Field Application of the H9M2e Enzyme-Linked Immunosorbent Assay for Differentiation of H9N2 Avian Influenza Virus-Infected Chickens from Vaccinated Chickens\textsuperscript{\textdagger}

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Vaccination for control of H9N2 low-pathogenicity avian influenza (LPAI) in chickens began in 2007 in South Korea where the H9N2 virus is prevalent. Recently, an enzyme-linked immunosorbent assay (ELISA) using the extracellular domain of the M2 protein (M2e ELISA) was developed as another strategy to differentiate between vaccinated and infected chickens. Here, an ELISA using the extracellular domain of the M2 protein of H9N2 LPAI virus (H9M2e ELISA) was applied to differentiate infected from vaccinated chickens using the H9N2 LPAI virus M2 peptide. The specificity and sensitivity of the optimized H9M2e ELISA were 96.1% and 83.8% (the absorbance of the sample to the absorbance for the positive control [S/P ratio] ≥ 0.6), respectively, with the cutoff value (S/P ratio = 0.6), and the criterion of avian influenza (AI) infection in a chicken house was established as >20% reactivity of anti-M2e antibody per house with this cutoff value. After infection in naïve chickens and once-vaccinated chickens with a hemagglutination inhibition (HI) assay titer of 9.25 ± 0.75 log\textsubscript{2} units, the sera from infected chickens were confirmed as AI infected when the chickens were 1 week old in both groups, and AI infection lasted for 24 weeks and 9 weeks in naïve and once-vaccinated chickens, respectively, although in twice-vaccinated chickens with a higher HI titer of 11.17 ± 0.37 log\textsubscript{2} units, anti-M2e antibody in infected sera did not reach a level indicating AI infection. In field application, anti-M2e antibody produced in infected chickens after vaccination or in reinfected chickens could be identified as AI infection, although HI test could not distinguish infected from vaccinated sera. These results indicate the utility of H9M2e ELISA as a surveillance tool in control of H9N2 LPAI infections.

Avian influenza (AI) is an important infectious disease in both animals and humans. Since the late 1990s, H9N2 low-pathogenicity avian influenza (LPAI) virus (LPAIV) has been reported from South Asia, South Africa, Europe, and the Middle East (1–5, 16, 21, 23, 26), and infection has become increasingly prevalent in domestic poultry (16). H9N2 LPAI virus can be transmitted from poultry to mammalian species, including humans, by direct and indirect contact (4, 25). Therefore, the H9N2 LPAI virus is considered an important candidate source of future human pandemic influenza. In South Korea, the first H9N2 LPAI outbreak in chickens occurred in 1996 and caused slight to moderate mortality (5 to 30%) with apparent clinical signs of depression, edema in the head of the chicken, drop in egg production, and cyanosis in the comb and legs of the chicken (15, 20). H9N2 LPAI reappeared in late 1999 and has become prevalent in South Korea and has caused appreciable economic losses in the poultry industry.

Vaccination is being considered more commonly as an alternative control measure to prevent avian influenza, although it can affect serologic surveillance for H9N2 LPAI infection and negatively affect international trade. H9N2 LPAI vaccination in several countries has been a promising control measure (15, 17, 21, 34); inactivated vaccines prevent clinical signs and increase resistance to infection despite the risk of masking circulation due to the shedding of field virus, if vaccinated poultry become infected with a sufficiently high dose of virus (6, 29, 30). In South Korea, an H9N2 inactivated vaccine consisting of inactivated-virus whole-virus vaccine (in oil emulsion) has been used since March 2007 in an effort to combat the widespread distribution of H9N2 LPAI since 2004, with increasing prevalence of clinical signs and infection-related mortality (7). However, an important limitation in the use of homologous inactivated vaccine is that vaccinated chickens cannot be differentiated from naturally infected chickens using serologic tests such as the agar gel immunodiffusion test (AGD), commercial enzyme-linked immunosorbent assay (ELISA), and hemagglutination inhibition (HI) tests (29).

Strategies of differentiation of infected from vaccinated animals (DIVA) that have been proposed include the use of unvaccinated sentinel birds, subunit vaccines, vaccination with inactivated virus and a homologous hemagglutinin (HA) to the circulating field virus but a heterologous neuraminidase (NA) and the measurement of the serologic response to nonstructural protein 1 (NS1) (28). In South Korea, unvaccinated sentinel chickens have been placed in vaccinated flocks to determine whether flocks have been exposed to H9N2 LPAI. Although sentinels are thought to provide a sensitive measure of infection of a vaccinated flock, this strategy carries practical difficulties; the sentinel poultry must be marked, and these naive poultry may increase the risk of infection of the flock.
(28). Therefore, development of a diagnostic method(s) in the DIVA strategy for control of H9N2 LPAI is needed for use of the H9N2 LPAI vaccine.

Recently, an alternative to DIVA strategy was reported using the measurement of a differential immune response to the extracellular domain of the third integral membrane protein (M2 protein) of the influenza virus (14). The M2e protein (extracellular domain of M2), like NS1, is also associated with actively replicating virus which is exuberantly synthesized in infected cells but is not incorporated into the infectious viral particle, and M2 protein shares with NS1 protein the potential for elaborated DIVA testing, due to differential epitope density on the surfaces of infected cells and on infectious viral particles (10, 18, 22, 35). This M2 protein consists of 97 amino acids (aa), and 24 N-terminal amino acids (M2e) are exposed outside the membrane surface (9); this M2e region is highly conserved in all human, swine, and avian influenza A viruses, with host restriction specificities (18).

The main goal of the present study was to evaluate an ELISA using the extracellular domain of the M2 protein (M2e) of H9N2 LPAI virus (H9M2e ELISA) on the basis of the production of anti-M2e antibodies readily detectable in sera from infected chickens in the differentiation of H9N2 LPAIV-vaccinated chickens from infected chickens with the aim of further field application and, ultimately, replacement of the sentinel strategy used in vaccinated flocks in South Korea.

MATERIALS AND METHODS

Viruses. Four H9N2 low-pathogenicity avian influenza virus (LPAIV) strains, A/Ck/Kor/MS96/96, A/Ck/Kor/310/01, A/Ck/Kor/Q30/04, and A/Ck/Kor/116/04 (where A/Ck/Kor stands for A/Chicken/Korea [South Korea]), were selected for this experiment. A/Ck/Kor/MS96/96 was the first strain isolated in South Korea, and A/Ck/Kor/310/01 is the seed strain for a commercial H9N2 vaccine in South Korea. A/Ck/Kor/Q30/04 and A/Ck/Kor/116/04 were selected because they are different genotypes in the South Korean H9N2 viruses (16). The viruses were grown, the titers of the viruses were determined in 10-day-old specific-pathogen-free (SPF) chickens, and the median embryo infectious dose (EID₅₀) was calculated as previously described (27).

Vaccine. Commercial H9N2 AI vaccine (DaeSung Microbiological Labs, Uiwang, South Korea) was made from inactivated whole viruses prepared from South Korea. A/Ck/Kor/MS96/96 was made from inactivated whole viruses prepared from South Korea. A/Ck/Kor/MS96/96, A/Ck/Kor/310/01, A/Ck/Kor/Q30/04, and A/Ck/Kor/116/04 (where A/Ck/Kor stands for A/Chicken/Korea [South Korea]), were selected for this experiment. A/Ck/Kor/MS96/96 was the first strain isolated in South Korea, and A/Ck/Kor/310/01 is the seed strain for a commercial H9N2 vaccine in South Korea. A/Ck/Kor/Q30/04 and A/Ck/Kor/116/04 were selected because they are different genotypes in the South Korean H9N2 viruses (16). The viruses were grown, the titers of the viruses were determined in 10-day-old specific-pathogen-free (SPF) chicken embryos, and the median embryo infectious dose (EID₅₀) was calculated as previously described (27).

Peptide. M2e(1-22) peptide (M2e with amino acids 2 to 18) (1-SSLTEVETPIRNEWGCR-19), the M2e consensus sequence of H9N2 LPAI viruses isolated in South Korea, was synthesized by PEPTRON (Daejeon, South Korea). The M2e consensus sequence of H9N2 LPAI viruses isolated in South Korea, was synthesized by PEPTRON (Daejeon, South Korea).

Chickens. All experiments were performed after approval of the bioethics committee according to the standards set for performing animal experiments established by the National Veterinary Research and Quarantine (NVROS), South Korea. Six-week-old SPF White Leghorn chickens (Yangsung Labaratories, Yongin, South Korea) were used. They were housed in isolators that were ventilated under positive pressure with HEPA-filtered air. Appropriate feed and water were provided ad libitum. Before the experiments, all animals were confirmed to be free of AIV antibody by the hemagglutination inhibition (HI) test (24) and AIV antibody (Ab) ELISA (BioNote, Hwaseong, South Korea).

Experimental sera. (i) Sensitivity and specificity of H9M2e ELISA. To test sensitivity and specificity in SPF chickens, a serologic response experiment was conducted using seven groups of chickens (7 chickens per group). One group was immunized by a single intramuscular (i.m.) injection of 0.5 ml of commercial inactivated vaccine into the pectoral muscle. The second group was inoculated with 100 μl of 10⁶.0 EID₅₀ of A/Ck/Kor/MS96/96, A/Ck/Kor/310/01, A/Ck/Kor/Q30/04, or A/Ck/Kor/116/04. The control group was inoculated with 100 μl of allantoic fluid from healthy, uninfected chicken embryos. All serum samples were collected every 7 days from 2 weeks postinoculation/postinfection for 5 consecutive weeks. To test sensitivity and specificity in commercial uninfected or infected chickens, with the aim of field application, 3,017 chicken serum samples acquired from 1- to 80-week-old chickens from 203 houses on field farms were used as negative and positive sera in HI and H9M2e ELISA analyses. Among these samples, 1,185 samples from 77 houses were from nonimmunized farms where the chickens were unvaccinated and uninfected, and 1,792 samples from 120 houses were from immunized farms where the chickens were vaccinated once or twice. However, 40 samples from six houses were collected from chickens confirmed as having H9N2 LPAI infection.

(ii) Onset and persistence of anti-M2e antibody. The onset and persistence of anti-M2e antibody was assessed in infected and vaccinated groups (18 6-week-old SPF chickens per group). Group 1 was infected intranasally with 0.1 ml of 10⁶.0 EID₅₀ of A/Ck/Kor/310/01 strain. Group 2 was vaccinated with one dose of commercial H9N2 AIV vaccine, and 3 weeks later, the group was infected by intranasal administration of 0.1 ml of 10⁶.0 EID₅₀ of A/Ck/Kor/310/01 strain. The third group was vaccinated and boosted 3 weeks later with commercial H9N2 AIV vaccine, and 3 weeks after the boost, the group was infected intranasally with 0.1 ml of 10⁶.0 EID₅₀ of A/Ck/Kor/310/01 strain. Finally, the control group was inoculated with 100 μl of allantoic fluid from healthy, uninfected chicken embryos. Serum samples were collected every 7 days after inoculation until the chickens were 30 weeks old.

HI test. HI test was performed according to the World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (24). Detecting antibodies to H9N2 LPAIV was conducted in V-bottom, 96-well microtiter plates using 4 HA units of the A/Chicken/Korea/310/01 strain and 1% chicken erythrocytes.

H9M2e ELISA. The conditions used in the H9M2e ELISA were optimized by diluting the peptide, sera, and conjugate. Maxisorp microplates (Nunc, Roskilde, Denmark) were coated with the M2e(1-18) peptide diluted in phosphate-buffered saline (PBS) (pH 7.2) and incubated at 4°C overnight. The plates were then blocked with PBS containing 0.05% Tween 20 (PBST) supplemented with 3% skim milk (Becton Dickinson, Sparks, MD) for 4 h at 37°C. The serum samples were inactivated by being heated to 56°C for 30 min, cooled to 4°C, and treated to reduce nonspecific reactivity with PBS, a 12.5% suspension of acid-washed kaolin (11, 12), and PBS containing 2% skim milk. In brief, the serum samples were diluted 10 times in PBST, and 40 μl of the diluted serum sample and 60 μl of 12.5% suspension of acid-washed kaolin were completely mixed by vortexing. The mixed suspension was incubated for 1 h at room temperature, and then the kaolin was sedimented by centrifugation for 1 min at 13,000 rpm. Seventy microliters of the supernatant and 70 μl of PBST containing 2% skim milk were mixed well and incubated for 1 h at 4°C. After the blocked plate was washed, 100 μl of pretreated serum (1:50) was incubated for 30 min at room temperature. Binding of antibodies was revealed using horseradish peroxidase-conjugated anti-chicken immunoglobulin G (KPL, Gaithersburg, MD) diluted 1:2,000 and incubated for 30 min at room temperature. Peroxidase activity was revealed by adding 100 μl of tetramethylbenzidine peroxidase substrate (TMB; KPL) for 10 min at room temperature, and the reaction was stopped by the addition of 100 μl of 1 N hydrochloric acid (Merck, Darmstadt, Germany) and measured at 450 nm using an ELISA reader (TECAN, Männedorf, Switzerland), and the absorbance of the sample to the absorbance for the positive control (S/P ratio) was calculated. The cutoff values for the H9M2e ELISA were calculated from the results of sera from infected and uninfected SPF chickens and commercial chickens using two-graph receiver operating characteristic (TG-ROC) analysis. A serum sample was considered positive when its S/P ratio was greater than the cutoff value.

Field application of H9M2e ELISA. Serum samples of two monitoring commercial layer farms, GN and WS, were generously provided by Son Young-Ho (Bansuk Poultry Clinic and Lab, Eumseong, South Korea). For the GN farm, the chickens were vaccinated when they were 66 days old. Seras were regularly collected from chickens when they were 57 to 469 days old, and the number of eggs produced was counted every 7 days from 133-day-old chickens. A decrease in egg production was experienced twice, when the chickens were 294 and 434 days old. For the WS farm, there were two flocks, flock 081021 and flock 081024. The chickens in flock 081021 and flock 081024 were vaccinated when they were 99 and 92 days old, respectively. When the chickens on the GN and WS farms were 104 and 101 days old, respectively, urate deposition on the surfaces of visceral organs, hemorrhage and ulceration on the proventriculus, and increase of mortality were assessed in both flocks, and then the farm was confirmed as positive for H9N2 LPAI by virus isolation. Serum samples were collected when the chickens in flocks 081024 and 081021 were 57 and 54, 92 and 89, 144 and 141, and 197 and 194 days old, respectively, to monitor the farms.
RESULTS

Establishment of H9M2e ELISA conditions. (i) Nonspecificity. The optimal concentration of the coating peptide and the optimal dilutions of serum and conjugate were determined to be 750 ng/100 μl per well, 1:50, and 1:2,000, respectively, for sera from infected and vaccinated chickens. Nonspecificity of uninfected field sera (2,977 sera) was measured on unvaccinated and vaccinated sera every 10 weeks until the chickens were 80 weeks old (Fig. 1). Nonspecificity increased with age and was highest when the chickens were 51 to 80 weeks old. The nonspecific reactivities of the H9M2e ELISA in uninfected commercial chickens were 6.17% (S/P ratio ≤ 0.4), 4.61% (S/P ratio ≤ 0.5), 4.01% (S/P ratio ≤ 0.6), 3.34% (S/P ratio ≤ 0.7), and 2.81% (S/P ratio ≤ 0.8) in all serum samples.

(ii) Sensitivity and specificity. Using the optimized H9M2e ELISA conditions, 3,055 AIV-uninfected and 180 AIV-infected sera from SPF and commercial chickens were analyzed to verify the sensitivity and specificity of H9M2e ELISA using TG-ROC. As shown in Fig. S1 in the supplemental material, the cutoff value of H9M2e ELISA was determined to be an S/P ratio of 0.6 in consideration of nonspecific sera in commercial chickens, and the sensitivity and specificity were determined to be 83.8% and 96.1%, respectively.

Criteria of AIV infection in chicken houses by H9M2e ELISA. H9M2e ELISA was applied to sera from chickens in 77 houses (1,185 unvaccinated and uninfected sera), 120 houses (1,792 vaccinated and uninfected sera), and 6 houses (40 unvaccinated and infected sera) in the field using the cutoff value (S/P ratio = 0.6) (Table 1). In the unvaccinated and uninfected groups, 75 of 77 houses (97.4%) were observed with ≤15% per house and two of 77 houses (2.6%) with 20% per house using the cutoff value. In addition, in the vaccinated and uninfected groups, 116 of 120 houses (96.7%) were observed with ≤15% per house and four of 120 (3.3%) with 20% per house. However, >20% per house was not observed in all uninfected groups. In contrast to the uninfected group, all the houses with infected chickens displayed >20% per house using the cutoff value. Almost all infected sera (38/40 [95%]) were identified at a level higher than the S/P ratio for the cutoff value, and a positive reaction in each house containing infected chickens was identified with 75 to 100% in six infected houses. Therefore, the criterion of AI infection in any chicken house was established as >20% per house using the cutoff value (S/P ratio = 0.6).

Anti-M2e antibody response after vaccination or infection in SPF chickens. To validate the usefulness of H9M2e ELISA in the serological differentiation of vaccinated or infected chickens, sera collected from chickens before and after vaccination or infection were tested on the basis of the cutoff value (S/P ratio = 0/6) (see Table S1 and Fig. S2 in the supplemental material). Almost all of the sera from infected chickens at 2 weeks after infection were positive (27/28 [96.4%]), and the S/P ratio of each group was markedly higher (0.72 to 10.17).

FIG. 1. Age-dependent nonspecific reactivity of H9M2e ELISA on serum samples from commercial chickens not infected with avian influenza virus. The age of the chickens is shown in weeks (w) below the graph, and the number of serum samples is shown below the age of the chickens.

TABLE 1. H9M2e ELISA reactivity in uninfected and infected commercial chickens

| Group | No. of houses (no. of serum samples) | AI positive rate (%) per house by H9M2e ELISA | No. of houses with the indicated AI positive rate |
|-------|-------------------------------------|---------------------------------------------|-----------------------------------------------|
| Uninfected, unvaccinated chickens | 77 (1,185) | ≤5 | 57 |
| | | ≤10 | 12 |
| | | ≤15 | 6 |
| | | ≤20 | 2 |
| | | >20 | 0 |
| Uninfected, vaccinated chickens | 120 (1,792) | ≤5 | 65 |
| | | ≤10 | 32 |
| | | ≤15 | 19 |
| | | ≤20 | 4 |
| | | >20 | 0 |
| Unvaccinated and infected chickens | 6 (40) | ≤5 | 0 |
| | | ≤10 | 0 |
| | | ≤15 | 0 |
| | | ≤20 | 0 |
| | | >20 | 6 |

*The commercial chickens were uninfected or infected with avian influenza (AI) virus. The H9M2e ELISA cutoff value was an S/P ratio of 0.6.*
than the cutoff value (see Fig. S2a in the supplemental material). Sera collected from chickens in each group 2 weeks after infection displayed a higher positive rate (85 to 100% per group) than those of 3 to 6 weeks, after which the positive rate of anti-M2e antibody gradually decreased to 42.9 to 85.7% (see Table S1 in the supplemental material). All of the infected groups 26 weeks after infection were identified as AIV infected (42.9 to 100% per group) using the H9N2 criterion of AI infection in a chicken house. However, all of the sera from chickens vaccinated once or twice were AI negative (0% per each group) 2 to 6 weeks postvaccination, although the HI antibody titer of the vaccinated group (8.43 to 11.57 log₂ units) was higher than that of all infected groups (5.14 to 7.43 log₂ units) (see Table S1 and Fig. S2b in the supplemental material). Control sera also were AI negative (0% per each group) when the chickens were 2 to 6 weeks old (see Table S1 in the supplemental material).

Onset and persistence of anti-M2e antibodies. The onset and persistence of anti-M2e antibodies were determined in infected and vaccinated chickens (Fig. 2). In the nonvaccinated, infected group (Fig. 2a), anti-M2e antibody was first detected 1 week after infection, and at that time, the positive rate was identified as 83.3% with the cutoff value, although HI antibody was detected 1 week after infection (5.61/1.69). All infected chickens in this group displayed a positive reaction 2 to 4 weeks after infection by H9M2e ELISA. The positive reaction gradually decreased and lasted until 24 weeks after infection; this infected group was confirmed as having AIV infection until at least 24 weeks after infection on the H9M2e ELISA reactivity and average HI titers are shown. The black broken and solid arrows show the time points of vaccination and challenge, respectively. The horizontal broken line in each panel shows the H9M2e ELISA cutoff value.

FIG. 2. Onset and persistence of anti-M2e antibody after infection with or without vaccination of 6-week-old SPF chickens. (a) Chickens were infected with 10⁶.₀ EID₅₀/0.1 ml of A/Ck/Kor/310/01 when the chickens were 6 weeks old. (b) Chickens were vaccinated once with the South Korean commercial H9N2 vaccine when they were 6 weeks old and challenged with 10⁶.₀ EID₅₀/0.1 ml of A/Ck/Kor/310/01 when they were 9 weeks old. (c) Chickens were vaccinated twice with the South Korean commercial H9N2 vaccine when they were 6 and 9 weeks old. They were challenged with 10⁶.₀ EID₅₀/0.1 ml of A/Ck/Kor/310/01 when they were 12 weeks old. The H9M2e ELISA was performed using an S/P ratio of >0/6. H9M2e ELISA reactivity and average HI titers are shown. The black broken and solid arrows show the time points of vaccination and challenge, respectively. The horizontal broken line in each panel shows the H9M2e ELISA cutoff value.
tified at 22% using the cutoff value. The positive rate was highest (66%) 2 weeks after challenge and then gradually decreased. In this group, AI infection was observed for 9 weeks after challenge on the /H11022 criterion of AI infection in a chicken house (Fig. 2b). However, the twice-vaccinated group revealed antibody levels about four times higher (11.17/0.37 log2 units) than the once-vaccinated group prior to challenge and was not identified as AI infected despite challenge on the criterion of AI infection in a chicken house (Fig. 2c). The control group was AIV negative during this experiment by both H9M2e ELISA and HI testing (data not shown).

Identification of AI infection from field monitoring sera. For the GN farm, after vaccination of the chickens when they were 66 days old, the antibody titer in HI test peaked at 98 days and gradually decreased (Fig. 2a). The egg production rate was normal for 294 days (Fig. 3b). At the same time, AI infection was not identified until day 294 by H9M2e ELISA based on the criterion of AI infection in a chicken house (Fig. 3a). After the chickens were bled at 294 days, the egg production rate decreased severely from 90.7% to 76.7%, which is related to AI infection confirmed from sera collected just after the drop in egg production at 306 and 320 days (86.7% and 53.3%, respectively) by H9M2e ELISA (left gray band in panel a). There was another drop in the egg production rate, a smaller decrease from 80.3% to 77.8% (right gray band in panel b) and the sera at day 445 were detected with a reactivity of 40% by H9M2e ELISA (right gray band in panel a). The broken line in panel a shows the H9M2e ELISA cutoff value.

For the WS farm, HI antibody was not detected in flock 081021 or 081824 before the chickens were vaccinated when...
they were 99 days and 92 days old, respectively (Fig. 4a and b). H9N2 LPAI infection was identified from sera from chickens at both 104 days (flock 081021) and 101 days (flock 081824) after infection based on clinical signs, appearance of gross lesions, and virus isolation. Both flocks could not be distinguished by HI antibody titer with sera 144 days (flock 081021) and 141 days (flock 081024) after infection, because the HI antibody could be produced by vaccination or infection. However, sera examined at days 144 and 141 were confirmed to be AIV positive (87.7% for flock 081021 and 33.3% for flock 081824) based on the criterion of AI infection in a chicken house by H9M2e ELISA (Fig. 4a and b).

**DISCUSSION**

H9N2 LPAI virus has caused great economic losses to the poultry industry in South Korea since the first outbreak in the mid-1990s. The South Korean government permitted the use of an inactivated whole-virus vaccine derived from one selected H9N2 Korean strain (Ck/Kor/310/01) in 2007 (7). Before permission was granted for this vaccine to be used, conventional diagnostic methods, including agar gel immunodiffusion test (AGP), hemagglutination inhibition (HI) test, and ELISA had been used to detect antibodies to H9N2 LPAI infection. However, vaccination was found to interfere with the surveillance of domestic chickens using conventional serologic tests. Moreover, management of sentinel chickens in flocks has proven to be difficult in farms where the chickens were vaccinated.

Differentiation of infected from vaccinated animals (DIVA) strategies using NS1 and M2 protein have been reported to overcome limitations caused by whole-virus vaccine with homologous HA and NA. At first, NS1 protein was considered the most useful marker of virus replication for DIVA strategies. Anti-NS1 antibodies to the NS1 protein using *Escherichia coli*-expressed recombinant NS1 and two NS1 peptides, NS1\(^{149-160}\) and NS1\(^{206-219}\) as the antigen, were detected by ELISA in serum samples of experimentally infected chickens but not in vaccinated chickens for H1, H3, H5, H7, and H9 subtypes (3, 8, 31, 32, 36). However, in our study, anti-NS1 antibodies were not sufficient to differentiate infected from vaccinated sera in experimentally infected and vaccinated chickens using insect cell-expressed recombinant NS1 protein and NS1 peptides as the antigen (data not shown). Recently, the extracellular domain of the M2 protein (M2e) has been chosen as another putative differential diagnostic marker for H5 and H7 subtypes (14). Lambrecht et al. (14) reported that M2e-specific antibodies were positive in chickens and ducks experimentally infected with H7 or H5 HPAI viruses, respectively, by M2e ELISAs but not in chicken and ducks inoculated with inactivated-AIV vaccines (14). In addition, ducks inoculated with inactivated vaccine and challenged with an HPAI H5N1 virus were differentiated from ducks inoculated with inactivated vaccine (14). A few changes were observed in the M2e segment of different influenza viruses, although the M2 protein is remarkably conserved among type A influenza viruses (14, 18, 19). The reactivity of M2e ELISA was a little different for M2e sequences as an antigen peptide; the reactivity was better for the same peptide sequence than with different peptides (14). Therefore, in this study, H9M2e sequence based on H9N2 influenza viruses isolated in South Korea was selected for H9M2e ELISA for the situation in South Korea, although the M2e sequence was the same as that of H7N1 influenza viruses.

Prior to the practical field application of the H9M2e ELISA, testing positive and negative sera obtained from chickens of different ages was done in the field, since nonspecific reactions seem to occur with sera obtained from the field as opposed to sera from SPF chickens reared in experimental conditions (13, 33). Nonspecific reactivity may be due to cross-reactive antibodies to a common antigen of host-expressed target protein, particularly in the sera of older chickens in the field (31). Another nonspecific factor could be the presence of lipoprotein in sera; by removing nonspecific lipoprotein inhibitors, kaolin, heparin-MnCl\(_2\), or dextran sulfate-CaCl\(_2\) have been reported to be found in sera (2). Among kaolin, heparin-MnCl\(_2\), and dextran sulfate-CaCl\(_2\), kaolin efficiently reduced nonspecific reactivity (data not shown) and increased the optical density difference in positive/negative sera (see Fig. S3 in
the supplemental material). The cutoff value was established at the S/P ratio of 0.6 in consideration of the specificity and sensitivity of H9M2e ELISA, 96.1 and 83.8%, respectively (see Fig. S1 in the supplemental material), because nonspecific reactivity was not eliminated in the all negative sera in the field despite the use of kaolin (Fig. 1). Generally, 15 to 20 sera from chickens in each chicken house were collected for AIV serological monitoring, and the nonspecific reactivity in each house was identified up to 20% in six of the 197 uninfected houses (3.05%) on the basis of the cutoff value (Table 1). However, all houses with infected chickens were identified with 75 to 100% in each house (Table 1). The results were consistent with the suggestion that >20% of positive/total sera of the cutoff value would be appropriate for the positive criterion of AI infection in each house.

H9M2e ELISA would be useful in differentiating infected from vaccinated chickens using sera from chickens infected with H9N2 LPAI viruses even though the chickens had previously been vaccinated. In experimentally infected SPF chickens, differentiating infected from vaccinated chickens was impossible by HI test, because all infected groups displayed HI antibody titers as high as those of vaccinated chickens (see Fig. S2b in the supplemental material). However, H9M2e ELISA could efficiently distinguish infected from vaccinated chickens by detecting anti-M2e antibody produced only in sera from infected chickens (42.9 to 100% per house) 2 to 6 weeks old (see Table S1 and Fig. S2a in the supplemental material). In addition, the onset and duration of antibody response to M2e protein after infection were evaluated. AI infection in the infected chicken group was identified by H9M2e ELISA 1 week after infection and lasted for at least 24 weeks (Fig. 2a), and in the challenged chicken group after a single vaccination, it was also identified 1 week after infection and lasted for 9 weeks (Fig. 2b). However, in chickens that were vaccinated twice and challenged, anti-M2e antibody levels due to AI infection were just below the criterion of AI infection in a chicken house, which was confirmed as AI negative despite AI infection (Fig. 2c). These results suggest that H9M2e ELISA would be effective in the long-term in unimmunized chickens or chickens immunized with a low dose in detecting anti-M2e antibody caused by the viral replication because infected virus could be more easily replicated in unimmunized chickens or chickens immunized with a low dose than in chickens immunized with a high dose.

H9M2e ELISA would also be useful to confirm AI infection serologically in AI-infected field cases. In the field on the GN farm (Fig. 3), a drop in egg production was experienced two times during the laying period. The first drop in egg production was confirmed to be caused by AI infection by H9M2e ELISA analysis of sera, although a high HI antibody titer was suspected to be caused by AI infection taking into consideration the HI titer by regular monitoring (left gray bands of Fig. 3a and b). The second drop in egg production was also confirmed to be due to AI infection, despite infection in chickens with high HI antibody titer (6.67 ± 1.23) at 418 days, while the HI test could not identify AI infection, because the HI antibody titer (6.67 ± 1.23) before infection was similar to the titer after infection (7.07 ± 1.21) (right gray bands of Fig. 3a and b). In another field case on the WS farm (Fig. 4), AI infection was identified through clinical signs and virus isolation just after vaccination of the chickens from the history of the farm; chickens from flocks 081021 and 081024 were infected just before and after producing AI antibody, respectively (Fig. 4a and b). AI exposure could not be identified with sera obtained 144 (flock 081021) and 141 (flock 081024) days after infection by the HI test, because the HI antibody titers on those days could be produced by vaccination. However, the sera from chickens in both flocks were identified as AI infected by H9M2e ELISA. In the case of flock 081021, AI infection was confirmed with 86.7% at day 40 and 46.7% at day 93 after infection, which would seem to be similar to the reaction pattern caused by infection in naive chickens as shown in Fig. 2a. However, for flock 081024, AI infection was confirmed (33.3% at day 40), and the reactivity and length of time the anti-M2e antibody could be detected were less than those of chickens in flock 081021, which would seem to be similar to the reaction pattern caused by infection in immunized chickens as shown in Fig. 2b or the first or second infection as shown in Fig. 3a. Therefore, these results suggest that the length of time the anti-M2e antibody was detected could be affected by the level of AI protective antibody in immunized chickens.

In conclusion, this study demonstrates that chickens infected with H9N2 LPAI viruses could be differentiated from chickens vaccinated with commercial vaccine on the basis of detection of anti-M2e antibody. Use of H ELISA for AI surveillance could be a valuable diagnostic tool for the control of H9N2 LPAI infections.

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