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Citation	Archives of Virology, 158(12), 2473-2478 https://doi.org/10.1007/s00705-013-1762-z
Issue Date	2013-12-01
Doc URL	http://hdl.handle.net/2115/57623
Rights	The final publication is available at link.springer.com.
Type	article (author version)
File Information	AVIROL-D-13-00212.pdf

Factors responsible for pathogenicity in chickens of a low pathogenic H7N7 avian influenza virus isolated from a feral duck

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Key words: avian influenza, chicken, pathogenicity

Running head: Acquisition of pathogenicity of an H7 influenza virus

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Abstract

Highly pathogenic avian influenza viruses have poly-basic amino acid residues at the cleavage site in hemagglutinin (HA). Although this poly-basic region is a pre-requisite factor for pathogenicity in chickens, not much is known about additional factors responsible for the acquisition of pathogenicity of the duck influenza virus in chickens. Here, we introduced poly-basic amino acid residues into the HA cleavage site of the A/duck/Hokkaido/Vac-2/2004 (H7N7) strain of avian influenza virus, which has low pathogenicity in chickens; the resultant Vac2sub-P0 strain was not intravenously pathogenic in chickens. In contrast, the Vac2sub-P3 strain, which was recovered from 3 consecutive passages of Vac2sub-P0 in chicks, was intravenously pathogenic in chickens. Six amino acid substitutions were identified by comparison of *Vac2sub-P3* with *Vac2sub-P0* genomic sequence: Lys123Glu in PB2, Asn16Asp in PB1, Glu227Gly and Ile388Thr in HA, Gly228Arg in M1, and Leu46Pro in M2. The results of intravenous inoculations of chickens with recombinant virus indicated that all 6 amino acid substitutions were required to varying degrees for Vac2sub-P3 pathogenicity with Glu227Gly and Ile388Thr in HA being particularly essential. These results reveal the roles of additional viral factors in the acquisition of pathogenicity in addition to the previously characterized role of the poly-basic amino acid residues at the HA cleavage site.

Introduction

All known sub-types of influenza A viruses (H1–H16 and N1–N9) have been isolated from water birds, particularly migratory ducks [1,2]. Migratory water birds bring viruses that are either non-pathogenic or have low pathogenicity for chickens from their nesting lakes in the northern territory such as Siberia, Alaska and Canada. Although influenza viruses from ducks cannot directly infect chickens, pathogenicity for chickens is obtained by transmission from feral ducks to chickens using domestic water birds such as ducks and geese and then from terrestrial birds such as quails and turkeys [3,4]. A highly pathogenic avian influenza virus (HPAIV) was selected by repeated multiple infections in the chicken population [5,6]. HPAIV hemagglutinin (HA) differs from HA in low pathogenic avian influenza viruses (LPAIVs) by the presence of more than a pair of di-basic amino acids at the cleavage site [7]. This structure permits ubiquitous proteases that recognize multiple basic amino acids, such as furin and PC6, to cleave the HA and cause systemic infection in chickens [8]. In contrast, LPAIV HA is cleaved only by the trypsin-like proteases expressed in the respiratory or intestinal epithelia, leading to only mild or asymptomatic local infections. The poly-basic amino acid residues are essential factors for the fatal pathogenicity of HPAIV in chickens. Previous reports suggest that H2, H3, H4, H5, H6, H8, H9 and H14 influenza viruses acquired pathogenicity in chickens after introduction of poly-basic amino acid residues at the HA cleavage site [9-12]. However, additional pathogenicity factors are unknown.

Recently, H7 HPAIV was isolated from poultry in Italy, the Netherlands, Spain, Canada and Pakistan [13-17]. Compared with H5 HPAIVs, H7 HPAIVs have different motifs in the HA cleavage site and exhibit different pathogenicity in chickens [18]. Here, we have used site-directed mutagenesis and reverse genetics to introduce poly-basic amino acid residues at the HA cleavage site of A/duck/Hokkaido/Vac-2/2004 (H7N7), a reassortant virus between A/duck/Mongolia/736/2002 (H7N7) and A/duck/Hokkaido/49/1998 (H9N2) [19]; consecutive passaging of the recombinant strains in the air sacs of chicks allowed us to identify novel factors responsible for pathogenicity in chickens.

Material and methods

Viral strains

Influenza virus A/duck/Hokkaido/Vac-2/2004 (H7N7), which is a reassortant strain between A/duck/Mongolia/736/2002 (H7N7) and A/duck/Hokkaido/49/1998 (H9N2) [19], was propagated *in ovo* in the allantoic cavities of embryonic day 10 chicks at 35 °C for 2 days and at −80 °C until used.

Reverse genetics

Viral RNA was extracted from the allantoic fluid of A/duck/Hokkaido/Vac-2/2004 (H7N7)-infected chicks and reverse-transcribed with the Uni12 primer [20] according to Soda *et al* [10]. Whole genome amplification by PCR of the 8 gene segments was performed with universal primer sets [21]. PCR products were cloned into pGEM-T Easy (Promega, Mannheim, After confirmatory sequencing, T-vector clones were digested with *Bsm*BI and inserted into pHW2000 [22]; some segments of the passaged virus were cloned into pHW2000 using HD Cloning Kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. Plasmids were transfected into co-cultures of 293T and MDCK cells using TransIT-293 (Mirus LLC, WI, USA) according to the manufacturer’s directions. At 48 h post-transfection, culture supernatant was collected and re-propagated *in ovo,* as described earlier.

Site-directed-mutagenesis

Viral strains containing specific mutations were introduced using QuickChange II site-directed mutagenesis kit (Stratagene, CA, USA) into the basic motif at the HA cleavage site 1) and specific regions of *PB2, PB1, HA, M1* and *M2*, according to manufacturer’s instructions. The mutant viruses were rescued by reverse genetics as described above, and the entire genome of the 8 gene segments were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Consecutive passages of Vac2sub-P0 in chick air sacs

The Vac2sub-P0 mutant virus contains poly-basic amino acid residues in the HA cleavage site, which is characterisitic of A/duck/Hokkaido/Vac-2/2004 (H7N7). Three 3-day-old chicks were each inoculated with 200 µl of Vac2sub-P0 into the caudal thoracic air sac. According to previous studies [6,10], the chicks were sacrificed, and their brains were collected 3 days post-inoculation (dpi). A pooled 10% tissue suspension of infected organs was serially passed into the air sac of 3–6 3-day-old chicks. Passaged viruses were propagated *in ovo* in the allantoic cavities of 10-day-old embryonated chicks for 48 h at 35°C.

Infection of chickens with mutant virus strains

Pathogenicity of mutant viruses was tested in 4-week-old chickens. To calculate the intravenous pathogenicity index (IVPI) [23], 8 chickens were intravenously inoculated with 100 µl of each virus (1:10 dilution in allantoic fluid) and examined for clinical signs at 24 h intervals for 10 days. Similarly, 100 µl of allantoic fluid containing each virus at 10^6 50% egg infectious dose (EID_{50}) was intranasally inoculated into 6 chickens and observed for 14 days. Specific antibodies against homologous viruses were detected in the serum at 14 dpi using the hemagglutination inhibition (HI) test. All experiments were carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility. The institutional animal care and use committee of the Graduate School of Veterinary Medicine in Hokkaido University approved the experimental protocols (approval number: 09-0072) and all experiments were performed according to the approved guidelines.

Results

Pathogenicity of viruses recovered from chicks

The pathogenicity of each passaged virus was tested by intravenous inoculation of 4-week-old chickens (Fig. 2a). Chickens inoculated with Vac2sub-P0 had no clinical signs and survived for 10 days with an IVPI of 0.00. Some chickens inoculated with Vac2sub-P1 had only slight clinical signs with an IVPI of 0.05. In contrast, chickens inoculated with Vac2sub-P2 showed some clinical signs (e.g. depression, diarrhoea and nervous symptom), and 7 of the 8 chickens died by 10 dpi with an IVPI of 2.01. All 8 chickens inoculated with Vac2sub-P3 died by 6 dpi with an IVPI of 2.54 (Table 1). Four-week-old chickens were inoculated intranasally with the virus (Fig. 2b). Vac2sub-P0 or Vac2sub-P1 inoculation did not lead to any clinical signs, whereas 2 of 6 chickens inoculated with either Vac2sub-P2 or Vac2sub-P3 died by 14 dpi.

Amino acid changes of the viruses that acquired pathogenicity in chickens

Nucleotide sequences of the 8 genome segments of the air sac-passaged viruses were obtained and the deduced amino acid sequences were compared with the parental virus (Table 1). Vac2sub-P0 and Vac2sub-P3 were found to differ by 6 substitutions: 1 each in PB2, PB1, M1 and M2, and 2 in HA. Lys123Glu in PB2 and Asn16Asp in PB1 substitutions were found after the first passage; Glu227Gly and Ile388Thr in HA (equivalent to position 218 and 378 of the H3 HA) were found after the second passage; Gly228Arg in the M1 and Leu46Pro in the M2 were found after the third passage. In addition, 3 temporary substitutions were identified: Ile4Asn in the PB2, Glu113Gly in the HA and Arg45His in the M2.

Intravenous pathogenicity of rgVac2sub-P0, rgVac2sub-P3 and mutant viruses in chickens

To determine amino acid changes involved in the acquisition of pathogenicity, rgVac2sub-P0, rgVac2sub-P3 and 12 additional mutant strains were generated using site-directed-mutagenesis and reverse genetics, and inoculated intravenously into chickens (Table 2). RgVac2sub-P0 inoculation had no clinical effects, whereas all chickens inoculated with either rgVac2sub-P3 or Vac2sub-P3 died by 10 dpi. Inoculations with mutant strains that had only single amino acid substitution, led to less mortality than with rgVac2sub-P3, which contained more than 1 substitution. In contrast, all chickens inoculated with rgP3/P0-HA-227 and rgP3/P0-HA-388 survived for 10 days. These results indicate that amino acid substitutions in PB2, PB1, M1 and M2 were important in the acquisition of pathogenicity in chickens via the intravenous route, in addition to HA substitutions, which were the most influential.

Discussion

HPAIV is selected by transmission of non-pathogenic virus from migratory birds to chickens; pathogenicity is acquired following repeated multiple infections within the chicken population [5,6]. The presence of poly-basic amino acid residues at the HA cleavage site in HPAIV [7] allows cleavage of HA by ubiquitous proteases, which enable HPAIV to cause systemic infection [8]. In addition to the poly-basic motif in HA, PB2, PB1, NP and NS1 are also involved in the pathogenicity of influenza viruses [24-26], although the nature of their involvement is not well understood. Here, we demonstrate that the H7N7 influenza virus isolated from a feral duck and serially passaged in chicks acquired pathogenicity in chickens through introduction of poly-basic amino acid residues at the HA cleavage site. Six amino acid substitutions were found between Vac2sub-P0 and Vac2sub-P3 (Table 1). Because Vac2sub-P0 was not pathogenic, the introduced poly-basic residues were necessary but not sufficient for pathogenicity in chickens, indicating that other viral factors were also involved.

As shown in Table 2, both amino acid changes at positions 227 and 388 in the HA of Vac2sub-P3 were essential for intravenous pathogenicity; since position 227 is in the vicinity of the receptor binding site, this residue may be involved in affinity of the receptor for its ligand, sialic acid. To better understand the acquisition of viral pathogenicity, it will be necessary to investigate not only its affinity for sialic acid-containing carbohydrates but also the identity of the sialic acid ligand. The amino acid at position 388 in the HA locates in the HA2 subunit and may be involved in the step of membrane fusion [27].

We observed that substitutions in PB2, PB1, M1 and M2 were involved in the acquisition of Vac2sub-P3 pathogenicity (Table 2). The polymerase complex of influenza viruses comprises PB2 and PB1 with PA and replicate viral RNA [28]. However, the polymerase activities of Vac2sub-P0 and Vac2sub-P3 were not significantly different using luciferase assays in 293T (human kidney cell line), CEF (chicken embryo fibroblast) and QT6 (quail fibroblast) cells (data not shown). As position 123 in PB2 and position 16 in PB1 are both responsible for bonding of the polymerase complex [29,30], the efficiency of polymerase complex formation will be assessed in further studies. Position 46 in M2 is in the vicinity of the amphipathic helix involved in virus budding [31]. M1 and M2 both act in combination with HA in particle formation of influenza viruses [32]. It will be necessary to assess the roles of M1 and M2 in viral particle stability and the efficiency of viral budding. Other viral proteins besides HA are responsible for the acquisition of pathogenicity in chickens; therefore, several viral factors are responsible for efficient replication in chickens.

Although all chickens inoculated intravenously with Vac2sub-P3 died, only 2 of the 6 intranasally inoculated chickens died. Intravenously inoculated virus replicated systemically, particularly in the brain and the kidneys (data not shown), leading to the death of birds. In contrast, the virus inoculated intranasally replicated mildly in the organs (data not shown) and was then eliminated by the acquired immune response. Considering the natural infection route of influenza viruses, it may be necessary for Vac2sub-P3 to accumulate additional amino acid substitutions before it is as pathogenic as wild HPAIVs.

We demonstrated that H7 avian influenza viruses isolated from a duck acquired pathogenicity through reverse genetics method and serial passaging in chicks. Early detection of H5 and H7 low pathogenic avian influenza viruses by culling and monitoring is important to avoid an outbreak of highly pathogenic avian influenza.

Acknowledgements

We are grateful for the support of the Global Center of Excellence (GCOE) Program of Hokkaido University, founded by the Japan Society for the Promotion of Science (JSPS). This work was also partially supported by JSPS KAKENHI 23780304 and J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases.

References

1. Kida H, Yanagawa R, Matsuoka Y (1980) Duck influenza lacking evidence of disease signs and immune response. Infect Immun 30 (2):547-553

2. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56 (1):152-179

3. Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR (2003) Replication and transmission of influenza viruses in Japanese quail. Virology 310 (1):8-15. doi:S0042682203000941 [pii]

4. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, Webster RG (2003) Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol 77 (5):3148-3156

5. Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ (2001) Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 146 (5):963-973

6. Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, Kuwayama M, Kawaoka Y, Otsuki K (2001) Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol 75 (9):4439-4443. doi:10.1128/jvi.75.9.4439-4443.2001

7. Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Süss J, Lipkind M, Kida H, Webster RG (1996) Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis 40 (2):425-437

8. Feldmann A, Schäfer MK, Garten W, Klenk HD (2000) Targeted infection of endothelial cells by avian influenza virus A/FPV/Rostock/34 (H7N1) in chicken embryos. J Virol 74 (17):8018-8027

9. Munster VJ, Schrauwen EJ, de Wit E, van den Brand JM, Bestebroer TM, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA (2010) Insertion of a multibasic cleavage motif into the hemagglutinin of a low-pathogenic avian influenza H6N1 virus induces a highly pathogenic phenotype. J Virol 84 (16):7953-7960. doi:JVI.00449-10 [pii] 10.1128/JVI.00449-10

10. Soda K, Asakura S, Okamatsu M, Sakoda Y, Kida H (2011) H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens. Virol J 8 (1):64. doi:1743-422X-8-64 [pii] 10.1186/1743-422X-8-64

11. Stech O, Veits J, Weber S, Deckers D, Schröer D, Vahlenkamp TW, Breithaupt A, Teifke J, Mettenleiter TC, Stech J (2009) Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. J Virol 83 (11):5864-5868. doi:JVI.02649-08 [pii] 10.1128/JVI.02649-08

12. Veits J, Weber S, Stech O, Breithaupt A, Gräber M, Gohrbandt S, Bogs J, Hundt J, Teifke JP, Mettenleiter TC, Stech J (2012) Avian influenza virus hemagglutinins H2, H4, H8, and H14 support a highly pathogenic phenotype. Proc Natl Acad Sci U S A 109 (7):2579-2584. doi:1109397109 [pii] 10.1073/pnas.1109397109

13. Aamir UB, Naeem K, Ahmed Z, Obert CA, Franks J, Krauss S, Seiler P, Webster RG (2009) Zoonotic potential of highly pathogenic avian H7N3 influenza viruses from Pakistan. Virology 390 (2):212-220. doi:10.1016/j.virol.2009.05.008

14. Capua I, Mutinelli F, Pozza MD, Donatelli I, Puzelli S, Cancellotti FM (2002) The 1999-2000 avian influenza (H7N1) epidemic in Italy: veterinary and human health implications. Acta Trop 83 (1):7-11. doi:S0001706X02000578 [pii]

15. Iglesias I, Martinez M, Munoz MJ, de la Torre A, Sanchez-Vizcaino JM (2010) First case of highly pathogenic avian influenza in poultry in Spain. Transboundary and emerging diseases 57 (4):282-285. doi:10.1111/j.1865-1682.2010.01145.x

16. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, 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 (9409):587-593. doi:S0140-6736(04)15589-X [pii] 10.1016/S0140-6736(04)15589-X

17. Pasick J, Berhane Y, Hisanaga T, Kehler H, Hooper-McGrevy K, Handel K, Neufeld J, Argue C, Leighton F (2010) Diagnostic test results and pathology associated with the 2007 Canadian H7N3 highly pathogenic avian influenza outbreak. Avian Dis 54 (1 Suppl):213-219

18. Lee CW, Lee YJ, Senne DA, Suarez DL (2006) Pathogenic potential of North American H7N2 avian influenza virus: a mutagenesis study using reverse genetics. Virology 353 (2):388-395. doi:10.1016/j.virol.2006.06.003

19. Soda K, Sakoda Y, Isoda N, Kajihara M, Haraguchi Y, Shibuya H, Yoshida H, Sasaki T, Sakamoto R, Saijo K, Hagiwara J, Kida H (2008) Development of vaccine strains of H5 and H7 influenza viruses. Jpn J Vet Res 55 (2-3):93-98

20. Desselberger U, Racaniello VR, Zazra JJ, Palese P (1980) The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 8 (3):315-328. doi:0378-1119(80)90007-4 [pii]

21. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146 (12):2275-2289

22. Hoffmann E, Webster RG (2000) Unidirectional RNA polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids. J Gen Virol 81 (Pt 12):2843-2847

23. OIE (2011) AVIAN INFLUENZA. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf. Accessed 1st April 2013.

24. Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H (2006) The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 80 (22):11115-11123. doi:JVI.00993-06 [pii] 10.1128/JVI.00993-06

25. Tada T, Suzuki K, Sakurai Y, Kubo M, Okada H, Itoh T, Tsukamoto K (2011) NP body domain and PB2 contribute to increased virulence of H5N1 highly pathogenic avian influenza viruses in chickens. J Virol 85 (4):1834-1846. doi:JVI.01648-10 [pii] 10.1128/JVI.01648-10

26. Wasilenko JL, Lee CW, Sarmento L, Spackman E, Kapczynski DR, Suarez DL, Pantin-Jackwood MJ (2008) NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens. J Virol 82 (9):4544-4553. doi:JVI.02642-07 [pii] 10.1128/JVI.02642-07

27. Klenk HD, Rott R (1988) The molecular biology of influenza virus pathogenicity. Adv Virus Res 34:247-281

28. Horisberger MA (1980) The large P proteins of influenza A viruses are composed of one acidic and two basic polypeptides. Virology 107 (1):302-305

29. Biswas SK, Nayak DP (1996) Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. J Virol 70 (10):6716-6722

30. Perez DR, Donis RO (2001) Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. J Virol 75 (17):8127-8136

31. Zhou HX, Cross TA (2013) Modeling the membrane environmenthas implications for membrane protein structureand function: Influenza a M2 protein. Protein science : a publication of the Protein Society. doi:10.1002/pro.2232

32. Roberts PC, Lamb RA, Compans RW (1998) The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 240 (1):127-137. doi:S0042-6822(97)98916-9 [pii] 10.1006/viro.1997.8916

Figure legends

Fig. 1 Nucleotide and amino acid sequences at the HA cleavage site of each virus

Poly-basic amino acid substitutions were introduced into the HA cleavage site of Vac2 (H7N7) by site-directed mutagenesis. Mutation sites are underlined and the deduced amino acid sequences are depicted in italics. Basic amino acids are in bold. Numbers correspond to the amino acid positions in HA (methionine encoded by the AUG start codon is defined as position 1).

Fig. 2 Survival curves of chickens inoculated with passaged viruses via intravenous and intranasal routes

(A) Eight 4-week-old chickens were intravenously inoculated with 100 μl of each virus (1:10 diluted allantoic fluid) and observed for 10 days. (B) Six 4-week-old chickens were inoculated intranasally with 100 μl of each virus at 10^6 EID_{50} and observed for 14 days.

Figure 1

							←HA1	/	HA2→
Vac2:	CCC	GAG	ATT	CCA	AAG	GGA	AGA	/	GGC
	P	*E*	*I*	*P*	***K***	*G*	***R***		*G*
Vac2sub:	CCC	AAG	AGG	CGA	AGG	AGA	AGA	/	GGC
	P	***K***	***R***	***R***	***R***	***R***	***R***		*G*
No. of amino acid:	333	334	335	336	337	338	339	/	340

Figure 2

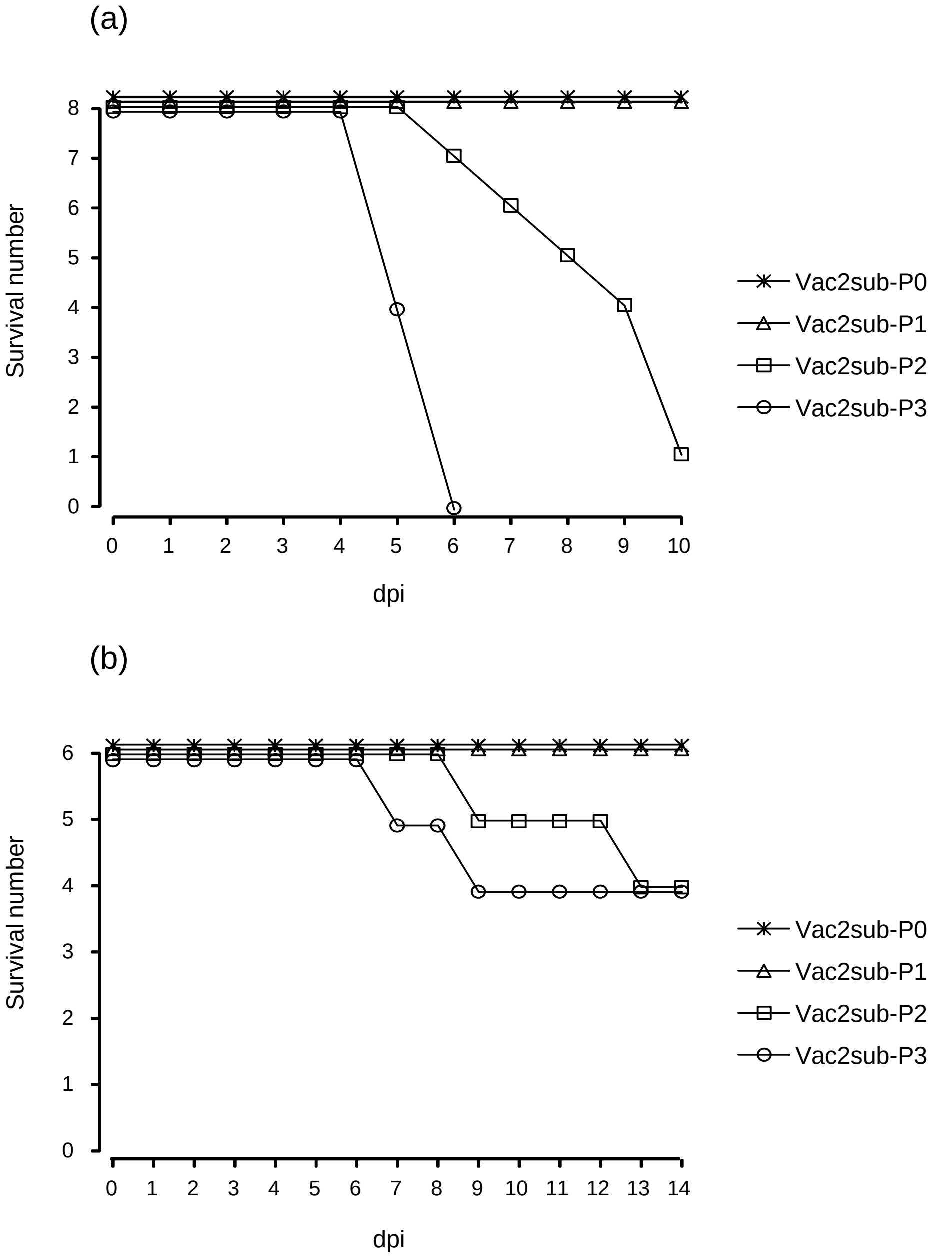
(a)
Survival number
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
10
dpi
Vac2sub-P0
Vac2sub-P1
Vac2sub-P2
Vac2sub-P3
(b)
Survival number
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
dpi
Vac2sub-P0
Vac2sub-P1
Vac2sub-P2
Vac2sub-P3

Table1 Amino acid changes during consecutive passages of Vac2sub

Passaged viruses		PB2		PB1	HA			M1	M2		Mortality		IVPI[c]
		4[a]	123	16	113 (105)	227 (218)	388 (378)	228	45	46	Intravenous	Intranasal	
Vac2sub-	P0	Ile	Lys	Asn	Glu	Glu	Ile	Gly	Arg	Leu	0/8	0/6	0.00
	P1	Asn	Glu	Asp	Gly	.	.	.	His	.	0/8	0/6	0.05
	P2	Asn	Glu	Asp	.	Gly	Thr	.	His	.	7/8	2/6	2.01
	P3	.[b]	Glu	Asp	.	Gly	Thr	Arg	.	Pro	8/8	2/6	2.54

[a] Methionine encoded by the AUG start codon is defined as position 1 (equivalent to the H3 numbering).

[b] Periods indicate the same amino acids as Vac2sub-P0.

[c] IVPI: intravenous patogenicity index.

Table 2

Table2 Pathogenicity of each mutant virus for chicken via intravenous route

Viruses	PB2	PB1	HA		M1	M2	Mortality	IVPI[c]
	123[a]	16	227	388	228	46		
rgVac2sub-P0	Lys	Asn	Glu	Ile	Gly	Pro	0/8	0.00
rgVac2sub-P3	Glu	Asp	Gly	Thr	Arg	Leu	8/8	2.21
rgP3/P0-PB2	.[b]	Asp	Gly	Thr	Arg	Leu	4/8	1.83
rgP3/P0-PB1	Glu	.	Gly	Thr	Arg	Leu	3/8	1.44
rgP3/P0-HA-227	Glu	Asp	.	Thr	Arg	Leu	0/8	0.00
rgP3/P0-HA-388	Glu	Asp	Gly	.	Arg	Leu	0/8	0.15
rgP3/P0-M1-228	Glu	Asp	Gly	Thr	.	Leu	2/8	0.75
rgP3/P0-M2-46	Glu	Asp	Gly	Thr	Arg	.	4/8	1.66
rgP0/P3-PB2,HA	Glu	.	Gly	Thr	.	.	2/8	0.70
rgP0/P3-PB1,HA	.	Asp	Gly	Thr	.	.	6/8	1.50
rgP0/P3-HA,M	.	.	Gly	Thr	Arg	Leu	6/8	1.21
rgP0/P3-HA	.	.	Gly	Thr	.	.	2/8	0.68
rgP0/P3-HA-227	.	.	Gly	.	.	.	0/8	0.05
rgP0/P3-HA-388	.	.	.	Thr	.	.	2/8	0.54

[a] Methionine encoded by the AUG start codon is defined as position 1.

[b] Periods indicate the same amino acids as rgVac2sub-P0.

[c] IVPI: intravenous patogenicity index.