Cloning of *M* and *NP* Gene of H5N1 Avian Influenza Virus and Immune Efficacy of their DNA Vaccines*

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Abstract: The M and NP genes of H5N1 avian influenza virus (A/chicken/Hubei/489/2004) were ampli-fied by RT-PCR from viral RNA, and cloned into pMD18-T vector respectively. The expression plasmid containing the M gene (pHM6-m) or the NP gene (pHM6-np) was then constructed by inserting the M or NP gene into the pHM6 eukaryote expression vector; the constructed plasmid was then sequenced. 32 BALB/c mice (6-week-old) were divided into four groups at random. Three groups of BALB/c mice were inoculated one time the intramuscular route with either 30 μg of plasmid pHM6-m, 30 μg of plasmid p HM6-np or the mixture of plasmid pHM6-m (15 μg) and pHM6-np(15 μg) respectively. A additional group of mice were injected with 100 μl PBS as controls. Two weeks later, all mice were challenged with homologous H5N1 avian influenza virus, and observed in the following 12 days. The survival rates of mice in the pHM6-m group, the pHM6-np group and mixed plasmids group were 62.5%, 25.0% and 50.0%, respectively. Results showed that effective protection could be provided by either pHM6-m or pHM6-np, but pHM6-m provided a better protective effect than pHM6-np.

Key words: H5N1 influenza virus; M gene; NP gene; Cloning; DNA vaccine

Avian influenza virus (AIV), a member of the *Ortho-myxoviridae* family, is a kind of segmented, negative-stranded RNA virus. It is highly mutational, and can be divided into different serotypes. AIV is a serious threat to most kinds of birds, as well as to public health. In particular, in recent years, H5N1 AIV , has not only done great harm to poultry (2), but also caused many lethal infection cases in human (4, 9). The human infection cases caused by H5N1 AIV, as reported to the World Health Organization, is 230 in total, from January 1 in 2003 to July 14 in 2006, of which 132 were lethal cor-responding to a mortality rate of 57.4%.

Current vaccines against H5N1 AIV in the world are mainly inactivated vaccine, recombinant live virus vaccine and a DNA vaccine. Among those three vaccines, the DNA vaccine, a new vaccine based on nucleic acid, which was developed in the 1990s, can induce high level CTL ac-tivity and antibody response. It also has many other advan-tages compared with traditional vaccines (10). For examp le, the DNA vaccine can induce lasting immune respon-

se, while the plasmid vector composing DNA vaccine has no immunogenicity; additionally, the DNA vaccine can be used repeatedly, easily produced, stored and transported because of its stability. Thus, in recent years, DNA vaccines against H5N1 AIV has held the attention of many researchers in view of its potential application in the future.

The genome of H5N1 AIV is composed of eight RNA segments with negative polarities,which encode 10 proteins, including PB1, PB2, PA, HA, NP, NA, M1, M2, NS1 and NS2. At present, domestic research on DNA vaccine against H5N1 AIV has mainly focused on hemagglutinin (HA) and neuraminidase (NA), and a few studies response, while theplasmid vector composing DNA vaccine has no immunogenicity; additionally, the DNA vaccine can be used repeatedly, easily produced, stored and transported because of its stability. Thus, in recent years, DNA vaccines against H5N1 AIV has held the attention of many researchers in view of its potential application in the future.

The genome of H5N1 AIV is composed of eight RNA segments with negative polarities, which encode 10 proteins, including PB1, PB2, PA, HA, NP, NA, M1, M2, NS1 and NS2. At present, domestic research on DNA vaccine against H5N1 AIV has mainly focused on hemagglutinin (HA) and neuraminidase (NA), and a few studies have provided convincing evidence that a DNA vaccine encoding HA or NA of H5N1 AIV could induce protective immunity effectively in experimental animals (4, 5, 11). However, a DNA vaccine encoding the *M* gene or the *NP* gene remains to be studied. In order to know whether an *M*-gene DNA vaccine or *NP*-gene DNA vaccine can provide sufficient protective effects against H5N1 AIV, we amplified the *M* and *NP* genes of H5N1 AIV (A/chicken/Hubei/489/2004) and constructed eukaryote expression plasmids pHM6-m and

pHM6-np as DNA vaccines. Then we detected the protective effects of the two DNA vaccines in BALB/c mice.

1. Materials and methods

1.1 Virus, vectors and enzynes

H5N1 AIV,A/chicken/Hubei/489/2004, used in this study, was provided by Professor Shu-wen WU and stored in our laboratory. pMD18-T vector was purchased from TaKaRa(Dalian),and Eukaryote expression vector pHM6 was purchased from Roche. Competent cells DH5α were developed by our laboratory. Reverse transcriptase M-MuLV and restriction endonucleases (*Hin*d III and *Eco*R I) were purchased from MBI Fermentas. KOD Plus DNA polymerase and T_4 DNA ligase were purchased from TOYOBO (Shanghai). Taq DNA polymerase was purchased from TaKaRa (Dalian).

SPF embryo eggs and BALB/c mice: 9-day-old SPF embryo eggs were purchased from Wuhan institute of biological products. BALB/c female mice (6-week-old) were purchased from the School of Medicine in Wuhan University.

1.2 Proliferation and purification of the virus

H5N1 AIV, A/chicken/Hubei/489/2004, was inoculated into the allantoic cavities of 9-day-old SPF embryo eggs. Inoculated eggs were incubated for 72 hours at 34℃ before collection of the allantoid fluid. The allantoid fluid was harvested and stored at -70℃ for the use followed.

Further to information provided in previous reports (3,6,7), *M* and *NP* gene sequences of H5N1 AIV in GenBank, universal primers for H5N1 AIV reverse transcription and two pairs of specific primers (containing *Hin*d III and *Eco*R I restriction sites) for respective amplification of viral *M* gene and *NP* gene were designed. All the primers were synthesized by Sangon (Shanghai). The expected size of the gene seg-

ments amplified was ~ 1.1 kb (*M*) and 1.6 kb (*NP*), re-
spectively.

Universal primer for H5N1 AIV reverse transcrip-
tion: 5'-AGCGAAAGCAGG-3'

M forward primer: 5'-AAAGCTTAGCAAAAGCA
GGTAGATATTGAA-3'

M reverse primer: 5'-AGAATTCAGTAGAAACAAG
GTAGTTTTTTACTC-3'

NP forward primer: 5'-AAAGCTTCAGCGAAAG
CAGGGTAGATAATCA-3'

NP reverse primer: 5'-AGAATTCAGTAGAAACA
AGGGTATTTTTCTTA-3'

1.3   RNA isolation

Viral RNA was isolated from the allantoid fluid
containing H5N1 AIV(A/chicken/Hubei/489/2004)
according to the instruction for Trizol reagent.

1.4   RT-PCR and extraction of products

With the two-step RT-PCR method, the *M* gene and
NP gene were amplified, using their specific primers.
The first cycle of the PCR program consisted of a 4-min
period at 94℃ and was followed by 30 cycles with the
following conditions: 94℃ for 30 sec, 60℃ for 30 sec,
and 70℃ for 2min. The program ended with one cycle
at 70℃ for 10 min. KOD Plus DNA polymerase was
used in the PCR reaction. The products amplified were
confirmed by gel electrophoresis, and then extracted
with the Gel Extraction Kit purchased from OMEGA.

1.5   Adding A to 3' end of PCR products

An adenine deoxyribonucleic acid was added to the 3'
end of the two kinds of extracted PCR products by Taq
DNA polymerase, in virtue of its characteristic of adding
an adenine deoxyribose nucleotide acid to the 3' end of
the DNA segment. The reaction was performed at 72℃
for 30 minutes. Then the products were extracted with
the Gel Extraction Kit.

1.6   Cloning PCR products into pMD18-T vector

Two kinds of PCR products, to whose 3' end an A
had been added, were inserted into the pMD18-T
vector; followed this, the two kinds of recombinant
plasmids were introduced into competent cells (DH5α),
respectively. Competent cells who took up the right
recombinant plasmid were selected on Xgal/IPTG/Amp
LB plates. Two kinds of plasmids were obtained from
transformed competent cells, and identified by restriction
enzyme digestion (*Hin*d III and *Eco*R I) and gel electro-
phoresis. These plasmids were named pMD18-T-m and
pMD18-T-np, respectively, and sequenced at UNITED
GENE HOLDINGS LTD. (Shanghai).

1.7   Construction of eukaryote expression plasmids

After the digestion of the pMD18-T-m, pMD18-T-np
and pHM6 with *Hin*d III and *Eco*R I, the *M* gene, *NP*
gene and linear pHM6 vector were obtained respectively
by gel electrophoresis and extraction. The *M* gene was
then linked to the linear pHM6 vector, forming orbicular
eukaryote expression plasmid pHM6-m. A similar pro-
cess using the *NP* gene, resulted in expression plas-mid
pHM6-np. pHM6-m and pHM6-np were introduced into
competent cells DH5α. The competent cells were then
incubated in LB/ampicillin(50 μg/ mL) medium. Two
kinds of recombinant plasmids were respectively ex-
tracted from the LB culture, identified by restriction en-
zyme digestion (*Hin*d III and *Eco*R I) and gel electro-
phoresis. Both eukaryote expression plasmids pHM6-m
and pHM6-np were sequenced at UNITED GEN
E HOLDINGS LTD. (Shanghai).

1.8   Preparation of abundant eukaryote expression
plasmids

The E.coli cells containing plasmid pHM6-m or
pHM6-np were cultured in 200 mL LB/ampicillin(50 μg/
mL) medium. Then pHM6-m and pHM6-np were ex-

tracted respectively with the Plasmid Rapid Isolation Kit (BioDev, Beijing). The two kinds of plasmids were quantified with a UV-VIS Spectrophotometer, dissolved in PBS buffer, and stored at -20℃ for later use.

1.9　Vaccine formulations and immunization protocol

　　32 BALB/c female mice (6-week-old) were divided into four groups at random, 8 mice in each group. In the pHM6-m plasmid group, each mouse was injected with 30 μg pHM6-m (dissolved in 100 μL PBS), while in the pHM6-np group, each mouse was injected with 30 μg pHM6-np (dissolved in 100 μL PBS). In the mixed plasmids group each mouse was injected with 15 μg pHM6-m and 15 μg pHM6-np (dissolved in 100 μL PBS together). The control group of mice were injected with 100 μL PBS. All mice were injected via intramuscular route under ether anesthesia.

1.10　Infection

　　Two weeks after immunization, all the mice were challenged with H5N1 AIV A/chicken/Hubei/489/2004 ($10^{5.6}$ ELD$_{50}$(50% egg lethal dose H5N1 AIV A/chicken/Hubei/489/2004), by intranasal administration of 100 μL of the viral suspension (allantoid fluid collected in 2.2.1). The mice were observed every day, and the number of mice died was recorded.

2.　Results

2.1　Amplification of *M* gene and *NP* gene by RT- PCR

　　The *M* gene and *NP* gene of A/chicken/Hubei/489/2004 (H5N1) were successfully amplified by RT-PCR with the designed primers, fragments for *M* (1.1 kb) and *NP* gene (1.6 kb) were obtained. The results from PCR reaction are shown in Fig.1.

2.2　Cloning *M* gene and *NP* gene into pMD18-T vector and identification of products

　　The two kinds of recombinant plasmids pMD18-T-m

and pMD18-T-np were extracted and then confirmed by restriction enzyme digestion (*Hin*d III and *Eco*R I) and gel electrophoresis. The results from gel electrophoresis are shown in Fig.2. Products of expected size (1.1 kb and 1.6 kb) were obtained respectively after pMD18-T-m and pMD18-T-np were digested. Both plasmids were sequenced at United Gene Holdings Ltd. (Shanghai). The intact open reading frames of M1 and M2 could be found in pMD18-T-m and the intact open reading frame of NP was also found in pMD18-T-np. This confirms that the M gene and NP gene were cloned into pMD18-T vector successfully.
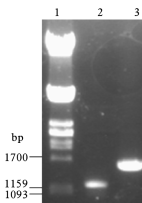

Fig. 1. Gel electrophoresis of the RT-PCR products. 1,λ/Pst I DNA marker; 2, RT-PCR product of M gene; 3, RT-PCR product of NP gene.

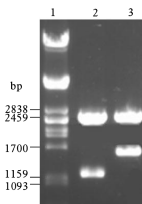Fig. 2. Gel electrophoresis of recombinant plasmids pMD18-T-m and pMD18-T-np digested with *Hin*d III and *Eco*R I. 1:λ/Pst I DNA marker; 2: pMD18-T-m (digested with *Hin*d III and *Eco*R I); 3: pMD18-T-np (digested with *Hin*d III and *Eco*R I).

2.3 Construction and identification of eukaryote expression plasmids pHM6-m and pHM6-np

M fragments and NP fragments were respectively inserted into the multiple cloning site (between *Hin*d III and *Eco*R I restriction sites) of the pHM6 vector (Fig.3, Fig. 4). The eukaryote expression plasmids pHM6-m and pHM6-np were confirmed by restriction enzyme digestion (*Hin*d III and *Eco*R I) and gel electrophoresis. Results from gel electrophoresis (Fig.5) show that two kinds of fragments of the expected size (5.4 kb and 1.1 kb) were obtained after plasmid pHM6-m was digested. Similarly, digestion of pHM6-np created two fragments with expected size (5.4 kb and 1.6 kb). Digestion of pHM6-np created two fragments with expected size (5.4 kb and 1.6 kb). Plasmids pHM6-m and pHM6-np
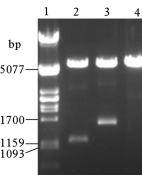

Fig. 5. Gel electrophoresis of recombinant eukaryote expression plasmids pHM6-m and pHM6-np, digested with *Hin*d III and *Eco*R I. 1:λ/Pst I DNA marker; 2: pHM6-m (digested with *Hin*d III and *Eco*R I); 3: pHM6-np (digested with *Hin*d III and *Eco*R I); 4: pHM6 vector(digested with *Hin*d III and *Eco*R I).
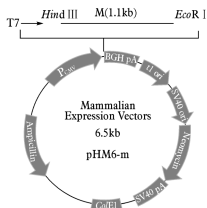
were then sequenced at United Gene Holdings Ltd. (Shanghai). Results confirmed that pHM6-m contains an intact *M* gene while pHM6-np contains an intact *NP* gene. We could therefore draw the conclusion that construction of the eukaryote expression plasmids pHM6-m and pHM6-np was successful.

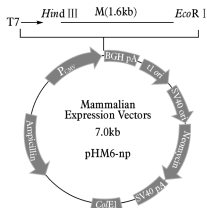Plasmids pHM6-m and pHM6-np were then sequenced at United Gene Holdings Ltd.(Shanghai). Results confirmed that pHM6-m contains an intact M gene while pHM6-np contains an intact NP gene. We could therefore draw the conclusion that construction of the eukaryote expression plasmids pHM6-m and pHM6-np was successfu

2.4 Survival rates of mice after H5N1 AIV chall enge

Two weeks after immunization, all mice were challenged with $10^{5.6}$ ELD$_{50}$ (50% egg lathal dose) H5N1 AIV, A/chicken/Hubei/ 489/2004 , and observed for the following 12 days. Table 1 and Fig. 6 showed that all mice in the control group died within 8 days. The final survival rates of mice in pHM6-m group and pHM6-np group were 62.5% and 25.0%, respectively. When mixed plasmids of pHM6-m and pHM6-np were immunized, 50% protection was observed (Fig.6).

Fig. 3. Genetic map of pHM6-m
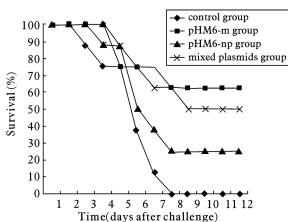

Fig. 4. Genetic map of pHM6-np

Fig.6. The survival rates of mice in different groups after A/chicken/Hubei/489/2004(H5N1) challenge. All mice in control group died within 8 days. The final survival rates of mice in pHM6-m group, pHM6-np group and mixed plasmids group were 62.5%, 25.0% and 50.0%, respectively

3. Discussion

In this study, we amplified the full-length *M* gene and full-length *NP* gene of H5N1 AIV, A/ chicken / Hubei/ 489/2004, by RT-PCR, and inserted the *M* gene and the *NP* gene into pMD18-T vector respectively, and used these vectors for the study of the efficacy of a DNA vaccine containing the *M* gene or the *NP* gene against H5N1 AIV.

Previous research on DNA vaccines against H5N1 AIV have mainly focused on DNA expression plasmids containing the HA or the NA gene(4, 5, 11). We constructed pHM6-m and pHM6-np as DNA vaccines encoding the *M* gene and the *NP* gene respectively for the first time, and examined the protective effects of a *M* DNA vaccine and a *NP* DNA vaccine against homolog-

ous H5N1 AIV, A/chicken/Hubei/489/2004, were studied in BALB/c mice. Results show that a single intramuscular immunization with 30 μg pHM6-m could protect 62.5% of the mice challenged with homologous H5N1 AIV, whereas a single intramuscular immunization pHM6-np 30 μg gave 25.0% protection,suggesting that immunization with the *M*-gene DNA vaccine or the *NP*-gene DNA vaccine could protect against a homologous H5N1 AIV challenge. However, the *NP*-gene DNA vaccine provided weaker protection than the *M*-gene DNA vaccine. We also set a mixed plasmids group in which mice were immunized once with a mixture of 15 μg pHM6-m and 15 μg pHM6-np so as to investigate whether the *M*-gene DNA vaccine or *NP*-gene DNA vaccine is better. We expected a result that mixed DNA vaccines could protect against homologous H5N1 AIV more effectively than any single kind of DNA vaccine, but ultimately, the survival rate of mice in the mixed plasmids group only reached 50.0%, which was higher than the 25.0% in the pHM6-np group, but a little lower than the 62.5% observed in the pHM6-m group. That is, evidence of optimized protective effect was not observed when mixed DNA vaccines (M DNA vaccine and NP DNA vaccine) were used. However, the results provided convincing evidence that the protective effect of the *M*-gene DNA vaccine was better than that of the *NP*-gene DNA vaccine. It was also shown that the *M* gene and the *NP* gene could

Table 1. The survival rates of mice in different groups after H5N1 AIV challenge[a]

	Urvival/Total												Survival (%)
	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10	Day11	Day12	
Control group	8/8	8/8	7/8	6/8	6/8	3/8	1/8	0/8	0/8	0/8	0/8	0/8	0.0
pHM6-m group	8/8	8/8	8/8	8/8	6/8	6/8	6/8	5/8	5/8	5/8	5/8	5/8	62.5
pHM6 -np group	8/8	8/8	8/8	8/8	7/8	3/8	3/8	2/8	2/8	2/8	2/8	2/8	25.0
Mixed plasmids group	8/8	8/8	8/8	7/8	7/8	5/8	5/8	4/8	4/8	4/8	4/8	4/8	50.0

[a]Two weeks after immunized once via intramuscular route under aether anesthesia, all BALB/c mice were challenged with H5N1 AIV, A/chicken/Hubei/2004(105.6 ELD50), and the number of mice died was noted every day followed

both be candidates for DNA vaccines against H5N1 AIV.

The *M* gene of H5N1 AIV encodes viral matrix proteins (M1 and M2), which is in the majority of all viral proteins. The DNA sequence of the *M* gene, in particular M1, is highly conserved (8) and in of all the genes of H5N1 AIV, the *NP* gene is the most conserved. So the *M*-gene DNA vaccine (pHM6-m) and the *NP*-gene DNA vaccine (pHM6-*NP*) are subjects for further studies, and their wide application can be expected in the future.

H5N1 AIV can cause lethal infection in human, and the mortality is high among infected subjects(4, 9) and so it presents a serious threat to public health. Our study results, based on BALB/c mice, showed that the *M*-gene DNA vaccine and the *NP*-gene DNA vaccine could provide reasonably effective protection against homologous H5N1 AIV in mammalians. This suggests that the *M*-gene DNA vaccine and the *NP*-gene DNA vaccine have much potential in clinical applications.

In this study, mice were immunized with pHM6-m or pHM6-np once via the intramuscular route. Although their protective efficacy could not meet the requirement for clinical application, results showed that pHM6-m and pHM6-np could provide obvious protection against H5N1 AIV. Previous research(1,4,8,9,11) has show that protective efficacy of DNA vaccines can be improved by constructing DNA vaccines containing target gene with optimized codons, performing immunization several times,improving the immunization route(for example, immunization by *in vivo* electroporation or gene gun) , or immunized together with traditional vaccines. We can also try to improve the protection efficacy of *M*-gene DNA vaccine or *NP*-gene DNA vaccine by adding or deleting part of nucleotides of *M* gene or *NP* gene. Thus, based on this study, we think it is worthwhile to continue further research on a DNA vaccine

encoding the *M* or *NP* genes of H5N1 AIV.

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