A novel M2e multiple antigenic peptide providing heterologous protection on mice

Feng Wen†, Ji-Hong Ma†, Hai Yu*, Fu-Ru Yang, Meng Huang, Yan-Jun Zhou, Ze-Jun Li, Xiu-Hui Wang, Guo-Xin Li, Yi-Feng Jiang, Wu Tong, Guang-Zhi Tong*

Division of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China

*Corresponding authors: Tel: +86-21-34293436; Fax: +86-21-54081818; E-mail: gztong@shvri.ac.cn (Tong GZ), haiyu@shvri.ac.cn (Yu H)

†The first two authors contributed equally to this work.
Swine influenza viruses (SwIVs) cause considerable morbidity and mortality in domestic pigs, resulting in a significant economic burden. Moreover, the pigs have been considered to be a possible “mixing vessel” in which novel strains looms. Here, we developed and evaluated a novel M2e multiple antigenic peptide (M2e-MAP) as a supplemental antigen for inactivated H3N2 vaccine to provide cross-protection against two main subtypes, H1N1 and H3N2, of SwIVs. The novel tetra-branched MAP was constructed by fusing four copies of M2e to one copy of foreign T helper (Th) cell epitopes. A high-yield reassortant H3N2 virus was generated by plasmid based reverse genetics approach. The efficacy of the novel H3N2 inactivated vaccines with or without M2e-MAP supplementation was evaluated in a mouse model. M2e-MAP conjugated vaccine induced strong antibody responses in mice. Complete protection against heterologous swine H1N1 virus was observed in mice vaccinated with M2e-MAP combined vaccine. Moreover, this novel peptide confers protection against lethal challenge of A/Puerto Rico/8/34(PR8) (H1N1). Taken together, our results suggest the combined immunization of reassortant inactivated H3N2 vaccine and the novel M2e-MAP provided cross-protection against swine and human viruses and may serve as a promising approach for influenza vaccine development.

**Keywords:** H3N2, M2e-MAP, high-yield, inactivated vaccine, swine influenza virus
Introduction

Currently, three subtypes (H1N1, H3N2 and H1N2) of swine influenza virus (SwIVs) are circulating in swine populations throughout the world. The 1968 Hong Kong pandemic was caused by the outbreak of a H3N2 strain, which has been transmitted to domestic pigs and has undergone frequent reassortment [18]. However, H3N2 SwIV infection was reported sporadically in swine population until a swine-adapted reassortant virus emerged in 1998, which rapidly spread through the US swine population and caused severe respiratory and reproductive diseases [20]. Most recently, a novel reassortant H3N2 SwIV variant with the M gene from the 2009 pandemic virus were isolated in humans in multiple U.S. States [1].

Currently, influenza vaccine strains for humans are selected annually based on global epidemiological surveillance data. However, since there is no comprehensive surveillance on SwIVs, the demand to develop universal vaccines with broad cross-reactivity against SwIVs is an important and urgent issue.

The extracellular domain of influenza M2 protein (M2e) is highly conserved among human influenza A viruses and considered as an appropriate target for the development of influenza vaccine with broad-spectrum protection [4]. Vaccination with M2e could protect animals against different subtypes of influenza viruses, including avian H5N1 influenza virus [19]. The protection was mediated by M2e-specific antibodies via antibody dependent cytotoxicity [10]. In addition, M2e monoclonal antibody could reduce plaque size of influenza A virus and inhibit the growth of virus in vitro [8, 17, 24]. However, the human body could not induce high levels of antibody against M2e due to the poor immunogenicity of M2e. Therefore, developing the conjugate vaccine that could increase the production of antibody level against M2e in swine population is a promising way to develop M2e based universal influenza vaccine.

As the surface protein hemagglutinin (HA) and neuraminidase (NA) are the major antigens of
influenza virus and the production of antibodies against HA is crucial for immune protection [5], we selected the HA and NA of a prevailing H3N2 isolate and six internal genes from PR8 to generate a high-growth reassortant H3N2 influenza seed virus (rgH3N2) [21]. We designed a novel tetra-branched MAP based vaccine, which were constructed by fusing four copies of M2e to one copy of foreign T helper (Th) cell epitope. In this study, mice were vaccinated with rgH3N2 inactivated vaccine combined with the novel M2e-MAPs to determine whether supplementation of M2e-MAP could improve the immune response and provide cross-protection against swine and human viruses.

Materials and Methods

Mice

Six-week-old female BALB/c mice were purchased from SLAC laboratory animal Co. Ltd. (Shanghai, China) and housed in specific pathogen-free (SPF) facility following the approved animal care protocols. The mice were acclimated to their new environment for one week prior to immunization. All experimental protocols involving mice were approved by the Chinese Ministry of Agriculture and the Review Board of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (permit number SYXK 2011–0116).

Viruses and peptide

A/Swine/Guangdong/164/06 (H3N2, SwGD164), A/Swine/Heilongjiang/1/05 (H3N2, SwHLJ1) and A/Swine/Guangdong/96/06 (H1N1, SwGD96) were isolated from pig farms in China [23]. A/Puerto Rico/8/34 (H1N1, PR8) is a mouse-adapted virus. All viruses were grown for 48 hours at 37°C in the allantoic cavities of 10-day-old SPF chicken embryo eggs. The novel tetra-branched M2e-MAP was constructed by fusing four copies of a universal sequence, which is an extracellular part of the M2 protein, with one copy of promiscuous T helper (Th) cell epitope by a lysine tree with two additional Lys residues defining a putative
cleavage site for cathepsin D [13]. It was synthesized by Genscript Co., Ltd (China). M2e-MAP was prepared at 10 mg/ml in distilled water and stored at -20°C. The construction and sequence of M2e-MAP is shown in Figure 1.

**Generation of a high growth reassortant virus (rgH3N2)**

The rgH3N2 harboring the HA and NA genes from SwGD164 and six internal genes from PR8 was generated by plasmid-based reverse genetics as previously described [21]. Briefly, one microgram of each plasmid was added into 250 µL Opti-MEM (Invitrogen, USA) and vortexed. Sixteen microliters of the transfection reagent Lipofectamine 2000 (Invitrogen) were then added into 250 µL Opti-MEM and mixed gently. Five minutes later, the diluted transfection reagent was mixed with the diluted plasmids. The DNA-transfection reagent mixture was kept at room temperature for 20 min and then added directly to a monolayer of 293T cells in a 6-well plate (Costar; Corning, USA). After 6 h of incubation at 37°C in 5% CO2, the medium was replaced with 2 mL fresh Opti-MEM, and the plate was then incubated as described above for 48 h. The supernatant was subsequently inoculated into the allantoic cavity of 10-day-old embryonated SPF eggs. The allantoic fluid was harvested after 48 h of incubation at 37°C, after which the virus was identified by hemagglutination assay. Reverse transcription PCR (RT-PCR) and re-sequencing confirmed that the genome of the rescued virus was identical in sequence to the cDNA in the plasmids used for its rescue. The rescued viruses were then confirmed by sequencing. The titer of the rgH3N2 virus was determined in 10-day-old SPF embryonated chicken eggs, and the 50% Embryo Infective Dose (EID$_{50}$) was calculated by the Reed-Muench method.

**Vaccine preparation**

The rgH3N2 virus was propagated in the allantoic cavity of 10-day old embryonated SPF chicken eggs and inactivated with 0.1 % (v/v) formalin for 48 h at 4°C. The virus was then purified by sucrose density gradient centrifugation [12].
The novel M2e-MAP was synthesized and stored at -20 °C. One dose of M2e-MAP combined vaccine containing 10 μg of each inactivated rgH3N2 and M2e-MAP (in 50μl sterile PBS) was mixed and emulsified with the same volume of Freund's incomplete adjuvant (FIA, sigma) or Freund's complete adjuvant (FCA, sigma).

**Immunization and virus challenge**

A total of 120 female BALB/c mice were randomly divided into 4 groups (n=30 in each group) and were immunized subcutaneously (s. c.) with immunogen plus FCA and boosted with the same amount of immunogen in FIA at 2-week intervals. Mice in group 1 (M2e-MAP+rgH3N2+Freund) received one dose of M2e-MAP combined vaccine. Mice in group 2 (rgH3N2+Freund) were immunized with one dose of the vaccine as prepared above. Mice in group 3 (rgH3N2) and group 4 (Freund) were immunized with inactivated virus and Freund alone as controls, respectively. The immunization schedule of mice in the experiment is shown in Table 1. Serum samples from 5 mice of each group were collected at 0, 1, 2, 3 and 4 weeks post the first immunization. Mice (n=10 for each virus) were anesthetized and challenged intranasally (i.n.) with $10^6$ EID$_{50}$ of A/Swine/Guangdong/96/06 and A/Swine/Heilongjiang/1/05 as well as $10$ LD$_{50}$ of A/Puerto Rico/8/34 on day 14 post the booster. The loss of body weight and symptom were monitored daily and the survival curves were analyzed by Kaplan-Meier methods.

**Antibody detection**

Enzyme-Linked Immunosorbent Assay (ELISA) was applied to determine titers of antibody specific to rgH3N2 and M2e-MAP in serum samples collected on days 7, 14, 21 and 28 post primary immunization as described previously [22]. Hemagglutination inhibition (HI) assay was performed according to the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf).
Neutralization test (NT) was performed according to Kida et al. [11].

**Virus titers in lung tissues**

Lung tissues from euthanized mice were removed on 3 days post infection (dpi) and homogenized in 500μl of ice cold PBS. Virus titers in mice lungs were determined by EID$_{50}$ titration and quantitative real-time PCR method as described [21]. The specific primers and labeled fluorescent-probe were as follows: InfA-F: 5-GACCGATCCTGTCACCTCTGAC-3; InfA-R: 5-AGGGCATTCTGGACAAAGCGTCTA-3; TaqMan probe:5-FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ-3.

**Histopathological analysis**

The lung tissues of challenged mice were collected on day 4 and fixed in 10% neutral buffered formalin, and histopathological changes of the mouse lungs were examined by hematoxylin-eosin (HE) stain and observed under light microscope.

**Statistical analysis**

Data was analyzed and charted with Excel and Graphpad Prism 5. The survival curves were analyzed by Kaplan-Meier methods, and the significance of differences in overall survival between groups was determined by log-rank test. Statistical significance was analyzed with two-way ANOVA.

**Results**

**Characterization of the reassortant virus rgH3N2**

The rgH3N2 virus harboring the HA and NA genes from SwGD164 and six internal genes from PR8 was generated by plasmid-based reverse genetics and confirmed by sequencing analysis. The HA titer of rgH3N2 reached 512 HAU/50μl, 2 folds higher than SwGD164. The rgH3N2 virus ($10^{6.7}$EID$_{50}$/ml) grows to a higher titer than SwGD164 in embryonated chicken eggs.
M2e-MAP combined vaccine induced strong antibody responses

M2e-MAP combined vaccine induced strong M2e-specific and rgH3N2-specific antibody responses in mice (Fig 2). The titer of M2e-specific antibody reached 1:10³ at 7 days post primary immunization and increased to the highest titer over 1:10⁵ at 14 days post the boost. In contrast, only background level of antibody was detected in mice vaccinated with Freund’s alone (Fig 2). Both the antibody titers to rgH3N2 and M2e-MAP were increased after the boost. Antibody titer of rgH3N2 in group 3 was lower than the one emulsified with Freund (Group 1 and 2) at each sampling point.

The HI and NT tests were performed on the serum collected on 2 and 4 weeks post the primary immunization. As shown in Table 2, HI antibodies and neutralizing antibodies were detected in all groups except for the control group. The HI and NT titers were almost equal in groups 1 and 2, where the average titer of HI and NT were found to be 256 and 10³ after the first immunization, respectively. The mean titers of HI and neutralizing antibodies were up to 1152 and 10⁴ until viral infection, respectively. In group 3, the average titer of HI antibodies was 160 and the one of neutralizing antibody was 10² after first immunization, and until viral infection, mean titers of HI and neutralizing antibody were up to 512 and 10³, respectively. The above results indicated that combined immunization with M2e-MAP combined H3N2 inactivated vaccine induced high level of humoral responses in mice.

M2e-MAP vaccination limits viral replication

Mice lungs were collected on 3 dpi and the virus titers were determined by EID₅₀ titration and also by quantitative real-time PCR method. No detectable influenza virus was present in the lungs of mice challenged with SwHLJ1 (H3N2) or SwGD96 (H1N1) (<1.0 log₁₀ EID₅₀/ml), and the titer decreased to 10³.25±0.35 EID₅₀/ml in PR8 challenged mice (Table 2). The addition of M2e-MAP significantly increased the ability of the vaccine to reduce heterologous virus replication in group 1. Meanwhile, real-time quantitative PCR results indicated M2e-MAP
combined vaccine provides strong protection against PR8 (H1N1), SwGD96 (H1N1) and SwHLJ1 (H3N2) with significant differences of viral titers among four groups ($P < 0.001$).

**M2e-MAP vaccination reduces lung lesions**

Mice lungs were collected for histopathologic examination at 3 dpi. In groups 2, 3 and 4, moderate to severe broncho-interstitial pneumonia was observed (Fig. 4). In groups of mice challenged with PR8, lung tissues were damaged and inflammatory cell infiltration was observed in large areas in the control group (Fig.4j). However, the lung tissues of mice that were challenged with PR8 in group 1 appeared relatively normal aside from slight alveolar dilatation (Fig.4a). No histopathologic changes were observed in the group 1 mice challenged with SwGD96 (H1N1) (Fig.4b) or SwHLJ1 (H3N2) (Fig.4c).

**Body weight and survival curve**

The body weight and survival rate of the mice that received lethal challenge (10LD$_{50}$) of PR8 were monitored for 14 days post challenge (Fig.5). Survival rates of mice in groups 1, 2 and 3 were 100 %, 40 % and 30 %, respectively ($P = 0.0002$) (Fig. 5A). All mice in group 4 died on day 9. As shown in Fig 5B, the body weight of mice in group 1 descended slightly at first and increased on day 10. Symptoms of lethargy loss of appetite, fever and shiver were observed in groups 2, 3 and 4 at 2 dpi.

**Discussion**

Influenza A viruses infect a broad range of species, with avian and swine strains presenting the greatest potential for interspecies transmission. Since the 1990s, H3N2 triple-reassortant viruses with genes derived from human (HA, NA, and PB1), swine (N, M, and NS), and avian (PB2 and PA) influenza viruses have caused outbreaks of respiratory disease in pigs throughout North America [15, 26]. Yu et al. summarized and reported for the first time the co-existence of wholly human-like H3N2 viruses, double-reassortant H3N2 viruses, and
triple-reassortant H3N2 viruses in pigs in China by analyzing the homology among eight
genes of swine influenza A (H3N2) viruses found in China from 1970 to 2006 [23]. The HA
and NA genes in SwGD164 that used in this study showed high homology with the prevailing
H3N2 SwIV, but its fecundity in chicken eggs was low. In order to improve the replication
ability of SwGD164 in chicken eggs, we generated a high-growth rgH3N2 virus that contains
the HA and NA from SwGD164 and 6 internal genes from the PR8 virus by plasmid based
reverse genetics. The HA titer of rgH3N2 was significantly improved compare to SwGD164.
It has been reported that cross-protective immunity might serve an important role in
protection against emerging influenza pandemics [6]. The specific conserved epitopes of
cytotoxic T lymphocytes play a role in heterologous protection [14]. The vaccination of
seasonal influenza vaccine (H3N2/H1N1) provided cross protection to heterologous virus
(H5N1) and that CD4T cells were involved [7]. The antibody to conserved nucleoprotein can
also provide heterologous protection [2]. The highly conserved M2 protein of influenza virus
is an ideal target of universal vaccine with cross protection; however, influenza virus infection
or immunization cannot induce specific antibody to M2 protein [10, 19], which could not be
largely synthesized in infected cells and some were packaged into nucleocapsid. It has been
reported that anti-M2e IgG can eliminate infected cells by antibody-dependent cell mediated
cytotoxicity (ADCC) or by complement-mediated phagocytosis, in which the natural killer
cells, alveolar macrophages and dendritic cells play an important role[3, 9]. Thus, M2e-based
vaccines were developed and have reported to provide broad cross protection against
divergent or heterologous influenza viruses [13, 16, 25].

In the present study, we developed a novel tetra-branched M2e-MAP and combined it with a
high rgH3N2 inactivated vaccine. The mice that received the M2e-MAP combined vaccine
were then infected with 10^6 EID_{50} of SwGD96 and SwHLJ1 and 10LD_{50} of PR8 through nasal
inoculation. High titer of HI and neutralizing antibody induced by the vaccine provided
effective suppression of replication of homologous SwHLJ1 virus (H3N2) and pathological changes in lung, with protection rate of 100%. In addition, reassortant H3N2 vaccine also provided certain cross protection, determined by the internal gene, against heterologous virus PR8 (H1N1) (40%). These results suggested the addition of M2e-MAP did not affect the generation of rgH3N2 specific HI and neutralizing antibodies. Meanwhile, vaccination of M2e-MAP combined vaccine reduced virus replication and pathological changes in the lungs, which further indicated the M2e-MAP may improve immune protection of vaccine against heterologous virus effectively. High titer of HI and neutralizing antibody induced by rgH3N2+M2e can provide effective suppression to the replication of homologous SwHLJ1 virus (H3N2) and pathological changes in lung, with protection rate of 100%. In addition, reassortant H3N2 vaccine also produced some cross protection, determined by internal gene, against heterologous virus PR8 (H1N1) (40%).

Taken together, the approach of developing M2e-MAP combined vaccines with cross-protection is feasible. Our study provided solid evidence that M2e-MAP can significantly enhance the protective ability of traditional inactivated vaccine against heterologous viruses. The M2e-MAP combined high growth reassortant H3N2 inactivated vaccine that developed in this study could provide cross-strain protection and thus to be served as a candidate for SI vaccine in China.

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Conflict of Interest

There is no conflict of interest.
**References**


17. Roberts PC, Lamb RA, and Compans RW. The M1 and M2 proteins of influenza A


Fig. 1. Structure and sequence of synthetic M2e-MAP.

Fig. 2. Antibodies response induced by M2e-MAP combined vaccine. M2e-specific antibody and rgH3N2-specific antibody titers were determined by end-point ELISA. Mice were vaccinated with M2e-MAP+rgH3N2+Freund、rgH3N2+Freund、rgH3N2 (s.c.) respectively.
Mice receiving Freund were used as negative controls. Sera were collected at 1, 2, 3, 4 weeks post first immunization. The titers were expressed as the highest serum dilution which is greater than twice average absorbance value at OD450 nm of pre-vaccination sera. The data are expressed as geometric mean titer (GMT) ± standard deviation (SD) of 5 mice per group. The lower limit detection (1:20 in A and 1:50 in B) are indicated by a dotted line. Experiments were repeated three times.

Fig. 3. Viral amounts in lungs on 3 dpi. Five mice of every challenge group were euthanized on 3 dpi and the viral amounts of lungs were determined by real time PCR. The values were expressed as the mean log10 viral copies/μL ± S.D of 5 mice per challenge group. *** means in M2e-MAP+rgH3N2+Freund immunized and PR8 challenged group \( P < 0.001 \) compared to the other 4 immunized groups; ** means in M2e-MAP+rgH3N2+Freund immunized and SwGD96 challenged group \( P < 0.001 \) compared to the rest 4 groups; * indicates in M2e-MAP+rgH3N2+Freund immunized and SwHLJ1 challenged group \( P < 0.001 \) compared to the other 4 groups.
Fig. 4. Histopathological changes in the lungs of virus challenged mice. Immunized mice were challenged by PR8(a,d,g,j), SwGD96(b,e,h,k) and SwHLJ1(c,f,i,l) and lungs were collected for histopathological analysis on 3 dpi (HE stain; bar = 50 μm). The figure indicates the representative images of histopathological observations of M2e-MAP+rgH3N2+F, rgH3N2+F, rgH3N2 and Freund, respectively.

Fig. 5. Survival and body weight curve in PR8 challenged mice. Mice were challenged with 10LD50 of PR8 virus intranasally and monitored daily for 2 weeks post challenge. A. Survival rate. The difference of the four group is very significant (P = 0.0002). B. Percentage (%) of mouse body weight. Each point represents mean of 5 mice per group.
Table 1. Immunization schedule of mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mice number</th>
<th>Dose (μL)</th>
<th>Immun e time</th>
<th>Virus challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)M2e-MAP+rgH3N2+Freund</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>PR8/SwGD96/SwHLJ1</td>
</tr>
<tr>
<td>(2)rgH3N2+Freund</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>PR8/SwGD96/SwHLJ1</td>
</tr>
<tr>
<td>(3)rgH3N2</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>PR8/SwGD96/SwHLJ1</td>
</tr>
<tr>
<td>(4) Freund</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>PR8/SwGD96/SwHLJ1</td>
</tr>
</tbody>
</table>

Table 2. Results of HI, NT and virus titer of lungs on 3 dpi

<table>
<thead>
<tr>
<th>Immunogena</th>
<th>HI antibody titerb</th>
<th>Neutralization antibody titerc</th>
<th>Protection against challenge and virus titerd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 1</td>
<td>Dose 2</td>
<td>Dose 1</td>
<td>Dose 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2e-MAP+rgH3N2+Freund</td>
<td>224</td>
<td>1152</td>
<td>2.58±0.34</td>
</tr>
<tr>
<td>rgH3N2+Freund</td>
<td>256</td>
<td>1024</td>
<td>2.8±0.19</td>
</tr>
<tr>
<td>rgH3N2</td>
<td>160</td>
<td>512</td>
<td>1.96±0.23</td>
</tr>
<tr>
<td>Freund</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a. Groups of mice were injected s.c. with 2 doses of the M2e-MAP+rgH3N2+Freund, rgH3N2+Freund, rgH3N2 and Freund, respectively.

b. Serum samples were collected at two time points: 2 two weeks after dose 1 and 2 weeks after dose 2. The mean values of influenza-specific IgG antibody titers in serum were calculated and represented means±S.D.

c. Neutralization antibody titer of serum collected at the same time as HI titers were calculated and represented as log10 means±S.D.

d. Mice were challenged intranasally 2 weeks post 1st boost with 10⁶EID₅₀ of SwGD96, SwHLJ1 and 10LD₅₀ of PR8 viruses. Virus titer of lungs was determined on 3 dpi. The values were calculated by the method of Reed-Muench and expressed as the mean log₁₀EID₅₀/ml±S.D. The lower limit of detection of virus was 1.0 log₁₀EID₅₀/ml.