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Molecular characterization of mammalian-adapted Korean-type avian H9N2 virus and evaluation of its virulence in mice

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Avian influenza A virus (AIV) is commonly isolated from domestic poultry and wild migratory birds, and the H9N2 subtype is the most prevalent and the major cause of severe disease in poultry in Korea. In addition to the veterinary concerns regarding the H9N2 subtype, it is also considered to be the next potential human pandemic strain due to its rapid evolution and interspecies transmission. In this study, we utilize serial lung-to-lung passage of a low pathogenic avian influenza virus (LPAI) H9N2 (A/Ck/Korea/163/04, WT163) (Y439-lineage) in mice to increase pathogenicity and investigate the potential virulence marker. Mouse-adapted H9N2 virus obtained high virulence (100% mortality) in mice after 98 serial passages. Sequence results show that the mouse adaptation (ma163) possesses several mutations within seven gene segments (PB2, PA, HA, NP, NA, M, and NS) relative to the wild-type strain. The HA gene showed the most mutations (at least 11) with one resulting in the loss of an N-glycosylation site (at amino acid 166). Moreover, reverse genetic studies established that an E627K substitution in PB2 and the loss of the N-glycosylation site in the HA protein (aa166) are critical virulence markers in the mouse-adapted H9N2 virus. Thus, these results add to the increasing body of mutational analysis data defining the function of the viral polymerase and HA genes and their roles in mammalian host adaptation. To our knowledge, this is first report of the generation of a mammalian-adapted Korea H9N2 virus (Y493-lineages). Therefore, this study offers valuable insights into the molecular evolution of the LPAI Korean H9N2 in a new host and adds to the current knowledge of the molecular markers associated with increased virulence.

Keywords: influenza A virus, H9N2, mammalian adaptation, reverse genetic method, virulence marker

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Introduction

For the past decade, avian influenza (AI) viruses, especially the H9N2 subtype, have not only been enzootic in Asia but have also been isolated from different types of terrestrial poultry worldwide (Naeem *et al*., 1999; Cameron *et al*., 2000; Guo *et al*., 2000; Saito *et al*., 2001; Liu *et al*., 2003a, 2003b). Based on antigenic and molecular properties, three lineages of H9N2 influenza viruses have been identified among avian species in Southeastern China including: A/Quail/Hong Kong/G1/97-like (G1-like), A/Chicken/Hong Kong/Y280/97-like (Y280-like) (Mo *et al*., 1997; Choi *et al*., 2005), and A/Duck/Hong Kong/Y439/97-like (Y439-like) lineages. Of these avian H9N2 virus lineages, viruses genetically closely related to the Dk/Hong Kong/Y439/97-like lineage from aquatic birds were transmitted in Korean avian species and distinctively formed the Korean-like lineage of H9N2 viruses (Guo *et al*., 1999; Lee *et al*., 2000; Choi *et al*., 2005). The first confirmed low pathogenic avian influenza (LPAI) virus outbreak in Korean poultry was caused by an H9N2 A/Chicken/Kor/MS96/96 virus, which was detected in and isolated from several broiler breeders in Gyeonggi-do (Mo *et al*., 1997). Typically, infection with H9N2 AI viruses causes mild disease among terrestrial birds; however, these viruses can also be transmitted to poultry farms where they may cause severe outbreaks (Brown *et al*., 2005).

Since 2000, LPAI H9N2 has become endemic in Korean poultry, especially in laying farms, causing economic losses due to reductions in egg production and sometimes severe morbidity and mortality of infected chickens. Since vaccination is the most promising control measure for the LPAI H9N2 virus, the Korean government permitted the use of an inactivated vaccine derived from a Korean H9N2 isolate (Ck/Korea/01310/01) to be used among laying poultry in October, 2007 (Choi *et al*., 2008). However, a recent study conducted with virus isolates collected between January 2008 and December 2009 from selected meat bird slaughterhouses revealed the evolution and emergence of novel genotypes of H9N2 viruses (Park *et al*., 2011). Most recent H9N2 isolates were the reassortants between the previous H9N2 and H3N2 Korean aquatic bird influenza viruses, and serologic analysis showed that the isolates have undergone significant antigenic drift and shift with wild aquatic avian influenza A viruses that somehow altered their pathogenicity in experimental animals (Park *et al*., 2011).

While generally considered to be more a burden to the farming industry, the LPAI H9N2 subtype virus has also been transmitted to humans on several occasions. Two pediatric cases with mild respiratory disease were identified in Hong Kong in 1999 and several more human H9N2 infections

were reported in mainland China between 1998 and 1999 (Peiris *et al*., 1999; Lin *et al*., 2000; Uyeki *et al*., 2002). Furthermore, two additional H9N2 virus infections in children were documented in Hong Kong in 2003 and 2007 (Katz *et al*., 2009). Thus, with the rapid spread of H9N2 worldwide and its rapid evolution and transmission to various hosts there is a potential threat of this virus becoming the next pandemic. Therefore, in this study we analyzed potential markers of virulence and pathogenicity of a Korean linage LPAI A/Ck/Korea/04163/04 virus after serial lung-to-lung passage in mice. To this end, we utilized an established reverse genetics (RG) system to generate recombinant viruses in order to compare the parental and mouse-adapted strains. Our results show that a characteristic Glutamic acid (E)-to-Lysine (K) substitution in PB2 is the main molecular determinant for increased virulence of the ma163 strain. However, the contribution of additional mutations in other gene segments, namely a loss of an N-glycosylation site in HA (amino acid 166), with respect to enhanced pathogenicity and virulence could not be ruled out.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (EMEM) with 5% fetal bovine serum (FBS), while 293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium containing 5% FBS. Media and additives were purchased from Lonza. All cells were incubated at 37°C in 5% CO_2.

Virus strain A/Ck/Korea/04163/04 (WT163) was isolated in 2004 from routine avian influenza virus surveillance and diagnostic services conducted for poultry farms in Korea. This wild-type virus was then infected into mice intranasally. At 2 days post-inoculation (dpi), lungs of the infected mice were harvested and smashed in the presence of 50% antibiotics. Supernatant was isolated and then serially passaged in mice until a mouse-adapted (ma), highly virulent strain termed ma163 was generated. The reverse genetic (RG) ma163 virus is phenotypically and genetically identical to the ma163 virus. While RG ma163 $PB2_{627E}$ and RG-ma163 $HA_{166N\text{-}glyco}$ have a K627E substitution in its PB2 gene and an N-glycosylation site and amino acid 166 in HA gene via site-directed mutagenesis inserted by site-directed mutagenesis system, respectively (Invitrogen). These virulent viruses were isolated by plaque purification, described below, and were selected based on their phenotypes and sequence differences relative to the parental wild-type (163) virus.

Plaque purification and selection

To isolate a single-phenotype virus that causes mortality similarly to the mouse-adapted virus, lung isolates of the virulent mouse-adapted strain were plaque-purified in MDCK cells as previous study (Gubareva *et al*., 1997). Briefly, supernatants of lung tissue homogenates were serially diluted by 10-fold in appropriate media. MDCK cells were infected with the dilution samples in six-well plates. After 1 h of incubation, the cells were washed with PBS and overlaid with a 0.7% agarose-medium mixture with L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin. Sixty hours later, twelve single-plaque colonies were picked, resuspended in medium, and injected into 11-day-old embryonated chicken eggs. After 48 h of incubation, viruses were harvested and the 50% egg infectious dose (EID_{50}) was calculated by the method of Reed and Muench (1938). Virulence of the twelve plaque-purified viruses (5.0 $\log_{10}$ EID_{50}/ml) was re-evaluated in mice. Based on the highly virulent phenotype and sequence analysis, one out of the eight purified plaques was selected for further studies.

Genomic sequencing and molecular analysis

Viral RNA was extracted from infected embryonated chicken egg isolates using a QIAamp Viral RNA Mini kit (QIAGEN). RT-PCR was carried out under standard conditions using influenza A virus-specific primers as previously described (Hoffmann *et al*., 2000). A 50 μl PCR reaction contained 5 U of Enzynomics *n*Taq DNA polymerase (Enzynomics), 6 μl of 20 mM Mg^{2+}, and 3 μl of 2.5 mM of each dNTP, appropriate concentrations of template cDNA, and 1 μl of 10 pM primer mixture. Each PCR product was amplified using the following conditions: denaturation step for 5 min at 94°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min 30 sec, followed by a final extension step at 72°C for 10 min. Amplicons were purified using the GeneAll gel extraction kit (Gene All) and sent to Cosmo GeneTech. DNA sequences were compiled and analyzed using Lasergene sequence analysis software package 5.0 (DNASTAR).

Plasmids and rescue of reverse genetics viruses

To clone all eight genes of the A/Ck/Korea/04163/04 and ma163 H9N2 virus, we amplified each segment by multiplex RT-PCR from isolated viral RNAs and cloned them into the pHW2000 vector as described previously (Hoffmann *et al*., 2000). All recombinant and point mutation viruses were rescued in a six-well plate of co-cultured 293T and MDCK cell mixtures (3:1 ratio) transfected with the corresponding eight viral plasmids, each containing 0.5 μg of the respective gene segment, using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Transfection medium was removed after 6 h and replaced with Opti-MEM I (Gibco) containing 0.3% bovine serum albumin and 0.01% FBS. After 30 h, 1 ml of Opti-MEM I containing 0.2 g/ml of TPCK-trypsin was added to the transfected cells. Supernatant was harvested after 48 h and injected into 11-day-old embryonated chicken eggs for virus propagation.

Experimental infection of recombinant viruses in mice

Sixteen groups of 12, 5-week-old female BALB/c mice were intranasally infected with 30 μl of 5.0 $TCID_{50}$/ml recombinant viruses. Mice were caged individually by groups and were observed for 12 days to compare mortality. The mice were housed in a facility that maintained consistent temperature and humidity. Survival rate was monitored within the 12 day period and 2 mice for each group were sacrificed at 3, 5, 7 dpi for lung virus titer determination. Lung samples were stored and at -80°C.

Statistical analysis

The data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software). *P* values of less than 0.05 ($P<0.05$) were considered to be statistically significant.

Results

Generation of mouse-adapted (ma) H9N2 virus

Strain A/Ck/Korea/04163/04 (H9N2, WT163) is an LPAI virus that does not bear a polybasic amino acid motif at the HA proteolytic cleavage site. This virus was isolated in 2004 during routine surveillance of domestic poultry and is able to infect but is otherwise avirulent in mice. In contrast, mouse-adapted variants obtained after serial lung-to-lung passage (98 passages) in mice replicated well and showed highly lethality in mice (Fig. 1).

To obtain a single, purified mouse-adapted H9N2 virus, plaque purification was conducted on passage 98 mouse lungs in MDCK cells. From the plaque-purified viruses 12 plaques were randomly selected and the virulence confirmed in mice. Four of the twelve viruses (plaques 3, 6, 9, and 11) showed high lethality (100%) (Fig. 1) and full-length sequences of all segments of these four purified viruses were compared with those of the wild-type parental virus (WT163). Results indicated that three of the highly lethal purified viruses (plaques 3, 6, and 11) share the same sequences and only one purified virus showed minor amino acid sequence deviations from the other three (plaque 9). Of the three highly-lethal purified viruses, one plaque (plaque 6) (ma163) was chosen for further analysis. Several base differences were observed between the parental and mouse-adapted virus (plaque 6), affecting seven of the eight viral gene segments. These included two coding changes (627 and 740) in the polymerase basic protein 2 (PB2), three changes (160, 549, and 616) in polymerase acidic protein (PA), and one coding change (120) in the neuraminidase (NA) gene. Moreover, there were two coding changes in the each non-structural protein (NS) and the matrix protein (M). There was no amino acid mutations observed in the polymerase basic protein 1 (PB1) (Table 1). It is also worth noting that the observed frequency of mutations in the HA gene is relatively high as compared to other gene segments (11 amino acid mutations), which includes mutations in the cleavage site, A335I, and an N-glycosylation site, N166S. An E627K mutation was also found in the PB2 gene, and is believed to be one of the virulence-affecting mutations. To further verify the frequency of a particular amino acid at the substituted sites, we analyzed the single amino acid polymorphism in avian and human influenza sequence databases obtained from the Influenza Research Database. As reported, the proportion of K627 in PB2 is much higher in human influenza isolates than in avian isolates (Table 1) indicating the potential role for this substitution as a marker of mammalian adaptation. Interestingly, the proportion of H52 in NP is 8.3% in avian isolates and 25.2% in human isolates implying that the mutation may be preferentially selected during avian influenza viral adaptation in human.

Mouse-adapted H9N2 virus is highly virulent and pathogenic in mice

To identify potential virulence markers linked to increased replication and mortality of ma163, we attempted to generate recombinant ma163 and parental viruses using reverse genetics. To this end, only ma163 viruses that showed high viral titers in mouse lungs rescued growth while parental WT163 failed to rescue. To compare the virulence of WT163, ma163, and RG-ma163 viruses, groups of mice were infected intranasally with 5.0 $\log_{10}$ EID_{50}/ml of each of the viruses and survival was monitored. For the parental virus strain (WT163), there were no observable clinical signs of infection and all infected mice survived for the duration of the study (100% survival rate). On the other hand, for the ma163 and RG-ma163 viruses mortality were observed as early as days 4 and 5 post-inoculation and all mice succumbed to death by 9 dpi, (Fig. 2A). Moreover, ma163 and RG-ma163 infected mice showed clinical signs of infection such as decreased musculature, erect hair, and hunched backs (data not shown). These results suggest that the ma163 and RG-ma163 viruses acquired high virulence during serial passage in mice. To investigate whether the increased virulence correlates with viral growth in lungs, three mice in each group were euthanized on days 1, 3, 5, 7, and 9 dpi (most mice inoculated with ma163 and RG ma163 died before day 9), and the viral titer in the lungs was measured by EID_{50}/ml. WT163 virus could replicate in the lungs of infected mice as evidenced by the

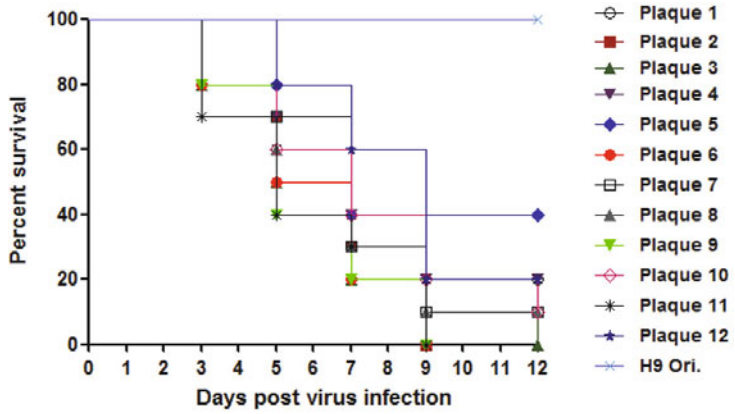

Fig. 1. Virulence tests of plaque-purified mouse-adapted H9N2 viruses. After 98 serial lung passages in mice, the highly virulent H9N2 viruses were plaque-purified in MDCK cells. To confirm virulence of the selected plaques, each plaque virus was intranasally infected into 10 mice (30 μl of 5.0 $\log_{10}$ EID_{50}/ml). Four of the plaques (P3, 6, 9, 11) showed 100% mortality after 12 days of infection and these plaques were fully sequenced afterwards for comparison with wild-type H9N2 (see Table 1).

Table 1. Amino acid differences between the parental wild-type and the mouse-adapted strains and their amino acid sequence variations in avian and human influenza viruses

Gene	Position (aa)	Wild type virus	Mouse-adapted virus	Polymorphism(s) in database [frequency (%)]			
				Avian viruses	No. of sequences	Human/mammalian viruses	No. of sequences
PB2	627	E	K	E(94.82), K(3.795), T(0.210), V(0.916), L(0.130), R(0.036), A(0.021), G(0.021), Q(0.014), M(0.014), D(0.007), I(0.007)	13752	K(56.88), E(42.87), Q(0.143), R(0.082), A(0.015)	13265
	740	D	N	D(99.333), L(0.0074), A(0.5918), N (0.0374), E(0.0149), G(0.0149)	13347	D(99.298), N (0.6861), A(0.0155)	12825
PA	160	V	D	D(98.840), E(0.7856), N (0.3051), G(0.0381), K(0.0152), V(0.0076), Y(0.0076)	13110	D(99.939), E(0.0456), A(0.0076), N(0.0076)	13139
	549	L	I	L(99.504), F(0.7392), I(0.4657), M(0.0147), P(0.0073)	13527	L(99.740), F(0.7628), I(0.1602), V(0.0839), S(0.0076)	13108
	616	S	P	S(99.198), F(1.4845), V(0.3859), T(0.1261), L(0.1187), A(0.0742), P(0.0519), I(0.0148), C(0.0074), W(0.0074)	13472	S(99.847), L(0.0913), P(0.0304), T(0.0304)	13138
HA	87 (79)[a]	L	S	L(87.869), M(4.2677), P(3.7708), Q(1.9877), I(1.0523), R(0.0876), S(0.8769), V(0.0584), T(0.0292)	3421	L(94.202), Q(4.3478), G(1.4492)	69[b]
	127 (119)	R	H	S(64.412), R(26.493), N(7.6945), G(0.8743), K(0.3206), M(0.0874), I(0.0291), P(0.0291), T(0.0291), C(0.0291)	3431	S(91.304), R(7.2463), G(1.4492)	69
	138 (130)	R	H	T(89.539), S(4.6037), R(3.7004), A(1.5442), K(0.5244), H(0.0291), E(0.0291), G(0.0291)	3432	T(95.652), R(2.8985), E(1.4492)	69
	166[c] (158)	N	S	N(72.732), S(17.267), D(8.0232), R(0.6686), T(0.0581)	3440	N(72.463), S(18.840), D(8.6956)	69
	179 (171)	D	E	N(93.994), T(2.9881), D(2.8140), I(0.0580), K(0.0290)	3447	N(97.101), D(2.8985)	69
	225 (217)	I	L	I(98.789), V(1.1258), K(0.0562), T(0.0281)	3553	I(100)	69
	246 (238)	K	R	K(99.549), R(0.3380), N(0.0845), I(0.0281),	3550	K(100)	69
	335[d] (327)	A	I	R(81.762), K(8.6930), A(7.7239), T(1.3215), I(0.2643), S(0.1174), G(0.0881), N(0.0293)	3405	R(97.058), A(2.9411)	68
	382 (374)	V	A	A(99.907), T(0.0614), S(0.0307)	3253	A(100)	63
	514 (506)	V	L	V(97.529), I(2.4073),L(0.0316), A(0.0316)	3157	V(96.491), I(3.5087)	57
	554 (546)	C	R	C(99.647), S(0.1566), R(0.0783), G(0.0783), Y(0.0391)	2554	C(97.560), I(2.4390)	41
NP	52	Y	H	Y(82.619), H(8.3221), N (7.0727), F(0.7437), Q(1.7626), C(0.1710), S(0.0446)	13446	Y(74.062), H(25.156), N(0.4936), Q(0.2726), S(0.0073), C(0.0073)	13571
	433	S	T	T(95.738), A(2.4382), N (0.9971), F(0.7789), I(0.3115), M(0.0389), P(0.2492), S(0.2103), G(0.0077)	12837	T(56.463), N(41.041), A(2.2588), M(0.1103), S(0.0735), K(0.0220), I(0.0220), V(0.0073)	13591
NA	120	P	S	P(99.888), S(0.0431), T(0.0370), L(0.0308)	16204	P(99.774), L(0.1761), H(0.0306), S(0.0153), R(0.0038)	26117
M	36	N	S	N(99.718), S(0.1714), T(0.0274), K(0.0411), D(0.0274), I(0.0068) , H(0.0068)	14581	N(99.887), S(0.0918), T(0.0051), Y(0.0051), H(0.0051), D(0.0051)	19595
	167	T	I	T(94.973), A(4.8091), I(0.0884), S(0.0884), N (0.0340), V(0.0068)	14701	T(55.741), A(44.107), I(0.1106), N(0.0251), S(0.0100), E(0.0050)	19881
NS	5	S	T	T(99.854), L(0.0582), P(0.0291), S(0.0218), A(0.0072), H(0.0072), K(0.0072), C(0.0072), R(0.0072),	13740	T(99.978), I(0.0219)	13669
	180	A	V	V(73.041), I(26.276), T(0.3131), A(0.2644), D(0.0626), G(0.0278),M(0.0069), S(0.0069)	14370	V(98.300), I(1.6274), F(0.7200), T(0.0216), L(0.0144), D(0.0144), A(0.0072),G(0.0072)	13887

[a] the number according to H9 HA
[b] H9 sequences contain all mammalian host isolates found in Influenza Research Database.
[c] N-glycosylation site.
[d] at the cleavage site.

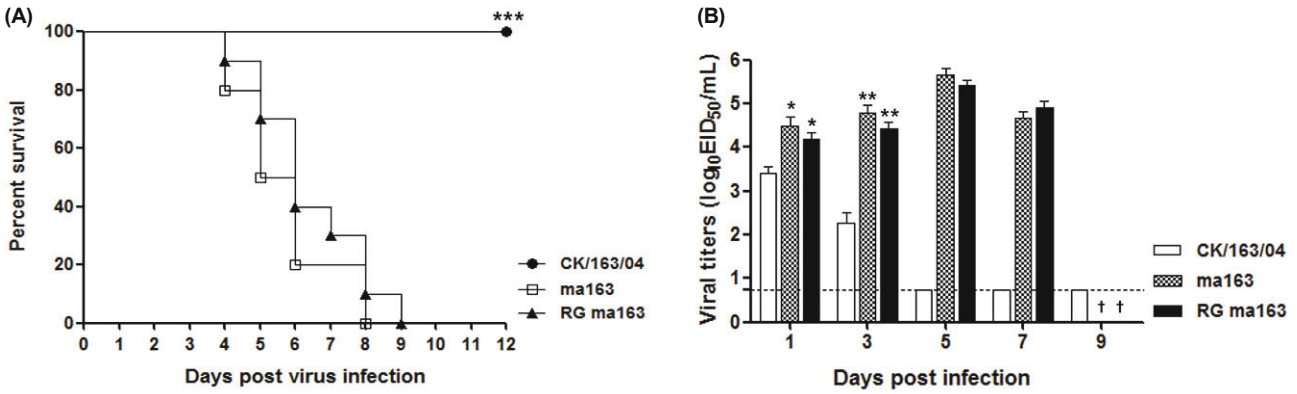

Fig. 2. Survival rate (A) and lung virus titers (B) of the WT163, ma-163, and RG ma-163 viruses in mice. (A) Each group of mice was inoculated intranasally with a 30 μl volume of 5.0 $\log_{10}$ EID_{50}/ml of WT163, ma163 or RG-ma163 viruses and survival was monitored daily for 12 days. ***, $P<0.0001$ compared to the ma163 and RG ma163 groups by Log-rank (Mantel-Cox) Test. (B) Mouse lungs (n=5) were collected at 1, 3, 5, 7, and 9 dpi and virus titers were determined from lung homogenates. Error bars indicate standard error of the mean (SEM) of triplicate assays. The lower limit of detection (0.75 $\log_{10}$ EID_{50}/ml) is represented by a broken line. * indicates $P<0.01$ compared to the CK/163/04 group; **, $P<0.001$ compared to the CK/163/04 by Student *t*-test. Mice that died are indicated by †.

viral titer of 2.3 EID_{50}/ml at 3 dpi, while no virus was detected from 5 dpi on (Fig. 2B). In contrast, the ma163 and RG-ma163 viruses showed significantly higher viral titers at all-time points (peaked 5.5 to 5.7 EID_{50}/ml at 5 dpi) compared with the WT163 virus. These data demonstrate that the increased virulence of ma163 is associated with high virus growth in infected mice.

The role of the E627K mutation within the PB2 gene in mice

The PB2 E627K mutation is a widely known mutation that contributes to pathogenicity and virulence of influenza A viruses (Shinya *et al.*, 2004). As shown above, the HA gene of the ma163 virus was found to be the most mutated (at least 11 mutations) with the loss of an N-glycosylation site (amino acid No. 166). In order to verify the importance of these mutations and their contribution to virulence, we utilized reverse genetics to develop a mouse-adapted virus bearing a glutamic acid residue at site 627 instead of lysine (RG ma163 $PB2_{627E}$), or an N-glycosylation site and amino acid 166 (RG-ma163 $HA_{166N\text{-}glyco}$) via site-directed mutagenesis. To compare the virulence of these recombinant viruses in mice, groups of mice (n=10) were infected intranasally with 5.0 EID_{50}/ml of each virus and survival was monitored daily for a 12-day observation period (Fig. 3). As expected, the RG ma163 infecteion resulted in 100% mortality by 8 dpi, while the RG ma163 $PB2_{627E}$ virus exhibited significant attenuated virulence (20% mortality) compared with the RG ma163 virus (Fig. 3). Further, the addition of an N-glycosylation site within the HA protein, at amino acid 166, (RG ma163 $HA_{166N\text{-}glyco}$) also attenuated the virulence of the RG ma163 virus (70% vs 100% mortality). These data clearly demonstrate that the 627K substitution in PB2 and the N-glycosylation site at amino acid 166 of the H9 HA protein of A/Ck/Korea/04163/04 appear to be critical for the increased virulence in mice; however, additional roles for other mutations within internal genes cannot be ruled out.

Discussion

Serial lung-to-lung passage of influenza viruses in animal models allows competition between many potential mutants and results in the selection of optimal genotypes with the highest replicative fitness in the host. Although there has

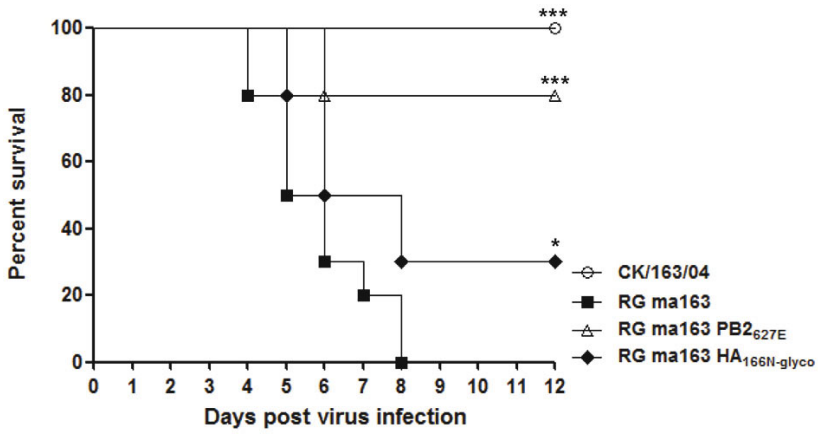

Fig. 3. Virulence of each recombinant virus in mice. Groups of mice (n=10) were intranasally infected with a 30 μl volume of 5.0 $\log_{10}$ EID_{50}/ml of each RG ma-163, RG $PB2_{627E}$, and RG ma163 $HA_{166N\text{-}glyco}$, and the survival rate was compared to that of the WT163-infected group. RGma163 showed 100% mortality at 8 dpi, while the RG ma163 $PB2_{627E}$ and RG ma163 $HA_{116N\text{-}glyco}$ viruses showed significantly attenuated virulence in infected mice. * indicates $P<0.05$ compared to the RG ma163 group; ***, $P<0.0001$ compared to the RG ma163 groups by Log-rank (Mantel-Cox) Test.

been a vast amount of research pertaining to mouse adaptations arising through serial lung passage, it was confined to virus strains such as H1, H2, H3, HPAI H5, LPAI H5, and H7 (Lipatov *et al*., 1995; Gabriel *et al*., 2005; Mase *et al*., 2007; Smee *et al*., 2007; Narasaraju *et al*., 2009; Song *et al*., 2009). In our surveillance analysis of avian influenza viruses among poultry farms in South Korea, the H9N2 AI subtype was found to be the most abundant isolate (unpublished data). Many studies have shown that H9N2 viruses are virulent in chickens and other poultry, causing severe disease and high mortality (Guo *et al*., 2000; Capua and Alexander, 2004; Choi *et al*., 2004; Aamir *et al*., 2007). Importantly, cases of human infection with H9N2 influenza viruses have been reported in both Hong Kong and mainland China, although sustained human-to-human transmission has not been documented (Guo *et al*., 1999, 2000; Peiris *et al*., 1999; Butt *et al*., 2005). However, previous studies indicated that at least 2.0% of the human population in southern China has H9 antibodies suggesting that a low level of human-to human-transmission of this virus may have occurred (Guo *et al*., 1999; Peiris *et al*., 1999; Butt *et al*., 2005). Thus, there is concern that the H9N2 influenza A virus might have the capacity to undergo genetic evolution resulting in enhanced replication and transmission to humans. It should be noted, however, that the genetic lineage of Korean H9N2 (Y439-lineage) is quite different compared to viruses from South Eastern Asia, including Hong Kong and mainland China (G1- or Y280 lineages).

Although there are no known cases of human infection with the Y439 lineages of H9N2 viruses, given the zoonotic concerns surrounding H9N2 viruses, we focused more on the ability of the H9 virus to adapt to a mammalian host. To generate the 100% mouse lethal Korean H9N2 virus, we conducted 98 lung-to-lung serial passages of LPAI A/Ck/Korea/04163/04 H9N2. In our experience, 98 passages is an unusually high number of passages required to obtain a high virulence LPAI strain in mice. For example, H5 and H7 viruses can obtain 100% mortality in mice within 20 passages (Smeenk and Brown, 1994; Park *et al*., 2011). Nonetheless, our studies show that ma163 is able to grow with a high viral titer and reach 100% mortality by 8 dpi (Fig. 2). Furthermore, this finding confirms the potential of this avian virus strain to replicate in a mammalian host, and suggests it may also be able to replicate in humans.

Genetic and molecular analyses revealed that most of the differences in the amino acid sequences of WT163 and ma163 viruses were contained within the HA gene (11 sites) (Table 1). However, no amino acid changes were observed in the PB1 gene segment. At the molecular level, one strong indicator of adaptation is convergent evolution, which is characterized by the repeated and independent occurrence of common mutations in adapted variants (Brown *et al*., 2001). However, it should be noted that not all adaptive mutations are indicative of increased virulence in specific hosts. In the HA gene, mutations were observed at the cleavage site (A335I) and in addition there was the loss of an N-glycosylation site at amino acid residue 166 (N166S). The HA gene is known to be responsible for host-specificity as well as for fusion with the host cell (Brown, 1990; Basler *et al*., 1999) and the mutation we observed in the cleavage site of HA is an important determinant of pathogenicity (Lee *et al*., 2006). Moreover, the presence of N-glycosylation sites could greatly interfere with the host immune response and therefore, can contribute to increased virulence. Three mutations were localized within the HA2 region, V382A, V514L, and C554R. HA2 acts during uncoating to promote fusion of the viral envelope with the endosomal membrane (Smeenk and Brown, 1994; Brown *et al*., 2001) and thus, mutations in this region might affect this process and contribute to increased virulence. The NP protein plays an important role in both viral assembly and RNA synthesis (Brown, 1990, 2000; Gabriel *et al*., 2005; Wu *et al*., 2009). The NP Y52H substitution reported here is located within the RNA-binding region, and the frequency of Histidine residues at this site is much higher in human influenza isolates than in avian isolates (25.2 vs 8.3%) suggesting a putative site for mammalian adaption. This substitution (Y52H) is also commonly found in recently-isolated human H3N2 viruses (the A/California/7/04(H3N2)-like isolates) (Bragstad *et al*., 2008) implying that the mutation may be preferentially selected during avian influenza viral adaptation in human. However, the precise roles of the Y52H substation during mammalian adaption in NP gene need to be verified as a further study.

One of most important mutations identified is the E627K substitution within PB2 since multiple studies of mouse adaptations have suggested that E627K is a primary contributor to virulence (Gao *et al*., 1999; Hatta and Kawaoka, 2003, 2005; Shinya *et al*., 2004; De Wit *et al*., 2010; Yamada *et al*., 2010). Because of this, we attempted to confirm the importance of E627K using a recombinant virus. Infection with WT163, ma163, and RG-ma163 $PB2_{627E}$ viruses in mice demonstrated that only the ma163 virus was 100% lethal at 8 dpi while the RG-ma163 $PB2_{627E}$ and WT163 showed significantly attenuated virulence with 80% and 100% survival rates, respectively (Fig. 2). This result implies that $PB2_{627K}$ does indeed contribute to virulence in the Korean H9N2 virus since the revertant virus (RG-ma163 $PB2_{627E}$) exhibited a survival rate as high as 80%. In addition, the RG ma163 $HA_{166N\text{-}glyco}$ virus, which contains an additional N-glycosylation site at amino acid 166 within the HA protein is commonly found in avian H9 viruses, could also alter the virulence of the RG ma163 virus (mortality 70% vs 100%) (Fig. 2). Although we cannot rule out the possible contribution of additional adaptive changes within other gene segments of ma163 to virulence, these data demonstrate that the 627K substitutions in PB2 and N-glycosylation at the 166 site of the H9 HA protein of A/Ck/Korea/04163/04 play critical roles for the increased growth and enhanced virulence characteristics of this virus in mice. Taken together, our results demonstrates the critical roles of the viral polymerase and HA genes for virulence and mammalian host adaptation of avian influenza A virus. To our knowledge, this is first report of the generation of a mammalian-adapted Korean-lineage of avian H9N2 virus and defining the molecular markers associated with increased virulence. Further analysis of additional amino acid mutations, specifically in the HA gene, which showed the highest number of mutations, will be necessary in order to better understand the evolutionary adaptation of H9N2 as well as the nature of its transmission to mammalian hosts.

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