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# Development and Identification of a Live Attenuated Influenza B Virus Vaccine Candidate*

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**Abstract** A cold-adapted (*ca*), temperature sensitive (*ts*), live attenuated influenza B virus strain B/Ann Arbor/1/66 was chosen for influenza virus rescue research, in which six internal gene segments, PB1, PB2, PA, NP, M, NS, were fully synthesized and nine amino acid substitutions were artificially alter by human intervention. The resultant B/Ann Arbor/1/66 plasmids were named as pAB121-PB1, pAB122-PB2, pAB123-PA, pAB124-HA, pAB125-NP, pAB126-NA, pAB127-M and pAB128-NS, respectively. A recombinant influenza A virus was previously generated entirely from cloned cDNA. An infectious recombinant influenza B virus was generated here, and designated as rMDV-B, by plasmid-based reverse genetics. The rMDV-B virus contained HA and NA genes from an epidemic influenza B virus strain B/Malaysia/2506/2004 in the background of internal genes derived from influenza B virus strain B/Ann Arbor/1/66. HA titer of rMDV-B in MDCK cells and embryonated chicken eggs ranged from 1∶64 to 1∶512. The results may allow an effective live influenza B vaccine to be produced from a single master strain, providing a model for the design of future live human influenza vaccines.

**Key words** influenza B virus, reverse genetics, eight-plasmid system, reassorted influenza virus
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Influenza is one of the most important respiratory pathogens. It is reported by WHO that influenza is responsible for approximately 250 000～500 000 deaths each year worldwide (http://www.cdc.gov/). Influenza B virus is one of two types of influenza virus that cause substantial morbidity and mortality in humans, the other being influenza A virus. Influenza A and B viruses each contain eight segments of single stranded RNA with negative polarity. The eight genome segments of influenza B encode 11 proteins. The three largest genes code for components of the RNA polymerase, PB1, PB2, and PA. Segment 4 encodes the HA protein. Segment 5 encodes NP. Segment 6 encodes the NA protein and NB protein. Both proteins, NB and NA, are translated from overlapping reading frames of a biscistronic mRNA. Segment 7 of influenza B also encodes two proteins: M1 and BM2. The smallest segment encodes two proteins: NS1 is translated from the full length RNA, while NS2 is translated from a spliced mRNA variant.

To date, vaccine is still a principal and effective means for influenza prophylaxis. Vaccines to prevent influenza generally contain the surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins from the two currently circulating influenza A subtypes (i.e., H3N2 and H1N1) and one circulating influenza B strain. The inability to provide lasting protection to humans against influenza virus infection is due, in part, to antigenic drift or antigenic shift in the HA. Hence,

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the vaccines must be frequently updated to ensure coverage against virus strains.

FluMist is a live attenuated influenza vaccine (LAIV) composed of the three dominant circulating strains of human influenza virus. This vaccine has been shown to be efficacious and safe for delivery in adults and children and has been licensed for use in the United States in 2003[1].In recent years, reverse-genetics systems for the rescue of recombinant influenza virus have proven to be of great value for influenza virus research and vaccine development.

In our previous study, a reverse genetics system for the generation of recombinant influenza A virus was well established that facilitates the generation of vaccine viruses without the need for time-consuming co-infection and selection procedures currently required to produce reassortants. Moreover, the system successfully contributes to vaccine production. Here, the strategy we applied is based on that described for recovery of recombinant influenza A virus. In this report, we described the establishment of an eight-plasmid system that enables the generation of infectious, recombinant influenza B vaccine candidate by reverse genetics.

## 1 Materials and methods

### 1.1 Viruses, cells and eggs

Influenza virus strain B/Malaysia/2506/2004 were received from China CDC and propagated in 10-day-old specific pathogen-free embryonated research grade eggs (Center of laboratory animal, Beijing). COS-1 cells and Madin-Dardy canine kidney (MDCK) cells were obtained from Yangzhou University and maintained in essential medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS) at 37℃ in an atmosphere with 5% $CO_2$.

### 1.2 Virus RNA extraction, PCR amplification, and gene cloning

A cold-adapted (*ca*), temperature sensitive (*ts*), live attenuated influenza virus strain B/Ann Arbor/1/66 was used as the master donor virus (MDV) for virus rescue, in which six internal gene fragments were fully synthesized. Meanwhile, nine amino acid substitutions at positions, $PB2^{630}$ (S630R), $PA^{431}$ (V431M), $PA^{497}$ (Y497H), $NP^{55}$ (T55A), $NP^{114}$ (V114A), $NP^{410}$ (P410H), $NP^{510}$ (A510T), $M1^{159}$ (H159Q), $M1^{183}$ (M183V), have been artificially altered by human intervention and six pairs specific primers were designed according to references[2,3](Table 1). The 5′-ends have recognition sequences for the restriction endonucleases *Bsm*BⅠ (Bm) or *Bsa*Ⅰ (Ba) (New England Biolabs). B/Malaysia/ 2506/2004 was propagated in 10-day-old embryonated chicken eggs and concentrated by density gradient centrifugation on sucrose. Total RNA was extracted from infected allantoic fluid with the RNeasy kit (Qiagen) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni9 primer (5′ AGCAGAAGC 3′)[4] and AMV reverse transcriptase (Invitrogen). Expand High Fidelity PCR System (RoChe) was used and 8～10 clones were sequenced in order to confirm the authenticity of 5′ and 3′ ends. Finally, we spliced the 8 whole gene

**Table 1 RT-PCR primers for amplification of the eight vRNAs of influenza *ca* B/AA/1/66**

| | Primer | Sequence( 5′to 3′) | Size/bp |
|---|---|---|---|
| PB2 | Bm -BPB2-1 | TATTCGTCTCAGGGAGCAGAAGCGGAGCGTTTTCAAGATG | 2 396 |
| | Bm -BPB2-2 | ATATCGTCTCGTATTAGTAGAAACACGAGCATT | |
| PB1 | Bm-BPB1-1 | TATTCGTCTCAGGGAGCAGAAGCGGAGCCTTTAAGATG | 2 369 |
| | Bm-BPB1-2 | ATATCGTCTCGTATTAGTAGAAACACGAGCCTT | |
| PA | Bm-BPA-1 | TATTCGTCTCAGGGAGCAGAAGCGGTGCGTTTGA | 2 308 |
| | Bm-BPA-2 | ATATCGTCTCGTATTAGTAGAAACACGTGCATT | |
| NP | Bsa-BNP-1 | TATTGGTCTCAGGGAGCAGAAGCACAGCATTTTCTTGT | 1 842 |
| | Bsa-BNP-2 | ATATGGTCTCGTATTAGTAGAAACAACAGCATTTTT | |
| M | Bm-BM-1 | TATTCGTCTCAGGGAGCAGAAGCACGCACTTTCTTAAAATG | 1 190 |
| | Bm-BM-2 | ATATCGTCTCGTATTAGTAGAAACAACGCACTTTTTCCAG | |
| NS | Bm-BNS-1 | TATTCGTCTCAGGGAGCAGAAGCAGAGGATTTGTTTAGTC | 1 098 |
| | Bm-BNS-2 | ATATCGTCTCGTATTAGTAGTAACAAGAGGATTTTTAT | |
| HA | Bm-Nab-1 | TATTCGTCTCAGGGAGCAGAAGCAGAGCA | 1 844 |
| | Bm-Nab-2 | ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTT | |
| NA | Bm-Nab-1 | TATTCGTCTCAGGGAGCAGAAGCAGAGCA | 1 516 |
| | Bm-Nab-2 | ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTT | |

sequences containing the non-coding region of 3′ and 5′ ends by BLAST. cDNA fragments with *Bsm*BⅠ and *Bsa*Ⅰ of B/Ann Arbor/1/66 and B/Malaysia/2506/2004 were cloned between the two *Bsm*BⅠ sites of the vector pAD3000 (a derivative of pHW2000 which allows the transcription of negative sense vRNA and positive mRNA). Eight transcription/expression plasmids were obtained and named as pAB121-PB1, pAB122-PB2, pAB123-PA, pAB124-HA, pAB125-NP, pAB126-NA, pAB127-M and pAB128-NS, respectively. Sites of the two restriction endonucleases in six internal genes were as followed (Table 2). All cloned were confirmed by full-length sequencing.

**Table 2 Sites of the two restriction endonucleases in six internal genes**

| Restriction endonucleases | BPB2 | BPB1 | BPA | BNP | BM | BNS |
|---|---|---|---|---|---|---|
| *Bsa*Ⅰ | — | 799, 1831 | 242 | — | — | — |
| *Bsm*BⅠ | — | — | — | 1176, 1416 | — | — |

### 1.3 Transfection and generation of virus

The rescue of infection virus from cloned cDNA was done under GMP conditions. MDCK and COS-1 cells were co-cultured in 6-well plate at a ratio of 1∶1 and the cells were used for transfection at a confluency of approximately 70%～80%. Plasmid DNA transfection was performed using PolyFect transfection reagent (Qiagen) by mixing 0.2 μg of each of the eight recombinant plasmids (BPB2, BPB1, BPA, BHA, BNP, BNA, BM, BNS)with 10 μl of PolyFect diluted in 100 μl DMEM. The DNAs and transfection reagent mixture were incubated at room temperature for 5～10 min followed by addition of 500 μl DMEM. The transfection mixture was then added dropwise to the co-cultured MDCK/COS-1 cells. The transfection cells were incubated at 33℃ for 48 h, 1 ml of DMEM containing 1 mg/L TPCK-trypsin(PIERCE) was added to the cells. At 96 h post-transfection, 1 ml of DMEM containing 1 mg/L TPCK-trypsin was added to the cells. Six days post-transfection, transfected culture supernatants were harvested. The recovered virus was then amplified in confluent MDCK cells or directly amplified in embryonated chick eggs. The amplified viruses were stored at −80℃. 200 μl of the clarified supernatant was injected into the allantoic cavity of individual 10-day-old embryonated chicken eggs. After 72 h incubation at 33℃, each egg was candled to determine embryo viability before chilling at 4℃. Allantoic fluid was harvested from each egg and was tested for haemagglutination activity.

### 1.4 Identification and analysis of recombinant virus

**1.4.1** RT-PCR. RT-PCR was performed to map the genotypes of the recovery viruses. Virus RNA was isolated from the infected cell culture supernatant or allantoic fluid from the second passage using the RNeasy mini kit and the eight internal gene segments were amplified by RT-PCR using specific primers of each MDV-B gene segment (Table1).

**1.4.2** Electronic microscope. The allantoic fluid from the second passage was concentrated and purified by density gradient centrifugation on sucrose. Then, morphology of the rescued virus and wild-type virus strain were observed by electronic microscope.

**1.4.3** Titration of viral infectivity in eggs and MDCK. Infectivity in eggs ($EID_{50}$) was determined by inoculating eggs with 0.2 ml of 10-fold dilutions of allantoic fluid (4 eggs per dilution). After incubation at 33℃ for 72 h, the presence of virus was determined by hemagglutination. The hemagglutination titer (HA titer) was measured by standard method using 1.0% chicken red blood cells (RBC) in phosphate-buffered saline (PBS, pH 7.2) (Palmer *et al*., 1975). The values of HA titers were determined as an average from 4 eggs. To determine virus infectivity in MDCK ($TCID_{50}$) cells grown to confluence in 96-well plates were washed with PBS and inoculated with serial 10 fold dilutions of virus (eight replicates for each sample). After one hour at 33℃, MDCK cells were washed and DMEM containing 1 mg/L TPCK-trypsin was added. The endpoint for calculation of $TCID_{50}$ was cytopathic effect (CPE) after the incubation of the inoculated cells for 3～4 days at 33℃ in an atmosphere with 5% $CO_2$. $TCID_{50}$ titers were calculated by the method of Reed and Muench (1938).

**1.4.4** SDS-PAGE. To verify the main component of the rescue influenza B virus, the allantoic fluid from the second passage, after concentrated and purified were analyzed by 15% SDS-PAGE using conventional techniques.

**1.4.5** Stability testing in eggs. In order to test the stability of the vaccine virus on propagation, ten consecutive passages of the virus were made in embryonated chicken eggs. The virus was adjusted to $10^{-3}$ dilution with PBS, and 0.2 ml of the solution was injected into the allantoic cavities of four 10-day-old embryonated chicken eggs and then reinjected into another four eggs. To confirm the genetic stability of the rescue virus, we compare the HA, NA and NP gene sequences of the rescued virus from the second, fourth, sixth, eighth and tenth passage.

## 2 Results

### 2.1 Generation of recombinant influenza B virus from eight plasmids

Six days post-transfection, culture supernatants with eight recombinant plasmids incorporating the 6 MDV-B internal genes, and HA and NA derived from B/Malaysia/2506/2004 (6∶2 reassortant, rMDV-B), were used to infect fresh MDCK cells. Then, the infected cells were incubated at 33℃ for 3～4 days in DMEM with 1 mg/L TPCK-trypsin. The cytoplasmic effect of the recombinant virus on infected MDCK cells was observed using a microscope. Expression of viral hemagglutinin was monitored using a standard hemagglutination assay (HA). HA titers were detected to be 1∶64 approximately from the amplified 6∶2 reassortant virus in the first passage. Meanwhile, no virus was detected in supernatants derived from cells transfected with only seven plasmids. These transfection reactions were as follows (Table 3).

**Table 3 Generation of infectious influenza B virus from eight plasmids**

| Segment | rMDV-B | Control |
|---|---|---|
| BPB1 | pAB121-PB1 | — |
| BPB2 | pAB121-PB2 | pAB121-PB2 |
| BPA | pAB121-PA | pAB121-PA |
| BHA | pAB121-HA | pAB121-HA |
| BNP | pAB121-NP | pAB121-NP |
| BNA | pAB121-NA | pAB121-NA |
| BM | pAB121-M | pAB121-M |
| BNS | pAB121-NS | pAB121-NS |
| HA | 1∶64 | — |
| CPE | + | — |

### 2.2 RT-PCR

As shown in Figure 1, PCR products were generated for all segments. After a subsequent passage on MDCK cells, RT-PCR of the supernatant of infected cells was used to confirm the authenticity of the generated virus. RT-PCR was performed with segment specific primers for all eight segments. The full-length sequence of the recombinant virus after passage into embryonated chicken eggs was identical to that of the input plasmids. Eight desired fragments were amplified by RT-PCR from the second passage, they were 2.3 kb, 2.3 kb, 2.3 kb, 1.8 kb, 1.8 kb, 1.5 kb, 1.1 kb and 1.0 kb, respectively. However, HA and NS gene couldn't be amplified from allantoic fluid exclude contamination of plasmids.

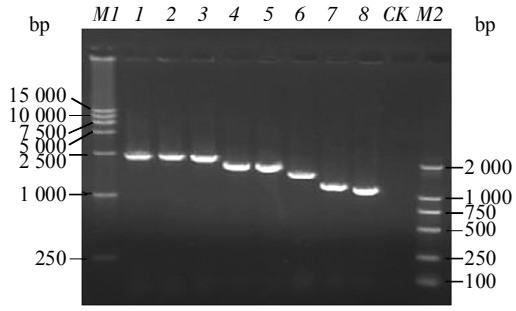


**Fig. 1 Eight gene segments of rMDV-B amplified by PCR**
*M1*: DL15000 marker; *M2*: DL2000 marker; *1*～*8*: PB2, PB1, PA, HA, NP, NA, M, NS; *CK*: Negtive control.

### 2.3 Electronic microscope

Influenza viral particle with capsule could be observed in two samples (Figure 2). The virion morphology of the rescued viruses was verified to be identical to that of the wild-type influenza B virus strain B/Malaysia/2506/2004. There were no obvious differences between influenza A and B strain in virion morphology.

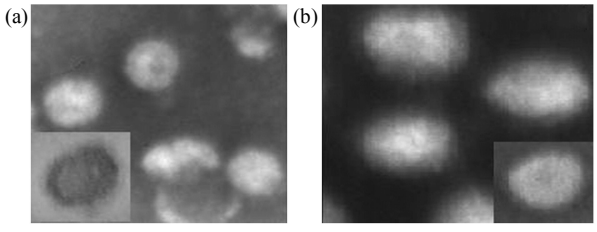


**Fig. 2 Morphology of influenza B viruses**
(a)Recovered rMDV-B reassortant (× 30 000). (b)Wild-type B/Malaysia/2506/2004 (× 93 000). At the bottom of picture (a) or (b) is a single virion (× 65 000).

### 2.4 SDS-PAGE

After rMDV-B was concentrated, there were many bands appeared and dosage increase obviously.

Even after rMDV-B was purified, three bands still exist in lane one. According to the molecular mass, they were the NP, NA and M protein respectively (Figure 3). These results showed that the main component of influenza virus did not lose during the processing.

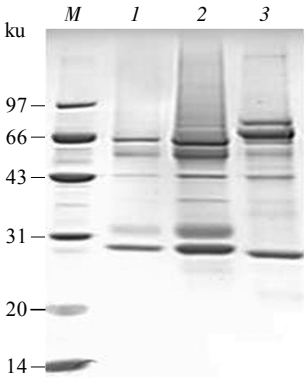


**Fig. 3 SDS-PAGE of rMDV-B**

*1*: Purified rMDV-B; *2*: Concentrated rMDV-B; *3*: rMDV-B; *M*: Molecular mass marker.

### 2.5 Stability of rMDV-B

From the HA, NA and NP gene sequence of different passages, we found virus titer ranged from 1∶64 to 1∶512. Moreover, the homology of HA, NA and NP gene were above 99.99% in the allantoic fluid of the second, fourth, sixth, eighth and tenth passages. This result demonstrates that passage of rMDV-B does not lead to changes in the HA, NA and NP gene.

## 3 Discussion

Influenza is undoubtedly the most important human respiratory pathogen worldwide, causing considerable morbidity and an often-underestimated mortality. Moreover, it imposes a huge burden on the economy. Only an effective and safe vaccine offers the possibility of protecting the human population from the vagaries of almost yearly epidemic. Twice a year, the World Health Organization (WHO) meets to update the vaccine strains to match the strains in the community in each hemisphere. Recent discoveries in influenza pathogenicity and reverse genetics have the potential to revolutionize the way pandemic and interpandemic influenza vaccines are prepared and manufactured. So far, attenuated live vaccine plays an important role for influenza control.

Since 1977, influenza A subtypes have co-circulated along with influenza B viruses[1]. A live attenuated vaccine (FluMist™) was licensed in 2003 in the USA. The vaccine viruses were produced by selecting HA and NA segments from currently circulating viruses and allowing them to reassort with all internal gene segments from either master donor virus (cold-adapted A/Ann Arbor/6/60 for type A (H1N1 and H3N2) and B/AnnArbor/1/66 for type B viruses)[5]. The resultant vaccine viruses were then combined and sprayed into the nostrils of vaccinees. The clinical studies showed that these vaccines are attenuated, safe and efficacious in adults and children. In 2002, David, *et al*. and Erich, *et al*. reported the rescue of influenza B virus entirely from cDNA respectively[2, 3]. Recently, Seo, *et al*.[6] published the development of recombinant influenza B virus by reverse genetics in South Korea. However, it is a blank field in China. We previously generated a recombinant influenza A virus entirely from cloned cDNA. Here we described the generation and identification of reassortant influenza B viruses that carried functional HA and NA from an epidemic influenza B virus strain B/Malaysia/2506/2004 in the background of internal genes derived from influenza B virus strain B/Ann Arbor/1/66, yielding rMDV-B.

As for influenza vaccine production, high virus yield is a critical factor, because it can reduce cost and sustain longer immune protection periods by providing enough antigens[7, 8]. Vaccine production procedures must allow the virus produced to retain the antigenic properties of the parent viruses. Any benefit from an increase in yield would be negated by changes in the antigenicity of a vaccine. The rescued viruses were antigenically indistinguishable from the corresponding wild-type viruses. To confirm the genetic stability of the rescued viruses, the HA gene sequences of the rescued and wild-type viruses were compared. And we found the homology of HA, NA and NP gene sequence were above 99.99% during each passage of recombinant virus. In addition, the injected eggs gave a positive HA titer ranging from 1∶64 to 1∶512 with passage increasing. After rescued virus was concentrated and purified, the main component did not lose.

In principle, plasmid-only systems allow the manipulation of the non-coding and coding regions of the viral RNA. The non-coding regions contain *cis*-acting signals for the regulation of transcription and replication of viral RNA. Compared with influenza A virus, the NCRs of influenza B viruses are relatively

large, extending up to 100 nt. A base mutation may cause failure for generation of recombinant virus. So, these sequences play an important role in rescuing of recombinant virus. In order to assure the authenticity and accuracy of gene fragment, each recombinant plasmid must be identified and sequence repeatedly.

In summary, we have rescued a cold-adapted recombinant influenza B virus by co-transfecting eight transcription plasmids entirely from cDNA. The plasmid rescue system for influenza B will enable molecular genetic studies of influenza B viruses. In addition, the establishment of cold-adapted influenza virus rescue system by RG will contribute to development of cold-adapted human influenza virus vaccine candidates and mucosal immune mechanisms against influenza.

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# 冷适应减毒 B 型流感病毒疫苗候选株的制备及鉴定 *

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**摘要** 以冷适应、温度敏感、减毒的 B/Ann Arbor/1/66 流感病毒株作为重配病毒骨架，对其 6 个内部基因片段进行了全基因合成，同时人工引入 9 个氨基酸突变．构建了 8 个基因的拯救载体，经测序获得序列准确的拯救质粒，命名为：pAB121-PB1, pAB122-PB2, pAB123-PA, pAB124-HA, pAB125-NP, pAB126-NA, pAB127-M 和 pAB128-NS．在成功拯救冷适应 A 型流感病毒的基础上，利用反向遗传学技术成功获救了具有感染性的重配 B 型流感病毒株，命名为 rMDV-B．该重配病毒株以 B/Ann Arbor/1/66 为病毒骨架，其中 HA 和 NA 来源于 2006～2007 年当年流行株 B/Malaysia/2506/2004．rMDV-B 在鸡胚尿囊液和 MDCK 细胞中的 HA 效价可达 1∶64～1∶512．实验结果暗示：从单一供体病毒株可以产生有效的减毒活 B 型流感病毒疫苗候选株，能够为将来人用流感疫苗的设计提供可借鉴的模型．

**关键词** B 型流感病毒,反向遗传学,8 质粒拯救系统,重组流感病毒
**学科分类号** R183.3，R392.1 **DOI:** 10.3724/SP.J.1206.2008.00542

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