Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains

Yuan-Pin Huang, Ching-Ho Wang*

Department of Veterinary Medicine, National Taiwan University

Street address: No 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan

Tel: 886 2 2369 0628

Fax: 886 2 2363 1542

E-mail: chingho@ntu.edu.tw

*Corresponding author
Abstract

Due to variations in serotypes among different strains of avian infectious bronchitis viruses (IBV), vaccination of chicks by imported vaccines fails to protect them from IBV infections in Taiwan. Therefore, we develop attenuated vaccines from local strains in Taiwan. A Taiwan Group I (TW I) strain was passaged 74 times through specific pathogen-free (SPF) chicken embryonated eggs, and then tested in SPF chickens. The attenuated vaccine was not pathogenic in 1-week-old chicks, had a neutralization index (NI) of greater than 4.4 and efficacy of 90% when inoculated birds were challenged with a field IBV strain. Similar results were obtained for a vaccine made from a Taiwan Group II IBV strain. Additionally, the TW I attenuated vaccine strain had no reversion to virulence after five back passages in chicks. In conclusion, these attenuated vaccines were efficacious in controlling local Taiwanese IBV infections in chickens.

Keywords: Attenuated vaccine; Infectious bronchitis virus

1. Introduction

Avian infectious bronchitis (IB) has been identified in many parts of the world and
is an economically important disease of chickens [4]. Chickens of all ages may be infected, and the virus replicates in many tissues. The infected chickens show signs of depression, coughing, sneezing, nasal discharge, polyuria and death [3]. Less commonly, some strains of IBV exhibit tropisms for the alimentary tract and kidneys, and the major IBVs isolated in Taiwan are mostly nephrotropic strains. Based on restriction fragment length polymorphism, the IBVs in Taiwan may be divided to two groups, Taiwan Group I (TW I) and Taiwan Group II (TW II), and are different from other genotypes in the world [13, 14]. Although vaccines have generally been used extensively, outbreaks of IB occur frequently due to serotype differences. It is well known that little or no cross protection occurs between different serotypes of IBV [2, 7], and emergence of new variant strains occurs frequently. Therefore, to develop vaccines from local strains is a must for IB control. In this study, we passaged strains 2575/98 (TW I) and 2296/95 (TW II) in embryonated eggs, and then tested its attenuation, safety, and efficacy according to the Standard Requirements for Animal Drugs [1].

2. Materials and methods

2.1 Genetic grouping of published IBV strains and sequence analysis

Grouping of IBV strains based on HVR1 in subunit S1, named the rC2U-rC3L
region [11], and in the N gene, named the NP1-NP2 region [12], was compared. The sequences of rC2U and rC3L primers were rC2U: 5'TGGTT GGCA(T/C) TTACA (A/C/T)GG(A/G/T) 3' and rC3L: 5'(A/G)CAAT GTGTA ACAAA (T/C)ACT3'. The rC2U-rC3L region was from nt 114 to nt 341 in the N-terminus of the S1 subunit, and the size of expected product was about 229 bp. The sequences of the NP1 and NP2 primers were NP1: 5'GGTAG (C/T)GG(C/T)G TTCCT GATAA3' and NP2: 5'TCATC TTGTC (A/G)TCAC CAAAA3'. The NP1-NP2 fragment was from nt 157 to nt 775 in the N gene, and the size of expected product was about 619 bp. To compare the sequences between and after passage, amplification of the entire S1 gene was performed using the modified forward oligo5' (5'AAACT GAACA AAAGA CAGAC TTAG3') and reverse IBVc2 (5'GCCAT AACTA ACATA TGGAC AAC3') primer pairs [15]. Molecular evolutionary analyses were conducted using the computer program MEGA version 2.1 [6]. Using the Jukes-Cantor distance correction method, an evolutionary phylogenetic tree was generated.

2.2 RNA extraction

Viral RNA was extracted using TRIzol Reagent (Life Technologies, Frederick, MD). A total of 250 µL of visceral or allantoic fluid harvested from inoculated embryonated eggs were collected and mixed with 750 µL of TRIzol reagent. The mixture was
mixed well and left for 5 minutes at room temperature. After adding 200 µL of chloroform (Merck, Darmstadt, Germany), the capped sample tubes were mixed by inversion and shaken vigorously for 15 seconds. The mixture was incubated for 3 minutes at room temperature and centrifuged at 12,000 × g for 15 minutes at 4°C to separate into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The upper aqueous phase at which viral RNA remained was transferred to a fresh microcentrifuge tube. Next, 500 µL of isopropyl alcohol (Merck) was added to the sample and then vortexed and incubated for 10 minutes at room temperature. To precipitate viral RNA, the mixture was centrifuged at 12,000 × g for 10 minutes at 4°C, and the supernatant was removed. Next, 1000 µL of 75% ethanol (Merck) was added to wash the RNA pellet. After centrifugation at 7,500 × g for 5 minutes at 4°C, the supernatant was removed. The RNA pellet was dried in a vacuum desiccator for 3 to 5 minutes. The RNA was dissolved in 50 µL of sterile diethyl pyrocarbonate-treated water (DEPC) (Sigma, St. Louis, Missouri), and used immediately or stored at –20°C.

2.3 Reverse transcriptase-polymerase chain reaction and direct sequencing

A total volume of 50 µL of reaction mixture was prepared by adding 5 µL of 10X DNA polymerase buffer, 0.5 µL of Taq DNA polymerase (Promega, Madison, WI), 16
µL of 1.25 mM dNTPs (Promega), 0.5 µL of 50 pmole/µL upstream primer, 0.5 µL of 50 pmole/µL downstream primer, 0.4 µL of 40 U/µL Recombinant Rnasin ribonuclease inhibitor (Promega), 0.1 µL of 10 U/µL of Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (RT) (Invitrogen, Carlsbad, California), 20 µL of viral RNA solution and 7 µL of DEPC-treated water. RT-polymerase chain reaction (PCR) was performed in one step and conducted in the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California). Reverse transcription was performed at 42°C for 30 minutes. PCR was then performed for 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and polymerization at 72°C for 1 minute and 30 seconds. The initial denaturation step was conducted at 94°C for 3 minutes, and the final polymerization step was at 72°C for 10 minutes. The amplified RT-PCR products were analyzed on a 1.5% agarose gel (electrophoresis grade, Gibco BRL, Life Technologies, Grand Island, NY) and stained with ethidium bromide. Sequencing was performed using a commercial service (Mission Biotech Company, Taipei, Taiwan).

2.4 Viruses

IBV strain 2575/98, a TW I isolate, was isolated in Changhua, Taiwan in July 1998. It was purified by three consecutive limit dilutions. In addition, the IBV strain
2993/02, a pathogenic IBV isolate (TW I group), was isolated in Yilan, Taiwan in January 2002, and used as a challenged virus. IBV strain 2296/95, a group TW II isolate, was isolated in Taoyuan, Taiwan in September 1995, and was also purified.

2.5 Attenuation

IBV strains 2575/98 and 2296/95 were passaged by inoculating 9 to 11-day-old embryonated specific-pathogen-free (SPF) chicken eggs (Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan) by the allantoic sac route. Inoculated eggs were then incubated for 48 hours at 37°C, and the allantoic fluid was harvested for subsequent passage. Eggs that died within 24 hours of inoculation were discarded. At every 10th passage, the eggs were examined using RT-PCR for the presence of virus. The viruses were titrated by inoculating 10-fold serial dilutions with phosphate-buffered saline (PBS) of the virus stocks into the allantoic sac of 10-day-old SPF embryonated eggs, and the titre was calculated by the method of Reed and Muench [9].

2.6 Safety

Safety testing was conducted according to the Standard Requirements for Animal Drugs [1], which states that if none of 10 vaccinated birds show respiratory signs
and/or death, the vaccine is considered to be safe. At least 20 SPF 7-day-old chicks (AHRI), given feed and water *ad libitum*, were divided into two groups with at least 10 chicks in each group. The chicks in the experimental group were vaccinated intranasally with 10 doses ($1 \times 10^{4.5}$ 50% embryo infectious dose [EID$_{50}$]/0.1 mL) of the passage 74 of IBV 2575/98 or passage 76 of IBV 2296/95 at 7 days of age and were observed twice daily for clinical signs for 21 days. In the control group, chicks were inoculated with 0.1 ml PBS. At 28 days of age, sera were collected for enzyme-linked immunosorbent assay (ELISA) (IDEXX, Westbrook, Maine).

### 2.7 Efficacy

At least 20 SPF chicks aged 7 days were divided into two groups. The birds were given feed and water *ad libitum*. Chicks in the inoculated group were vaccinated intranasally with the passage 74 of IBV 2575/98 or passage 76 of IBV 2296/95. Twenty-one days postvaccination, sera were collected, and the birds were challenged intranasally with at least $10^{3.5}$ EID$_{50}$/0.1 mL of pathogenic IBV 2993/02 or passage 4 of IBV 2296/95. All of the birds were then killed and necropsied at 7 days postchallenge. At necropsy, sera were collected for ELISA, and kidneys and tracheas were collected for virus detection by RT-PCR and for virus isolation by inoculating 9 to 11-day-old embryonated SPF chicken eggs. The least significant difference of the
means was statistically calculated using Student’s $t$-test for each pair.

2.8 Antibody titre

Sera collected 21 days postvaccination and were incubated 30 minutes in a 56°C water bath for inactivation. The challenge viruses, IBV 2993/02 or pass 4 of IBV 2296/95, were 10-fold serially diluted with PBS. Then, the dilutions were divided into three groups. Group 1 was mixed with sera from the inoculated group. Group 2 was mixed with sera from the control group, and group 3 was mixed with PBS. The mixtures were allowed to stand at 4°C for 18 to 24 hours. At least five 8-day-old embryonated eggs were inoculated with 0.1 mL of the challenge virus by the allantoic sac route. Inoculated eggs were incubated for 8 days at 37°C, and eggs that died within 24 hours were discarded. At the end of the experimental period, the remaining live eggs were examined for lesions, and the neutralizing index (NI) was calculated.

2.9 Virulence reversion

One- to 10-day-old chicks were divided into two groups of at least three chicks. The inoculated group were inoculated intranasally with 0.1 mL ($1.0 \times 10^{4.5}$ of 50% embryo infectious dose [EID$_{50}$]/0.1 mL) of passage 74 of IBV 2575/98 and were observed twice daily for clinical signs for 5 days. In the control group, chicks were
inoculated with 0.1 mL of PBS. All of the birds were killed and necropsied at 5 days postinoculation. At necropsy, kidney and trachea were collected for virus detection by RT-PCR. The homogenates were inoculated into the next group of chicks and the experiment was repeated five times.

3. Results

3.1 RT-PCR and direct sequencing

RNA from allantoic fluid from inoculated SPF eggs tested positive for IBV by RT-PCR. IBV was also detected in the viscera of SPF chickens in the efficacy test.

Based on the data sequences, a phylogenetic tree was developed as shown in Fig. 1, which describes the relationships between nucleotide sequences of the rC2U-rC3L region in the S1 gene of IBV variants isolated in Taiwan and published data. The deduced amino acid sequences of S1 glycoprotein gene of passage 74 of IBV 2575/98 and passage 76 of IBV 2296/95 were determined and compared with those of low passage number strains (Table 1).

3.2 Attenuation

At every 10th passage, the virus was examined by RT-PCR for the presence of virus (Fig. 2). Sequence analysis verified that the molecular type of the virus had not
changed. The virus titres from passages 65 and 74 of IBV 2575/98 and passages 4 and 76 of IBV 2296/95 were determined in 10-day-old embryonated eggs. The titre was calculated at $1 \times 10^7 \text{EID}_{50}/0.1 \text{mL}$ for passage 65 of IBV 2575/98 and $1 \times 10^{7.4} \text{EID}_{50}/0.1 \text{mL}$ for passage 74 of IBV 2575/98, $1 \times 10^{9.7} \text{EID}_{50}/0.1 \text{mL}$ for passage 4 and passage 76 of IBV 2296/95.

In the pretest (data was not shown), 1-day-old birds given $1 \times 10^7 \text{EID}_{50}$ of passage 65 of IBV 2575/98 via the intranasal route had 20% (2/10) mortality. Because the attenuation of passage 65 of the IBV stock was not complete, the safety and efficacy studies were not conducted on that passage. Therefore, we chose passage 74 of IBV 2575/98 for the safety and efficacy studies. Accordingly, we chose the passage 76 of IBV 2296/95 for safety and efficacy studies.

### 3.3 Safety

At least 10 7-day-old SPF chicks were given $1 \times 10^{4.5} \text{EID}_{50}/\text{bird}$ (10 doses) of passage 74 of IBV 2575/98 or passage 76 of IBV 2296/95 via the intranasal route. No clinical sign was observed in any bird throughout the safety study. Serum samples collected prior to inoculation with the virus were negative for IBV antibodies in the ELISA test. The ELISA serum antibody titres measured 3 weeks postinoculation in the inoculated group (average titre = 380.7 for the IBV 2575/98 group and 215.6 for
the IBV 2295/95 group) were not statistically different from that of the control group (average titre = 145.2 for IBV 2575/98 and 72.9 for IBV 2296/95) or that in the 7-day-old SPF chicks prior to inoculation (average titre = 185.3 for IBV 2575/98 and 15.2 for IBV 2296/95).

3.4 Efficacy

On the basis of virus detection by RT-PCR, in the IBV 2575/98 group, 100% (10/10) of the birds inoculated with passage 74 of IBV 2575/98 were protected from challenge with IBV 2993/02. Ninety percent (9/10) of the non-vaccinated (control) birds challenged with IBV 2993/02 were positive for virus detection, indicating adequate challenge (Table 2). In the IBV 2296/95 group, 100% (13/13) of the birds inoculated with passage 76 of IBV 2296/95 were protected from the challenge. One hundred percent (14/14) of the non-vaccinated challenged control birds were positive for virus detection (Table 2). Results of virus recovery are presented in Table 2. ELISA serum antibody titres measured 7 days postchallenge in inoculated group (average titre = 665.6 in the IBV 2575/98 group and 244.5 in the 2296/95 group) were not statistically different from that in control group (average titre = 593.3 in the IBV 2575/98 group and 204.9 in the IBV 2296/95 group).
3.5 Antibody titre

In the IBV 2575/98 group, the antibody titre in group 1 was less than $3 \log_{10}\text{EID}_{50}$, in group 2, it was greater than $7 \log_{10}\text{EID}_{50}$, and in group 3, it was $7.4 \log_{10}\text{EID}_{50}$. The results showed that the NI of the experimental group was greater than 4.4, and that in the control group was less than 0.4 (Table 3). This data conformed to the related Taiwanese rules that the NI in an inoculated group must be greater than 2.0, and that of the control group must be less than 1.0. In the IBV 2296/95 group, the antibody titre for group 1 was less than $3 \log_{10}\text{EID}_{50}$, in group 2, it was $7 \log_{10}\text{EID}_{50}$, and in group 3, it was $9.7 \log_{10}\text{EID}_{50}$. The results showed that the NI of inoculated groups was greater than 6.7, and that of the control group was 2.7 (Table 3).

3.6 Virulence reversion

In five necropsies and five virus detections, we detected IBV in the viscera of inoculated groups, but not in control group. During the observation period, no clinical signs or deaths were observed. The results showed that no virulence reversion of passage 74 of IBV 2575/98 occurred in these chickens. After five passages in chickens, there was no nucleotide mutation of IBV 2575/98 in the rC2U/rC3L region or in the NP1/NP2 region.
4. Discussion

According to the phylogenetic tree, we know that various differences between Taiwanese strains and foreign strains exist (Fig. 1). Furthermore, even IBV strains isolated from China, which is quite close to Taiwan geographically, are quite different from Taiwanese strains. Based on the comparison of the deduced amino acid sequences before and after high numbers of passage, attenuation does not appear to correlate with mutation after high numbers of passage. One possible reason for this is that the attenuation in this study may have been due to mutations in other genes, not in the S1 subunit. Another possibility is that the attenuation might be related to many factors, and not limited to specific loci in the S1 subunit. However, since the S1 subunit is known to contain regions related to viral neutralizing, serotype-specific, and haemagglutination-inhibiting antibodies [5, 8], greater numbers of passages using several IBV strains will be needed to further characterize the attenuation of IBVs.

On the basis of clinical signs, the attenuation of passage 65 of the IBV 2575/98 stock was not complete. When the passage 74 of IBV 2575/98 was given to 7-day-old SPF chicks, no clinical signs were observed. On the basis of these findings, passage 74 of IBV 2575/98 was tested for safety and efficacy. Meanwhile, we chose IBV 2993/02 for the challenge strain because it was recently isolated, and is also in group TW I. Referring to the passage number of IBV 2575/98, we chose passage 76 of IBV
2296/95 for safety and efficacy studies, and chose a low passage embryo-adapted
strain, passage 4 of IBV 2296/95, for challenge virus.

The titres of passage 74 of IBV 2575/98 and passage 76 of IBV 2296/95 were
determined to be $1 \times 10^{7.4}$ and $1 \times 10^{9.7}$ EID$_{50}$/0.1 mL, and 10 doses ($1 \times 10^{4.5}$
EID$_{50}$/bird) were given to each of at least 10 SPF chicks. No clinical signs were
observed after vaccination.

When IBV 2993/02 was used to challenge the birds inoculated with passage 74 of
IBV 2575/98, all of the birds were protected on the basis of virus detection (0/10
negative), and the numbers were statistically different from those of uninoculated
controls (9/10 positive). In the efficacy study, serum antibody titres of the inoculated
birds were not statistically higher than those of the uninoculated controls. Similar
results were observed for IBV 2296/95. These findings were typical of birds
vaccinated for IBV and were not unexpected. Low serum antibody titres do not
always correlate with lack of protection against IBV because local antibody responses
and cell mediated immunity play a significant role total protection against IBV [10].

In the Standard Requirements for Animal Drugs [1], an effective vaccine strain is
defined as an NI of an inoculated group greater than 2.0, and in uninoculated groups,
the NI should be less than 1.0. In our study, the NI of the birds inoculated with
passage 74 of IBV 2575/98 was greater than 4.4, and in uninoculated controls, the NI
was less than 0.4. This result indicates that inoculated birds could produce enough antibodies to neutralize the challenge virus. In contrast to the IBV 2575/98 group, in the IBV 2296/95 group, the NI in uninoculated controls was not less than 1.0, and the NI for birds inoculated with passage 76 of IBV 2296/95 was much greater than 2.0 (NI = 6.7). However, the result still indicates that inoculated birds could produce enough antibodies to neutralize the challenge virus.

Virulence reversion testing is important for evaluating an attenuated virus, and in this study we detected IBV in homogenates in the birds inoculated with passage 74 of IBV 2575/98, but not the control group. During the observation period, no clinical signs or deaths were observed. The results showed that no virulence reversion of pass 74 of IBV 2575/98 occurred in chickens in these five passages. In the sequence alignment, no nucleotide mutation was observed in the sequence of rC2U-rC3L region or of NP1/NP2 region after five passages in chickens (data not shown). Although this vaccine was developed for commercial use, we have not tested it experimentally in the field, nor tested it for the effectively minimal dosage.

Acknowledgements

We thank Mr. Chia-Ju Chung and Mr. Feng-Yi Heich for technical help of the project. The financial support of the National Science Council and Council of
Agriculture, Taiwan is greatly appreciated.

References


Fig. 1. Phylogenetic tree describing the relationships between nucleotide sequences of rC2U-rC3L regions of the S1 gene of infectious bronchitis viruses (IBV) variants isolated in Taiwan and published data. Interior branch values represent the percent
occurrence of the clade per 3,000 bootstrap replicates. TW I = Taiwan group I, TW II = Taiwan group II, A = American group, E = Europe group, M = Massachusetts group.
**Fig. 2.** Reverse transcriptase-polymerase chain reaction (RT-PCR) of the rC2U-rC3L region and the NP1-NP2 region from passage 74 of infectious bronchitis virus (IBV) 2575/98. 1: = 100 bp molecular weight marker (GeneCraft); 2 = rC2U-rC3L region (219 bp); 3 = NP1-NP2 region (619 bp).
Table 1. Comparison of the deduced amino acid sequences of S1 glycoprotein gene of infectious bronchitis viruses (IBV) before and after high numbers of passages.

<table>
<thead>
<tr>
<th>IBV</th>
<th>Passage</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number</td>
<td></td>
</tr>
<tr>
<td>2575/98</td>
<td>5</td>
<td>P A</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>T S</td>
</tr>
<tr>
<td>2296/95</td>
<td>7</td>
<td>N L S S H S</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>Y F P A Y C</td>
</tr>
</tbody>
</table>
Table 2. Infectious bronchitis virus (IBV) detection by reverse transcriptase-polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Group</th>
<th>Chick number</th>
<th>Virus isolation&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2575/98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>X</td>
</tr>
<tr>
<td>2296/95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>X</td>
</tr>
</tbody>
</table>

<sup>a</sup>Chicks were vaccinated intranasally with passage 74 of IBV 2575/98 or passage 76 of IBV 2296/95. Twenty-one days postvaccination, the birds were challenged intranasally with at least $10^{3.5} \text{EID}_{50}/0.1 \text{mL}$ of pathogenic IBV 2993/02 or passage 4 of IBV 2296/95. All of the birds were killed and necropsied at 7 days postchallenge. At necropsy, the kidneys and tracheas were collected for virus detection by reverse transcriptase polymerase chain reaction (RT-PCR) and for virus isolation.

<sup>b</sup>Significantly different from the control, $P < 0.01$.

<sup>c</sup>X = not done.

<sup>d</sup>Individual kidney and trachea material inoculated into 9 to 11-day-old embryonated SPF chicken eggs and examined for virus by RT-PCR.
### Table 3. Neutralization index (NI) for antibody titre test

<table>
<thead>
<tr>
<th>Serum</th>
<th>Virus dilution (log(_{10}))</th>
<th>Log(<em>{10}) EID(</em>{50})^d</th>
<th>NI^e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2</td>
<td>-3</td>
<td>-4</td>
</tr>
<tr>
<td>2575/98^b</td>
<td>0/5^c</td>
<td>1/4</td>
<td>1/5</td>
</tr>
<tr>
<td>Control^b</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>2296/95^b</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Control^b</td>
<td>3/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

^aTitre calculated by method of Reed & Muench [6].

^bSera collected 21 days postvaccination were neutralized with challenge virus (IBV 2993/02 for the IBV 2575/98 group or passage 4 of IBV 2296/95 in the IBV 2296/95 group), and then the mixture was inoculated into 8-day-old specific pathogen-free embryonated eggs for calculation of the neutralization index (NI).

^cValues in body of table are number infected/number inoculated.

^dEID\(_{50}\) = median embryo infective dose.

^eNI = virus titre – antibody titre. The virus titre in the IBV 2575/98 group was 7.4, and for the IBV 2296/95 group, it was 9.7.