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Poultry vaccination directed evolution of H9N2 low pathogenicity avian influenza viruses in Korea

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ABSTRACT

Significant economic losses in the poultry industries have resulted from H9N2 low pathogenic avian influenza virus infections across North Africa, the Middle East and Asia. The present study investigated the evolutionary dynamics of H9N2 viruses circulating in Korea from 1996 to 2012. Our analysis of viral population dynamics revealed an increase in genetic diversity between the years 2003 and 2007, corresponding to the spread and diversification of H9N2 viruses into multiple genetic groups (named A and B), followed by a sudden decrease in 2007, which was associated with implementation of vaccination using a Clade A virus. Implementation of the H9N2 vaccination program in Korea has dramatically reduced the diversity of H9N2 virus, and only one sub-lineage of clade B has survived, expanded, and currently circulates in Korea. In addition, the antigenic drift of this new genetic group away from the current vaccine strain suggests the need to update the vaccine seed strain.

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Introduction

Sixteen hemagglutinin (HA) subtypes (H1–H16) and nine NA subtypes (N1–N9) have been identified among avian influenza viruses (Swayne et al., 2013). The H9N2 low pathogenic avian influenza virus (LPAIV) was first identified in poultry in the 1960s and became widespread in Asian poultry in the 1990s (Guan et al., 1999). The first outbreak of the H9N2 LPAI in China occurred in Guangdong province of Southern China during November 1992 to May 1994 and rapidly spread to become the most prevalent LPAIV in domestic poultry (Lee and Song, 2013; Sun and Liu, 2015; Zhang et al., 2009). This H9N2 LPAIV lineage has resulted in great economic losses due to decreased egg production and increased mortality. In addition, the H9N2 LPAIV has caused sporadic human infections in Asia since 1998, raising concerns about a pandemic potential with this lineage of virus (Butt et al., 2005; Lin et al., 2000; Matrosovich et al., 2001). Phylogenetic and antigenic analysis have identified several groups of H9N2 LPAI virus in Eurasia: the G1 lineage, represented by A/quail/Hong Kong/G1/97 (G1-like); the Y280 lineage, represented by three prototype viruses A/duck/Hong Kong/Y280/97 (Y280-like), A/chicken/Beijing/1/94 (BJ94-like), and A/chicken/Hong Kong/G9/97 (G9-like); and the Y439 or Korean lineage, represented by A/duck/Hong Kong/Y439/97 (Y439-like) and A/chicken/Korea/38349-p96323/96 (Korean-like) (Butt et al., 2010; Guan et al., 1999; Matrosovich et al., 2001).

The first field outbreak of the H9N2 LPAIV in Korea occurred in 1996 with A/chicken/Korea/96006/96 (H9N2) being the reference strain, a virus genetically closely related to the Y439-like lineage virus later isolated from aquatic birds. Since then, H9N2 LPAIV has become endemic in domestic poultry in Korea and has formed the distinct Korean-like lineage (Kwon et al., 2006; Lee et al., 2000, 2007; Lee and Song, 2013). The Korean-like lineage has continued to evolve, exhibiting antigenic drift of the hemagglutinin and reassortment of internal gene segments with other LPAIV circulating in the Korean live bird markets (Choi et al., 2005; Kim et al., 2010, 2006; Lee et al., 2010, 2007; Park et al., 2011). To control H9N2 LPAI outbreaks, the Korean veterinary authorities utilized government stamping-out and compensation policies between 1996 and 1999, but complete eradication was not achieved. Since 2007, the veterinary authority has permitted the use of the inactivated oil adjuvant H9N2 LPAI vaccine derived from a Korean H9N2 isolate (A/chicken/Korea/01310/2001) in commercial layer and broiler breeder chickens (Choi et al., 2008).

A major determinant of variation in substitution rates among hemagglutinin genes of influenza A viruses seems to be the strength of immune selection pressure; at approximately one mutation per each genome replication, this translates into substitution rates of 10^{-4}–10^{-3} nucleotide substitutions per site per year (Nelson and Holmes, 2007). Thus, strong humoral immunity induced by vaccination can be an important factor promoting

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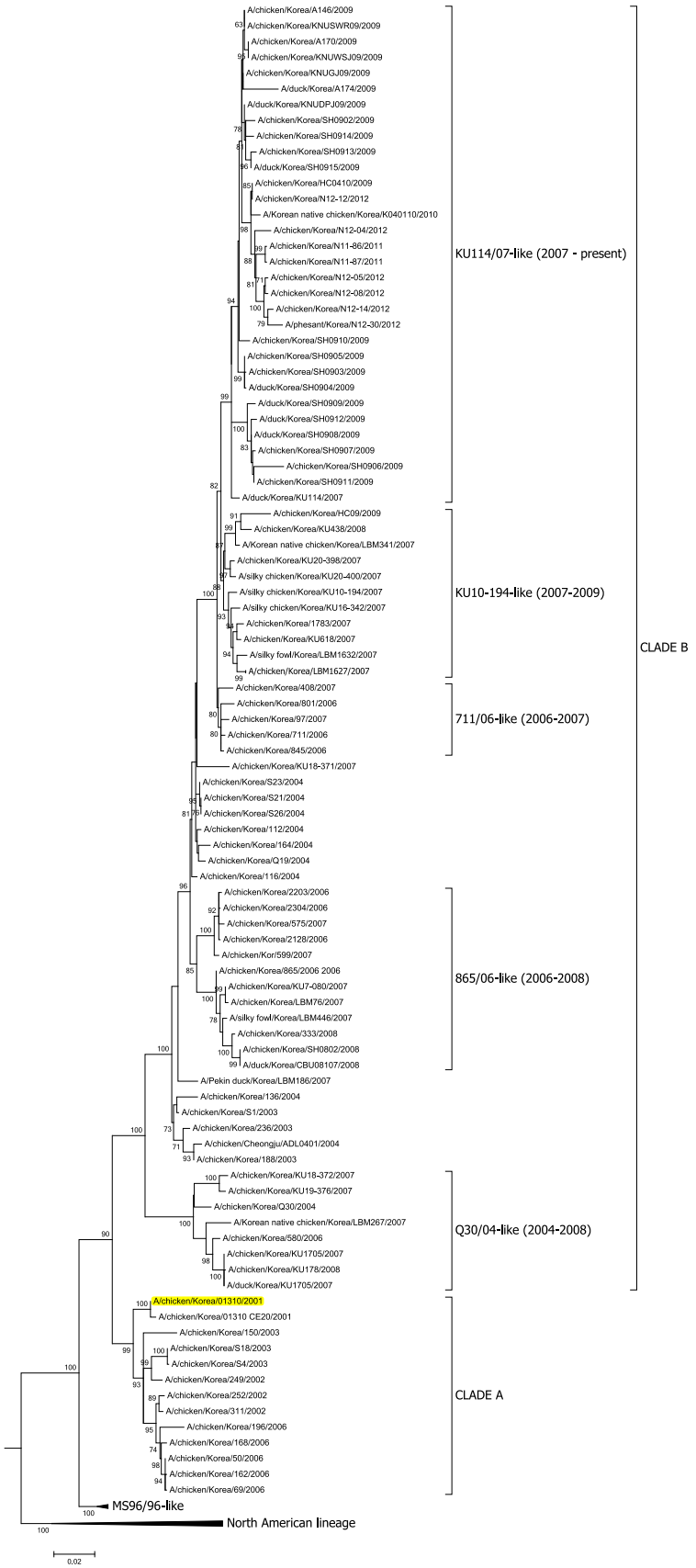

Fig. 1. Phylogenetic tree created by the Maximum-likelihood algorithm for the HA gene of H9N2 avian influenza viruses from Korea. The current commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), is highlighted.

selection of escape mutants in vaccinated animals. In a previous study, Cattoli et al. proposed a difference in evolutionary dynamics of H5N1 high pathogenicity avian influenza viruses (HPAIV) among countries where vaccination was or was not adopted. Particularly, evolutionary rates and the number of positively selected sites were higher in virus populations from countries applying vaccine for H5N1 HPAIV, compared to viruses populations in countries which had never used vaccination (Cattoli et al., 2011a). In the present study we investigated the evolutionary change and phylodynamics of Korean H9 HA genes isolated from 1996 through 2012, and analyzed selection pressure and point mutations related to antigenic features before and after the introduction of vaccination in Korea.

Results

The topology of the HA tree of representative H9N2 lineages (351 sequences) indicated that the Korean viruses formed a well-supported monophyletic group within the Y439-like or Korean-like lineage (Supplemental Fig. S1). To explore the evolutionary dynamics of the HA gene in Korea, a ML phylogenetic tree was inferred for a total of 100 H9N2 LPAIV identified from 1996 to 2012, before and after implementation of field vaccination (Fig. 1). The Korean H9N2 viruses can be mostly divided into four distinct clades, defined by high bootstrap values (> 80%) and long branch lengths in the HA phylogeny: MS96/96-like, 01310/01-like (Clade A), 116/04-like (Clade B), and North American (Fig. 1). The MS96/

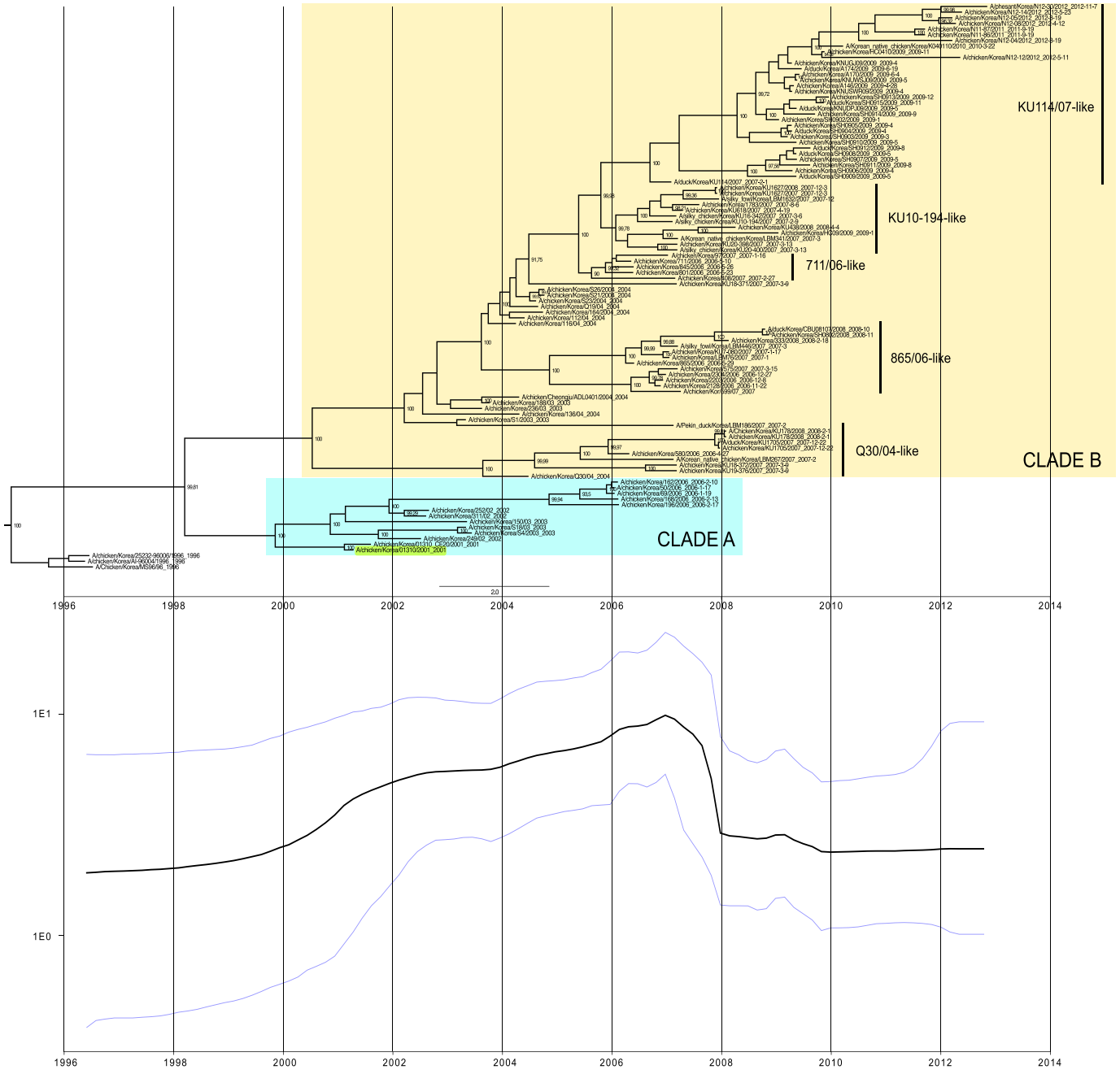

Fig. 2. Temporally structured maximum clade credibility phylogenetic tree and Bayesian Skyline plot of the HA gene showing changes in genetic diversity in Korean H9N2 viruses (1996–2012). A measure of genetic diversity (Net) in Bayesian Skyline plot is given on the *y*-axis with 95% HPD values shown in thin line. The current commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), is highlighted.

Table 1
Evolutionary profiles of Korean H9N2 LPAI viruses.

Year	No. of sequences	Evolutionary rate sub/site/year $\times 10^{-3}$ (95% HPD)	Mean d*N*/d*S*	Positively selected sites (p-value < 0.1)	
				N	Amino acid position (H9 numbering)
1996–2012	100	5.60(4.67–6.49)	0.236	7	12, 131, 133, 153[a], 166[a,b], 287, 498
2001–2006	34	5.79(3.60–8.21)	0.214	1	230[a]
2007–2012	63	5.80(3.55–8.42)	0.219	4	131, 133, 437, 498

[a] Sites for previously reported antigenic escape mutant.
[b] Potential glycosylation sites.

96-like group contained H9N2 LPAI viruses that caused the first H9N2 outbreak on Korean chicken farms in 1996, represented by A/chicken/Korea/96006/96 (H9N2) which was closely related to the A/duck/Hong Kong/Y439/97(H9N2) (Lee et al., 2000). H9N2 viruses isolated in Korea from 2001 to 2012 fell within two separate clades, designated as clade A (01310/01-like lineage), which contains viruses collected from 2001 to 2006, and clade B (116/04-like lineage), which comprises the majority of the recent isolates (2003–2012). Clade B can be further divided into five clusters, named Q30/04-like, 865/06-like, 711/06-like, KU10-194/-like, and KU114/07-like. Interestingly, these clades include only H9N2 viruses from Korea, suggesting that they were originally derived from a single viral introduction into poultry. By contrast, multiple introductions of H9N2 viruses with some genes of the North American lineage were found in Korea in wild birds [A/wild bird/Korea/8g-39/2005(H9N2), A/white-fronted goose/Korea/20-36/2007(H9N2), and A/bean goose/Korea/220/2011(H9N2)], but with only a limited spread across the country and the lack of transmission to poultry (Fig. 1) (Lee et al., 2014, 2013a, b).

As shown in Fig. 2, Korean H9N2 LPAI viruses were distinctly separated into clades A and B in MCC tree, consistent with the phylogenetic tree obtained by the ML algorithm. The time-scaled phylogeny indicated that clade A disappeared during 2006, and was replaced with clade B that evolved into several sub-lineages. However, except for KU114/07-like viruses, none of the clade B sub-lineages were detected after early 2009. The analysis of virus population dynamic revealed a gradual increase in genetic diversity from the beginning of the epidemic to the end of 2006 followed by a sudden decrease during 2007. The increasing population size corresponded to the appearance of clade A and B viruses and their diversifications into multiple sub-lineages, while the sudden decrease corresponded to the start of mass vaccination and the extinction of clade A viruses (Fig. 2), the source of the seed strain used in the vaccination campaign.

The evolutionary rate estimated for the HA gene of the Korean H9N2 viruses was 5.6×10^{-3} substitutions/site/year (95%highest posterior density, HPD, from 4.67×10^{-3} to 6.49×10^{-3}). The mean time of the most recent common ancestor (tMRCA) of H9N2 viruses was September 1994 (95% HPD, January 1992–March 1996), when H9N2 virus was first isolated in Southern China. To characterize the viral population dynamics of each clade, we calculated the evolutionary rates of the clades A and B separately. Interestingly, the rates of the two clades were different: a mean rate of 1.97×10^{-3} substitutions/site/year (95% HPD from 1.23×10^{-3} to 3.45×10^{-3}) for clade A, and a rate of 5.28×10^{-3} substitutions/site/year (95% HPD from 4.22×10^{-3} to 6.36×10^{-3}) for clade B, making the latter clade the faster evolving group. The estimated tMRCA was October 1999 (95% HPD, May 1998–November 2000) for clade A and June 2000 (95% HPD, September 1998–December 2001) for clade B, suggesting that the detection of these two clades occurred several months after their appearance in the country.

We analyzed the selection profiles of the HA protein of Korean H9N2 viruses. Overall, the ratio of nonsynonymous to synonymous substitutions per site (d*N*/d*S*) was 0.236, indicating that Korean H9N2 viruses had evolved under purifying selection. However, we found seven individual codons (p-value < 0.1) that may be under positive selection in the HA protein of the Korean H9N2 viruses, one putatively positive selected residues in viruses collected before the implementation of vaccination (2003–2006) and four in viruses collected from vaccinated poultry populations (2007–2012) (Table 1, Fig 3). In reference to the antigenic sites described in an earlier report, three positively selected sites (positions 153, 166 and 230) were antigenic escape mutant sites; the position 153 and 166 were located in antigenic site II and I in H9, respectively, and the position 230 was located in the vicinity of the trimeric interface of the globular domains of HA1 (Kaverin et al., 2004; Okamatsu et al., 2008; Wan et al., 2014). In addition, the position 166 fell within a potential glycosylation site (166-168) (Supplemental Table S2). The KU114/07-like lineage had different amino acid sequences at antigenic escape mutant sites (positions N153G, N201S, and L230I) compared to most of the viruses isolated between 1996 and 2007 and compared to vaccine strain 01310_CE20/2001 (N145T, N201S, N145T, N166S, L230I) (Supplemental Table S1).

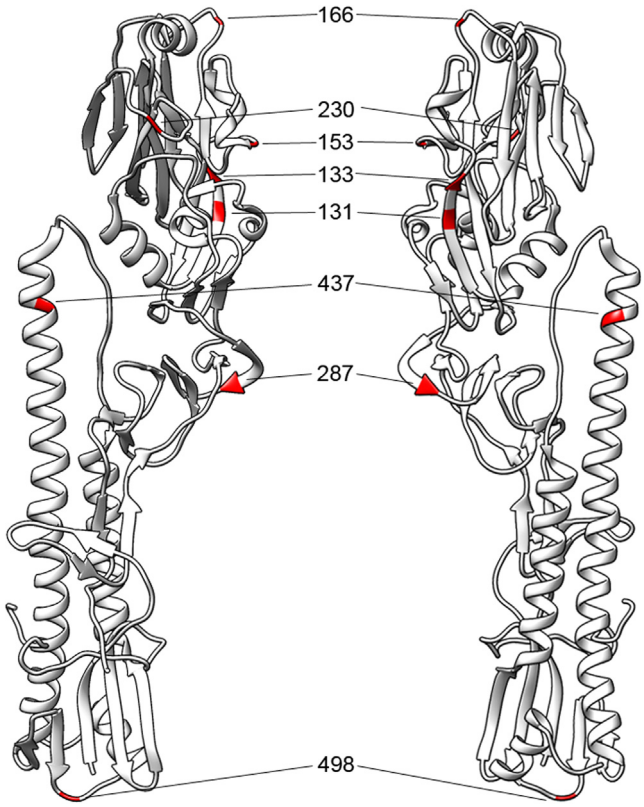

Fig. 3. Ribbon diagram of the monomer of H9 hemagglutinin. Front (a) and back (b) views are shown. Locations of positively selected amino acid changes of Korean H9 isolates are labeled with red color.

Discussion

Our phylogenetic analysis of the HA gene segment of the Korean H9N2 viruses collected between 1996 and 2012 revealed the occurrence of different introductions within the country by poultry or wild birds. However, only a single lineage of virus evolved and circulated extensively in Korean poultry, eventually giving rise to clades A and B. The origin dates of the most recent common ancestors for clades A and B were May 1998–November 2000 and September 1998–December 2001, respectively, suggest that they emerged almost simultaneously probably from the MS96/96-like group. No evidence of new introductions of H9N2 strains from Asia to Korean poultry during 2001–2015 was detected, as shown by our neighbor-joining phylogenetic tree of the HA sequences representative of the H9 lineages (Supplemental Fig. S1).

The analysis of population dynamic revealed a gradual increase of genetic diversity between the years 1996 and 2007 and showed a distinct decrease during 2007. The increase of genetic variability corresponds to the appearance of clade A and the multiple sub-lineages of clade B. The vaccine trials by Korean vaccine companies were conducted on chicken farms between the latter half of 2006 and early 2007 and widespread commercial vaccination commenced in February 2007. The timing of the remarkable decrease in genetic variability corresponded to the introduction and the widespread use of H9N2 clade A vaccine in Korea.

Implementation of H9N2 vaccine likely resulted in the selection and persistence of KU114/07-like lineage of clade B, and the loss of clade A and other sub-lineages of clade B. Clade A and most sub-lineages of clade B disappeared after vaccination, suggesting that the H9N2 vaccination program in Korea dramatically reduced the diversity of the H9N2 lineage. In particular, no clade A viruses were detected after the beginning of the vaccination program. The fact that the vaccine strain belongs to this clade may explain the sudden disappearance of this genetic group. However, KU114/07-like lineage of clade B survived after implementation of vaccination, and expanded in importance, including accumulation of three mutations at antigenic escape mutant sites. Similar changes to HA gene evolution have been reported after H5N2 LPAI vaccine implementation in Mexico (Lee et al., 2004). In 1995, widespread vaccination program was applied to commercial poultry for control of H5N2 HPAIV in Mexico, and has continued against H5N2 LPAIV. The antigenic variants that existed prior to implementation of vaccine were well controlled by vaccine-induced immunity, but new lineages arose after vaccination that replaced the original viruses. These newly emerged viruses in Mexico were antigenically distinct and commercial vaccination was not able to prevent virus shedding when chickens were challenged with these isolates. In addition, the long-term utilization of vaccines against H5 HPAI has been associated with emergence of vaccine resistant field viruses in China (Chen, 2009; Swayne, 2012), Egypt (Cattoli et al., 2011b; Grund et al., 2011), Hong Kong (Connie Leung et al., 2013), Indonesia (Swayne et al., 2015), and Vietnam (Cha et al., 2013).

A previous study reported that H9N2 LPAI virus which belonged to KU114/07-like lineage [A/chicken/Korea/K040110/10 (H9N2)], was isolated from a severe outbreak at a Korean chicken farm in 2010. Interestingly, this isolate replicated well and caused clinical signs (facial edema and diarrhea) in H9N2-vaccinated birds (Lee et al., 2011). In addition, Park et al. (2011) also showed that Korean H9N2 viruses belonging to KU114/07-like lineage showed lower hemagglutination inhibition titer (geometric mean titer (GMT)=20–160) against vaccine strain than that of clade A (GMT=640) and 865/06-like sub-lineage of clade B (GMT=320). Moreover, these isolates were able to replicate in H9N2-vaccinated birds under experimental condition. These reports suggested the KU114/07-like lineage arose following implementation of vaccination, antigenically drifting rapidly away from the commercial vaccine strain, A/chicken/Korea/01310/2001(H9N2), resulting in the recent KU114/07-like viruses being poorly protected by the vaccine.

Vaccine immunity can exert selective pressure for point mutations in the HA gene. Consequently, rapid antigenic evolution in the HA gene with a slightly changed antigenic structure may prevent effective immunity with existing vaccines (Hensley et al., 2009). Based on our results, three positively selected sites (positions 153, 166 and 230) identified in the HA gene segment were antigenic escape mutant sites identified in previous studies and KU114/07-like lineage had different amino acid sequences at these sites compared to viruses isolated before vaccination. In particular, the 2001 vaccine strain and a few field isolates possessed an additional potential glycosylation site at position 166-168 compared to most of the field isolates (Supplemental Tables S1 and S2). The H9N2 viruses collected after vaccination showed slightly higher mean d*N*/d*S* ratio and number of positively selected sites than before vaccination. Overall, these results suggest that Korean H9N2 viruses isolated after 2007 are undergoing increased antigenic drift that is most likely due to vaccination pressure. Although phenotypic effect of these positively selection and mutations in antigenic sites remains unclear, these sites should be closely monitored to identify the relatedness of antigenicity and genetic features.

Implementation of the H9N2 vaccination program in Korea has dramatically reduced the incidence and severity of H9N2 disease in poultry and reduced the genetic and antigenic diversity of H9N2 virus. However, KU114/07-like lineage of clade B has survived, evolving to a subclade that has shown poor antigenic match with the current 2001 vaccine strain. Enhanced surveillance of H9N2 viruses is needed to identify further increments in viral evolution and such data will help in more timely update of vaccine strain to antigenically more closely match the circulating field viruses. Implementation of timely change in vaccine seed strains to more closely match field viruses could reduce viral divergence and better control H9N2 associated poultry disease.

Materials and methods

Nucleotide sequencing

Viral RNA was extracted from 22 H9N2 virus stocks using the RNeasy Mini kit (Qiagen) and reverse transcribed with the Omniscript Reverse Transcriptase kit (Qiagen). PCR amplifications of the HA gene segment were performed as described previously (Hoffmann et al., 2001). The amplified DNA products were electrophoresed in a 1.0% agarose gel. Pieces of the gel containing DNA bands of the expected sizes were extracted using MEGA quick-spin (INtRON Biotechnology, Korea). Nucleotide sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit and products were analyzed on the ABI PRISM 3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

A total of 103 HA gene segments were used in this study. Specifically, the nucleotide sequences of 22 H9N2 viruses from 2007 to 2012 generated for this study (accession numbers KT157792-KT157809 and KT165007-KT165010) were analyzed together with the HA segment (sequence length > 1590) of all H9N2 viruses isolated in Korea that were available in the GenBank (n=81) from 1996 to 2011. The nucleotide sequences of Korean H9 LPAIV were aligned using MUSCLE (Edgar, 2004) and manual editing of alignments were performed in MEGA 6 software

(Tamura et al., 2013). The maximum likelihood (ML) tree was estimated by the MEGA 6 software (Tamura et al., 2013) using the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites (with four rate categories, Γ4), and proportion of invariant sites (I) were estimated. Statistical analysis of phylogenetic tree was determined by bootstrap analysis with 1000 replicates. Additionally, to investigate whether sub-lineages diversified within Korea or some of variants represent novel introductions into Korea from other countries, we constructed a neighbor-joining phylogenetic tree using 351 representative H9 sequences identified available in the GenBank.

Molecular evolution and skyline plot

Molecular evolution rates and genetic diversity were analyzed as previously demonstrated (Davidson et al., 2014; Fusaro et al., 2011) for the complete dataset (100 sequences), as well as for clade A and B viruses. We excluded three viruses that had North American lineage hemagglutinin gene that were isolated in Korean wild birds because they were unrelated to the Korea-like H9N2 lineage. Rates of nucleotide substitution per site per year and time of most recent common ancestor (TMRCA) were estimated using the BEAST program version 1.8.1 (Drummond and Rambaut, 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) approach. For each analysis, we employed a codon-based SRD06 nucleotide substitution model and an uncorrelated lognormal relaxed clock. In addition, we utilized a Skyline coalescent tree prior (10 piece-wise constant groups), as this is the best descriptor of the complexity of the population dynamics of the H9N2 viruses (Drummond et al., 2005). Maximum clade credibility (MCC) phylogenetic tree was estimated from the posterior distribution of trees generated by BEAST using the program TreeAnnotator v1.8.1 (Drummond and Rambaut, 2007). The MCC tree was visualized using the program FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). A Bayesian Skyline plot was used to infer the population dynamics of Korean H9N2 viruses in terms of changing level of relative genetic diversity [$N_e t$] through time, in which N_e represents the effective population size and t the generation time.

Analysis of selection pressures and glycosylation

Gene- and site-specific selection pressures for the AIV HA protein of the Korean H9N2 viruses were measured as the ratio of non-synonymous (dN) to synonymous (dS) nucleotide substitutions per site for the complete dataset (100 sequences), as well as for viruses collected before (1996–2006) and after (2007–2012) the vaccination campaign. The dN/dS ratios and the selection pressures at individual codons were estimated using the single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), and mixed effects model of episodic diversifying selection (MEME) available at the DataMonkey online version of the HY-Phy package http://www.datamonkey.org (Kosakovsky Pond and Frost, 2005; Pond and Frost, 2005). All analyses utilized the GTR model of nucleotide substitution and employed input NJ phylogenetic trees. Positively selected sites that confirmed by at least two different methods were included in this study.

The positions of positively selected amino acids on the HA molecule were examined on the 3-dimentional structure obtained from the Protein Databank (PDB accession number, 1JSD) with the Chimera 1.10 program. Potential N-glycosylation sites were predicted using NetNGlyc server 1.0.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.023.

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