Protection of Chickens against Very Virulent Marek’s Disease Virus (MDV) by an Infectious Clone of Meq-Null MDV Vaccination

Ning Cui¹, Yanpeng Li², Shuai Su³*, Zhizhong Cui¹, Jiabo Ding³, Mengjiao Kang³, Peng Sun¹ and Hongfei Zhu²

¹College of Veterinary Medicine, Shandong Agricultural University, Tai’an, Shandong, 271018, China; ²Biotechnology research institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China; ³China Institute of Veterinary Drug Control, Beijing, 100081, China

*Corresponding author: ssu6307@163.com

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ABSTRACT
To evaluate the immune-protective effect of GX0101∆meq bacterial artificial chromosome (BAC) which contains an infectious meq-null Marek’s disease virus genome, 1-day-old SPF chickens were reared separately in isolators with positive filtered air. On 1 day of age, chickens were immunized with 20 µg of GX0101∆meq BAC suspended in PBS, and challenge infection with 500 PFU very virulent rMd5 were performed at day 5 and 12 post-immunization separately. During 90 days after challenge, all chickens were recorded and checked for necropsy. The protective index of the two vaccines used was 80 and 40 for CVI988/Rispens and GX0101∆meq BAC, respectively, after challenged with the very virulent (vv) virus rMd5 at day 5 post-immunization. When challenged with rMd5 at day 12 post-immunization, the protection index of GX0101∆meq BAC increased to 67%. Except that GX0101∆meq BAC can confer protection against vv MDV, a delay in the development of MD could be observed in some chickens vaccinated with GX0101∆meq BAC. On the other hand, compared with CVI988/Rispens, the rescue of GX0101∆meq BAC in the body is a prerequisite for access to protection. Therefore, there is a blank period after immunization, which provides a chance for infection with the wild MDV.

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INTRODUCTION
Marek’s disease virus (MDV), an alphaherpesvirus, causes Marek’s disease (MD), which is a lymphoproliferative disease of chickens resulting in T cell lymphomas and immunosuppression (Calnek, 2001). MDV is transmitted by air, causing high mortality in unvaccinated susceptible chickens (Gimeno et al., 1999; Zhang et al., 2015). Vaccine is the main way to prevent and control MD. Commercial MD vaccine at present include attenuated type I CVI988/Rispens strain, avirulent type II SBI strain and type III herpesvirus of turkey (HVT) FC126 strain, of those CVI988/Rispens is most effective and widely used (Islam et al., 2013; Lupiani et al., 2013; Walkden Brown et al., 2013; Liu et al., 2015).

Traditional MDV vaccine can control MD effectively, but there existed many inconveniences. MDV is cell-dependent virus, and CEF cells must be used in production of the vaccine, which have to be stored and transported in liquid nitrogen to keep ultralow temperature (Lupiani et al., 2013), thus making high production cost and inconvenience of storage, transportation and use. What is worrying most is the possible pollution of virus and bacteria in SPF embryos (Wei et al., 2012). HVT vaccine can be lyophilized preserved while its bad protection efficacy and character of ease to be affected by material antibodies have limited its widespread use. What is more serious is that the virulent of MDV is continuously growing, and the present MDV vaccine could not provide adequate immune protective efficacy against super virulent MDV (Witter, 1997). As the acceleration development of molecular biology of MDV, especially in the aspect of gene function research (Jarosinski et al., 2005; Brown et al., 2006), gene recombination technology has provided a new way to obtain effective vaccine, in which construction of MDV
pathogenic gene deletion strain and DNA vaccine provided good perspective to get more effective vaccine as much as possible (Tischer et al., 2002; Lee et al., 2008, 2010, 2012).

MDV Chinese strain GX0101, isolated in 2001, is the first reported recombinant MDV field strain with one reticuloendotheliosis virus (REV) long terminal repeat (LTR) insert (Zhang et al., 2005; Su et al., 2013). We constructed an infectious BAC clone of GX0101 (Sun et al., 2009, 2010), and knocked off meq gene in GX0101 genome using recombination technology to construct a meq deletion strain, GX0101Δmeq (Li et al., 2011). GX0101Δmeq is able to replicate in cell cultures stably, and induce better protective immunity against vv MDV challenge than commercial vaccine CVI988/Rispens without any pathogenicity and oncogenicity in chickens (Su et al., 2010; Li et al., 2011). In the present study, SPF chickens were vaccinated with DNA of infectious clone GX0101Δmeq with meq gene deleted. It could provide certain immune protection against vv MDV rMd5 and offer a new perspective to develop MDV DNA vaccines.

MATERIALS AND METHODS

Chickens and cells: Specific-pathogen-free (SPF) chickens and chicken embryos for preparation of chicken embryo fibroblast (CEF) cultures were purchased from SPAFAS Co. (Jinan, China; a joint venture with Charles River Laboratory, Wilmington, MA, USA). They were free of avian leukosis virus (ALV), reticuloendotheliosis virus (REV) and chicken infectious anemia virus (CAV).

Viruses and plasmids: Recombinant Md5 virus (rMd5) was generated from cosmids derived from the very virulent (vv) Md5 strain as previously described (Reddy et al., 2002). Infectious clone GX0101Δmeq with meq gene deleted and pDS-pHAI-US2 containing BAC backbone as immune control were preserved in our lab (Sun et al., 2009; Li et al., 2011). CVI988/Rispens were commercial vaccine.

Vaccination experiments: GX0101Δmeq BAC DNA was isolated from E. coli strain DH10B using commercially available kits (Qiagen) according to the standard protocols (Sambrook and Russell, 2001). Seventy five 1-day-old SPF chickens were randomly divided into five equal groups (15 in each group) and reared separately in isolators with positive filtered air. At day 1, each chicken in group 1 was immunized by intramuscular (i.m.) injection with 20ug of GX0101Δmeq DNA, diluted in 200ul of phosphate buffer saline (PBS) (PH7.2). Two thousands PFU commercial vaccine CVI988/Rispens were intra-abdominally (i.a.) into each chicken in group 2. Chickens in group 3 were immunized by i.m. injection with 20ug of plasmid pDS-pHAI-US2 in PBS (200ul), as an immune control group. Chickens in groups 4 and 5 were immunized by i.m. injection with 200ul PBS, as control groups. Five days later, chickens in group 1, 2, 3 and 4 were challenged i.a. with 500 PFU of vv MDV rMd5. During 90 days after challenges, all dead chickens were recorded and necropsied. The tumor-suspected tissues were examined by histo-sections. At the end, all survived chickens were killed and necropsied. Vaccinal immunity to MD was expressed as a protective index (PI) calculated as the percentage of gross MD in non-vaccinated challenged control chickens minus the percentage of gross MD in vaccinated, challenged chickens divided by the percentage of gross MD in non-vaccinated challenged control chickens × 100.

REFERENCES:

Statistics analysis: Statistical analysis was performed with the SPSS statistical software package for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were examined for statistical significance by a two-tailed Student T-test. P<0.05 were considered statistically significant.

RESULTS

GX0101Δmeq virus was reconstituted in vivo from GX0101Δmeq BAC DNA: At 1 day of age, chickens were immunized by i.m. injection with GX0101Δmeq DNA. Four days later, six chickens were randomly selected for sterile acquisition anticoagulant for virus isolation and MDV were isolated from four chickens. To confirm the virus, MDV special plaques were examined by immunofluorescence assay (IFA) with monoclonal antibody (mAb) H19 specific for the MDV-unique protein pp38 or mouse anti-Meq polyclonal serum (Cui et al., 1991). The virus that expressed pp38 but not Meq was GX0101Δmeq (Fig. 1). The results were further verified by PCR.

Clinical symptoms and autopsy of chickens infected with rMd5 after immunized with GX0101Δmeq DNA: Chickens were challenged with rMd5 5 days post immunization. In the whole trial, seven chickens died in GX0101Δmeq DNA vaccine group with two chickens developed typical MDV tumor nodules; three chickens died in CVI988/Rispens vaccine group with one chicken developed typical MDV tumor nodules; all chickens were died in pDS-pHAI-US2 or PBS vaccine group with four and five chickens developed typical MDV tumor nodules respectively; no chickens died in negative control group. When chickens were challenged with rMd5 12 days post immunization, during the whole trial: three chickens died in GX0101Δmeq DNA vaccine group with two chickens developed typical MDV tumor nodules; one chicken died in CVI988/Rispens vaccine group with typical MDV tumor nodules; fourteen or thirteen chickens died in pDS-pHAI-US2 or PBS vaccine group with four and five chickens developed typical MDV tumor nodules respectively; no chickens died in negative control group (Fig. 2, Table 1).

Protective efficacy of GX0101Δmeq DNA vaccination against rMd5 in SPF chickens: To evaluate immune protection efficacy of GX0101Δmeq as a vaccine for SPF chickens, vv MDV rMd5 were challenged at different time after immunized with GX0101Δmeq DNA. In the whole trial, challenged control group and pDS-pHAI-US2 plasmid immunization group all showed 100% MDV-
DNA in different immune forms (dissolved in PBS, researches also compared the protection effect of plasmid of CVI988/Rispens (Tischer et al., 2000). Tischer and Petherbridge showed that infectious virus was rescued and played its role after chickens were immunized with MDV BAC plasmid, which indicated that MDV BAC DNA induce immune protection efficacy as DNA vaccine in virus form in essence.

Therefore, the most important factor that influenced the immune protection effect of MDV BAC DNA as DNA vaccine was the construction of MDV infectious clone. Currently, except several gene deletion strains, there was no better vaccine strain than CVI988/Rispens (Witter et al., 2004). Meq gene deletion strain GX0101∆meq constructed in our lab lost its pathogenicity completely in SPF chickens and could induce better immune protection efficacy than CVI988/Rispens (Su et al., 2010; Li et al., 2011). Therefore, we speculated that GX0101∆meq BAC DNA could provide better protection effect than pCVI988. Compared with Petherbridge’s experiments, the dose and virulent of the virus used for challenge were different, but both of us have used CVI988/Rispens as control. In Petherbridge’s experiments, CVI988/Rispens could provide 100% protection rate, while in the present study, it was only 87%, which demonstrated that the virus we used were more virulent. But even so, the protection efficacy of GX0101∆meq BAC against MDV challenged on 5 days post immunization was superior to that of pCVI988, which was challenged on seven days post immunization. The results have confirmed our above inference.

Evaluation of immune protection efficacy against challenge on 12 days post immunization was also carried out. The results demonstrated that the immune protection efficacy against challenge on 12 days post GX0101∆meq BAC immunization were obviously better than challenge on 5 days post immunization. It is mainly because that chicken of six days old was more susceptible to MDV. On the other hand, it takes some time for GX0101∆meq to rescue enough viruses to exert its immune efficacy in vivo. This showed that compared with CVI988/Rispens; there will be a certain immune phase space for GX0101∆meq BAC as a vaccine.

The study found that the protection efficacy of GX0101∆meq-BAC was no better than cell-dependent MDV vaccine CVI988/Rispens. On one hand, it needs some time to rescue enough viruses in vivo, and on the other hand, it has a great relationship with the dose of DNA in-taken by body cells. Therefore, further improvement and optimization of immunization dose and pathways for DNA vaccine are necessary. Although the protection efficacy of GX0101∆meq BAC as a DNA vaccine may not be ideal under laboratory conditions, it might be better under natural infection cases in consideration of the longtime of natural infection and the lower dose than challenge in experiment. But such specific death and pathological symptom; CVI988/ Rispens vaccine group showed 20% and 13% MDV-specific death and pathological symptom respectively in the trial challenged on 5 and 12 days post immunization; GX0101∆meq DNA vaccine group showed 60% and 33% MDV-specific death and pathological symptom respectively in the trial challenged on 5 and 12 days post immunization; Therefore, based on MD incidence, the protection index of GX0101∆meq DNA was 40% and 67% in the trial challenged on 5 days and on 12 days post immunization while the protection index of CVI988/Rispens was 80 and 87%, respectively (Table 2).

**DISCUSSION**

MDV is a double stranded DNA virus, and it can be rescued by transfecting cells with BAC clone containing MDV genome (Schumacher et al., 2000). Tischer and others evaluated the immune protective efficacy of attenuated MDV strain 584A p80C infectious clone BAC20 as a vaccine. The results showed that BAC20 can provide certain immune protection as a vaccine even though its immune efficacy (PI: 42%) was lower than that of CVI988/Rispens (Tischer et al., 2002). Their researches also compared the protection effect of plasmid DNA in different immune form (dissolved in PBS, calcium phosphate precipitation, chitosan package, in DH10B E. coli and use gene gun, etc) in immunized chickens. The immune effect of plasmid DNA dissolved in PBS was best, and it may mainly because plasmid was in parcel or precipitation state in other forms, which blocked virus being rescued. Petherbridge demonstrated that the immune protection efficacy of CVI988/Rispens infectious clone pCVI988 as a DNA vaccine in 2003 (Petherbridge et al., 2003). Results from Tischer and Petherbridge showed that infectious virus was rescued and played its role after chickens were immunized with MDV BAC plasmid, which indicated that MDV BAC DNA induce immune protection efficacy as DNA vaccine in virus form in essence.

| Table 1: Preventive effect of GX0101∆meq BAC DNA vaccination on tumorigenesis induced by vv MDV |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Vaccines | Challenge with rMD5 | Challenge with rMD5 | Died | Tumors | Died | Tumors |
|-------------------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| GX0101∆meq | rMD5 | 7 (47) | 2 (13) | 3 (20) | 2 (13) |
| CVI988/Rispens | rMD5 | 3 (20) | 1 (7) | 1 (7) | 1 (7) |
| pDS-pHAI-US2 | rMD5 | 15 (100) | 4 (27) | 14 (93) | 4 (27) |
| PBS | rMD5 | 15 (100) | 5 (33) | 13 (87) | 4 (27) |
| Control | - | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

Samples tested in each case were 15. Values in parenthesis indicate percentage.

**Fig. 1:** Immunofluorescence analysis of the recombinant virus. The mAb H19 specific for the MDV-unique protein pp38, and mouse serum against Meq were used for IFA. Parental virus, GX0101 expressed Meq protein, whereas the deletion mutant virus GX0101∆Meq did not. The recombinated virus GX0101∆Meq was confirmed by staining of MDV-specific pp38 protein. (A) GX0101 with anti-pp38 mAb; (B) recombinated virus with anti-pp38 mAb; (C) GX0101 with anti-Meq serum; (D) recombinated virus with anti-Meq serum.
assumption relies on simulation of natural infection under laboratory conditions. The study also showed that disease time of chickens in GX0101Δmeq BAC immunization group was significantly delayed compared with challenge control group and pDS-pHAI-US2 plasmid immunization control group. Compared with traditional MD vaccine, BAC DNA is cost saving as it can be prepared in a large-scale by E. coli; it prevents any exogenous pathogen contamination in CEF cells used for production of MD vaccine; the produced vaccine can be stored under 4ºC or -20, which is convenient to store and transport, showing protective efficacy in vivo, but also provide good immune protection efficacy in vivo.

Conclusions: GX0101Δmeq BAC could not only delay disease time of chickens when infected with very virulent strain, but also provide dual protective efficacy as DNA vaccine. The rescue of GX0101Δmeq BAC virus in vivo is a prerequisite for access to protection.

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REFERENCES


Fig. 2: Survival curves after rMD5-challenge in chickens of each group. (A) Each chicken was immunized at 1 day of age and maintained in isolation for 13 weeks. Non-immunization group served as the negative control. Chickens were challenged with 500 PFU of rMD5 strain at day 6. The mortalities of different groups were recorded weekly. Dead chickens during the experiment were evaluated for MDV-specific gross lesions. (B) Each group was significantly delayed compared with challenge control group or pDS-pHAI-US2 plasmid immunization control group. Compared with traditional MD vaccine, BAC DNA can provide a new perspective and attempt for the current and future prevention of MDV (Suter et al., 1999).

Table 2: Preventive effect of GX0101Δmeq BAC vaccination against vv MDV challenge

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Challenge with rMD5 at day 6</th>
<th>Challenge with rMD5 at day 13</th>
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<tbody>
<tr>
<td></td>
<td>Mortality (%)</td>
<td>MD lesions (%)</td>
</tr>
<tr>
<td>GX0101Δmeq</td>
<td>rMD5</td>
<td>7 (47)</td>
</tr>
<tr>
<td>CVI988/Rispens</td>
<td>rMD5</td>
<td>3 (20)</td>
</tr>
<tr>
<td>pDS-pHAI-US2</td>
<td>rMD5</td>
<td>15 (100)</td>
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<tr>
<td>PBS</td>
<td>rMD5</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Control</td>
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One-day-old SPF chickens (n=15 in each case) were vaccinated with the GX0101Δmeq BAC or CVI988/Rispens vaccine and challenged 5 or 12 days later with rMD5 strain. Mortality was observed for 13 weeks after chickens challenged with rMD5 strain, and both dead and survival chickens necropsies were subjected to examinations. PI=protection index. α indicates significant difference (P<0.05) in PI among the two experimental groups.


