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The nucleoprotein is responsible for intracerebral pathogenicity of A/duck/Mongolia/47/2001 (H7N1) in chicks

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Abstract

Avian influenza viruses, A/duck/Mongolia/47/2001 (H7N1) (47/01) and A/duck/Mongolia/867/2002 (H7N1) (867/02), were defined as low pathogenic avian influenza viruses (LPAIVs) by intravenous pathogenicity test in chickens. On the other hand, the intracerebral pathogenicity indices of 47/01 and 867/02 were 1.30 and 0.00, respectively. A series of reassortant viruses were generated between 47/01 and 867/02, and their intracerebral pathogenicity was compared in one-day-old chicks to identify the protein(s) responsible for the intracerebral pathogenicity of 47/01. The results indicate that the amino acid positions at 50 and 98 of the nucleoprotein are related to the pathogenicity of 47/01 in chicks by intracerebral inoculation. A significant association was found between mortality of the chicks inoculated intracerebrally with 47/01 and virus replication in the lungs and/or brains. These results indicate that the NP of avian influenza viruses may be responsible for intracerebral pathogenicity in hosts.

Introduction

Highly pathogenic avian influenza viruses (HPAIVs) cause severe systemic infection with high mortality in chickens. It is known that low pathogenic avian influenza viruses (LPAIVs) of H5 or H7 subtypes acquire pathogenicity during repeated passages among chicken populations [9].

The pathogenicity of avian influenza viruses is basically defined by an intravenous pathogenicity test using 4- to 8-week-old chickens. Insertion of multiple basic amino acids at the cleavage site of the haemagglutinin (HA) is well-established as an indicator of potential pathogenicity for H5 and H7 influenza viruses in chickens [4, 18]. In 1992, the criteria for the pathogenicity of avian influenza viruses were defined on the basis of an intravenous pathogenicity index (IVPI), growth in cultured cells in the absence of trypsin, and amino acid residues at the cleavage site of the HA protein by the European Union (EU), and World Organization for Animal Health (OIE) has also adopted these criteria [4, 18]. It is generally recognized that the HA subtypes of HPAIVs are restricted to H5 or H7, although it was reported that the IVPIs of two H10 subtypes were over 1.20 [29]. These H10 viruses do not have multiple basic amino acids at the cleavage site of the HA and do not cause severe diseases when inoculated intranasally. On the other hand, four H5 avian influenza viruses had multiple basic amino acids at the cleavage site of the HA but showed some pathogenicity (IVPI <1.2) when the viruses were inoculated into chickens intravenously [13].

H5N1 HPAIVs have been isolated from aquatic migratory birds as well as terrestrial poultry since 2005 [3, 12]. The ducks experimentally infected with the viruses isolated during the highly pathogenic avian influenza outbreaks in Japan, 2004, of which the HA gene belongs to Clade 2.5, did not show any disease signs [8, 10, 23] whereas the ducks inoculated with the isolates from the dead migratory birds in Mongolia, of which the HA genes belong to Clade 2.2 or 2.3.2, showed severe neurological signs, such as depression, blindness, and intermittent head-shaking

[23]. Chickens infected with H5 HPAIVs die due to systemic infection. On the other hand, ducks infected with H5 HPAIVs show neurological signs, resulting in death [23]. Histopathological studies indicated that neurological signs of the birds infected with HPAIVs, of which the HA genes belong to Clade 1, 2.5, and 8, were associated with inflammation and necrosis in the brain [21, 26, 30]. Nevertheless, the virus factor(s) responsible for intracerebral pathogenicity of HPAIVs have not been identified.

A/duck/Mongolia/47/2001 (H7N1) (47/01), isolated from a fecal sample of a duck that migrated from nesting lakes in Siberia in autumn, was defined as an LPAIV by the IVPI [25]; however, eight of the 10 one-day-old chicks intracerebrally inoculated with 47/01 died and the intracerebral pathogenicity index (ICPI) was 1.30. Taking into account that the highest ICPI of 91 LPAIVs isolated from wild birds was 0.83 by Otsuki et al. [20], the ICPI of 47/01 was apparently high. It was reported that an H7N1 LPAIV spread systemically in chickens after a combined intranasal and intratracheal inoculation [22]. Introduction of intracerebral pathogenicity factor(s) found in 47/01 into LPAIVs which can spread systemically in host may exert a severe intracerebral pathogenicity via natural infection route. In the present study, the pathogenicity of a series of reassortant viruses generated between 47/01 and intracerebrally low pathogenic virus, A/duck/Mongolia/867/2002 (H7N1) (867/02), was compared in one-day-old chicks. The results indicate that the amino acids at positions 50 and 98 of the nucleoprotein (NP) of 47/01 are related to the pathogenicity in chicks by intracerebral inoculation.

Materials and Methods

Viruses and animals

A/duck/Mongolia/47/2001 (H7N1) and A/duck/Mongolia/867/2002 (H7N1) were isolated from

fecal samples of migratory ducks in Mongolia in 2001 and 2002, respectively. These two viruses were propagated in 10-day-old embryonated chicken eggs at 35 ^{o}C for 48 hours and the infectious allantoic fluids were used as viral stocks.

Boris brown chickens and their fertile eggs were purchased from a hatchery vendor (Hokuren Chuou Syukeijo). Chicks were hatched from fertile eggs in our laboratory. These animals were housed in self-contained isolator units (Tokiwa Kagaku) at a BSL 3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Sequencing

Virus RNAs were extracted by TRIzol LS reagent (Invitrogen) and reverse-transcribed with the Uni12 primer [6] and M-MLV Reverse Transcriptase (Invitrogen). The full-length genome of each gene segment was amplified by polymerase chain reaction (PCR) with gene-specific primer sets [6]. Direct sequencing of each gene segment was carried out using an auto sequencer, CEQ 2000XL (Beckman Coulter). The nucleotide sequences of these two viruses in the present study were registered in DDBJ/EMBL/GenBank (GenBank ID: AB473548, AB302788, AB268552-AB268557, and AB473540-AB473547, respectively)

Generation of recombinant viruses

Two recombinant viruses, rg-A/duck/Mongolia/47/2001 (H7N1) (rg-47) and rg-A/duck/Mongolia/867/2002 (H7N1) (rg-867) were generated according to Hoffmann et al. [5, 7]. Each of the PCR products of the 8 gene segments of 47/01 and 867/02 was cloned into pGEM®-T easy vector (Promega). Each of the 8 segments of 47/01 and 867/02 was cloned into a dual-promoter plasmid pHW2000 [5]. The plasmids carrying the NP gene of either 47/01 or 867/02 were manipulated using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) in order to generate variant plasmids introducing single or double amino acid

substitution(s) into the NP of each virus. Each of the resulting 14 plasmids which had single or double amino acid substitution(s) in the NP of either 47/01 or 867/02 was obtained and designated as: pHW-47-NP-G34S, pHW-47-NP-N50S, pHW-47-NP-K98F, pHW-47-NP-N319K, pHW-47-NP-A350T, pHW-47-NP-M352V, pHW-47-NP-R384Q, pHW-867-NP-S34G, pHW-867-NP-S50N, pHW-867-NP-F98K, pHW-867-NP-K319N, pHW-867-NP-T350A, pHW-867-NP-V352M, pHW-867-NP-Q384R, pHW-47-NP-N50S-K98F, and pHW-867-NP-S50N-F98K, respectively.

Reverse genetic viruses were generated by DNA transfection according to Tsuda et al. [27]. Briefly, 293T cells and Mardin-Darby Canine Kidney (MDCK) cells were co-cultured and transfected with 1 μg of each of the 8 plasmids and 16 μl of TransIT-293 (Mirus Bio) in a total volume of 1 ml of Opti-MEM (Gibco). After 30 hours, 1 ml of Opti-MEM with 5 μg/ml of Trypsin Acetylated (Sigma) was added and incubated at 35 ^{o}C for 48 hours. One hundred microliters of the supernatant was inoculated into 10-day-old embryonated chicken eggs and incubated at 35 ^{o}C for 48 hours.

Pathogenicity tests in chicken embryos and chickens

Mean death time (MDT) of chicken embryos was determined according to Waterson et al. [28]. Ten-fold serially diluted viruses with phosphate-buffered saline (pH 7.2) (PBS) were inoculated into 10 nine-day-old embryonated chicken eggs. The eggs were examined every 8 hours by candling; the integrity of the circulatory system and movement of the embryo were criteria for viability until 120 hours post-inoculation (p. i.). The MDT was calculated as the mean time in hours for the minimum lethal dose (MLD) to kill all 10 embryos.

Intravenous pathogenicity test for influenza viruses was carried out according to the OIE manual [18]. Two hundred microliters of viruses, ten-fold diluted with PBS, were inoculated into 8 six-week-old chickens intravenously. The birds were observed for disease signs at intervals

of 24 hours until 10 days p. i., and each bird was scored 0 if normal, 1 if sick, 2 if severely sick (including paralysis), and 3 if dead, at each observation. The IVPI was the mean score per bird per observation until 10 days p. i.

Intracerebral pathogenicity test was carried out basically according to the OIE manual of Newcastle Disease and Alexander et al. [1, 19]. One hundred microliters of viruses, ten-fold diluted with PBS or $10^{6.0}$ EID_{50} of wild-type or recombinant viruses generated by the reverse genetics method, were inoculated into 10 one-day-old chicks by intracerebral injection. The birds were observed for disease signs at intervals of 24 hours until 8 days p. i., and each bird was scored 0 if normal, 1 if sick, and 2 if dead, at each observation. The ICPI was the mean score per bird per observation until 8 days p. i. Birds were housed in self-contaminated isolator units (Tokiwa Kagaku, Tokyo) at a BSL 3 biosafety facility at Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were authorized by the committee of Animal Experiments in Graduate School of Veterinary Medicine, Hokkaido University.

Plaque assay

Viruses serially diluted ten-fold with PBS were inoculated into confluent monolayers of MDCK cells and incubated at 35 ^{o}C for 1 hour. The cells were washed with minimal essential medium (MEM). Cells were then overlaid with MEM containing 0.7 % Bact-Agar (Gibco) and 5 μg/ml of Trypsin Acetylated (Sigma). After 48 hours incubation at 35 ^{o}C, cells were stained with 0.005% neutral red.

Virus growth potentials in MDCK cells

Viruses were inoculated into MDCK cell monolayers at a multiplicity of infection of 0.01 and incubated at 35 ^{o}C for 1 hour. Each virus was inoculated into three wells of MDCK cells. Cells

were then washed with MEM. MEM with or without 5 μg/ml of Trypsin Acetylated was added to the cells and they were incubated at 35 ^{o}C for 72 hours. Culture supernatants were collected and each of the supernatants was titrated for its infectivity by plaque assay.

Virus recovery from the chicks intracerebrally challenged with H7N1 viruses

In order to assess the virus growth potential in one-day-old chicks, either $10^{6.0}$ EID_{50} of rg-47 or rg-867 was inoculated into 60 one-day-old chicks by intracerebral injection. Three chicks in each challenged group were sacrificed every 12 hours from 12 hours p. i. If more than three birds died in an observation, all the dead birds were sampled and no chicks were sacrificed at that time. When the birds were sacrificed or died, the brain and lung tissues were collected aseptically. In order to make a 10% suspension with MEM, the collected tissue samples were homogenized by Multi Beads Shocker (Yasui Kikai). Suspensions of each sample were titrated in triplicate by plaque assay and titers were expressed as the common logarithm of plaque forming units (PFU) per gram of the tissues.

Statistical analysis

To examine whether virus replication in the host is responsible for pathogenicity, one-tailed Fisher's exact tests were conducted at the 5% confidence level.

Results

Pathogenicity of two wild-type H7N1 avian influenza viruses

Pathogenicity of wild-type 47/01 and 867/02 in nine-day-old chicken embryos, one-day-old chicks, and six-week-old chickens was assessed. Both of the MLDs of the chicken embryos

inoculated with 47/01 and 867/02 were 10 EID_{50} (data not shown). The MDTs of the chicken embryos inoculated with 47/01 and 867/02 were 93.0 and 94.7 hours, respectively, indicating that both viruses were low pathogenic in chicken embryos (Table 1). Neither 47/01 nor 867/02 replicated in MDCK cells in the absence of trypsin. All of the 8 six-week-old chickens inoculated intravenously with 867/02 survived for 10 days without showing any disease signs, indicating that the IVPI of the virus was 0.00. Two out of the 8 chickens inoculated with 47/01 died, indicating that the IVPI of the virus was 0.58. In addition, insertion of multiple basic amino acids at the cleavage site of the HA was not found in either H7N1 virus. The above results indicate that both 47/01 and 867/02 are categorized as LPAIVs according to the OIE criteria.

None of the one-day-old chicks inoculated intracerebrally with 867/02 showed disease signs and all survived for 8 days after inoculation. On the other hand, eight of the 10 one-day-old chicks inoculated with 47/01 intracerebrally died within 6 days p. i. The ICPI of 47/01 was thus scored as 1.30. Insertion of multiple basic amino acids at the cleavage site of the HA was not found in the recovered viruses from the brain tissues of the dead chicks after inoculation of rg-47 intracerebrally (data not shown).

Virus recovery from the chicks intracerebrally inoculated with recombinant H7N1 viruses

In order to clarify whether the intracerebral pathogenicity of the recombinant viruses is associated with virus growth potential in the brain, virus growth in the brain and lungs of the chicks intracerebrally inoculated either with rg-47 or rg-867 was assessed. Each virus of $10^{6.0}$ EID_{50} was inoculated intracerebrally into 30 one-day-old chicks.

The viruses were recovered from the brains of 19 chicks inoculated with rg-47 (Table 2). Since a high titer of viruses was directly inoculated into chicks intracerebrally and virus titers of the brain from 12 to 36 hours p. i. were gradually getting low, high virus titers from the brains as well as lungs of the chicks inoculated with the viruses from 12 to 36 hours p. i. should not be

attributed to virus replication but should be residual viruses after challenging. Among the 21 chicks sampled after 48 hours p.i., 15 birds were dead and the other 6 birds were sacrificed in each observation period. Of the 15 dead chicks, challenge viruses were recovered from 14 birds and three out of the 6 sacrificed birds. Mortality of the chicks followed by intracerebral inoculation with rg-47 was significantly associated with virus replication either in the lungs or in the brain ($P < 0.05$). Virus replication in the brain was confirmed in 10 out of the 21 chicks inoculated with rg-47 between 48 and 108 hours p. i. Virus replications in the lungs were confirmed in 16 out of the 21 chicks inoculated with rg-47 between 48 and 108 hours p. i. One of the birds inoculated with rg-47 died at 48 hours p. i. Seven of the birds inoculated with rg-47 died at 60 hours p. i. and 7 other birds died between 72 and 96 hours p. i. Virus replication in the lungs was confirmed in 13 out of the 15 dead chicks and three out of 6 sacrificed chicks, and virus replication in the brain was confirmed in 8 out of the 15 dead chicks and two out of the 6 sacrificed chicks.

Between 48 and 120 hours p. i., virus replication in the brain and lungs was confirmed in three and 4 out of the 21 chicks inoculated with rg-867, respectively. One of the birds inoculated with rg-867 died at 72 hours p. i. and the virus was not recovered from the brain.

Intracerebral pathogenicity of recombinant viruses in chicks

In order to determine the virus protein(s) of 47/01 responsible for the pathogenicity in chicks, a series of recombinant viruses between 47/01 and 867/02 were generated. Two recombinant viruses, rg-A/duck/Mongolia/47/2001 (H7N1) (rg-47) and rg-A/duck/Mongolia/867/2002 (H7N1) (rg-867), showed pathogenicity similar to 47/01 and 867/02 in chicks, respectively (Table 1 and Fig. 1). All of the 10 chicks inoculated with rg-47-NP/867 (H7N1), which had the NP gene segment of 47/01 and the others of 867/02, died and the ICPI of rg-47-NP/867 (H7N1) was 1.63 (Fig. 1). A significant difference between the number of dead chicks inoculated with rg-867 and

rg-47-NP/867 was found ($P < 0.05$), indicating that the intracerebral pathogenicity of rg-47-NP/867 (H7N1) was apparently higher than that of rg-867. In contrast, all the other single gene reassortant viruses, which had the other 7 genes of 867/02, did not show severe intracerebral pathogenicity in chicks and no significant differences between the number of dead chicks inoculated with each of them and with rg-867 were found. Nine out of the 10 chicks inoculated with rg-867-NP/47 (H7N1), which had the NP gene segment of 867/02 and the others of 47/01, survived for 8 days p. i. and the ICPI of rg-867-NP/47 (H7N1) was 0.15. A significant difference between the number of dead chicks inoculated with rg-47 and with rg-867-NP/47 was found ($P < 0.05$). In contrast, all the other single gene reassortant viruses, which had the other 7 genes of 47/01, were highly pathogenic in chicks and no significant differences between the numbers of dead chicks inoculated with each of them and with rg-47 were found. The present results indicate that the NP is responsible for the pathogenicity followed by intracerebral inoculation of 47/01 in chicks.

Between the NPs of 47/01 and 867/02, seven amino acid differences were found at positions 34, 50, 98, 319, 350, 352, and 384. In order to identify the amino acid position(s) of the NP responsible for the pathogenicity of 47/1 by intracerebral inoculation in chicks, fourteen clones of recombinant 47/01 or 867/02, each of which had a single amino acid substitution in the NP, were generated. The three recombinant viruses, rg-867-NP-S50N/867 (H7N1), of which an amino acid substitution from serine to asparagine was introduced at position 50 in the NP of 867/02, rg-867-F98K/867 (H7N1), of which an amino acid substitution from phenylalanine to lysine was introduced at position 98 in the NP of 867/02, and rg-867-NP-T350A/867 (H7N1), of which an amino acid substitution from threonine to alanine was introduced at position 350 in the NP of 867/02, were more pathogenic than rg-867. Amino acid substitutions at positions 50, 98, or 350 were responsible for the pathogenicity of rg-867 by intracerebral inoculation, although the significant difference was only found between the number of dead chicks inoculated with

rg-867-NP-S50N/867 (H7N1) and rg-867 ($P < 0.05$). The three mutant viruses, rg-47-NP-N50S/47 (H7N1), of which an amino acid substitution from asparagine to serine was introduced at position 50 in the NP of 47/01, rg-47NP-K98F/47 (H7N1), of which an amino acid substitution from lysine to phenylalanine was introduced at position 98 in the NP of 47/01, and rg-47-NP-R384Q/47 (H7N1), of which an amino acid substitution from arginine to glutamine was introduced at position 384 in the NP of47/01, were less pathogenic than rg-47. Amino acid substitutions at positions 50, 98, or 384 were responsible for the pathogenicity of rg-47 by intracerebral inoculation, although the significant difference was only found between the number of dead chicks inoculated with rg-47-NP-K98F/47 (H7N1) and rg-47 ($P < 0.05$). Taken together, we concluded that the pathogenicity of 47/01 by intracerebral inoculation in chicks was influenced by the amino acid at position 50 or 98 of the NP, although no significant difference was found between the numbers of dead chicks inoculated with rg-867-F98K/867 (H7N1) and with rg-867, and between the numbers of dead chicks inoculated with rg-47-N50S/47 (H7N1) and with rg-47.

Since it was thus suggested that amino acid substitutions at positions 50 and 98 of the NP of rg-47 are related to the pathogenicity in chicks, two recombinant viruses with double amino acid substitutions were generated. The recombinant virus, rg-867-NP-S50N-K98F/867 (H7N1), showed higher pathogenicity in chicks than either rg-867-NP-S50N/867 (H7N1) or rg-867-NP-F98K/47 (H7N1), whereas the pathogenicity of rg-47-NP-N50S-K98F/47 (H7N1) was not obviously different from those of rg-47-NP-N50S/47 (H7N1) and rg-47-K98F/47 (H7N1).

Discussion

Avian influenza virus, 47/01, which possesses the typical monobasic motif of LPAIVs at the

cleavage site of the HA, showed high pathogenicity in chicks by intracerebral injection. In the present study, the amino acid sequence at the cleavage site of the HA of the viruses recovered from the dead birds was the same as that of the inoculum virus (data not shown), indicating that the intracerebral pathogenicity of 47/01 in chicks was not attributed to the fusion activities of the HA. These results support the report that an H5N1 influenza virus strain that met the criteria according to the cleavage site of the HA, but both of the IVPI and ICPI of the virus was 0.0 became more pathogenic after serial intracerebral passages in 1- or 2-day-old chicks [14].

In the present study, a significant association were found between virus pathogenicity and virus replication in the hosts, although specific organs associated with virus pathogenicity were not determined. Interestingly, although many chicks died following inoculation with rg-47 via intracerebral route, viruses were recovered from the lungs of 13 out of 15 dead chicks inoculated with rg-47, whereas no viruses were recovered from the brains of 7 out of 15 dead birds. Virus replication in the lungs is therefore responsible for exertion of pathogenicity of 47/01 in the hosts, though a significant difference was not observed ($P = 0.11$). The probability that severe pathogenicity in hosts is attributed to organ failure in the lungs subsequent to virus replication in there is high. However, taking into consideration that no viruses were recovered from one out of 15 dead chicks, there may be other causes of death in the dead birds after intracerebral inoculation of 47/01, like as organ failure in the brain or another organ which was not investigate in the present study, or systemic failure subsequent to virus replication in given organ(s) which is shown as systemic inflammatory response syndrome caused by HPAIV infection.

In the animal experiments using the recombinant viruses, the responsibility of the NP of 47/01 for the pathogenicity by the intracerebral injection in chicks was demonstrated. Furthermore, it was indicated that amino acids at positions 50 and 98 of the NP of 47/01 are related to the pathogenicity in chicks by intracerebral inoculation. The NP is known as a protein which encapsidates the virus genome, forming the nucleocapsid packaging of the nucleotides of virus

RNA. Structural analysis of the NP indicates that amino acids at positions 50 and 98 are located in the body domain of the NP [31]. Amino acids at positions 50 and 98 of the NP are in one of the three regions where the NP binds to the PB2 protein [2]. It was suggested that the efficiency with which a given nucleocapsid ensured transcription and replication of virus RNA in 293T cells related to the efficiency of the NP binding to the PB1-PB2-PA complex [11]. On the basis of virus RNA replication, the NP was assigned a major role in the switch from mRNA transcription to cRNA synthesis by interacting with one or more polymerase proteins through a conformational change in the polymerase complex [15-17, 24]. These reports indicate that the NP is indirectly responsible for virus replication and for determining cell/host tropism by controlling virus polymerase activity

In the present study, an LPAIV defined by the IVPI test caused severe pathogenicity in one-day-old chicks followed by intracerebral inoculation. Definitions of the pathogenicity of avian influenza viruses by the IVPI or amino acid sequence at the cleavage site of the HA are the results of interactions between pathogens and hosts, and may conceal other important interactions or factors. Though the HA should play a major role in pathogenicity of avian influenza virus, virus pathogenicity associated with the virus proteins other than the HA has also reported in many papers. We believe that the present results will be helpful to investigate the intracerebral pathogenicity or neuropathology of avian influenza viruses.

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Figure Captions

Fig. 1 Pathogenicity of recombinant virus to chicks after intracerebral inoculation A series of recombinant viruses between 867/02 and 47/01 were generated by the reverse genetic method. Each of the recombinant viruses was injected into 8 one-day-old chicks intracerebrally and the ICPI was calculated according to the OIE manual of Newcastle Disease. White and black boxes indicate the derivation of virus gene segments from 867/02 and 47/01, respectively. Single and double stars indicate significant differences between the number of dead birds inoculated with rg-47 and with rg-867-NP/47, and there was a significant difference between the number of dead birds inoculated with rg-867 and with rg-47-NP/867, respectively.

Fig. 2 Pathogenicity of recombinant virus containing single amino acid substitution in the NP after intracerebral inoculation Fourteen clones of recombinant 47/01 or 867/02, each of which had a single amino acid substitution in the NP, were generated. Each of the recombinant viruses was inoculated into 8 one-day-old chicks intracerebrally and the ICPI was calculated according to the OIE manual of Newcastle Disease. White and black boxes or lines indicate the derivation of virus gene segments from 867/02 and 47/01, respectively. The numbers indicate the positions of the amino acid differences. A significant difference between the number of dead birds inoculated with rg-867 and inoculated with the single-point mutant was marked with a single star. A significant difference between the number of dead birds inoculated with rg-47 and inoculated with the single –point mutant was marked with double single stars.

Table 1. Pathogenicity indices of two H7N1 influenza virus strains

Virus	MDT (h)	Growth potential in MDCK cell in the absence of trypsin	IVPI test		Amino acid sequence at cleavage site of the HA	ICPI test	
			IVPI	Dead / Total		ICPI	Dead / Total
A/duck/Mongolia/47/2001 (H7N1)	93.0	-	0.58	2 / 8	IPKGR / G	1.30	8 / 10
A/duck/Mongolia/867/2002 (H7N1)	94.7	-	0.00	0 / 8	IPKGR / G	0.00	0 / 10

*: Abbreviation name of pathogenicity indices, IVPI; intravenous pathogenicity index, MDT; mean death time of embryonated chicken eggs, ICPI; intracerebral pathogenicity index, Dead / Total; number of dead one-day-old chicks per ten chicks in the ICPI test

Table 2. Virus recovery from the dead chicks inoculated either with rg-47 or rg-867

Virus	Hours post inoculation	No. of birds [a]	Virus recovery from the following tissues			
			Brain		Lungs	
rg-47	12	3	3 [b]	(5.8) [c]	2	(2.5)
	24	3	3	(3.8)	3	(3.7)
	36	3	3	(3.1)	1	(2.5)
	48	1+	1	(5.3)	1	(6.2)
	48	2	0		0	
	60 - 96	7+	7	(3.7)	6	(5.0)
	60 - 84	6+	0		6	(3.5)
	60	1+	0		0	
	72 - 108	4	2	(2.8)	3	(3.6)
rg-867	12	3	3	(5.3)	2	(3.0)
	24	3	3	(4.2)	1	(2.5)
	36	3	3	(2.7)	0	
	48 - 120	17	0		0	
	72	1+	0		1	(6.1)
	72 - 108	3	3	(3.4)	3	(3.4)

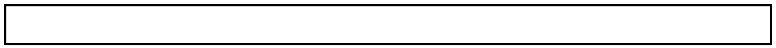
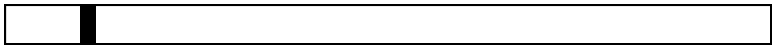
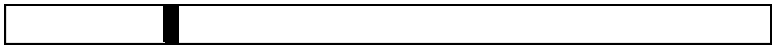
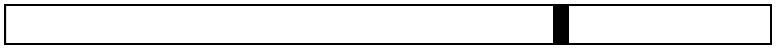
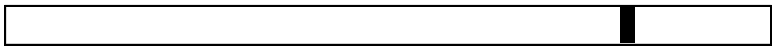
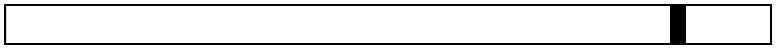
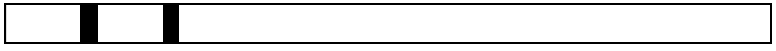
[a] Crosses indicate that all the birds died.

[b] Number of birds from which challenge viruses were recovered. Zero indicates no virus was recovered from any of the bird tissues

[c] Average of virus titers was indicated into the parenthesis (log PFU /g).

Virus	Virus gene segment								ICPI	Number of birds
	PB2	PB1	PA	HA	NP	NA	M	NS		Dead / Total
rg-47	■	■	■	■	■	■	■	■	0.95	7 / 10
rg-867	□	□	□	□	□	□	□	□	0.13	1 / 10
rg-867-PB2/47	□	■	■	■	■	■	■	■	1.11	6 / 10
rg-867-PB1/47	■	□	■	■	■	■	■	■	1.46	9 / 10
rg-867-PA/47	■	■	□	■	■	■	■	■	0.94	6 / 10
rg-867-HA/47	■	■	■	□	■	■	■	■	1.20	8 / 10
rg-867-NP/47	■	■	■	■	□	■	■	■	0.15	*1 / 10
rg-867-NA/47	■	■	■	■	■	□	■	■	1.23	8 / 10
rg-867-M/47	■	■	■	■	■	■	□	■	0.94	8 / 10
rg-867-NS/47	■	■	■	■	■	■	■	□	1.23	8 / 10
rg-47-PB2/867	■	□	□	□	□	□	□	□	0.06	1 / 10
rg-47-PB1/867	□	■	□	□	□	□	□	□	0.36	3 / 10
rg-47-PA/867	□	□	■	□	□	□	□	□	0.00	0 / 10
rg-47-HA/867	□	□	□	■	□	□	□	□	0.01	0 / 10
rg-47-NP/867	□	□	□	□	■	□	□	□	1.63	**10 / 10
rg-47-NA/867	□	□	□	□	□	■	□	□	0.00	0 / 10
rg-47-M/867	□	□	□	□	□	□	■	□	0.00	0 / 10
rg-47-NS/867	□	□	□	□	□	□	□	■	0.15	1 / 10

Figure 1. Isoda et al

Virus	Virus gene segment		ICPI	Number of birds
	NP	The other genes		Dead / Total
rg-47			0.95	7 / 10
rg-867			0.13	1 / 10
rg-867-NP-S34G/867			0.00	0 / 10
rg-867-NP-S50N/867			0.93	*8 / 10
rg-867-NP-F98K/867			0.61	5 / 10
rg-867-NP-K319N/867			0.15	1 / 10
rg-867-NP-T350A/867			0.48	3 / 10
rg-867-NP-V352M/867			0.18	1 / 10
rg-867-NP-Q384R/867			0.20	1 / 10
rg-47-NP-G34S/47			1.51	10 / 10
rg-47-NP-N50S/47			0.8	5 / 10
rg-47-NP-K98F/47			0.28	**2 / 10
rg-47-NP-N319K/47			1.20	9 / 10
rg-47-NP-A350T/47			1.58	10 / 10
rg-47-NP-M352V/47			1.49	10 / 10
rg-47-NP-R384Q/47			0.63	5 / 10
rg-47-NP-N50S-K98F/47			0.51	4 / 10
rg-867-NP-S50N-F98K/867			1.55	**10 / 10

*: There is a significant difference between the number of dead birds inoculated with rg-47 and that with this virus

**: There is a significant difference between the number of dead birds inoculated with rg-867 and that with this virus

Figure 2. Isoda et al