In poultry species, H9N2 low pathogenic avian influenza viruses (LPAI) infection has often caused slight to moderate mortality with apparent clinical signs that are characterized by depression, respiratory symptoms, and a decrease in egg production (Lee et al., 2000). Therefore, the frequent economic losses incurred with subtype H9N2 LPAI infection have raised serious concerns worldwide (Guo et al., 2000; Toroghi and Momayez, 2006; Lee et al., 2007; Monne et al., 2007; Wu et al., 2008; Nagarajan et al., 2009). In addition, acquired transmissibility to mammalian species, including humans, raises public concerns about an increasing pandemic potential (Lin et al., 2000; Butt et al., 2005).

In Korea, H9N2 LPAI was first documented in 1996 and it caused serious economic loss in the Korean poultry industry, including layer and broiler breeder farms. Since then, the H9N2 viruses have been prevalent in chicken farms and have continuously evolved through gene shift with wild bird and live bird market isolates (Lee et al., 2007, 2010; Moon et al., 2010). To control LPAI outbreaks, since 2007, the Korean veterinary authority has permitted the use of inactivated oil adjuvant H9N2 LPAI vaccine (Choi et al., 2010).

Although a 1-dose regimen with inactivated oil adjuvant H9N2 LPAI vaccine is very immunogenic and highly protective in laboratory trials using specific-pathogen-free (SPF) chickens (Choi et al., 2008), use of a 1-dose regimen in broiler breeder farms induced poor antibody response and could not prevent vaccinated broiler breeders from becoming infected and from shedding wild viruses. Therefore, improved vaccination strategy is required to control avian influenza outbreaks in farms.

Here, a study was conducted to determine whether a 2-dose regimen of inactivated H9N2 LPAI vaccine could enhance the immunologic response in chickens. Such gel-primed and mineral oil-boosted regimen has produced encouraging results associated with improved immune responses to an H9N2 LPAI. This strategy could be cost effective and helpful for preventing avian influenza virus in the poultry industry.

**MATERIALS AND METHODS**

For virus inactivation, formaldehyde solution was added to allantoic fluid of A/Chicken/Korea/01310/2001 (H9N2) virus-inoculated SPF embryonated eggs. Vaccines, with oil adjuvant, were prepared by emulsifying the $10^6.0$ 50% egg infectious dose/mL of formaldehyde-inactivated H9N2 antigen solution with ISA70 (Seppic, Puteaux, France) at a ratio of 30:70 (wt/wt). For the preparation of aluminum hydroxide...
gel vaccine, aluminum hydroxide gel was added to the inactivated antigen with a final concentration of 20% aluminum hydroxide gel (vol/vol).

In a laboratory trial, fifty 6-wk-old SPF chickens were divided into 5 groups (Table 1). With 1 or 2 doses of vaccines, 0.5 mL of inactivated oil adjuvant H9N2 vaccine or inactivated gel adjuvant H9N2 vaccine was injected via the intramuscular route. The second dose was administered 3 wk after the first dose. Two, 3, and 6 wk after vaccination, serum antibody titers were measured by hemagglutination inhibition (HI) test using homologous antigen. All experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of Konkuk University (Seoul, Korea).

In a field trial in a broiler breeder farm, 14- to 20-wk-old Ross broiler breeders were divided into 5 groups with 15 chickens each. With 1 or 2 doses of vaccines, 0.5 mL of inactivated oil adjuvant H9N2 vaccine or inactivated gel adjuvant H9N2 vaccine was injected via the intramuscular route. The second dose was administered 5 wk after the first dose. After vaccination, serum antibody titers were measured by HI test up to 44 wk. For statistical analysis, 2-tailed student t-test was performed.

**RESULTS AND DISCUSSION**

In a previous study, Chukiatsiri et al. (2009) demonstrated that the average antibody titers of chickens vaccinated with mineral oil adjuvant vaccine were higher than those of chickens vaccinated with aluminum hydroxide gel adjuvant vaccine. Based on our results, in a single-dose regimen, the oil adjuvant vaccine also produced higher antibody response than the gel adjuvant vaccine (Table 1; Figure 1). In particular, the oil adjuvant vaccine could produce faster and approximately 8-fold higher antibody response than the gel adjuvant vaccine in the broiler breeders. Mean antibody titers of vaccinated SPF chickens were higher than those of vaccinated broiler breeders. Difference of environmental conditions and species of chickens used in vaccine trials might influence the immune response of these groups.

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**Table 1. Immunogenicity of inactivated H9N2 vaccines in specific-pathogen-free chickens**

<table>
<thead>
<tr>
<th>Adjuvant type</th>
<th>First vaccination</th>
<th>Second vaccination</th>
<th>Mean HI titer2 (log 2 ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 wk after first</td>
<td>3 wk after first</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vaccination</td>
<td>vaccination</td>
</tr>
<tr>
<td>Oil adjuvant</td>
<td>ND</td>
<td>6.1 ± 0.8</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>Oil adjuvant</td>
<td>Oil adjuvant</td>
<td>5.8 ± 0.7</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Gel adjuvant</td>
<td>ND</td>
<td>5.1 ± 1.0</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>Gel adjuvant</td>
<td>Oil adjuvant</td>
<td>4.9 ± 0.9</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

1ND = not done.
2Hemagglutination inhibition (HI) test was done using chicken erythrocyte with homologous antigen.
3Second vaccination was conducted 3 wk after the first vaccination.

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**Figure 1.** Immunogenicity of inactivated H9N2 vaccine in broiler breeders. Ross broiler breeders (14–20 wk of age) were inoculated intramuscularly with 1 dose of oil-adjuvant H9N2 vaccine (●) or gel-adjuvant H9N2 vaccine (▲). Additionally, gel-primed and mineral oil-boosted regimen (▼) and oil-primed and oil-boosted regimen (■) were evaluated. Upward arrows indicate the time of vaccination. Serum antibody titers were measured by hemagglutination inhibition test using chicken erythrocyte with homologous antigen up to 44 wk. ***P < 0.0001 by 2-tailed student t-test compared with oil-primed and oil-boosted regimen group.
Particularly, the stress associated with the farm conditions and the heavy weight of broiler breeders are considered as causes of the decrease of the antibody response.

In the present study, in a 2-dose regimen, the gel-primed and oil-boosted regimen produced slower antibody response after the first vaccination but showed higher antibody response than the oil-primed and oil-boosted regimen. Memory B cells generated by aluminum hydroxide gel might play a crucial role in producing higher antibody response than the oil-primed and oil-boosted regimen could produce significantly higher antibody response after the first vaccination but showed slower antibody response than the oil-primed and oil-boosted regimen. Further, at 44 wk postvaccination, the gel-primed and oil-boosted regimen could produce significantly higher antibody response than the oil-primed and oil-boosted regimen. Memory B cells generated by aluminum hydroxide gel might play a crucial role in producing higher antibody response than the oil-primed and oil-boosted regimen could produce significantly higher antibody response than the oil-primed and oil-boosted regimen.

In conclusion, because a single-dose regimen of inactivated H9N2 vaccine provided not enough antibody levels in the broiler breeder flock, we recommend a 2-dose regimen of inactivated H9N2 vaccine rather than a single-dose regimen. Particularly, a gel-primed and oil-boosted regimen could provide high antibody titers in broiler breeders. In addition, gel adjuvants are more cost effective than oil adjuvants. Therefore, a gel-primed and oil-boosted regimen might be an economical and effective vaccine strategy for poultry producers.

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REFERENCES


