An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea

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The H9N2 subtype low pathogenic avian influenza is one of the most prevalent avian diseases worldwide, and was first documented in 1996 in Korea. This disease caused serious economic loss in Korea's poultry industry.

In order to develop an oil-based inactivated vaccine, a virus that had been isolated in 2001 (A/chicken/Korea/01310/2001) was selected based on its pathogenic, antigenic, and genetic properties. However, in animal experiments, the efficacy of the vaccine was found to be very low without concentration of the antigen (2^7 to 2^{10} hemagglutinin unit). In order to overcome the low productivity, we passaged the vaccine candidate virus to chicken eggs. After the 20th passage, the virus was approximately ten times more productive compared with the parent virus. For the most part, the passaged virus maintained the hemagglutinin cleavage site amino acid motif (PATSGR/GLF) and had only three amino acid changes (T133N, V216G, E439D, H3 numbering) in the hemagglutinin molecule, as well as 18 amino acid deletions (55-72) and one amino acid change (E54D) in the NA stalk region. The amino acid changes did not significantly affect the antigenicity of the vaccine virus when tested by hemagglutination inhibition assay. Though not complete, the vaccine produced after the 20th passage of the virus (01310 CE20) showed good protection against a homologous and recent Korean isolate (A/chicken/Korea/Q30/2004) in specific pathogen- free chickens.

The vaccine developed in this study would be helpful for controlling the H9N2 LPAI in Korea.

Keywords: AI, avian influenza, H9N2, inactivated vaccine, LPAI

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Introduction

Avian influenza virus (AIV) is an enveloped virus that belongs to the *Orthomyxoviridae* family and has an eight segmented, single stranded, negative sense RNA genome. Among the proteins encoded by the genome, there are two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). AIV is classified into subtypes according to the combination of 16 HA and 9 NA molecules [10,26].

Among the many subtypes, the H9N2 AIV is thought to have originated from shorebirds and gulls [30], and rapidly spread to become one of the most prevalent diseases in domestic poultry worldwide. It has also caused serious economic loss in the poultry industry [5,16,17]. In Korea, the first H9N2 low pathogenic avian influenza (LPAI) outbreak occurred in 1996 [15]. Since 2000, and it has become endemic (especially in layer farms) [13,16]. Many studies have demonstrated that there are several distinct H9N2 AIV lineages, and indicated the Korean H9N2 viruses formed a unique antigenic and phylogenetic cluster [13,15-18].

Although immunization with this vaccine is not complete, it is one of the most promising control measures for the H9N2 LPAI to date. Some countries have used vaccines for H9N2 LPAI [5,17,22,29]; however, vast antigenic variations exist even within the same subtype, and it is very difficult to select a vaccine strain that is effective on the virus in current circulation. In addition, some isolates do not grow to a high enough titer in the embryonated chicken eggs (ECEs) to achieve efficient vaccine productivity [31].

Korean animal health authorities took stamping-out and compensation control policies with regards to H9N2 LPAI when it occurred between 1996 and 1999. At that time, vaccines for subtypes of AIV, including H9N2 LPAI, were prohibited in Korea because they interfered in the discrimination of naturally infected birds from vaccinated birds. However, H9N2 LPAI became endemic, and the policy was not reliable enough to cover each outbreak.

According to the 2004 Avian influenza standard operating procedures, Korean animal health authorities permitted the use of the vaccine for LPAI (especially the H9N2 subtype), and the Committee on the National AI Vaccine Campaign determined that using a single vaccine strain was the most effective strategy with which to simplify the H9N2 AI situation in Korea [7,20].

In this study, we present the characterization of the Korean H9N2 LPAI vaccine strain, and evaluated the efficacy of the pilot vaccine in specific pathogen-free (SPF) chickens.

Materials and Methods

Viruses used in this study

All of the viruses were isolated by the National Veterinary Research and Quarantine Service using routine diagnostic practices. The infectious tissue homogenates were inoculated in the allantoic cavity of 9-11 day old SPF ECEs (Lohmann Valo SPF Cuxhaven, Germany) according to standard procedures [27]. The first H9N2 isolate in Korea, A/Chicken/Korea/MS96/1996 (MS96) [15] was used, and A/Chicken/Korea/99029/1999 (99029) and A/Chicken/Korea/01310/2001 (01310) were used as representative isolates of the 1999 and 2001 strain, respectively. The 2001 strain was eventually chosen as the vaccine candidate. In order to test the antigenicity and the efficacy of the vaccine, a recent isolate, A/Chicken/Korea/Q30/2004 (04Q30), was used [16].

Pathogenicity test of Korean isolates in SPF and commercial broiler chickens

In order to determine the pathogenicity of the selected viruses in SPF and commercial broiler chickens, MS96, 99029, and 01310 were inoculated via the intra-tracheal route in eight 7-week-old SPF chickens ($10^{6.5}$ EID$_{50}$/0.1 ml, $10^{5.6}$ EID$_{50}$/0.1 ml, and $10^{7.1}$ EID$_{50}$/0.1 ml, respectively) and fifteen 12-week-old commercial broiler chickens ($10^{5.2}$ EID$_{50}$/0.1 ml, $10^{5.7}$ EID$_{50}$/0.1 ml, and $10^{5.0}$ EID$_{50}$/0.1 ml, respectively), which were confirmed to be free of antibodies against H9N2 AIV. In the experiment with SPF chickens, tracheal and cloacal swab samples were taken at 3, 5, 7, and 9 days post-inoculation (dpi). The swab samples were suspended in 3 ml of gentamicin-PBS (1% gentamicin in PBS, pH 7.2), and were inoculated with 0.2 ml of samples into three 9-11 day old SPF ECEs via the allantoic cavity route. The inoculated broiler chickens were reared for 2 weeks, and the mortality was recorded.

Antigenic relationship between Korean H9N2 AIVs

After being propagated in ECEs, the viruses (MS96, 99029, and 01310) were inactivated by incubating them with 0.1% formalin at 20°C for 10 h. The inactivation was confirmed by injecting formalin-treated virus into the allantoic cavities of 10-day-old ECEs, two times serially.

Virus inactivation was determined by hemagglutination negativity, using 1% chicken red blood cells. The inactivated virus was emulsified with oil adjuvant (Montanide ISA 70 SEPPIC, France) at a ratio of 3：7, and wasinjected into eight 6-week-old SPF chickens. The antisera were obtained at 3 weeks after injection. In order to determine the antigenic relationship between the selected viruses, we performed a cross hemagglutination inhibition (HI) test with each of the antisera and virus antigens (four HA unit), and the r-value was subsequently calculated [2].

HA and NA gene sequencing for comparison with recent Korean isolates

Viral RNA was extracted from infectious allantoic fluid using the Viral Gene-Spin Viral DNA/RNA extraction kit (iNtRON Biotechnology, Korea) and amplified using gene-specific primer sets by RT-PCR with a Qiagen one-step RT-PCR kit (Qiagen, USA) according to the method described by Hoffmann *et al.* [12]. The amplified product was excised from agarose gel and eluted using the GENECLEAN SPIN kit (Qbiogene, USA). The nucleotide sequences were analyzed by direct sequencing of the PCR products using ABI PRISM BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, USA). The HA and NA gene nucleotide sequences of 01310 CE3 have been deposited in GenBank under accession number EU253561 and EU253562, respectively.

The nucleotide and deduced amino acid sequences of the HA and NA molecules were aligned by the Clustal W method with the MegAlign software (Lasergene 7.0; DNASTAR, USA). The similarity of the HA and NA amino acid sequences were compared with recently studied Korean H9N2 AIVs [16].

Efficacy testing of vaccine candidate virus according to the antigen contents and virus passage in ECEs

The selected vaccine candidate (01310 CE3, 2^7 HA unit) virus based on the above pathogenic, serologic, and molecular data (refer to the Results section) was used to prepare the vaccine according to the previously mentioned procedures. The preparation of the high HA content vaccine (2^{10} HA unit) involved the concentration of the virus by centrifugation (18,000 rpm, 4 h; Beckman, USA). The vaccines were injected into ten 6-week-old SPF chickens via the intramuscular (IM) route. The serum samples were taken three weeks post-vaccination (wpv), and were followed by the performance of the HI test. The HI test results were analyzed using Student's *t*-test. Statistical significance was set *a priori* at $\alpha = 0.05$. The chickens were challenged with 01310 CE3 virus ($10^{6.0}$ EID$_{50}$/0.1 ml) at 3 wpv via the oral route. Next, oropharyngeal and cloacal swab samples were taken at 5 dpi for virus isolation. The Fisher's exact test was performed to compare the virus isolation rate ($\alpha = 0.05$).

In order to recover the highly growing vaccine virus in ECEs, the vaccine candidate virus was serially passaged through 9- to 11-day-old SPF ECEs and selected with high HA titer using chicken red blood cells. The passage number in ECEs was indicated as CE X (X stands for passage number) after the virus name.

Pathogenicity test of the selected vaccine virus in SPF ECEs and chickens

In order to test the changes in the pathogenicity of the passaged vaccine viruses in chicken eggs, the viruses (CE5, CE10, CE15, CE20, and CE40) were diluted in PBS and inoculated in the allantoic cavities of 10-day-old SPF ECEs. In addition, the mortality was checked after 48 h of incubation.

For the pathogenicity test of the selected vaccine virus in chickens, eight 6-week-old SPF chickens were intra-venously inoculated with 0.2 ml of 1/10 diluted virus, and we noted the mortality for 10 days according to standard procedures [31].

Antigenicity testing and the optimum growth condition of the vaccine virus

The vaccines of the antigenicity test were prepared with 01310 CE6, CE20, and CE40 viruses. In addition, the HA titers of the viruses were adjusted to 2^9 HA units. Eight 6-week-old SPF chickens were then vaccinated at 0.5 ml/chickenvia the IM route. After vaccination, serum samples were taken at 3 wpv, and the HI assay was then performed with the MS96 CE3, 01310 CE20, and 04Q30 CE3 viruses. A one-way ANOVA test was subsequently performed.

In order to determine the optimum growth conditions of the vaccine strain in ECEs, the virus was serially diluted 10-fold with 1% gentamicin-PBS (10^{-3}-10^{-7}), and was inoculated into 60 SPF ECEs. At 8, 16, 24, 32, 40, and 48 hours after incubation, 10 eggs were harvested during each dilution and the HA titer was determined with 1% chicken red blood cells [27].

Vaccine efficacy test

The vaccine was prepared with the 01310 CE20 (2^{10} HA unit) strain as per the procedures mentioned in the Materials and Methods section, and was injected at a concentration of 0.5 ml/chicken via the intramuscular route (thigh muscle) into each of eight 6-week-old SPF chickens. Three weeks after vaccination, the parent virus (01310 CE3, $10^{5.5}$ EID$_{50}$/0.1 ml) and recent Korean isolate (04Q30 CE3, $10^{5.5}$ EID$_{50}$/0.1 ml) were challenged intranasally. To isolate the challenged virus, oropharyngeal and cloacal swab samples were taken at 1, 3, and 5 dpi from each chicken and suspended in 3 ml of gentamicin-PBS. The samples were subsequently inoculated into three 9- to 11-day-old SPF ECEs respectively, followed by assessment of the virus growth from the allantoic fluids. At 5 dpi, the chickens were sacrificed and the various tissues were taken with different scissors in order to prevent contamination. Next, the 10% (w/v) tissue homogenates were tested for viral growth in three 9- to 11-day-old SPF ECEs. In order to titrate the virus from various tissues, tissue homogenates were pooled in a group of equal volume, and were then titrated using SPF ECEs.

Results

Vaccine candidate selection

In order to select the vaccine candidate, we chose the MS96, 99029, and 01310 viruses as the representative viruses of the year. In addition, we compared the virus replication potency and pathogenicity for SPF and commercial broiler chickens. For the SPF chickens, all of the viruses had a zero mortality rate; however, the viruses were isolated at 3 to 5 dpi from oropharyngeal swab samples, and 3 to 7 dpi from cloacal swab samples. At 5 dpi, the MS96, 99029, and 01310 viruses were isolated from 5/8, 7/8, and 6/8 oropharyngeal swab samples and

Table 1. Comparison of virus isolation and mortality of recent Korean H9N2 LPAIV

Virus	Virus isolation in SPF chickens*								Pathogenicity of commercial broiler chickens[†]
	3 dpi		5 dpi		7 dpi		9 dpi		
	op	cl	op	cl	op	cl	op	cl	
MS96	8/8	1/8	5/8	1/8	0/8	2/8	0/8	0/8	1/15 (6.7%)
99029	8/8	0/8	7/8	3/8	0/8	2/8	0/8	0/8	1/15 (6.7%)
01310	8/8	3/8	6/8	5/8	0/8	4/8	0/8	0/8	4/15 (26.7%)
Control	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/15 (0%)

*7-week-old SPF chickens were inoculated via the intra-tracheal route with MS96 ($10^{6.5}$EID$_{50}$), 99029 ($10^{5.6}$EID$_{50}$), and 01310 ($10^{7.1}$EID$_{50}$): virus isolation/total inoculated. [†]12-week-old AIV antibody-free commercial broiler chickens were inoculated via the intra-tracheal route with MS96 ($10^{5.2}$ EID$_{50}$), 99029 ($10^{5.7}$EID$_{50}$), and 01310 ($10^{5.0}$EID$_{50}$): number of dead/total inoculated (% mortality). op: oropharyngeal, cl: cloacal.

Table 2. Antigenic relationship between Korean isolates

Antiserum	Virus		
	MS96	99029	01310
MS96	169* (1.00[†])	60 (0.73)	274 (0.90)
99029	239	158 (1.00)	181 (0.61)
01310	274	181	549 (1.00)

*Mean HI titer from 8 chickens. [†]r-value, r = (r1 × r2)$^{1/2}$. r1 = heterologous titer with virus 2 / homologous titer with virus 1. r2 = heterologous titer with virus 1 / homologous titer with virus 2.

1/8, 3/8, and 5/8 cloacal swab samples, respectively. Furthermore, in commercial broiler chickens, the 01310 strain showed a 26.7% mortality rate, whereas a 6.7% mortality rate was recorded for each of the other two viruses (Table 1).

To elucidate the antigenic relationship between the selected Korean isolates, the r-value was calculated with a cross HI titer between the viruses. The values ranged from 0.61 to 1.00, with no significant differences between the viruses (Table 2).

Based on our pathogenic and serologicdata, we chose the 01310 virus as a vaccine candidate. Moreover, when we compared the HA and NA amino acid sequences deduced by nucleotide sequences, the 01310 CE3 virus was found to have 95.5-98.6% and 94.6-98.5% similarity with the recent Korean H9N2 AIVs isolated from 2002 to 2004, respectively (data not shown).

Efficacy of the selected vaccine candidate virus according to the antigen contents

In order to elucidate the efficacy of the vaccine candidate virus, ten SPF chickens were immunized with different antigen contents. Chickens immunized with a high antigen vaccine (2^{10} HA unit) showed a similar but statistically significant ($p < 0.05$) antibody titer compared to the low antigen content (2^7 HA unit) vaccine (Table 3). In addition, in the low antigen content vaccine group, the challenged viruses were recovered from 7/10 and 9/10 chickens from oropharyngeal and cloacal swab samples at 5 dpi, respectively. It was believed that the vaccine was not effective to protect against viral shedding. However, in high antigen content groups, the challenged virus was isolated from 1/10 or 3/10 oropharyngeal/cloacal swab samples, respectively. Although neither of the vaccines were able to completely protect against viral shedding, the high antigen content vaccine was more effective than the low antigen content vaccine upon comparison of the virus recovery rate in the oropharyngeal/cloacal swab samples ($p = 0.02$, respectively) (Table 3).

Table 3. Comparison of vaccine efficacy according to the antigen content

Antigen content (HA unit)	HI titer at 3 weeks post vaccination	Virus isolation at 5 dpi[†]	
		Oropharyngeal swab	Cloacal swab
01310 CE3 (2^7)	8.2 ± 0.6*	7/10[‡]	9/10[‡]
01310 CE3 (2^{10})	8.8 ± 0.4*	1/10[‡]	3/10[‡]
Control	−	10/10	10/10

*Mean ± SD of the titer analyzed with the Student's *t*-test, α = 0.05, *p* = 0.022. [†]The chickens were challenged intranasally with the homologous virus (01310 CE3, $10^{6.0}$EID$_{50}$/0.1 ml): virus isolation / total tested. [‡]Fisher's exact test, *p* = 0.020, respectively.

Table 4. Comparison of virus titer and mortality in ECEs and SPF chickens of passaged vaccine candidate

Passage No.	HA titer (log$_2$)	Virus titer (log$_{10}$EID$_{50}$/0.1 ml)	Mortality in ECEs*	Mortality in SPF chicken[†]
CE5	7	7.5	3/10	nt
CE7	8	nt[‡]	nt	nt
CE10	9	8.5	2/10	nt
CE15	10	8.4	2/10	nt
CE20	10	8.7	6/10	0/8
CE30	10	nt	nt	nt
CE40	10	nt	5/10	nt

*Mortality at 2 dpi incubation time. The viruses were inoculated at $10^{4.5}$EID$_{50}$/0.1 ml each. [†]The 8 SPF chickens were intravenously inoculated with 0.2 ml of 1/10 diluted ($10^{7.7}$EID$_{50}$/0.1 ml) infectious allantoic fluid and observed for 10 days. [‡]nt: not tested.

Biological characteristics of the vaccine virus

The vaccine candidate virus (01310 CE3) grew in ECEs at around 2^7 HA units and about $10^{7.0}$ EID$_{50}$/0.1 ml, as did most of the field isolates (personal observation, data not presented), and we were not able to determine the expected vaccine efficacy with unconcentrated antigen (Table 3). Therefore, we attempted to get highly growing phenotyped viruses through egg passage. When the 01310 strain was passaged in SPF ECEs as a vaccine candidate, the virus titer increased with each ECE passage (Table 4). After the 15th passage in ECEs, the virus titer showed 2^{10} HA units stably; this was approximately 10 times greater than that of the parent virus. Moreover, the 20th passaged virus showed the highest titer, $10^{8.7}$ EID$_{50}$/0.1 ml (Table 4). As shown in Fig. 1, the 01310 CE20 showed the highest HA titer when inoculated with $10^{4.7}$ EID$_{50}$/0.1 ml and incubated for 32 hours. The mean HA titer at that point was 9.7 ± 0.5 (log$_2$).

However, as the 01310 virus was passaged in SPF ECEs, the mortality of the chicken eggs also increased. During 48

h of incubation, sixof ten eggs infected with the 01310 CE20 virus were dead, whereas, two to three of ten eggs were dead due to the CE15 virus or other less passaged viruses. In spite of increased mortality in chicken eggs, the CE20 virus showed no mortality for the intravenous challenge experiment with SPF chickens, and was thought to maintain the LPAI characteristics according to the OIE criteria [31] (Table 4).

Molecular characterization of HA and NA gene of the vaccine virus

Upon comparison of HA amino acid sequences of the 01310 CE3, CE20, and CE40 to HA cleavage (PATSGR/GLF), receptor binding (183H, 190E, 226Q, 228G), and potential glycosylation sites (158N), we found no changes (even in the CE40 passaged virus). HA has only three amino acid changes in the CE20 virus (T133N, V216G in HA1 region, and E439D in HA2 region), and an additional change occurred (L531F) in the CE40 virus (H3

numbering). In the NA molecule, the CE20 and CE40 viruses have an 18 amino acid deletion in the stalk region (55-72); both of these viruses also contain a change in one amino acid (E54D) (data not shown).

Antigenicity of the vaccine virus

As shown in Table 5, the HI titers ($\log_2$) of the 01310 CE20 antisera with MS96 CE3, 01310 CE20, and the recent isolate, 04Q30 CE3, were 8.8 ± 0.5, 8.3 ± 0.5, and 9.1 ± 1.0, respectively. Moreover, the HI titers of antisera of 01310 CE6, CE20, and CE40 with the homologous Ag (01310 CE20) were 7.8 ± 0.2, 8.3 ± 0.5, and 8.4 ± 0.6, respectively. One-way ANOVA comparison of the HI titers revealed no significant difference.

Vaccine efficacy test

For the vaccine efficacy test, eight 6-week-old chickens were vaccinated with 01310 CE20 and challenged after 3 weeks with homologous parent vaccine virus (01310 CE3) or the recent Korean isolate (04Q30 CE3). Moreover, swab samples were taken at 1, 3, and 5 dpi, and were tested for virus isolation. The highest isolation number was obtained
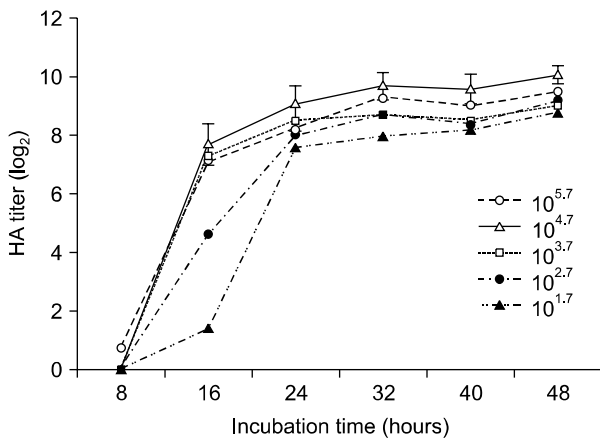

Fig. 1. Growth curves of the vaccine strain (01310 CE20) according to the virus titer ($\log_{10}EID_{50}/0.1$ ml) of the inocula.

Table 5. Immunogenicity and comparison of serological relationship with Korean isolates

Antisera	HI titer (mean ±SD, $\log_2$) with homologous / heterologous Ag		
	MS96	01310, CE20	04Q30, CE3
01310, CE6	$-$[‡]	7.8 ± 0.5[*]	$-$
01310, CE20	8.8 ± 0.7[†]	8.3 ± 0.7[*†]	9.1 ± 1.0[†]
01310, CE40	$-$	8.4 ± 0.7[*]	$-$
Control	0.0	0.0	0.0

The values were analyzed with a one-way ANOVA test. $\alpha = 0.05$, [*]$p = 0.12$, [†]$p = 0.15$. [‡] not tested.

Table 6. Vaccine efficacy test of the 01310 CE20 strain against the parent virus (01310 CE3) and the recent Korean isolate (04Q30 CE3)

Challenge virus	Vaccination*	Oropharyngeal (op) / cloacal (cl) swab sample[†]						Tissue homogenate (10% w/v)[‡]					
		1dpi		3 dpi		5 dpi		Brain	Trachea	Lung	Spleen	Kidney	Cecal Tonsil
		op	cl	op	cl	op	cl						
01310 CE3	+	0/7	0/7	0/7	0/7	0/7	0/7	0/7 (nt)	0/7 (0)	0/7 (0)	0/7 (0)	0/7 (0)	0/7 (0)
	−	2/8	0/8	7/8	0/8	7/8	6/8	0/8 (nt)	5/8 ($10^{1.4}$)	0/8 (0)	1/8 (0)	0/8 (0)	4/8 ($10^{5.4}$)
04Q30 CE3	+	2/8	0/8	2/8	0/8	0/8	0/8	0/8 (nt)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)
	−	6/8	0/8	8/8	3/8	8/8	5/8	0/8 (nt)	8/8 ($10^{4.4}$)	6/8 ($10^{2.0}$)	2/8 (0)	2/8 ($10^{1.6}$)	5/8 ($10^{6.0}$)

*Vaccine prepared as described in Materials and Methods with 01310 CE20 (2^{10} HA unit) and vaccinated intramuscularly at 0.5 ml/chicken. [†]Virus isolation / total tested. [‡]Tissues were taken at 5 dpi, virus isolation / total tested (virus titers of the pooled samples, $EID_{50}/0.1$ ml), nt: not tested.

for 5 dpi in both of the unvaccinated groups. The oropharyngeal and cloacal swab samples taken at 5 dpi showed isolation of 7/8 and 6/8 for the 01310 CE3 group and 8/8 and 5/8 for the 04Q30 CE3 group, respectively. On the other hand, the viruses for the vaccinated group, which were isolated only from the 04Q30 challenge group, showed isolation of 2/8 oropharyngeal swab samples taken at 1 and 3 dpi (Table 6).

In the tissue samples taken at 5 dpi, the challenged viruses were not isolated from the vaccinated group, whereas for the unvaccinated group, the 01310 CE3 virus was isolated from trachea (5/8, $10^{1.4}$ EID_{50}/0.1ml), spleen (1/8, 0), and cecal tonsil (4/8, $10^{5.4}$), but not from the brain, lung, or kidney. In addition, the 04Q30 CE3 virus was isolated from the trachea (8/8, $10^{4.4}$), lung (6/8, $10^{2.0}$), spleen (2/8, 0), kidney (2/8, $10^{1.6}$), and cecal tonsil (5/8, $10^{6.0}$), but not from the brain. In both of the unvaccinated groups, the cecal tonsil showed the highest viral titer (Table 6).

Discussion

Since the first H9N2 LPAI outbreak in 1996, numerous cases have occurred in Korea, and the H9N2 subtype LPAI has become one of the greatest problems of the country's poultry industry (especially for breeders and commercial layers) [13,16].

It is worth noting that avian influenza viruses in their natural hosts, which include wild waterfowl, gulls, and shorebirds, have shown a high rate of genetic conservation. Transmission to other species such as poultry may cause significant amounts of genetic and antigenic changes [12,16-18,25,30]. For a vaccine to be effective, it is necessary that the strain has genetic and antigenic traits similar to those of the currently circulating field viruses. Therefore, it is very important to choose the most effective vaccine strain to prevent the current circulation of viruses. Some countries such as China [17], Pakistan [22], Iran [29], and Israel [5] have already begun the use of H9N2 LPAI vaccines. Because of the above mentioned reasons, each of the countries used their own vaccine strains to control the H9N2 LPAI. Based on the properties of AIV, we chose a candidate vaccine virus with a relatively high pathogenicity in chickens, as well as antigenic and genetic similarity to recent field isolates. Despite this, the vaccine efficacy test of the selected virus (01310), according to the antigen contents using unconcentrated antigen (2^7 HA unit), showed unsatisfactory protective results compared with the high antigen content vaccine. As for other vaccines, the antigen contents are the critical component of an effective vaccine. Despite this, the antigen concentration requires an increased vaccine production cost, which may be a problem in terms of AI vaccination at the farm level. In order to overcome the low titer of the vaccine candidate strain, the virus was passaged in chicken eggs. Consequent

to the passages of the selected virus, the vaccine strain may have approximately ten times higher titers than the parent virus. Moreover, the rapid growth property and antigenicity of the virus were maintained through the fortieth passage.

In order to determine the optimum inactivation conditions, the virus was incubated in 0.1% formalin at 37°C. However, under these conditions, the HA titer of the virus was decreased by four times, even after 1 h of incubation (data not shown). HA is considered the major antigenic protein, and a decreased HA titer signifies decreased antigenicity. Therefore, the virus was treated at a lower temperature (20°C) and the inactivation conditions were determined. At 20°C, a 6-hour incubation time is enough to inactivate the virus and not show any HA titer changes, even after 12 h of incubation (data not shown).

Although the HA cleavage site (PATSGR/GLF) was well conserved and showed low pathogenic characteristics in the vaccine strain (01310 CE20), the pathogenicity in ECEs was increased. Therefore, we tested the pathogenicity of the vaccine strain in SPF chickens. When the vaccine strain was injected into SPF chickens via the intravenous route according to the standard procedures [31], no mortality was observed in the SPF chickens. Moreover, the vaccine strain has an amino acid sequence consistent withavian virus characteristics at the receptor binding site (Gln226), which confers a high binding affinity to the 2, 3-linked sialic acid (SA) moieties (abundant in avian and horses) rather than the 2, 6-linked SA moieties found in most mammals [19]. Although it is not sufficient, the pathogenicity for chickens and the low binding potential to 2, 6-linked SA secured the safety of the vaccine strain with regard to human infection (especially in vaccine producers).

We observed three amino acid changes (Thr133Asn, Val216Gly, Glu439Asp) compared to the parent virus (01310 CE3) in the HA molecules. The changed amino acids have similar traits (Thr and Asn have a polar uncharged side chain, Val and Gly have a non-polar side chain, Glu and Asp are acidic amino acids) with the parent sequences. However, the Thr133Asn change created an additional potential N-linked glycosylation site located on the right edge of the receptor binding pocket [11]. Many studies have demonstrated that the addition of oligosaccharides to the HA molecule changes the antigenicity of the virus [1,6,24,28]. However, in this study, the additional potential glycosylation site did not significantly affect the antigenicity of the vaccine strain.

A number of studies have shown that a balance between the HA and NA activities is critical for the efficiency of viral growth in host cells [3,4,8,21,23]. In this study, the HA molecule of the vaccine virus (01310 CE20) has a potential glycosylation site on amino acid 158, and represents an additional potential glycosylation site in the vicinity of the receptor binding site (133Asn), in addition

to the 18 amino acid deletion in the NA stalk region. It is not clear that such changes exclusively affect the high growth trait of the vaccine virus. Further studies would be needed to clarify the mechanism for the modification of this trait of the vaccine virus as the virus is being passaged in ECEs. In addition, it is believed that the 54 base pair deletion in the NA gene could be used as a vaccine strain marker to identify the vaccine virus.

Although the vaccine candidate virus was isolated in 2001 and may be thought of as outdated, the virus showed high antigenic and genetic similarity to the recent Korean H9N2 LPAIVs. Moreover and most importantly, the vaccine efficacy test with SPF chickens showed that the vaccine candidate sufficiently protected the chickens from the virus [both the homologous (01310 CE3) and recent Korean isolate (04Q30)] (Table 6). The challenge virus was recovered from only two of the eight oropharyngeal swab samples taken at 1 and 2 dpi in the 04Q30 challenged group, respectively, whereas the challenged virus was recovered throughout the experiment and peaked at 5 dpi for the unvaccinated group. Moreover, in subsequent repeated animal experiments, we were able to obtain a reliable and constant virus recovery rate for the cecal tonsil (data not shown). So far, no standards exist for the evaluation of the inactivated AI vaccine efficacy. Based on the animal challenge experiments (the experiments conducted in our lab and the data not presented), the Committee on the National AI Vaccine Campaign determined that the minimum efficacy requirements for inactivated H9N2 LPAI vaccines was a greater than 80% inhibition of the virus recovery rate in cecal tonsils of the vaccinated group, compared to the rate observed in unvaccinated chickens at 5 dpi.

Despite these requirements, AIV is a continuously evolving virus [9,13,16,17,25,30], and as shown in this study, the inactivated LPAI vaccine cannot completely protect against viral infection and shedding into the environment. In addition, immune pressures (such as vaccination) have resulted further complication of the AI situation [14]. Therefore, the Korean government has conditionally issued a vaccine production license according to the following stipulations: 1) The vaccine producers have to record all selling activity and submit records to the Korean animal health authority (NVRQS) as requested; 2) 20 to 30 sentinel birds must be deployed in every vaccinated poultry farm, and the sera from 10% of the vaccinated birds must be tested with those of unvaccinated sentinel birds in order to monitor LPAI infection at least twice a year, and the results must be submitted to the NVRQS. In addition, as a separate complementary measure, active surveillance has been deployed to monitor any changes in the antigenicity of the current circulating H9N2 LPAIV; if changes in antigenicity are detected, the vaccine virus will be changed to a more appropriate virus.

Acknowledgments

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