetics (rg) as described by Hoffman *et al.* [41]. In brief, the RNA of viruses was extracted using TRI® Reagent LS (Sigma, USA). The cDNAs were amplified by reverse transcription of viral RNA using Uni 12 primer (5'-AGC AAA AGC AGG-3'). Full-length genes of Ck/Yamaguchi/04 and Sw/Hokkaido/81 were amplified using gene-specific universal primer sets . The amplified genes were then sequenced using GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, USA) according to the manufacturer's instructions and analyzed with CEQ™ 2000XL sequencer (Beckman Coulter, USA). The amplified genes were first cloned in pCR 2.1 TOPO cloning vector

(Invitrogen, USA) and then in pHW2000 expression vector, except for PA, HA and NA genes of Ck/Yamaguchi/04 (H5N1) and PB2 and PB1 genes of Sw/Hokkaido/81 (H1N1), which were directly cloned in the pHW2000 expression vector. Genes cloned in pCR 2.1 TOPO vector or pHW2000 were sequenced and only those clones identical to the consensus sequence were selected. Ligation of the genes into pHW2000 expression vector was carried out using DNA Ligation Kit Version 2.1 (Takara, Japan) according to the manufacturer's instructions.

For generating viruses by reverse genetics using eight plasmids, 293T and MDCK cells were used as described earlier [42]. The rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) were inoculated into pigs to compare their potential to replicate in pigs with that of parent viruses. The rg-H5N1-PB2H1N1 virus (PB2 gene from Sw/Hokkaido/81 (H1N1) and remaining 7 genes from Ck/Yamaguchi/04 (H5N1)) was inoculated into pigs to evaluate the results obtained by *in vivo* selection of H5N1 reassortant viruses. The rg-H1N1-PB2H5N1 virus (PB2 gene from Ck/Yamaguchi/04 (H5N1) and remaining 7 genes from Sw/Hokkaido/81 (H1N1)) was inoculated into pigs to study the host-range restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) on 7 genes of Sw/Hokkaido/81 (H1N1) in pigs. The nasal swabs were collected and processed as described earlier.

Results

Selection of *in vivo*-generated reassortant viruses capable of replication in pigs

The nasal swabs, collected from pigs administered intranasally with inoculum prepared from co-inoculated eggs, were used for picking virus clones by plaque cloning. A total of 119 virus plaque clones were picked from nasal swabs collected from day 1-3 p.i. (Table 1). Of these, 11 virus clones were of H5N1 subtype. Interestingly all these clones were isolated from nasal swabs collected on day 1 p.i.; while all virus clones isolated from nasal swabs collected on day 2-3 p.i. were of H1N1 subtype. Therefore, nasal swabs collected from day 4-7 p.i. were not used for plaque cloning. The H5N1 subtype virus clones were plaque purified once again on MDCK cells. Partial genome sequencing of these virus clones revealed that two of these virus clones (clone 1 and 2) were single gene reassortants deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) and the remaining 7 genes from Ck/Yamaguchi/04 (H5N1). The remaining 9 clones (clone 3–11) derived all of their genes from Ck/Yamaguchi/04 (H5N1) (Table 2).

Virus clones recovered by *in vivo* selection in pigs are capable of replication in pigs

Virus clone 1, 2 (PB2 gene of Sw/Hokkaido/81 (H1N1) and the remaining 7 genes of Ck/Yamaguhi/04 (H5N1) origin) and virus clone 3, 4 (all 8 genes of Ck/Yamaguchi/04 (H5N1) origin) were re-inoculated into pigs to assess their replicative potential (Table 2). Virus clone 1 and 2 were recovered from nasal swabs and virus shedding occurred from day 1 to 5 p.i. while virus clone 3 and 4 were not recovered from nasal swabs. The 14 days p.i. sera collected from pigs inoculated with virus clone 1 and 2 showed sero-conversion while those inoculated with virus clone 3 and 4 did not show any sero-conversion

Table 1. Recovery and sub-typing of virus clones from nasal swabs

Days post-inoculation	Total plaques picked	HA subtype of virus clones	
		H5	H1
Day 1	61	11	50
Day 2	38	–	38
Day 3	20	–	20

(Table 2). This finding suggested that the PB2 gene of Sw/Hokkaido/81 (H1N1) conferred replicative potential to the reassortant virus possessing 7 genes of Ck/Yamaguchi/04 (H5N1) origin.

Potential of replication of viruses produced by reverse genetics is similar to that of parent viruses.

In order to compare the replicative potential of rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) in pigs with that of parent viruses, rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) were inoculated in the pigs and nasal swabs were collected. The rg-Ck/Yamaguchi/04 (H5N1) was not recovered from the nasal swabs and pigs did not show sero-conversion (Table 3) like that of Ck/Yamaguchi/04 (H5N1) as described by Isoda *et al.* [47]. No significant difference was observed in the virus shedding between rg-Sw/Hokkaido/81 (H1N1) and Sw/Hokkaido/81 (H1N1) [7].

A single gene reassortant virus (rg-H5N1-PB2H1N1) possessing gene constellation like that of virus clone 1 and 2 was generated by reverse genetics and was inoculated intranasally into the pigs. The rg-H5N1-PB2H1N1 was shed for 2–3 days as were the parent virus clone 1 and 2 (Table 3). This finding further supported the results obtained by re-inoculation of *in vivo* selected reassortant clones.

Amino acid substitutions found in the PB2 protein of rg-H1N1-PB2H5N1 after a single passage in pigs.

If the PB2 gene of Ck/Yamaguchi/04 (H5N1) restricted its replication in pigs, it should also restrict the replication of Sw/Hokkaido/81 (H1N1) in pigs. Therefore, rg-

H1N1-PB2H5N1, possessing 7 genes from Sw/Hokkaido/81 (H1N1) and the PB2 gene from Ck/Yamaguchi/04 (H5N1), was generated by reverse genetics. Two pigs (Pig No. 7 and 8) were inoculated intranasally with the rg-H1N1-PB2H5N1 virus. Interestingly, rg-H1N1-PB2H5N1 virus was first recovered on day 3 p.i. from nasal swabs, contrary to rg-Sw/Hokkaido/81 (H1N1) which was recovered on day 1 p.i. During the first 3 days of rg-H1N1-PB2H5N1 virus shedding, virus titers were 2-4 log less than those of rg-Sw/Hokkaido/81 (H1N1) (Table 3). Moreover, the duration of rg-H1N1-PB2H5N1 virus shedding was 3-4 days less that that of rg-Sw/Hokkaido/81 (H1N1). The full- length genes of isolates recovered from pigs on day 3 p.i. as well as rg-H1N1-PB2H5N1 (inoculum) were sequenced and compared (Table 4). The predicted amino acid sequences of all except PB2 gene were identical to that of rg-H1N1-PB2H5N1 (inoculum). PB2 protein of both isolates, Pig 7-day 3 and Pig 8-day 3, had glutamic acid 627 lysine (E627K) and aspartic acid 256 glycine (D256G) substitutions respectively.

Table 4. Comparison of amino acid sequences of PB2 genes of isolates recovered from pigs inoculated with rg-H1N1-PB2H5N1 virus

Name of virus isolate	PB2 gene	
	256[a]	627
rg-H1N1-PB2H5N1 (inoculum)	D	E
Pig 7-day 3	●	K
Pig 8-day 3	G	●
rg-Sw/Hokkaido/81 (H1N1)	●	K

[a] amino acid position

Discussion

It has been shown that avian and human H5N1 viruses isolated in 1997 [100] and 2004 [14] replicated to moderate levels in the upper respiratory tract of experimentally infected pigs. There is also evidence of co-circulation of avian and human influenza viruses in the pigs in China [86]. Therefore, it is reasonable to think that pigs can provide opportunity for the reassortment and subsequent emergence of new reassortant influenza viruses.

Kida *et al.* [58] inoculated pigs with A/duck/Hokkaido/8/1980 (H3N8) (non-replicable strain) and Sw/Hokkaido/81 (H1N1) (replicable strain). They recovered both parental gene constellation possessing viruses and reassortant viruses. The recovered H3N8 subtype viruses, after re-inoculation, did not replicate in the pigs; while reassortant viruses replicated. Similarly in the present study viruses with parental gene constellation were recovered and two of these virus clones (clone 3 and 4) were found to be identical to the parental and rg-Ck/Yamaguchi/04 (H5N1) virus in their capability to replicate in pigs; therefore, the isolation of entire H5N1 virus clones could be due to concurrent infection of cells lining the upper respiratory tract of the inoculated pigs with different combinations of reassortant viruses present in the inoculum and these reassortant viruses might have provided all 8 gene segments of Ck/Yamaguchi/04 (H5N1).

The role of PB2 protein in determining the host-range has been studied extensively using squirrel monkeys [17], mice [29, 71] or mammalian cells [121]. In the present study, we found that the PB2 gene of Ck/Yamaguchi/04 (H5N1) restricted its replication in pigs since its replacement with the PB2 gene of Sw/Hkkaido/81 (H1N1) enabled it to replicate in pigs as observed for naturally selected virus clones 1 and 2 and rg-H5N1-PB2H1N1

virus. Kida *et al*. [58] isolated triple gene reassortants deriving NP, NA and M or NP, NA and NS genes from the replicable strain Sw/Hokkaido/81 (H1N1) and remaining 5 genes from the non-replicable strain A/duck/Hokkaido/8/1980 (H3N8). In the present study single gene reassortant virus clones deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) were isolated. It could be due to differences in gene constellation of non-replicable strains bearing different host-range determinants [68, 101], used by Kida *et al*. [58] and in the present study.

The restrictive potential of the PB2 gene of Ck/Yamaguchi/04 (H5N1) was evaluated by studying the replication of rg-H1N1-PB2H5N1 virus in pigs. Interestingly, the virus was recovered on day 3 p.i. It replicated to moderate levels for shorter duration as compared to that of rg-Sw/Hokkaido/81 (H1N1) (Table 3). These findings indicate that during the first 2 days p.i., the virus might have undergone adaptive changes. This assumption was supported by examining the predicted amino acid sequence of the 2 virus isolates, Pig 7-day 3 and Pig 8-day 3, whose PB2 protein had E627K and D256G amino acid substitutions respectively. Amino acid substitution at position 256 in PB2 protein has not been reported previously while amino acid substitution at position 627 has been reported to be a host-range determinant. Li *et al.* [71] inoculated mice with two duck isolates of contrasting pathogenicity for mice. They found that more than 50% of the virus isolates recovered from mouse lungs had E627K substitutions in PB2 protein [71]. Similarly viruses recovered from mice inoculated with Ck/Yamaguchi/04 (H5N1) had E627K substitution in PB2 protein [74]; therefore, these studies suggested that the presence of E or K at position 627 is host-dependent and is indicator of avian-to-mammalian adaptation.

The PB2, PB1 and PA proteins make the viral RNA polymerase complex. Presence of overlapping PB1 and NP functional regions on PB2 protein has suggested their role in switching the transcriptase to replicase activity [87, 97]. The D256G substitution is located in the functional domain of PB2 protein. This region has been shown to be related to cap binding function [44, 87], interaction with NP protein [87] and interaction with PB1 protein [82]. Similarly E627K is located in the C-terminal region of PB2 protein which interacts with both PB1 and NP proteins [87]. Labadie *et al* [64] suggested that presence of K at position 627 in PB2 protein helps to stabilize the PB2-NP interaction in human cells through an un-known host cellular factor; while K at this position impairs this interaction in avian cells. Many host cell proteins have been shown to interact with different subunits of influenza virus polymerase complex and some of these were involved in either translocation of viral RNPs such as importin α [30] Ran binding protein 5 [21] or heat shock protein 90 [81] or in the regulation of polymerase activity [76, 77]. Recently, Jorba *et al* [52] identified many influenza virus polymerase interacting nuclear and cytosolic proteins; involved in transcription, modification and translocation. These findings suggest that interaction of polymerase components with each other to carry out the transcription or replication involves host-cellular factors; thus adaptive changes to host-cellular factors might play an important role in host-range determination. Taken together these findings, it can be speculated that the mutations D256G and E627K could have arisen as a result of the interaction of PB2 protein of Ck/Yamaguchi/04 (H5N1) with host-cellular proteins of swine origin or viral factors.

In the light of earlier and present findings, it is reasonable to conclude that PB2 protein of Ck/Yamaguchi/04 (H5N1) determines its host-range. However, the molecular

events which lead to the appearance of D256G and E627K substitutions are yet to be elucidated.

Brief summary

It is known that pigs act as mixing vessels since they possess avian-type (α2-3 Gal) and human-type (α2-6 Gal) receptors for hemagglutinin of influenza viruses. Evidences indicate that they acted as intermediate host in the emergence of Hong Kong pandemic strain in 1968; thus, it is believed that pigs should support the growth of all avian and human influenza A viruses. In contrast, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04 (H5N1)) do not replicate in pigs (Isoda *et al.* 2006). The genetic basis for such host-range restriction was determined using swine influenza virus as a donor virus, since swine influenza viruses have been reported to be circulating in pigs which can act as potential donors. The inoculum containing the reassortant viruses was prepared between A/swine/Hokkaido/2/1981 (H1N1) (Sw/Hokkaido/81 (H1N1)) and Ck/Yamaguchi/04 (H5N1) in embryonated eggs. The reassortant viruses capable of replication in pigs were selected by administering the inoculum intranasally into pigs. Two single gene reassortant virus clones of H5N1 subtype (clones 1 and 2) whose PB2 genes were derived from Sw/Hokkaido/81 (H1N1) and the remaining 7 genes from Ck/Yamaguchi/04 (H5N1), were recovered. These virus clones replicated in pigs upon re-inoculation. This finding was further supported by using rg-H5N1-PB2H1N1 virus of same gene constellation as virus clones 1 and 2. The rg-H1N1-PB2H5N1 virus was inoculated in pigs to study the host-range restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) on seven genes of Sw/Hokkaido/81 (H1N1) in pigs. It was interesting to find out that rg-H1N1-PB2H5N1 replicated to moderate levels and for shorter duration than that of rg-Sw/Hokkaido/81 (H1N1). Sequencing of entire genome of two isolates recovered from pigs inoculated with rg-H1N1-H5PB2H1 revealed two mutations, D256G and E627K, in the PB2 proteins of

respective isolates. These results indicated that the PB2 gene of Ck/Yamaguchi/04 (H5N1) determined the host-range and restricted replication of Ck/Yamaguchi/04 (H5N1) in pigs.

Conclusion

The author focused on the rapid diagnosis of avian influenza caused by H7 influenza viruses as well as molecular epidemiology of influenza viruses encompassing studies on the genetic analysis of influenza virus M genes and the host-range restriction of a highly pathogenic H5N1 avian influenza virus strain Ck/Yamaguchi/04 (H5N1).

The immunochromatography kit for the rapid diagnosis of avian influenza caused by H7 subtype viruses was developed. The kit detected specifically all of the tested H7 influenza viruses. The kit detected H7 HA antigen in swabs as well as tissue homogenates of experimentally infected chickens. The results in this study suggest that the present kit must be a valuable tool for the rapid diagnosis and consequent rapid control of avian influenza.

In 2000-2007, a total of 218 influenza viruses of 28 different HA and NA combinations were isolated from fecal samples of free-flying water birds in Hokkaido. The phylogenic analysis of M genes of 67 selected influenza viruses revealed that viruses belonging to different sublineages are independently circulating in the bird populations. The present study also revealed that contemporary H6N1 influenza viruses circulating in Taiwan appeared to originate from viruses in free-flying water birds traveling along the Pacific fly-way. The close phylogenetic relationship between viruses isolated in Hokkaido and those isolated in many countries of Europe and Asia indicated that these birds have played important role in the spread of influenza viruses not only along but also across their migratory routes.

The genetic basis of restriction in replication of Ck/Yamaguchi/04 (H5N1) in pigs was determined. The *in vivo* selection of reassortant viruses capable of replication in pigs

and reassortant virus generated by reverse genetics indicated that PB2 gene of Ck/Yamaguchi/04 (H5N1) is related to host-range restriction. This finding was further supported by the presence of two amino acid substitutions in PB2 protein of rg-H1N1-PB2H5N1 virus after a single passage in pigs. One of these substitutions is already known to control the host-range and pathogenicity.

Acknowledgements

The author deems it utmost pleasure to acknowledge Prof. H. Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for his auspicious and continuous guidance and encouragement during the present studies.

The author extends his profound indebtedness to Assoc. Prof. Y. Sakoda, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for his valuable guidance and support during the course of present studies.

The author also extends his profound gratitude to Prof. A. Takada, Department of Global Epidemiology, Research center for Zoonosis Control, Hokkaido University, and Prof. K. Ohashi, Laboratory of Infectious diseases, Graduate School of Veterinary Medicine, Hokkaido University, for their nice comments and suggestions to improve this dissertation.

The author is grateful to T. Mochizuki and Y. Namba, BL Co., Ltd., Japan, for their invaluable help, discussion and support to conduct the study on the development of immunochromatography kit.

The author would like to extend his sincere thanks to Assist. Prof. M. Okamatsu, his colleagues and friends in the Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for their help to complete these studies.

I would like to extend my heartiest thanks to my beloved wife, children, parents, brother and sister whose motivation proved an invaluable asset during my stay in Japan. I

would like to dedicate this work to my beloved deceased younger sister whose love and memories are everlasting for me.

References

1. Centers for Disease Control 2004 (Update): influenza activity-United States and world-wide, 2003-2004 season, and composition of the 2004-05 influenza vaccine. MMWR. Morb. Mortal. Wkly. Rep.:53: 547-552.
2. Alexander, DJ. 2000. A review of avian influenza in different bird species. Vet. Microbiol. 74:3-13.
3. Alexander, DJ. 2007. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian Dis. 51:161-166.
4. Almond, JW. 1977. A single gene determines the host range of influenza virus. Nature 270:617-618.
5. Aymard-Henry, M, Coleman MT, Dowdle WR, Laver WG, Schild GC, and Webster RG. 1973. Influenzavirus neuraminidase and neuraminidase-inhibition test procedures. Bull. World. Health. Organ. 48:199-202.
6. Bai, GR, Sakoda Y, Mweene AS, Fujii N, Minakawa H, and Kida H. 2006. Improvement of a rapid diagnosis kit to detect either influenza A or B virus infections. J. Vet. Med. Sci. 68:35-40.
7. Bai, GR, Sakoda Y, Mweene AS, Kishida N, Yamada T, Minakawa H, and Kida H. 2005. Evaluation of the ESPLINE INFLUENZA A&B-N Kit for the diagnosis of avian and swine influenza. Microbiol. Immunol. 49:1063-1067.
8. Bean, WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, and Webster RG. 1992. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J. Virol. 66:1129-1138.

9. Beare, AS, and Webster RG. 1991. Replication of avian influenza viruses in humans. Arch. Virol. 119:37-42.

10. Berthold, P. 2001. The phenomenon of bird migration p. 35-84. *In* C. M. Perrins (ed.), Birds Migration: A general Survey, vol. 2nd. Oxford University Press.

11. Campitelli, L, Di Martino A, Spagnolo D, Smith GJ, Di Trani L, Facchini M, De Marco MA, Foni E, Chiapponi C, Martin AM, Chen H, Guan Y, Delogu M, and Donatelli I. 2008. Molecular analysis of avian H7 influenza viruses circulating in Eurasia in 1999-2005: detection of multiple reassortant virus genotypes. J. Gen. Virol. 89:48-59.

12. Castrucci, MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, and Webster RG. 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology 193:503-506.

13. Chan, KH, Maldeis N, Pope W, Yup A, Ozinskas A, Gill J, Seto WH, Shortridge KF, and Peiris JS. 2002. Evaluation of the Directigen FluA+B test for rapid diagnosis of influenza virus type A and B infections. J. Clin. Microbiol. 40:1675-1680.

14. Choi, YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, Chaisingh A, Auewarakul P, Hanh NT, Ma SK, Hui PY, Guan Y, Peiris JS, and Webster RG. 2005. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J. Virol. 79:10821-10825.

15. Chua, TH, Ellis TM, Wong CW, Guan Y, Ge SX, Peng G, Lamichhane C, Maliadis C, Tan SW, Selleck P, and Parkinson J. 2007. Performance evaluation of five

detection tests for avian influenza antigen with various avian samples. Avian Dis. 51:96-105.

16. Claas, EC, Kawaoka Y, de Jong JC, Masurel N, and Webster RG. 1994. Infection of children with avian-human reassortant influenza virus from pigs in Europe. Virology 204:453-457.

17. Clements, ML, Subbarao EK, Fries LF, Karron RA, London WT, and Murphy BR. 1992. Use of single-gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of wild-type human influenza A virus for squirrel monkeys and adult human volunteers. J. Clin. Microbiol. 30:655-662.

18. Collins, RA, Ko LS, Fung KY, Chan KY, Xing J, Lau LT, and Yu AC. 2003. Rapid and sensitive detection of avian influenza virus subtype H7 using NASBA. Biochem. Biophys. Res. Commun. 300:507-515.

19. Collins, RA, Ko LS, So KL, Ellis T, Lau LT, and Yu AC. 2002. Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. J. Virol. Methods 103:213-225.

20. Connor, RJ, Kawaoka Y, Webster RG, and Paulson JC. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205:17-23.

21. Deng, T, Engelhardt OG, Thomas B, Akoulitchev AV, Brownlee GG, and Fodor E. 2006. Role of ran binding protein 5 in nuclear import and assembly of the influenza virus RNA polymerase complex. J. Virol. 80:11911-11919.

22. Dopazo, J. 1994. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J. Mol. Evol. 38:300-304.

23. Du Ry van Beest Holle, M, Meijer A, Koopmans M, and de Jager CM. 2005. Human-to-human transmission of avian influenza A/H7N7, The Netherlands, 2003. Euro. Surveill. 10:264-268.

24. Duan, L, Campitelli L, Fan XH, Leung YH, Vijaykrishna D, Zhang JX, Donatelli I, Delogu M, Li KS, Foni E, Chiapponi C, Wu WL, Kai H, Webster RG, Shortridge KF, Peiris JS, Smith GJ, Chen H, and Guan Y. 2007. Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. J. Virol. 81:7529-7539.

25. Dybkaer, K, Munch M, Handberg KJ, and Jorgensen PH. 2004. Application and evaluation of RT-PCR-ELISA for the nucleoprotein and RT-PCR for detection of low-pathogenic H5 and H7 subtypes of avian influenza virus. J. Vet. Diagn. Invest. 16:51-56.

26. FAO 2005, posting date. http://www.fao.org/ag/againfo/subjects/en/health/diseases-cards/migrationmap.html. [Online.]

27. Fouchier, RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, and Osterhaus AD. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79:2814-2822.

28. Fouchier, RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, and Osterhaus AD. 2004. Avian influenza A virus

(H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc. Natl. Acad. Sci. U S A. 101:1356-1361.

29. Gabriel, G, Dauber B, Wolff T, Planz O, Klenk HD, and Stech J. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. U S A. 102:18590-18595.

30. Gabriel, G, Herwig A, and Klenk HD. 2008. Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathog. 4:e11.

31. Gammelin, M, Mandler J, and Scholtissek C. 1989. Two subtypes of nucleoproteins (NP) of influenza A viruses. Virology 170:71-80.

32. Gorman, OT, Bean WJ, Kawaoka Y, and Webster RG. 1990. Evolution of the nucleoprotein gene of influenza A virus. J. Virol. 64:1487-1497.

33. Gorman, OT, Bean WJ, and Webster RG. 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Curr. Top. Microbiol. Immunol. 176:75-97.

34. Guan, Y, Shortridge KF, Krauss S, Li PH, Kawaoka Y, and Webster RG. 1996. Emergence of avian H1N1 influenza viruses in pigs in China. J. Virol. 70:8041-8046.

35. Guan, Y, Shortridge KF, Krauss S, and Webster RG. 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc. Natl. Acad. Sci. U S A. 96:9363-9367.

36. Hatta, M, Halfmann P, Wells K, and Kawaoka Y. 2002. Human influenza a viral genes responsible for the restriction of its replication in duck intestine. Virology 295:250-255.

37. He, Q, Velumani S, Du Q, Lim CW, Ng FK, Donis R, and Kwang J. 2007. Detection of H5 avian influenza viruses by antigen-capture enzyme-linked immunosorbent assay using H5-specific monoclonal antibody. Clin. Vaccine Immunol. 14:617-623.

38. Hinshaw, VS, Webster RG, Naeve CW, and Murphy BR. 1983. Altered tissue tropism of human-avian reassortant influenza viruses. Virology 128:260-263.

39. Hinshaw, VS, Wood JM, Webster RG, Deibel R, and Turner B. 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bull. World. Health. Organ. 63:711-719.

40. Hirst, M, Astell CR, Griffith M, Coughlin SM, Moksa M, Zeng T, Smailus DE, Holt RA, Jones S, Marra MA, Petric M, Krajden M, Lawrence D, Mak A, Chow R, Skowronski DM, Tweed SA, Goh S, Brunham RC, Robinson J, Bowes V, Sojonky K, Byrne SK, Li Y, Kobasa D, Booth T, and Paetzel M. 2004. Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg. Infect. Dis. 10:2192-2195.

41. Hoffmann, E, Krauss S, Perez D, Webby R, and Webster RG. 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 20:3165-3170.

42. Hoffmann, E, Neumann G, Kawaoka Y, Hobom G, and Webster RG. 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A. 97:6108-6113.

43. Hoffmann, E, Stech J, Guan Y, Webster RG, and Perez DR. 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146:2275-2289.

44. Honda, A, Mizumoto K, and Ishihama A. 1999. Two separate sequences of PB2 subunit constitute the RNA cap-binding site of influenza virus RNA polymerase. Genes Cells 4:475-485.

45. Imai, M, Ninomiya A, Minekawa H, Notomi T, Ishizaki T, Tashiro M, and Odagiri T. 2006. Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian influenza virus infection. Vaccine 24:6679-6682.

46. Imai, M, Ninomiya A, Minekawa H, Notomi T, Ishizaki T, Van Tu P, Tien NT, Tashiro M, and Odagiri T. 2007. Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method. J. Virol. Methods 141:173-180.

47. Isoda, N, Sakoda Y, Kishida N, Bai GR, Matsuda K, Umemura T, and Kida H. 2006. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch. Virol. 151:1267-1279.

48. Ito, T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, and Kawaoka Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. 72:7367-7373.

49. Ito, T, Gorman OT, Kawaoka Y, Bean WJ, and Webster RG. 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J. Virol. 65:5491-5498.

50. Ito, T, Okazaki K, Kawaoka Y, Takada A, Webster RG, and Kida H. 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch. Virol. 140:1163-1172.

51. Jackwood, MW, and Stallknecht DE. 2007. Molecular epidemiologic studies on North American H9 avian influenza virus isolates from waterfowl and shorebirds. Avian Dis. 51:448-450.

52. Jorba, N, Juarez S, Torreira E, Gastaminza P, Zamarreno N, Albar JP, and Ortin J. 2008. Analysis of the interaction of influenza virus polymerase complex with human cell factors. Proteomics 8:2077-2088.

53. Kaleta, EF, Hergarten G, and Yilmaz A. 2005. Avian influenza A viruses in birds -- an ecological, ornithological and virological view. Dtsch. Tierarztl. Wochenschr. 112:448-456.

54. Kawaoka, Y, Chambers TM, Sladen WL, and Webster RG. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163:247-250.

55. Kawaoka, Y, Krauss S, and Webster RG. 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. 63:4603-4608.

56. Keawcharoen, J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theambooniers A, Tantilertcharoen R,

Pattanarangsan R, Arya N, Ratanakorn P, Osterhaus DM, and Poovorawan Y. 2004. Avian influenza H5N1 in tigers and leopards. Emerg. Infect. Dis. 10:2189-2191.

57. Kida, H, Brown LE, and Webster RG. 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 122:38-47.

58. Kida, H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, and Webster RG. 1994. Potential for transmission of avian influenza viruses to pigs. J. Gen. Virol. 75:2183-2188.

59. Kida, H, Kawaoka Y, Naeve CW, and Webster RG. 1987. Antigenic and genetic conservation of H3 influenza virus in wild ducks. Virology 159:109-119.

60. Kida, H, Shortridge KF, and Webster RG. 1988. Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. Virology 162:160-166.

61. Kida, H, and Yanagawa R. 1979. Isolation and characterization of influenza a viruses from wild free-flying ducks in Hokkaido, Japan. Zentralbl. Bakteriol [Orig A]. 244:135-143.

62. Kida, H, Yanagawa R, and Matsuoka Y. 1980. Duck influenza lacking evidence of disease signs and immune response. Infect. Immun. 30:547-553.

63. Koopmans, M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, and Bosman A. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363:587-593.

64. Labadie, K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, and Naffakh N. 2007. Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology 362:271-282.

65. Lamb, RA. 1989. The genes and proteins of the influenza viruses, p. 1-87. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.

66. Lamb, RA, and Krug RM. 2001. Orthomyxoviridae: The Viruses and Their Replication. *In* D. M. Knipe and P. M. Howley (ed.), Fields-Virology, vol. 1.

67. Lamb, RA, and Lai CJ. 1981. Conservation of the influenza virus membrane protein (M1) amino acid sequence and an open reading frame of RNA segment 7 encoding a second protein (M2) in H1N1 and H3N2 strains. Virology 112:746-751.

68. Landolt, GA, Karasin AI, Schutten MM, and Olsen CW. 2006. Restricted infectivity of a human-Lineage H3N2 influenza A virus in pigs is hemagglutinin and neuraminidase gene dependent. J. Clin. Microbiol. 44:297-301.

69. Lee, MS, Chang PC, Shien JH, Cheng MC, Chen CL, and Shieh HK. 2006. Genetic and pathogenic characterization of H6N1 avian influenza viruses isolated in Taiwan between 1972 and 2005. Avian Dis. 50:561-571.

70. Lee, MS, Chang PC, Shien JH, Cheng MC, and Shieh HK. 2001. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J. Virol. Methods 97:13-22.

71. Li, Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, and Yu K. 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J. Virol. 79:12058-12064.

72. Liu, JH, Okazaki K, Bai GR, Shi WM, Mweene A, and Kida H. 2004. Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. Virus Genes 29:81-86.

73. Manzoor, R, Sakoda Y, Sakabe SM, T., Namba Y, Tsuda Y, and Kida H. 2008. Development of a Pen-Site Test Kit for the Rapid Diagnosis of H7 Highly Pathogenic Avian Influenza. J. Vet. Med. Sci. 70:557-562.

74. Mase, M, Tanimura N, Imada T, Okamatsu M, Tsukamoto K, and Yamaguchi S. 2006. Recent H5N1 avian influenza A virus increases rapidly in virulence to mice after a single passage in mice. J. Gen. Virol. 87:3655-3659.

75. Mase, M, Tsukamoto K, Imada T, Imai K, Tanimura N, Nakamura K, Yamamoto Y, Hitomi T, Kira T, Nakai T, Kiso M, Horimoto T, Kawaoka Y, and Yamaguchi S. 2005. Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. Virology 332:167-176.

76. Momose, F, Basler CF, O'Neill RE, Iwamatsu A, Palese P, and Nagata K. 2001. Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. J. Virol. 75:1899-1908.

77. Momose, F, Handa H, and Nagata K. 1996. Identification of host factors that regulate the influenza virus RNA polymerase activity. Biochimie 78:1103-1108.

78. Munster, VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, and Fouchier RA. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 3:e61.

79. Murphy, BR, Sly DL, Tierney EL, Hosier NT, Massicot JG, London WT, Chanock RM, Webster RG, and Hinshaw VS. 1982. Reassortant virus derived from avian and human influenza A viruses is attenuated and immunogenic in monkeys. Science 218:1330-1332.

80. Myers, KP, Olsen CW, and Gray GC. 2007. Cases of swine influenza in humans: a review of the literature. Clin. Infect. Dis. 44:1084-1088.

81. Naito, T, Momose F, Kawaguchi A, and Nagata K. 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J. Virol. 81:1339-1349.

82. Ohtsu, Y, Honda Y, Sakata Y, Kato H, and Toyoda T. 2002. Fine mapping of the subunit binding sites of influenza virus RNA polymerase. Microbiol. Immunol. 46:167-175.

83. OIE. 2005. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, http://www.oie.int/eng/normes/mmanual/A_00037.htm. OIE.

84. Okazaki, K, Takada A, Ito T, Imai M, Takakuwa H, Hatta M, Ozaki H, Tanizaki T, Nagano T, Ninomiya A, Demenev VA, Tyaptirganov MM, Karatayeva TD, Yamnikova SS, Lvov DK, and Kida H. 2000. Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch. Virol. 145:885-893.

85. Olsen, B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, and Fouchier RA. 2006. Global patterns of influenza a virus in wild birds. Science 312:384-388.

86. Peiris, JS, Guan Y, Markwell D, Ghose P, Webster RG, and Shortridge KF. 2001. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A

viruses in pigs in southeastern China: potential for genetic reassortment? J. Virol. 75:9679-9686.

87. Poole, E, Elton D, Medcalf L, and Digard P. 2004. Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology 321:120-133.

88. Reed, LJ, and Muench HA. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493.

89. Rogers, GN, and D'Souza BL. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology 173:317-322.

90. Rogers, GN, and Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127:361-373.

91. Sakabe, S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, Saijo K, Sawata A, Kume K, Hagiwara J, Tuchiya K, Lin Z, Sakamoto R, Imamura T, Sasaki T, Kokumai N, Kawaoka Y, and Kida H. 2008. A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens. Vaccine 26:2127-2134.

92. Scholtissek, C, Burger H, Bachmann PA, and Hannoun C. 1983. Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. Virology 129:521-523.

93. Scholtissek, C, Burger H, Kistner O, and Shortridge KF. 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147:287-294.

94. Scholtissek, C, Rohde W, Von Hoyningen V, and Rott R. 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87:13-20.

95. Schweiger, B, Bruns L, and Meixenberger K. 2006. Reassortment between human A(H3N2) viruses is an important evolutionary mechanism. Vaccine 24:6683-6690.

96. Sever, JL. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320-329.

97. Shapiro, GI, and Krug RM. 1988. Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J Virol. 62:2285-2290.

98. Sharp, GB, Kawaoka Y, Jones DJ, Bean WJ, Pryor SP, Hinshaw V, and Webster RG. 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. J. Virol. 71:6128-6135.

99. Shortridge, KF, and Webster RG. 1979. Geographical distribution of swine (Hsw1N1) and Hong Kong (H3N2) influenza virus variants in pigs in Southeast Asia. Intervirology 11:9-15.

100. Shortridge, KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A, and Webster RG. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252:331-342.

101. Snyder, MH, Buckler-White AJ, London WT, Tierney EL, and Murphy BR. 1987. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. J. Virol. 61:2857-2863.

102. Songserm, T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R, and Poovorawan Y. 2006. Fatal avian influenza A H5N1 in a dog. Emerg. Infect. Dis. 12:1744-1747.

103. Songsermn, T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, and Poovorawan Y. 2006. Avian influenza H5N1 in naturally infected domestic cat. Emerg. Infect. Dis. 12:681-683.

104. Spackman, E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, and Suarez DL. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256-3260.

105. Suarez, DL, Das A, and Ellis E. 2007. Review of rapid molecular diagnostic tools for avian influenza virus. Avian Dis. 51:201-208.

106. Subbarao, EK, London W, and Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. 67:1761-1764.

107. Swayne, DE, and Suarez DL. 2000. Highly pathogenic avian influenza. Rev. Sci. Tech. 19:463-482.

108. Tamura, K, Dudley J, Nei M, and Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

109. Tamura, K, Nei M, and Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U S A. 101:11030-11035.

110. Thompson, JD, Higgins DG, and Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

111. Tian, SF, Buckler-White AJ, London WT, Reck LJ, Chanock RM, and Murphy BR. 1985. Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/Mallard/NY/78 virus and its reassortants in squirrel monkey respiratory tract. J. Virol. 53:771-775.

112. Tsuda, Y, Sakoda Y, Sakabe S, Mochizuki T, Namba Y, and Kida H. 2007. Development of an immunochromatographic kit for rapid diagnosis of H5 avian influenza virus infection. Microbiol. Immunol. 51:903-907.

113. Tweed, SA, Skowronski DM, David ST, Larder A, Petric M, Lees W, Li Y, Katz J, Krajden M, Tellier R, Halpert C, Hirst M, Astell C, Lawrence D, and Mak A. 2004. Human illness from avian influenza H7N3, British Columbia. Emerg. Infect. Dis. 10:2196-2199.

114. Van Reeth, K. 2007. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet. Res. 38:243-260.

115. Wallace, RG, Hodac H, Lathrop RH, and Fitch WM. 2007. A statistical phylogeography of influenza A H5N1. Proc. Natl. Acad. Sci. U S A. 104:4473-4478.

116. Wang, G, Zhan D, Li L, Lei F, Liu B, Liu D, Xiao H, Feng Y, Li J, Yang B, Yin Z, Song X, Zhu X, Cong Y, Pu J, Wang J, Liu J, Gao GF, and Zhu Q. 2008. H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. J. Gen. Virol. 89:697-702.

117. Webster, RG, Bean WJ, Gorman OT, Chambers TM, and Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152-179.

118. Webster, RG, and Laver WG. 1972. The origin of pandemic influenza. Bull. World Health Organ. 47:449-452.

119. WHO 2008, posting date. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_06_19/en/index.html. [Online.]

120. Widjaja, L, Krauss SL, Webby RJ, Xie T, and Webster RG. 2004. Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses. J. Virol. 78:8771-8779.

121. Yao, Y, Mingay LJ, McCauley JW, and Barclay WS. 2001. Sequences in influenza A virus PB2 protein that determine productive infection for an avian influenza virus in mouse and human cell lines. J. Virol. 75:5410-5415.

122. Yasuda, J, Shortridge KF, Shimizu Y, and Kida H. 1991. Molecular evidence for a role of domestic ducks in the introduction of avian H3 influenza viruses to pigs in southern China, where the A/Hong Kong/68 (H3N2) strain emerged. J. Gen. Virol. 72:2007-2010.

和文要旨

H7 インフルエンザウイルスの感染に起因する鳥インフルエンザの迅速診断法の確立、鳥インフルエンザウイルス M 遺伝子の系統進化解析ならびに高病原性鳥インフルエンザウイルス Ck/Yamaguchi/04 (H5N1)の宿主域を決定する因子の解明を目指して研究した。

H7 亜型ウイルスの感染による鳥インフルエンザの迅速診断キットを開発した。このキットは検査に用いたすべての H7 ウイルスを特異的に検出した。さらに、このキットは、実験的に感染させたニワトリの組織乳剤および気管とクロアカのスワブから H7 ウイルスを検出した。以上の結果から、本キットが迅速診断に有用であり、鳥インフルエンザの制圧に貢献するものと期待される。

2000 から 2007 年の間に、北海道で渡り水禽から 28 通りの異なる HA と NA の組み合わせのインフルエンザウイルス 218 株が分離された。分離年と場所に基づき、67 株を選び、M 遺伝子の塩基配列を決定した。その結果、異なる亜系統に属するウイルスが自然界の水鳥の間で受け継がれて存続していることが明らかになった。さらに、台湾で分離された H6N1 インフルエンザウイルスが、渡り鳥の太平洋飛翔路に沿ってもたらされたものであることも明らかになった。また、北海道で分離されたウイルスがヨーロッパおよびアジアの多くの国で分離された株と近縁な系統関係にあることが明らかになった。この知見は、水禽が、その飛翔路に沿ってウイルスを運搬するばかりでなく、それを越えてウイルスを拡散する役割を演ずることを示している。

ブタが鳥インフルエンザウイルスとヒトのウイルスの遺伝子再集合体を産生する mixing vessel として、過去のヒトの新型インフルエンザウイルスが出現したことが証明されており、また、ブタは多くの亜型の鳥インフルエンザウイルスに感染することが知られている。ところで、日本でニワトリから分離された高病原性鳥インフルエンザウイルス Ck/Yamaguchi/04 (H5N1)株は、ブタに実験感染しなかった。この株がブタに感染しない分子基盤を明らかにするため、ブタ分離株との遺伝子再集合ウイルスをリバースジェネティクスによって作出して解析した。その結果、PB2 遺伝子のみがブタ分離株由来の遺伝子再集合体がブタで増殖することが判った。さらに、H5N1 ウイルスの PB2 タンパクの 2 アミノ酸残基の置換がブタにおける増殖と関連することが明らかになった。

Table 1. Influenza viruses isolated from fecal samples of free-flying water birds during 2000-2007

Location	Subtypes of influenza viruses isolated in following years							
	2000	2001	2002	2003	2004	2005	2006	2007
Sapporo	NC[a]	H1N1 (8)[b] H3N6 (1) H4N5 (1) H4N6 (2)	H3N8 (3) H5N3 (1) H11N9 (3)	H3N8 (11) H6N8 (2) H7N7 (18) H8N4 (1)	H1N1 (1) H3N8 (1) H4N2 (7) H5N3 (3) H6N1 (5) H6N8 (2) H10N5 (7)	H3N2 (1) H6N2 (4) H8N4 (2)	H3N8 (5) H4N6 (1) H6N2 (1) H9N2 (1) H11N9 (3)	H3N8 (2) H4N6 (2) H5N3 (2) H8N4 (2) H12N5 (1)
Wakkanai	H4N6 (1) H5N3 (2) H6N2 (2) H8N4 (1) H9N2 (1) H10N4 (12)	H2N2 (1) H2N3 (4) H3N8 (6) H6N2 (4) H12N5 (2)	NI[c]	NC	H4N6 (6) H6N2 (10) H6N8 (2) H7N7 (13) H8N4 (1) H9N2 (2) H10N6 (1) H11N9 (1) H12N5 (1)	H2N5 (1) H3N8 (3) H6N1 (1) H6N2 (3)	H3N6 (2) H3N8 (1) H4N9 (3) H6N1 (4) H6N5 (1) H9N2 (1) H10N8 (1) H11N9 (11) H13N6 (2)	H1N1 (1) H3N8 (1) H4N6 (2) H8N4 (1) H10N2 (1) H10N7 (1)

[a] Samples were not collected.

[b] Number of isolates of each antigenic subtype are shown in parenthesis.

[c] No virus was isolated.

Table 3. Genetic homologies and lineages of each gene of A/duck/Hokkaido/W95/2006 (H10N8)

Gene segment*	Nucleotide position sequenced (size in bp)	Viruses with highest homology		Homology	Lineage
		Name	Accession no.		
PB2	844 -1,385 (542)	A/duck/Hokkaido/120/2001 (H6N2)	AB286653	98%	Eurasian
PB1	1,279 -2,294 (1,016)	A/duck/Shiga/8/2004 (H4N6)	AB304145	99%	Eurasian
PA	1,110 -1,612 (503)	A/duck/Jiang-Xi/3345/2005 (H5N2)	EF597429	98%	Eurasian
HA	1,200 -1,683 (484)	A/duck/Hokkaido/18/2000 (H10N4)	AB274041	98%	Eurasian
NP	962 -1,497 (536)	A/duck/Hokkaido/262/2004 (H6N1)	AB358963	99%	Eurasian
NA	893 -1,419 (527)	A/duck/Hokkaido/228/2003 (H6N8)	AB294220	99%	North American
M	4 -976 (973)	A/pintal/Alberta/49/2003 (H12N5)	CY004339	98%	North American
NS	4 -547 (544)	A/aquatic bird/Korea/JN-2/2006 (H3N2)	EU301342	98%	Eurasian

* Accession number of each gene of A/duck/Hokkaido/W95/2006 (H10N8): PB2 (AB430756), PB1 (AB430749), PA (AB430750), HA (AB430751), NP (AB430752), NA (AB430753), M (AB428709) and NS (AB430754).

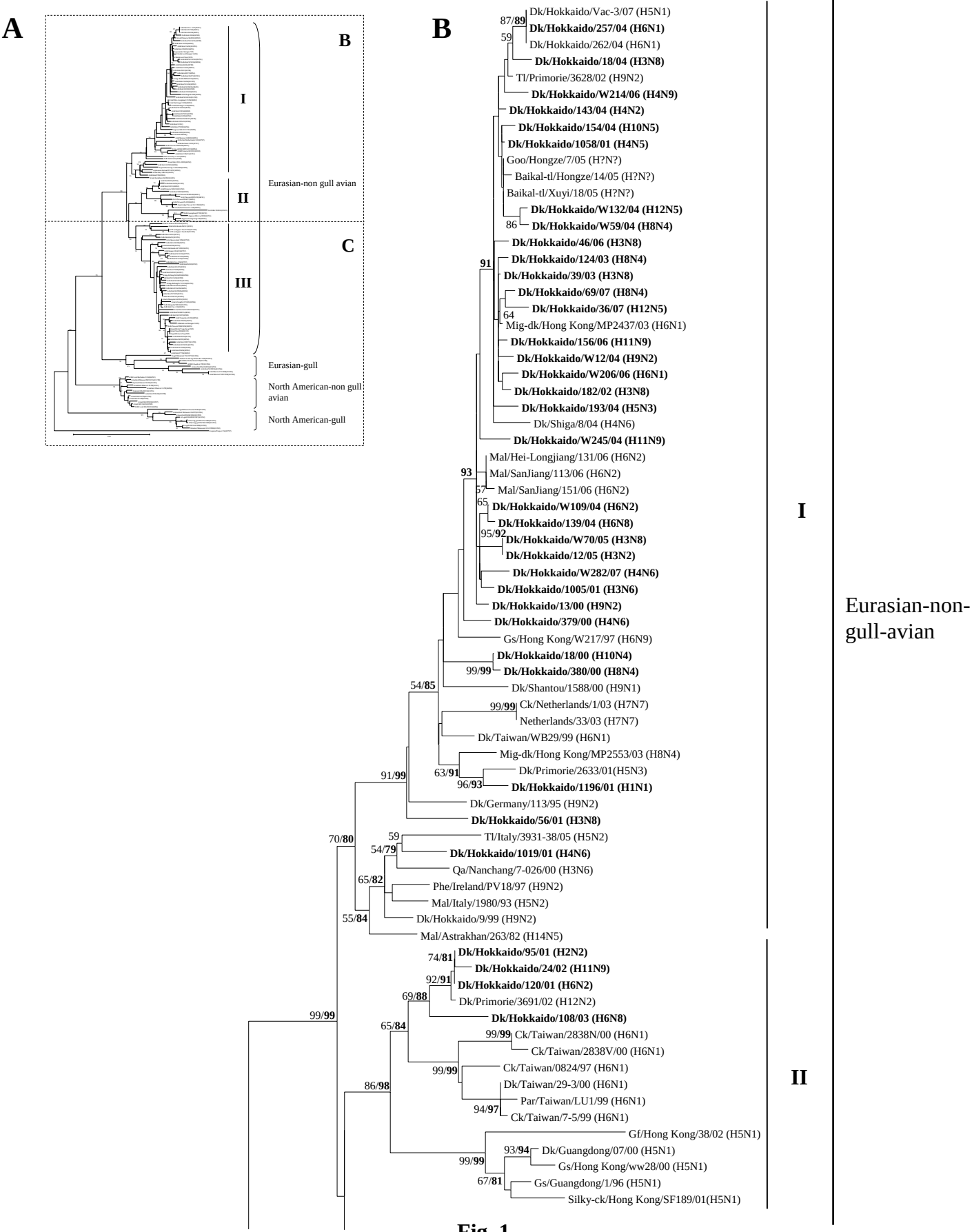
A
B
C
I
II
III
Eurasian-non gull avian
Eurasian-gull
North American-non gull avian
North American-gull
B
Dk/Hokkaido/Vac-3/07 (H5N1)
Dk/Hokkaido/257/04 (H6N1)
Dk/Hokkaido/262/04 (H6N1)
Dk/Hokkaido/18/04 (H3N8)
Tl/Primorie/3628/02 (H9N2)
Dk/Hokkaido/W214/06 (H4N9)
Dk/Hokkaido/143/04 (H4N2)
Dk/Hokkaido/154/04 (H10N5)
Dk/Hokkaido/1058/01 (H4N5)
Goo/Hongze/7/05 (H?N?)
Baikal-tl/Hongze/14/05 (H?N?)
Baikal-tl/Xuyi/18/05 (H?N?)
Dk/Hokkaido/W132/04 (H12N5)
Dk/Hokkaido/W59/04 (H8N4)
Dk/Hokkaido/46/06 (H3N8)
Dk/Hokkaido/124/03 (H8N4)
Dk/Hokkaido/39/03 (H3N8)
Dk/Hokkaido/69/07 (H8N4)
Dk/Hokkaido/36/07 (H12N5)
Mig-dk/Hong Kong/MP2437/03 (H6N1)
Dk/Hokkaido/156/06 (H11N9)
Dk/Hokkaido/W12/04 (H9N2)
Dk/Hokkaido/W206/06 (H6N1)
Dk/Hokkaido/182/02 (H3N8)
Dk/Hokkaido/193/04 (H5N3)
Dk/Shiga/8/04 (H4N6)
Dk/Hokkaido/W245/04 (H11N9)
Mal/Hei-Longjiang/131/06 (H6N2)
Mal/SanJiang/113/06 (H6N2)
Mal/SanJiang/151/06 (H6N2)
Dk/Hokkaido/W109/04 (H6N2)
Dk/Hokkaido/139/04 (H6N8)
Dk/Hokkaido/W70/05 (H3N8)
Dk/Hokkaido/12/05 (H3N2)
Dk/Hokkaido/W282/07 (H4N6)
Dk/Hokkaido/1005/01 (H3N6)
Dk/Hokkaido/13/00 (H9N2)
Dk/Hokkaido/379/00 (H4N6)
Gs/Hong Kong/W217/97 (H6N9)
Dk/Hokkaido/18/00 (H10N4)
Dk/Hokkaido/380/00 (H8N4)
Dk/Shantou/1588/00 (H9N1)
Ck/Netherlands/1/03 (H7N7)
Netherlands/33/03 (H7N7)
Dk/Taiwan/WB29/99 (H6N1)
Mig-dk/Hong Kong/MP2553/03 (H8N4)
Dk/Primorie/2633/01(H5N3)
Dk/Hokkaido/1196/01 (H1N1)
Dk/Germany/113/95 (H9N2)
Dk/Hokkaido/56/01 (H3N8)
Tl/Italy/3931-38/05 (H5N2)
Dk/Hokkaido/1019/01 (H4N6)
Qa/Nanchang/7-026/00 (H3N6)
Phe/Ireland/PV18/97 (H9N2)
Mal/Italy/1980/93 (H5N2)
Dk/Hokkaido/9/99 (H9N2)
Mal/Astrakhan/263/82 (H14N5)
Dk/Hokkaido/95/01 (H2N2)
Dk/Hokkaido/24/02 (H11N9)
Dk/Hokkaido/120/01 (H6N2)
Dk/Primorie/3691/02 (H12N2)
Dk/Hokkaido/108/03 (H6N8)
Ck/Taiwan/2838N/00 (H6N1)
Ck/Taiwan/2838V/00 (H6N1)
Ck/Taiwan/0824/97 (H6N1)
Dk/Taiwan/29-3/00 (H6N1)
Par/Taiwan/LU1/99 (H6N1)
Ck/Taiwan/7-5/99 (H6N1)
Gf/Hong Kong/38/02 (H5N1)
Dk/Guangdong/07/00 (H5N1)
Gs/Hong Kong/ww28/00 (H5N1)
Gs/Guangdong/1/96 (H5N1)
Silky-ck/Hong Kong/SF189/01(H5N1)
I
II
Eurasian-non-gull-avian
0.05

Fig. 1

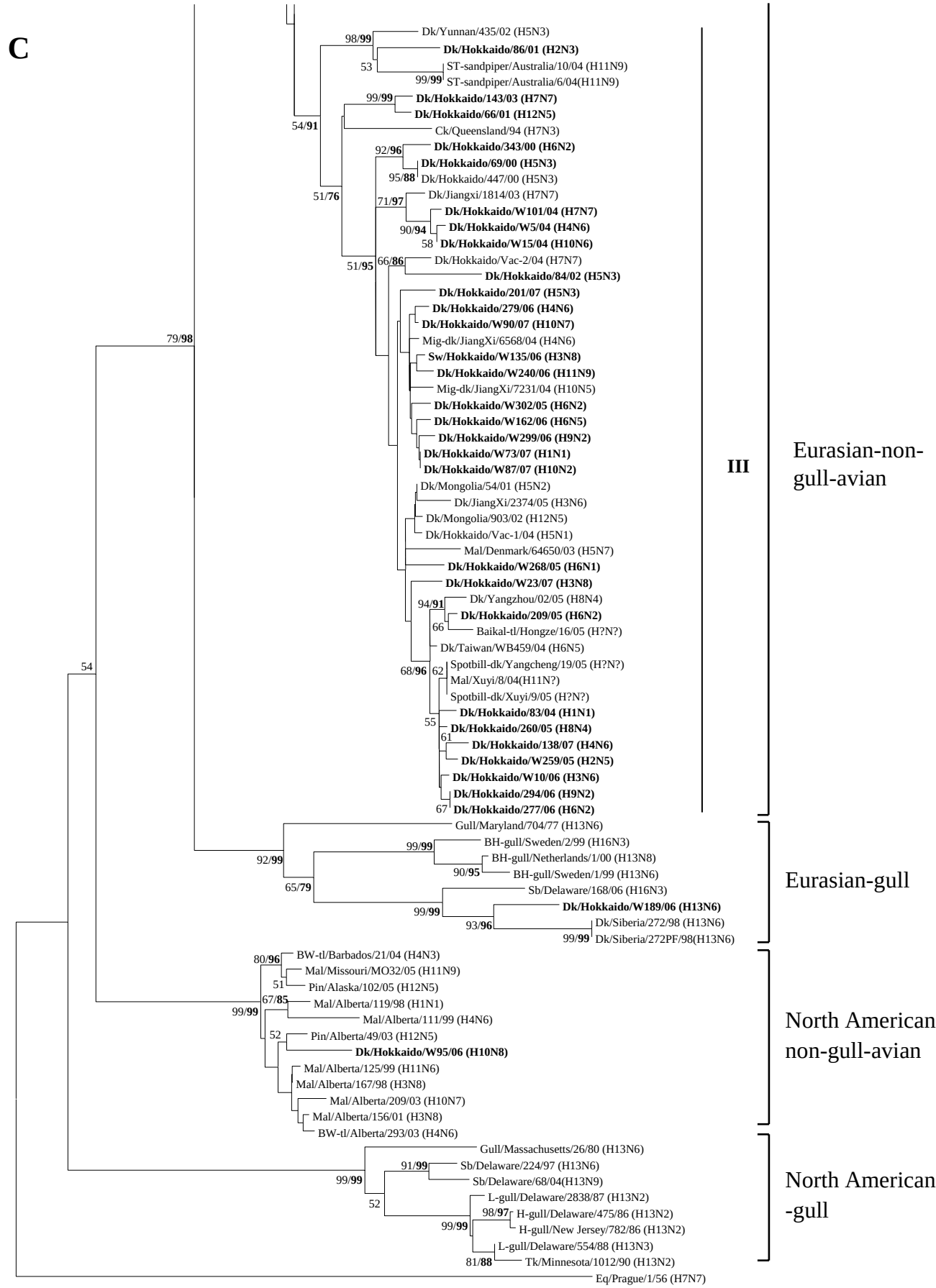

Fig. 1 *Continued*

Table 1. Virus recovery and subtyping of virus clones from nasal swab samples

Days post-inoculation	Total plaques picked	HA subtype of virus clones	
		H5	H1
Day 1	61	11	50
Day 2	38	–	38
Day 3	20	–	20

Table 2. Genome segment origin and susceptibility of pigs to H5N1 subtype virus clones recovered from nasal swabs of inoculated pigs

Virus Clone No.	Genome segment origin[a]								Susceptibility of pigs to virus clones	
	PB2	PB1	PA	HA	NP	NA	M	NS	Replication	Antibody response
1	Sw[b]	Ck[c]	Ck	Ck	Ck	Ck	Ck	Ck	+[d]	+
2	Sw	Ck	Ck	Ck	Ck	Ck	Ck	Ck	+[e]	+
3	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	–	–
4	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	–	–
5-11	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	ND[f]	ND

[a] Genome segment origin was determined by partial sequencing.
[b] Sw: Swine.
[c] Ck: Chicken.
[d] Virus was recovered from day 2-5 p.i. and titer ranged from $10^{1.5}$ – $10^{3.8}$ EID_{50}/ml.
[e] Virus was recovered from day 1-3 p.i. and titer ranged from $10^{1.5}$ – $10^{2.8}$ EID_{50}/ml.
[f] Not determined.

Table 3. Virus titers in nasal swabs of pigs inoculated with viruses produced by reverse genetics

Virus clone name	Pig No.	Virus titers expressed as $\log_{10}EID_{50}$/ml (days post-infection)										Antibody response
		1	2	3	4	5	6	7	8	9	10	
rg-Ck/Yamaguchi/04 (H5N1)	1-2	–[a]	–	–	–	–	–	–	ND[b]	ND	ND	–
rg- Sw/Hokkaido/81 (H1N1)	3	5.8	≥6.5	6.3	5.5	3.8	4.8	–	1.8	1.8	1.3	+
	4	5.3	≥6.5	6.3	5.8	2.5	3.3	–	–	3.5	2.3	+
rg-H5N1-PB2H1N1	5	3.5	3.8	1.3	–	–	–	–	ND	ND	ND	+
	6	–	1.5	1.3	–	–	ND	ND	ND	ND	ND	+
rg-H1N1-PB2H5N1	7	–	–	3.8[c]	5.8	4.3	4.8	2.5	2.8	1.8	–	+
	8	–	–	1.3[c]	3.5	2.3	4.8	3.5	2.8	–	–	+

[a] <1.5 $\log_{10}EID_{50}$/ml.

[b] Not determined.

[c] Virus isolates were sequenced and results are shown in Table 4.

Table 4. Comparison of amino acid sequence of PB2 genes of viruses recovered from pigs inoculated with rg H1N1-PB2H5N1 virus

	PB2 gene	
Virus isolate name	256[a]	627
rg-H1N1-PB2H5N1(inoculum)	D	E
Pig 7-day 3	●	K
Pig 8-day 3	G	●
rg-Sw/Hokkaido/81 (H1N1)	●	K

[a] amino acid position

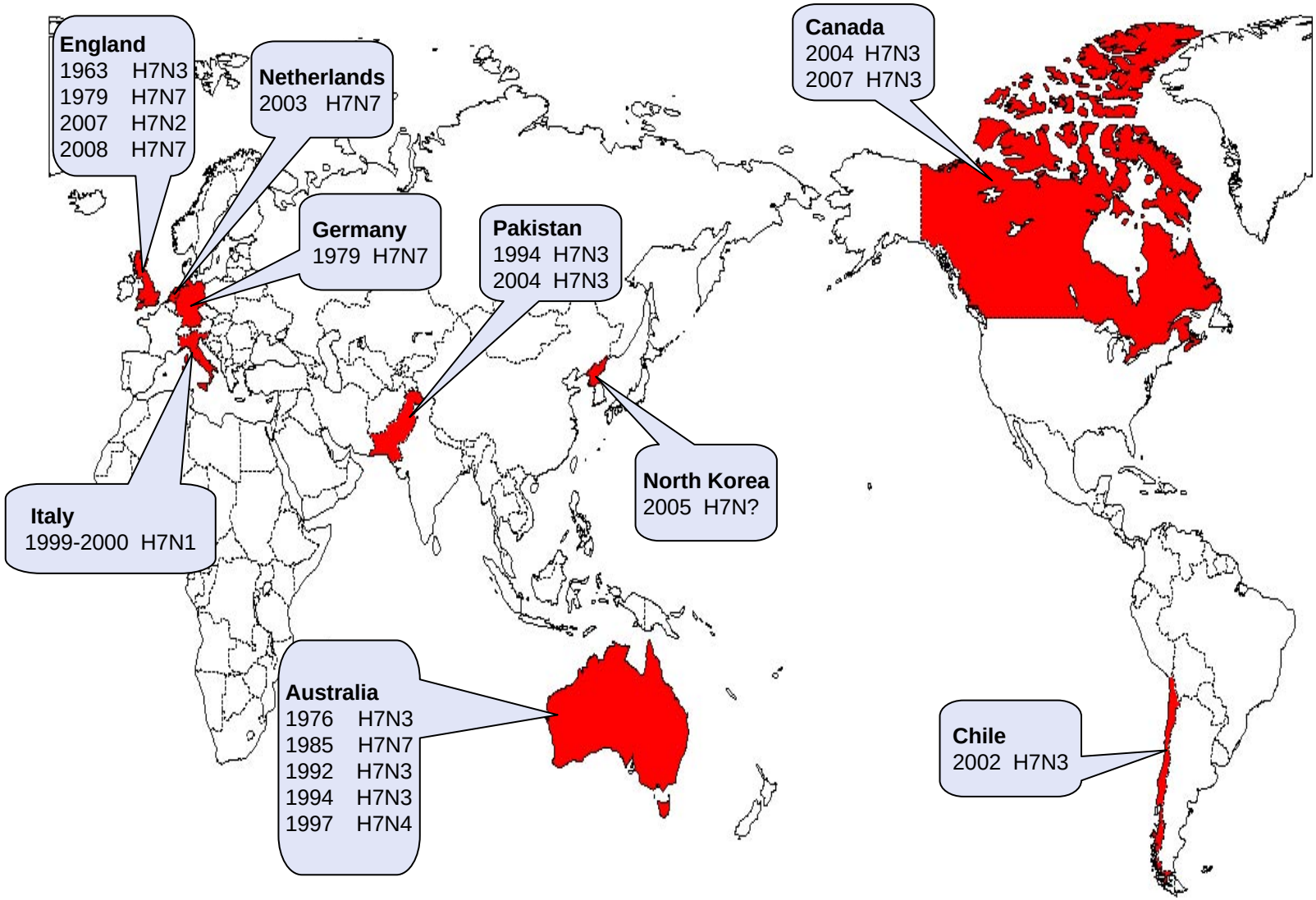

Fig. 1 Areas showing confirmed cases of avian influenza caused by H7 subtype influenza viruses.

Table 2. Detection limit of the kit to detect antigen of the H7 subtype viruses

Kit name	Detection limit ($\log_{10}EID_{50}$/test)			
	A/chicken/Netherlands /2586/2003 (H7N7)	A/chicken/Pakistan /447/1995 (H7N3)	A/seal/Massachusetts /1/1980 (H7N7)	A/duck/Hokkaido /Vac-2/2004 (H7N7)
H7 HA antigen detection kit	5.3	3.7	3.9	4.1
Capilia® Flu A+B kit	4.1	3.1	4.2	4.4

Table 3. Antigen detection and virus titers in the swabs and tissue homogenates of chickens infected with A/chicken/Netherlands/2586/2003 (H7N7)

Days p.i.	Clinical signs[a]	Swab samples		Tissue homogenates		
		Trachea	Cloaca	Trachea	Kidney	Colon
0	−	−[b]/ −[c]/ −[d]	− / − / −	− / − / −	− / − / −	− / − / −
	−	− / − / −	− / − / −	− / − / −	− / − / −	− / − / −
1	−	− / − / 1.8	− / − / −	− / − / −	− / − / −	− / − / −
	−	− / − / 2.5	+ / + / 2.3	− / − / 2.5	− / − / −	− / − / −
	−	− / + / 4.5	− / − / −	− / − / 1.5	− / − / −	− / − / 1.8
2	−	− / − / 1.5	− / − / 3.5	− / − / 3.3	− / − / 3.5	− / − / 3.5
	−	− / − / 2.3	− / − / −	− / − / 2.8	− / − / 2.8	− / − / 2.8
	−	− / − / 3.5	− / − / 1.5	− / − / 3.8	− / + / 3.8	− / + / 3.3
3	+	− / + / 3.5	− / − / 2.8	− / + / 3.5	− / + / 3.8	− / + / 3.3
	+	− / − / 1.5	− / − / 1.5	− / − / −	− / − / 3.3	− / − / 3.5
	+	− / + / 4.5	− / − / 1.8	− / + / 4.5	− / + / 3.8	− / − / 3.5
4	++	+ / + / 5.8	− / + / 2.5	+ / + / 5.5	+ / + / 5.5	− / + / 4.8
	++	− / + / 3.5	− / − / −	− / + / 3.8	− / + / 3.8	− / − / 2.5
	++	− / + / 3.5	− / − / 2.3	− / + / 4.3	− / + / 3.8	− / + / 4.5
5	+++[†]	+ / + / 4.8	− / + / 3.3	+/ + / 4.5	+ / + / 6.8	+ / + / 5.5
	++	− / + / 2.8	+ / − / −	− / + / 3.8	+ / + / 6.5	+ / + / 6.5
	++	− / + / 4.5	+ / + / 2.5	− / + / 4.5	+ / + / 7.8	− / + / 5.5
6	+++[†]	− / + / 2.3	+ / − / 2.5	− / + / 4.3	+ / + / 4.5	+ / + / 4.5
	+++	− / − / −	+ / − / 2.3	− / − / 3.3	+ / + / 7.5	+ / + / 4.5
	++	− / + / 3.5	− / − / 2.8	− / + / 3.3	+ / + / 6.5	+ / + / 4.8

[a] Clinical signs recorded on the day of sampling, − : No clinical signs, +: Huddling together but alert, ++: Gasping, ruffled feathers, reduced feed intake and depressed, +++ : In addition to respiratory signs, chicken developed paralysis. [b] Result of H7 HA antigen detection kit, [c] Result of Capilia® Flu A+B diagnosis kit, [d] Virus titer of swab sample ($log_{10}EID_{50}$/ml) and tissue homogenates ($log_{10}EID_{50}$/g). − : < 1.5 $log_{10}EID_{50}$/ml or g. [†] Dead chicken

Table 1. Specificity of the kit for the detection of the H7 HA antigen

Viruses	Subtypes	Results
A/duck/Tottori/723/1980	H1N1	–
A/duck/Hokkaido/17/2001	H2N3	–
A/duck/Mongolia/4/2003	H3N8	–
A/duck/Czechoslovakia/1956	H4N6	–
A/duck/Pennsylvania/10218/1984	H5N2	–
A/chicken/Yamaguchi/7/2004	H5N1	–
A/whooper swan/Mongolia/2/2006	H5N1	–
A/duck/Yokohama/aq-10/2003	H5N1	–
A/turkey/Massachusetts/3740/1965	H6N2	–
A/seal/Massachusetts/1/1980	H7N7	+
A/chicken/Netherlands/2586/2003	H7N7	+
A/duck/Hokkaido/Vac-2/2004	H7N7	+
A/turkey/England/1963	H7N3	+
A/turkey/Italy/4580/1999	H7N1	+
A/chicken/Pakistan/447/1995	H7N3	+
A/duck/Mongolia/47/2001	H7N1	+
A/duck/Hokkaido/143/2003	H7N7	+
A/gull/Shimane/91/1988	H7N8	+
A/duck/Taiwan/4201/1999	H7N7	+
A/swan/Shimane/42/1999	H7N8	+
A/duck/Hong Kong/47/1976	H7N2	+
A/turkey/Tennessee/1/1979	H7N3	+
A/duck/Hokkaido/W128/2004	H7N7	+
A/duck/Hokkaido/W182/2004	H7N7	+
A/turkey/Ontario/6118/1968	H8N4	–
A/turkey/Wisconsin/1/1966	H9N2	–
A/chicken/Germany/N/1949	H10N7	–
A/duck/England/1/1956	H11N6	–
A/duck/Alberta/60/1976	H12N5	–
A/gull/Maryland/704/1977	H13N6	–
A/mallard/Astrakhan/263/1982	H14N5	–
A/duck/Australia/341/1983	H15N8	–
A/black-headed gull/Sweden/5/1999	H16N3	–

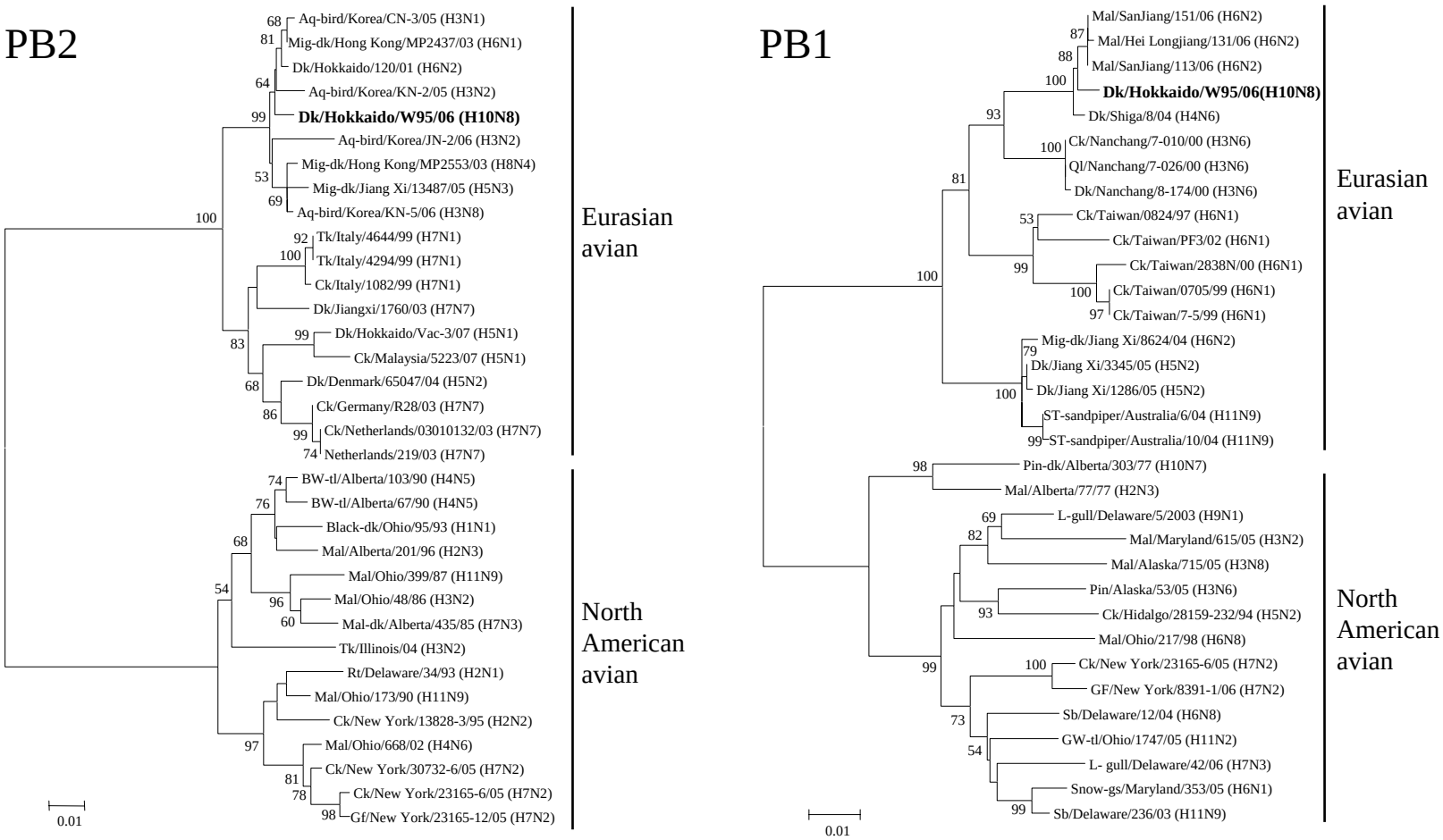
PB2
Aq-bird/Korea/CN-3/05 (H3N1)
Mig-dk/Hong Kong/MP2437/03 (H6N1)
Dk/Hokkaido/120/01 (H6N2)
Aq-bird/Korea/KN-2/05 (H3N2)
Dk/Hokkaido/W95/06 (H10N8)
Aq-bird/Korea/JN-2/06 (H3N2)
Mig-dk/Hong Kong/MP2553/03 (H8N4)
Mig-dk/Jiang Xi/13487/05 (H5N3)
Aq-bird/Korea/KN-5/06 (H3N8)
Tk/Italy/4644/99 (H7N1)
Tk/Italy/4294/99 (H7N1)
Ck/Italy/1082/99 (H7N1)
Dk/Jiangxi/1760/03 (H7N7)
Dk/Hokkaido/Vac-3/07 (H5N1)
Ck/Malaysia/5223/07 (H5N1)
Dk/Denmark/65047/04 (H5N2)
Ck/Germany/R28/03 (H7N7)
Ck/Netherlands/03010132/03 (H7N7)
Netherlands/219/03 (H7N7)
BW-tl/Alberta/103/90 (H4N5)
BW-tl/Alberta/67/90 (H4N5)
Black-dk/Ohio/95/93 (H1N1)
Mal/Alberta/201/96 (H2N3)
Mal/Ohio/399/87 (H11N9)
Mal/Ohio/48/86 (H3N2)
Mal-dk/Alberta/435/85 (H7N3)
Tk/Illinois/04 (H3N2)
Rt/Delaware/34/93 (H2N1)
Mal/Ohio/173/90 (H11N9)
Ck/New York/13828-3/95 (H2N2)
Mal/Ohio/668/02 (H4N6)
Ck/New York/30732-6/05 (H7N2)
Ck/New York/23165-6/05 (H7N2)
Gf/New York/23165-12/05 (H7N2)
0.01
Eurasian avian
North American avian
PB1
Mal/SanJiang/151/06 (H6N2)
Mal/Hei Longjiang/131/06 (H6N2)
Mal/SanJiang/113/06 (H6N2)
Dk/Hokkaido/W95/06(H10N8)
Dk/Shiga/8/04 (H4N6)
Ck/Nanchang/7-010/00 (H3N6)
Ql/Nanchang/7-026/00 (H3N6)
Dk/Nanchang/8-174/00 (H3N6)
Ck/Taiwan/0824/97 (H6N1)
Ck/Taiwan/PF3/02 (H6N1)
Ck/Taiwan/2838N/00 (H6N1)
Ck/Taiwan/0705/99 (H6N1)
Ck/Taiwan/7-5/99 (H6N1)
Mig-dk/Jiang Xi/8624/04 (H6N2)
Dk/Jiang Xi/3345/05 (H5N2)
Dk/Jiang Xi/1286/05 (H5N2)
ST-sandpiper/Australia/6/04 (H11N9)
ST-sandpiper/Australia/10/04 (H11N9)
Pin-dk/Alberta/303/77 (H10N7)
Mal/Alberta/77/77 (H2N3)
L-gull/Delaware/5/2003 (H9N1)
Mal/Maryland/615/05 (H3N2)
Mal/Alaska/715/05 (H3N8)
Pin/Alaska/53/05 (H3N6)
Ck/Hidalgo/28159-232/94 (H5N2)
Mal/Ohio/217/98 (H6N8)
Ck/New York/23165-6/05 (H7N2)
GF/New York/8391-1/06 (H7N2)
Sb/Delaware/12/04 (H6N8)
GW-tl/Ohio/1747/05 (H11N2)
L- gull/Delaware/42/06 (H7N3)
Snow-gs/Maryland/353/05 (H6N1)
Sb/Delaware/236/03 (H11N9)
0.01
Eurasian avian
North American avian

Fig. 2

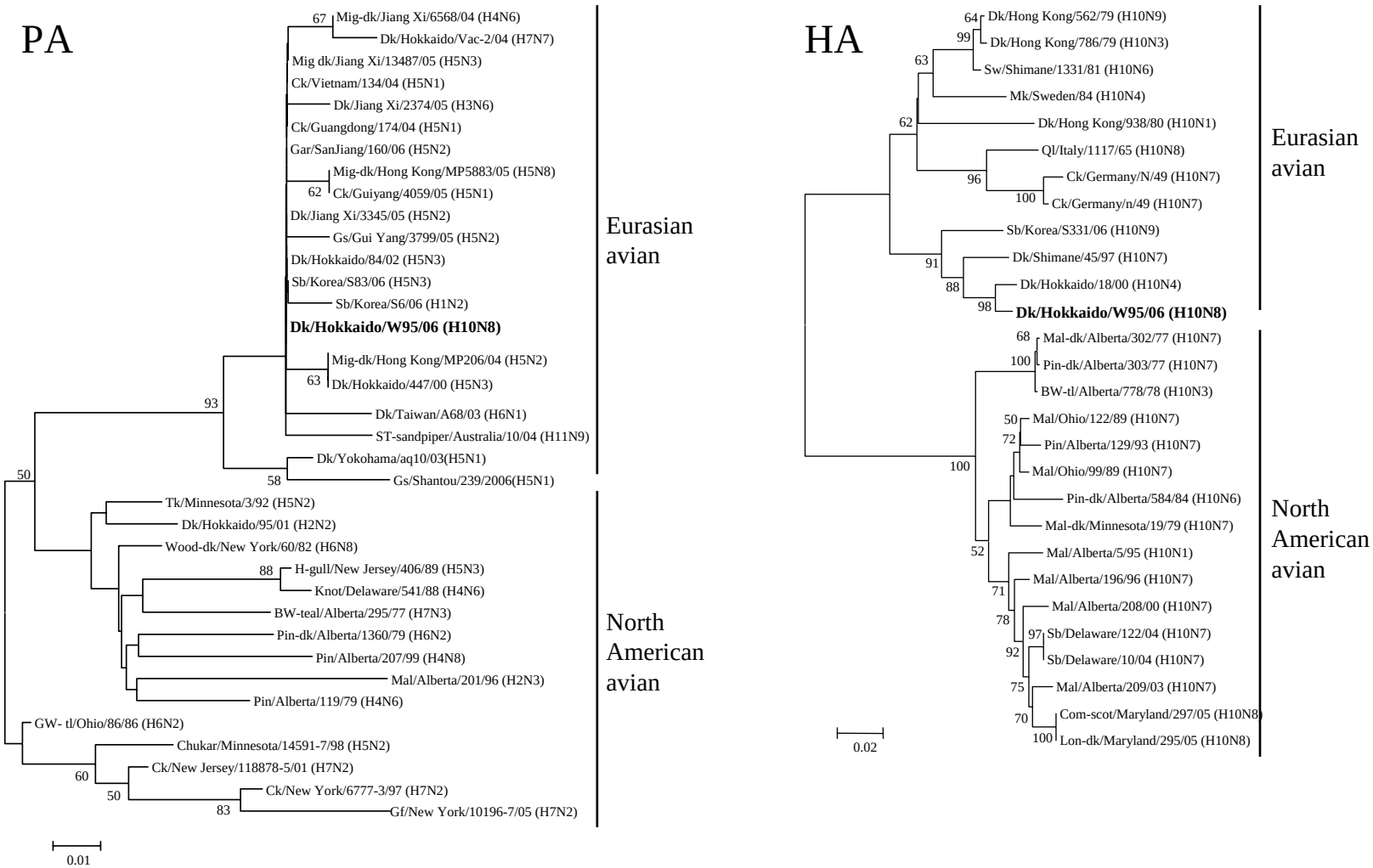

Fig. 2 ***continued***

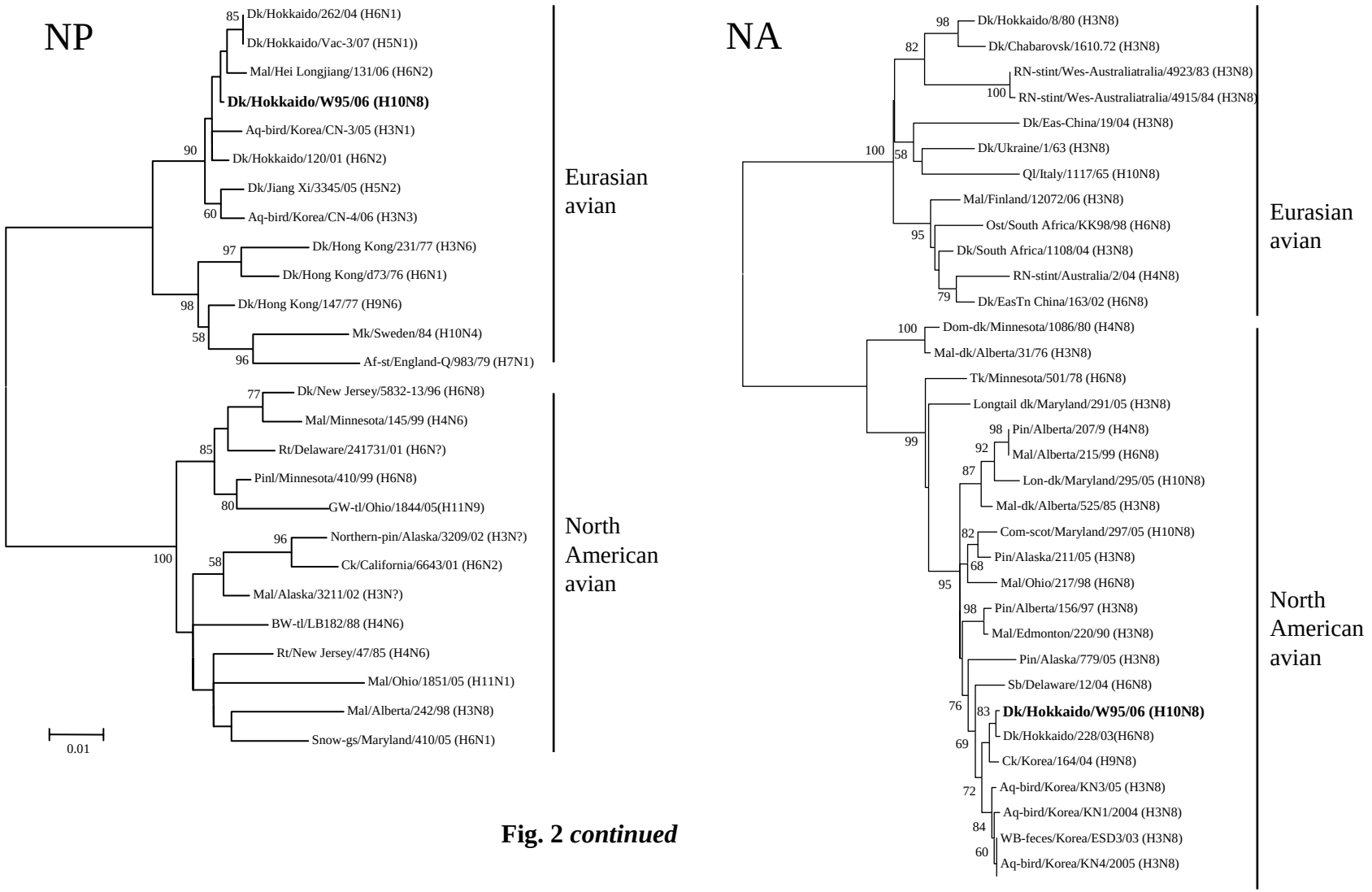

Fig. 2 *continued*

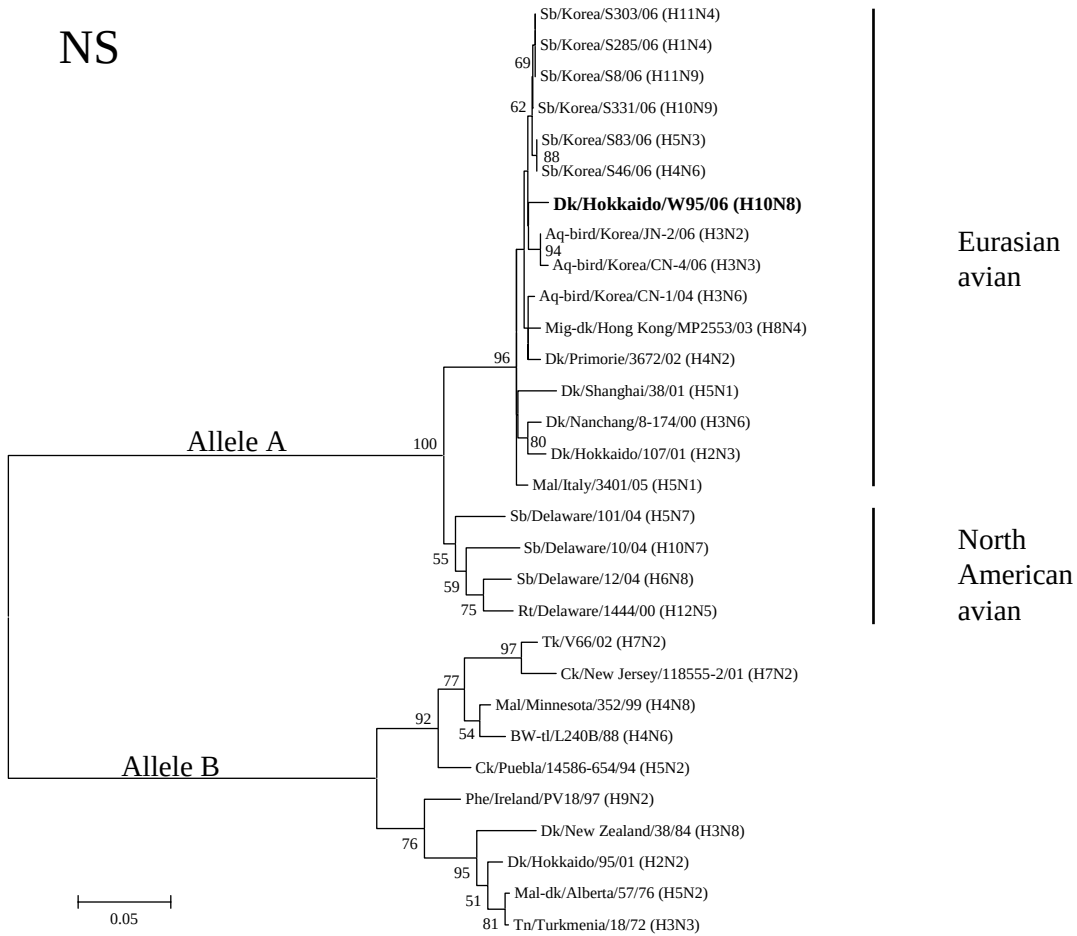

Fig. 2 ***continued***

Table 2. Influenza virus strains analysed in the present study

Virus strain[a]	Subtype	Accession number	Virus strain	Subtype	Accession number
A/duck/Hokkaido/13/2000	**H9N2**	**AB436804**	**A/duck/Hokkaido/W109/2004**	**H6N2**	**AB428693**
A/duck/Hokkaido/18/2000	**H10N4**	**AB282880**	**A/duck/Hokkaido/W132/2004**	**H12N5**	**AB428700**
A/duck/Hokkaido/69/2000	**H5N3**	**AB300041**	**A/duck/Hokkaido/W245/2004**	**H11N9**	**AB428694**
A/duck/Hokkaido/343/2000	**H6N2**	**AB428675**	A/duck/Hokkaido/12/2005	H3N2	AB428695
A/duck/Hokkaido/379/2000	**H4N6**	**AB370201**	A/duck/Hokkaido/209/2005	H6N2	AB428696
A/duck/Hokkaido/380/2000	**H8N4**	**AB436803**	A/duck/Hokkaido/260/2005	H8N4	AB428697
A/duck/Hokkaido/1005/2001	H3N6	AB370202	**A/duck/Hokkaido/W70/2005**	**H3N8**	**AB428698**
A/duck/Hokkaido/1019/2001	H4N6	AB370203	**A/duck/Hokkaido/W259/2005**	**H2N5**	**AB428701**
A/duck/Hokkaido/1058/2001	H4N5	AB266089	**A/duck/Hokkaido/W268/2005**	**H6N1**	**AB428702**
A/duck/Hokkaido/1196/2001	H1N1	AB370204	**A/duck/Hokkaido/W302/2005**	**H6N2**	**AB428703**
A/duck/Hokkaido/56/2001	**H3N8**	**AB428699**	A/duck/Hokkaido/46/2006	H3N8	AB428704
A/duck/Hokkaido/66/2001	**H12N5**	**AB428676**	A/duck/Hokkaido/156/2006	H11N9	AB428725
A/duck/Hokkaido/86/2001	**H2N3**	**AB428726**	A/duck/Hokkaido/277/2006	H6N2	AB428705
A/duck/Hokkaido/95/2001	**H2N2**	**AB428727**	A/duck/Hokkaido/279/2006	H4N6	AB428706
A/duck/Hokkaido/120/2001	**H6N2**	**AB286879**	A/duck/Hokkaido/294/2006	H9N2	AB428707
A/duck/Hokkaido/24/2002	H11N9	AB428677	**A/duck/Hokkaido/W10/2006**	**H3N6**	**AB428708**
A/duck/Hokkaido/84/2002	H5N3	AB300225	**A/duck/Hokkaido/W95/2006**	**H10N8**	**AB428709**
A/duck/Hokkaido/182/2002	H3N8	AB428678	**A/swan/Hokkaido/W135/2006**	**H3N8**	**AB428710**
A/duck/Hokkaido/39/2003	**H3N8**	**AB428680**	**A/duck/Hokkaido/W162/2006**	**H6N5**	**AB428711**
A/duck/Hokkaido/108/2003	**H6N8**	**AB428681**	**A/duck/Hokkaido/W189/2006**	**H13N6**	**AB428712**
A/duck/Hokkaido/124/2003	**H8N4**	**AB428682**	**A/duck/Hokkaido/W206/2006**	**H6N1**	**AB428716**
A/duck/Hokkaido/143/2003	**H7N7**	**AB428683**	**A/duck/Hokkaido/W214/2006**	**H4N9**	**AB428713**
A/duck/Hokkaido/18/2004	H3N8	AB428684	**A/duck/Hokkaido/W240/2006**	**H11N9**	**AB428714**
A/duck/Hokkaido/83/2004	H1N1	AB428685	**A/duck/Hokkaido/W299/2006**	**H9N2**	**AB428715**
A/duck/Hokkaido/139/2004	H6N8	AB428686	A/duck/Hokkaido/36/2007	H12N5	AB428717
A/duck/Hokkaido/143/2004	H4N2	AB428687	A/duck/Hokkaido/69/2007	H8N4	AB428718
A/duck/Hokkaido/154/2004	H10N5	AB428688	A/duck/Hokkaido/138/2007	H4N6	AB428719
A/duck/Hokkaido/193/2004	H5N3	AB299810	A/duck/Hokkaido/201/2007	H5N3	AB378693
A/duck/Hokkaido/257/2004	H6N1	AB428689	**A/duck/Hokkaido/W23/2007**	**H3N8**	AB428720
A/duck/Hokkaido/W5/2004	**H4N6**	**AB428690**	**A/duck/Hokkaido/W73/2007**	**H1N1**	AB428721
A/duck/Hokkaido/W12/2004	**H9N2**	**AB428691**	**A/duck/Hokkaido/W87/2007**	**H10N2**	AB428722
A/duck/Hokkaido/W15/2004	**H10N6**	**AB428692**	**A/duck/Hokkaido/W90/2007**	**H10N7**	AB428723
A/duck/Hokkaido/W59/2004	**H8N4**	**AB430755**	**A/duck/Hokkaido/W282/2007**	**H4N6**	AB428724
A/duck/Hokkaido/W101/2004	**H7N7**	**AB436898**			

[a] Name of the virus strains with corresponding accession number of M genes sequenced in this study. Virus strains isolated from lake Ohnuma, Wakkanai, Japan are in boldface and others are those isolated from Ohno-ike pond of Hokkaido University, Sapporo, Japan.