A safety assessment of a fowlpox-vectored Mycoplasma gallisepticum vaccine in chickens


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ABSTRACT A recombinant fowlpox virus vaccine expressing key protective Mycoplasma gallisepticum antigens could facilitate in the prevention both of fowlpox virus and M. gallisepticum infections. Vectormune FP-MG vaccine, a recombinant fowlpox virus expressing both M. gallisepticum 40k and mgc genes, was assessed for its safety in 8-wk-old specific-pathogen-free White Leghorn chickens. The vaccine virus was serially passed 5 times by wing-web inoculation. Based on the postinoculation clinical observation, gross pathological examination of air sacs and peritoneum, genetic stability evaluation, virus shedding and tissue distribution detection, horizontal transmission ability determination, and protection against fowlpox virus challenge, the Vectormune FP-MG vaccine possesses a high level of safety.

Key words: vaccine safety, fowlpox vector, Mycoplasma gallisepticum, assessment, passage

INTRODUCTION

The intensive poultry industries rely heavily upon the application of vaccines for disease control. Therefore, vaccine approaches to infectious diseases are widely welcomed and appreciated. For effective disease control in poultry, viral vectored vaccines offer new avenues in developing whole new generations of vaccines based on recombinant viruses. A live recombinant viral vector vaccine is a vaccine in which an immunogenic gene from one pathogen is inserted into the DNA of another pathogen designated as the vector, resulting in a recombinant strain that will protect against the disease of the gene insert and the disease caused by the vector (Bostock, 1990; Yamanouchi et al., 1998; Robert-Guroff, 2007).

The common vectors for human vaccine applications included poxvirus, adenovirus, alphavirus, rhabdovirus, and poliovirus (Polo and Dubensky, 2002). The engineering of fowlpox virus (FPV)-based vectors, which are presently the most widely used vectors, has direct application for recombinant vaccines in the poultry industry. Development of recombinant FPV (rFPV) vector vaccines began in the 1980s (Mackett et al., 1984; Skinner et al., 2005). Since then, many disease antigens expressed by rFPV, such as Newcastle disease virus (Boursnell et al., 1990; Taylor et al., 1990), avian influenza virus (Swayne et al., 2000; Senne, 2003; Chen, 2009; Qiao et al., 2009), infectious bronchitis virus (Wang et al., 2002), infectious bursal disease virus (Bayliss et al., 1991; Heine and Boyle, 1993), infectious laryngotracheitis virus (Davison et al., 2006), Marek’s disease virus (Lee et al., 2003), and reticuloendotheliosis virus (Calvert et al., 1993) have been developed or shown to be effective vaccines in poultry. Some rFPV vaccines have also been licensed for commercial use, such as rFPV for Newcastle disease virus and avian influenza virus in the United States (Boursnell et al., 1990; Taylor et al., 1990; Swayne et al., 2000) and rFPV against avian influenza virus in Mexico and China (Senne, 2003; Qiao et al., 2009; Villarreal, 2009).

All of these vaccines have to be proven safe as well as effective, not only before but also after they are licensed for use. The safety of the live recombinant vector vaccine, such as spread of the recombinant vector vaccine, tissue tropism, reversion to virulence, and horizontal transmission, should be investigated in the target species for the vaccine.

Vectormune FP-MG is a genetically engineered live FPV vaccine for use in chickens and turkeys. The FPV has been genetically modified to express key protective Mycoplasma gallisepticum antigens. Vectormune FP-MG is indicated as an aid in the prevention of fowlpox and M. gallisepticum infections and has received commercial licensure in the United States. In the present study, we assessed the safety of the vaccine by serial passage in specific-pathogen-free (SPF) chickens. The
trial included tissue distribution of recombinant virus, virus shedding, hereditary stability, cohabitation trial, environmental detection, and protection against FPV challenge.

MATERIALS AND METHODS

Vaccine

Vectormune FP-MG, which is produced by Ceva Biomune Animal Health Company (Lenexa, KS) and supplied by Ceva Animal Health China Representative Office (Beijing), is an rFPV-vectored vaccine with the 40k and mcg gene segments derived from *M. gallisepticum*. This vaccine is approved for wing-web administration to healthy, susceptible chickens at 8 wk of age or older as an aid in the prevention of FPV and *M. gallisepticum* infections.

Vaccination and Passage

Eigh-week-old SPF White Leghorn chickens were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (China) and were housed in isolators. All animal experiments were approved by the Beijing Administration Office of Laboratory Animal and Beijing Transgene Administration Committee (China). The vaccine virus was serially passaged 5 times in SPF chickens by wing-web inoculation. In the first passage, all of the chickens were given a single-dose vaccine of 0.03 mL. As a negative control, chickens received a dose of 0.03 mL of PBS. Two chickens were killed on d 5 postinoculation, and the skin at the injection site was collected to prepare a 10% (wt/vol) variolar crust suspension in PBS (containing 1,000 U/mL of penicillin and 1,000 U/mL of streptomycin, pH 7.2). The next passage was inoculated with 0.03 mL of suspension per bird. The procedure was repeated until the fifth passage was reisolated.

Clinical Observation

For each passage, the chickens were observed daily after the vaccination for 14 d. General health condition and local reactions at the injection site (swelling and scab formation) were examined and photographed. In addition, gross pathological examination of trachea and air sacs were also performed in 2 killed chickens. Skin suspension material from each passage was stored at −80°C and kept frozen until further processing.

Tissue Distribution of Recombinant Virus

Three birds from each group of the first passage (test and control) were randomly selected on d 1, 3, 5, 7, 9, 11, 14, and 21 postinoculation. Then they were humanely killed and were subjected to necropsy. Samples of different tissues including the skin at the injection site were collected.

### Table 1. Tissue distribution of recombinant virus in organs of specific-pathogen-free chickens after wing-web inoculation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Positivity at specified time postinoculation (no. positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 d</td>
</tr>
<tr>
<td>Skin</td>
<td>3/3</td>
</tr>
<tr>
<td>Liver</td>
<td>1/3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/3</td>
</tr>
<tr>
<td>Trachea</td>
<td>1/3</td>
</tr>
<tr>
<td>Brain</td>
<td>0/3</td>
</tr>
<tr>
<td>Bursa of Fabricius</td>
<td>0/3</td>
</tr>
</tbody>
</table>

1. Detected by PCR targeting a 4b gene fragment of fowlpox virus.
2. The local skin at the injection site.
site, trachea, liver, spleen, brain, and bursa of Fabricius were collected for PCR detection.

**Virus Shedding**

Ten cloacal swabs were randomly taken from each group of the first passage (test and control) on d 1, 3, 5, 7, 9, 11, 14, and 21 postinoculation. This was done for the rFPV detection by PCR targeting a 4b gene fragment of FPV.

**Hereditary Stability**

After 5 consecutive passages in SPF chickens, the nucleotide sequences of the 40k gene of *M. gallisepticum* were determined and compared with each other between the first and fifth passage. The median embryo infectious doses (EID$_{50}$) of recombinant virus were also tested by growth in 10-d-old embryonated SPF eggs from the first passage, the third passage, and the fifth passage.

**Cohabitation Trial**

On 24 h postinoculation in the first and fifth passage, 10 susceptible in-contact chickens were mingled with the inoculated chickens. This was done to evaluate horizontal transmission ability of the recombinant virus by protection test against FPV challenge.

**Environmental Detection**

Environmental feces, drinking water, and cage swabbing samples were collected from the isolator of the first passage on d 7 and 14 postinoculation. The purpose of this was for recombinant virus detection by using PCR targeting a 40k gene fragment of *M. gallisepticum*.

**Protection Against FPV Challenge**

After 14 d postinoculation in the fifth passage, 10 birds from the vaccinated chickens and in-contact chickens (the first and fifth passage) including unvaccinated control chickens were challenged with $10^3$ EID$_{50}$/50 μL virulent FPV through wing-web puncture. After challenge exposure, all of the chickens were kept in a separate isolator and observed up to 21 d for the development of clinical signs and symptoms.

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**DNA Extraction and PCR**

**DNA Extraction.** Deoxyribonucleic acid was extracted from each tissue sample with a DNAzol kit (Vigorous Biotechnology Beijing Co. Ltd., China) according to the manufacturer’s instructions. Tissue samples (25 to 50 mg) were homogenized in 1 mL of DNAzol by applying as few strokes as possible. Typically, 5 to 10 strokes are required for complete homogenization. The homogenate was sedimented for 10 min at 10,000 × $g$ at 4°C. After centrifugation, the resulting viscous supernatant was transferred to a fresh tube. Deoxyribonucleic acid was precipitated from the homogenate by the addition of 0.5 mL of 100% ethanol per 1 mL of DNAzol used for the isolation. The samples were mixed by inverting tubes 5 to 8 times and storing them at room temperature for 2 to 3 min. Deoxyribonucleic acid quickly became visible as a cloudy precipitate. The tubes were stored upright for about 1 min and the liquid was removed from the bottom of the tubes. The DNA precipitate was washed twice with 1.0 mL of 70% ethanol. At each wash, the DNA was suspended in ethanol by inverting the tubes 5 times. The tubes were stored vertically for 1 min to allow the DNA to settle to the bottom of the tubes and ethanol was removed by pipetting. The DNA was dissolved (without drying) in triple-distilled water. Cloacal swabs were directly swirled to mix in 1 mL of DNAzol. The suspension was precipitated for 10 min at 10,000 × $g$ at 4°C.

**PCR.** Polymerase chain reaction was performed in a PCR machine (Biometra, Goettingen, Germany) with 4 μL of tissue DNA as a template in a 20-μL reaction volume containing 0.5 μL of each primer and 10 μL of PCR TaqMix (Meilaibo, Beijing, China). Reactions were carried out at 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 53 to 60°C (depending on the special PCR reaction) for 45 s, 72°C for 1 to 2 min (depending

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<table>
<thead>
<tr>
<th>Group</th>
<th>1 d</th>
<th>3 d</th>
<th>5 d</th>
<th>7 d</th>
<th>9 d</th>
<th>11 d</th>
<th>14 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated</td>
<td>0/10</td>
<td>5/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>2/10</td>
<td>0/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Control</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

1Detected by PCR targeting a 4b gene fragment of fowlpox virus.
2The group in the first passage.

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<table>
<thead>
<tr>
<th>Item</th>
<th>Passage number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken embryos (log$<em>{10}$ EID$</em>{50}$/mL)</td>
<td>4.52</td>
</tr>
</tbody>
</table>

1The recombinant virus was serially passaged in specific-pathogen-free chickens.
2EID$_{50}$ = median embryo infectious dose.
on the size of the target gene), and, finally, 72°C for 10 min.

**RESULTS**

**Clinical and Gross Necropsy Findings**

Daily monitoring did not show any changes in clinical behavior of all of the groups, except local reactions at the injection site in inoculated birds (Figure 1). None of the chickens showed any gross lesions.

**Tissue Distribution of Recombinant Virus**

In the control chickens, there was no detectable recombinant virus in all of the examined tissue. Detected by PCR targeting a 4b gene fragment of FPV, the recombinant virus was demonstrated in the skin at the injection site on d 1, 3, 5, 7, 9, 11, 14, and 21 postinoculation; in the liver on d 1, 5, 11, and 21 postinoculation; in the trachea on d 1, 7, 9, and 21 postinoculation; and in the brain and bursa of Fabricius on d 7 postinoculation, as shown in Table 1.

**Shedding of Recombinant Virus**

The shedding level of the vaccine strain was assessed through collection of cloacal swabs on d 1, 3, 5, 7, 9, 11, 14, and 21 postinoculation from 10 birds in inoculated and control groups, respectively. Using detection by PCR targeting a 4b gene fragment of FPV, no detectable recombinant virus was found in all of the examined samples in the control chickens. In inoculated chickens, 5 out of 10 chickens were positive on d 3 postinoculation, 2 out of 10 chickens on d 11 postinoculation, and 1 out of 10 chickens on d 9 and 21 postinoculation (Table 2).

**Hereditary Stability**

After 5 consecutive passages in chickens, no mutations were observed in the 40k gene of *M. gallisepticum*. The EID50 of recombinant virus changed slightly in the first, the third, and the fifth passage, as seen in Table 3. The results indicated that the recombinant virus had a high level of genetic stability.

**Cohabitation Trial**

To evaluate the spread of the recombinant virus, non-vaccinated chickens were commingled with inoculated chickens 24 h after inoculation. Challenged by virulent FPV after 2 wk postinoculation in the fifth passage, there was not any protection against virulent FPV in in-contact chickens based on the presence of clinical symptoms (Table 4). The results showed that the recombinant virus could not transmit to in-contact chickens from inoculated chickens.

**Environmental Detection**

Using detection by PCR test and virus isolation, no detectable recombinant virus was found in all of the examined samples (Table 5). The results further indicated that the vaccine strain was safe for application in poultry production.

<table>
<thead>
<tr>
<th>Passage number</th>
<th>Postinoculation (d)</th>
<th>Inoculated</th>
<th>In-contact</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>9/10 (90)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>9/10 (90)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

1Challenged by wing-web puncture.

Figure 2. Protection against fowlpox virus challenge: (A) control group, 7 d postchallenge; (B) inoculated group (the fifth passage), 7 d postchallenge. Color version available in the online PDF.
Protection Against FPV Challenge

In the inoculated group (chickens vaccinated in the wing web with FP-MG vaccine), 9 out of 10 (90%) chickens were protected against FPV challenge. The control group had no protection based on the presence of clinical symptoms (chickens vaccinated in the wing web with PBS) (Table 4 and Figure 2).

DISCUSSION

Fowlpox virus is the best-studied member and type species of the Avipoxvirus genus of the Poxviridae. Development of rFPV vector vaccines began in the 1980s, for use not only in poultry but also in mammals including humans (Jones et al., 1997; Boyle et al., 2004; Skin- ner et al., 2005; Pozzi et al., 2009). Several important poultry viral pathogens became early targets for rFPV vaccines, especially avian influenza virus, Newcastle disease virus, Marek’s disease virus, infectious bronchi- tis virus, and infectious bursal disease virus (Boursnell et al., 1990; Taylor et al., 1990; Bayliss et al., 1991; Heine and Boyle, 1993; Swayne et al., 2002; Wang et al., 2002; Lee et al., 2003; Senne, 2003; Davison et al., 2006; Chen, 2009; Qiao et al., 2009). Some of them have been licensed for commercial use, although there are still some problems or limitations with the use of rFPV in poultry (Skinner et al., 2005).

Mycoplasma gallisepticum is the most economically significant mycoplasmal pathogen of poultry. Infection with M. gallisepticum is associated with chronic respiratory disease, reduced feed efficiency, decreased growth, and decreased egg production in chickens (Levisohn and Kleven, 2000). Mycoplasma gallisepticum can transmit horizontally or vertically in eggs. Some vaccines including the F strain and attenuated strains ts-11 and 6/85 are developed and used to prevent egg-production losses and reduce the effect of respiratory disease in commercial layers, and they can also aid eradication or reduce egg transmission in breeding flocks (Branton et al., 1988; Whithar, 1996; Bíró et al., 2005; Viscione et al., 2009). Compared with these conventional vaccines, the rFPV vector vaccine (Vectormune FP-MG) may have more benefit and safety, in which the FPV has been genetically modified to express key protective M. gallisepticum antigens and is recommended for wing-web application to healthy, susceptible chickens 8 wk of age or older to prevent fowlpox and M. gallisepticum infections.

This study demonstrates that vaccination with Vectormune FP-MG is safe in SPF chickens. No adverse vaccine reactions, vaccine-induced mortality, or clinical signs were observed, and all of the chickens remained clinically healthy. The genetic stability of the recombinant virus was examined after 5 serial passages in SPF chickens. It was shown that the vaccine strain of Vectormune FP-MG was extremely stable in vivo. After 5 consecutive passages, not a single mutation was observed in the nucleotide sequences of the 40k gene of M. gallisepticum. Wing-web inoculation targeted the recombinant virus to the skin at the injection site, occasionally in the trachea tissue, liver, brain, and bursa.

### Table 5. Detection of the recombinant virus in the living environment of inoculated chickens

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR detection(^1)</th>
<th>Virus isolation(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 d</td>
<td>14 d</td>
</tr>
<tr>
<td>Feces</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Drinking water</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cage swabbing</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^1\)Polymerase chain reaction targeted at a 4b gene fragment of fowlpox virus.
\(^2\)Inoculated in 10-d-old embryonated specific-pathogen-free eggs by chorioallantoic membrane route.

### Table 6. Serological reaction of Vectormune FP-MG (Ceva Biomune Animal Health Co., Lenexa, KS) vaccine in specific-pathogen-free chickens

<table>
<thead>
<tr>
<th>Week postvaccination</th>
<th>Mycoplasma gallisepticum SPA(^1) (positive/tested)</th>
<th>M. gallisepticum(^2) (positive/tested)</th>
<th>Mycoplasma synoviae SPA(^1) (positive/tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Vectormune FP-MG 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>Negative controls 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>3</td>
<td>Vectormune FP-MG 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>Negative controls 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>4</td>
<td>Vectormune FP-MG 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>Negative controls 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>5</td>
<td>Vectormune FP-MG 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>Negative controls 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>6</td>
<td>Vectormune FP-MG 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>Negative controls 0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

\(^1\)Serum plate agglutination (SPA) scored from 0 to 4, positive is ≥1. For each test, the positive control serum had an agglutination, which scored at least 3.
\(^2\)Commercial M. gallisepticum antibody ELISA kit for use in chickens. Positive has a titer ≥1.076.
of Fabricius (Table 1). Virus shedding was also detected positive by PCR, but the virus survived transiently in the environment confirmed by PCR and virus isolation. All of these results indicated that the Vectormune FP-MG vaccine had a high level of safety.

To evaluate the expression of *M. gallisepticum* antigens in SPF chickens, *M. gallisepticum* serum plate agglutination test and a commercial *M. gallisepticum* ELISA kit (IDEXX, Westbrook, ME) were used to detect *M. gallisepticum* antibody (Table 6). Serum plate agglutination and the *M. gallisepticum* ELISA kit used in this study did not detect *M. gallisepticum* antibody in response to the Vectormune FP-MG vaccine, similar with some previous studies (Whithear et al., 1990; Ley et al., 1997; Noormohammadi et al., 2002). The results reveal that seroconversion is not a reliable predictor; the local antibody response and cell-mediated immunity may be more significant in protecting against *M. gallisepticum* infection.

In summary, our study indicates that vaccination with Vectormune FP-MG is safe in chickens. The vaccine might be a good option for use in chicken flocks to prevent FPV and *M. gallisepticum* infections.

REFERENCES


