Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Vaccine 24 (2006) 785–791
Vaccine
www.elsevier.com/locate/vaccine

Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains

Yuan-Pin Huang, Ching-Ho Wang *

Department of Veterinary Medicine, National Taiwan University, No 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan

Received 16 June 2004; received in revised form 31 January 2005; accepted 12 August 2005
Available online 7 September 2005

Abstract

Due to variations in serotypes among different strains of avian infectious bronchitis viruses (IBV), vaccination of chicks with 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 have potential for controlling local Taiwanese IBV infections in chickens.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Attenuated vaccine; Infectious bronchitis virus

1. Introduction

Avian infectious bronchitis virus (IBV) is a member of the Coronaviridae, which is a diverse family of enveloped, positive-strand RNA viruses that cause intestinal and respiratory diseases. IBV is in Group 3 of the *Coronavirus* genus, and the other two groups comprise mammalian coronaviruses that differ extensively from IBV [8]. Besides the three groups, a novel coronaviruses (SARS-CoV) caused severe acute respiratory syndrome (SARS) emerged in humans in Guangdong Province, China, during November 2002, and globally disseminated within months. SARS-CoV appears to have arisen as a result of the zoonotic transmission of an animal coronavirus to humans, and patients often have evidence of multiple organ dysfunction and lower respiratory tract infection [15,20].

Infectious bronchitis (IB) has been identified in many parts of the world and is an economically important disease of chickens [8,24]. 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 [8,10]. 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 [25,26]. 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 [3,9,17], and emergence of new variant strains occurs frequently.

The S1 subunit is required to induce protective immunity, and small differences in S1 contribute to poor cross-protection [6,7]. Besides the S1 subunit, nucleocapsid protein primes protective immune responses in the chicken [1].

Because immunization of chickens with inactivated virus is less effective than that with live virus in spite of using a

* Corresponding author. Tel.: +886 2 2369 0628; fax: +886 2 2363 1542.
E-mail address: chingho@ntu.edu.tw (C.-H. Wang).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.08.081

considerably larger amount of antigen [13], and single applications of inactivated virus induces little or no protection against egg loss. Though two applications of inactivated IBV are much more efficacious, but this is not a commercial way in the poultry industry [2,18]. Because live vaccines are cheaper to make, and easier to apply, the poultry industry prefers to use live vaccines rather than inactivated ones.

Therefore, to develop attenuated 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 [4].

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 [12,23], and in the N gene, named the NP1–NP2 region (designed by Dr. PC Chang, National Chung Hsing University, Taiwan), 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 [27]. Molecular evolutionary analyses were conducted using the computer program *MEGA* version 2.1 [16]. 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 min 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 s. The mixture was incubated for 3 min at room temperature and centrifuged at 12,000 × *g* for 15 min 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 min at room temperature. To precipitate viral RNA, the mixture was centrifuged at 12,000 × *g* for 10 min 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 7500 × *g* for 5 min at 4 °C, the supernatant was removed. The RNA pellet was dried in a vacuum desecrator for 3–5 min. 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 10× 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 pmol/μL upstream primer, 0.5 μL of 50 pmol/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 min. PCR was then performed for 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and polymerization at 72 °C for 1 min and 30 s. The initial denaturation step was conducted at 94 °C for 3 min, and the final polymerization step was at 72 °C for 10 min. 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 (GenBank accession numbers: AY606314 for S1 and AY606327 for N), 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 (GenBank accession numbers: AY606316 for S1 and AY606329 for N), 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 (GenBank accession numbers: AY606321 for S1 and AY606334 for N), 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 9–11-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 h at 37 °C, and the allantoic fluid was harvested for subsequent passage. Eggs that died within 24 h 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 [21].

2.6. Safety

Safety testing was conducted according to the Standard Requirements for Animal Drugs [4], 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 9–11-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 min 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 18–24 h. 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 h 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 and N 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. The results showed that two point mutations caused amino acid substitutions of S1 glycoprotein gene in passage 74 of IBV 2575/98 and six substitutions in passage 76 of IBV 2296/95, but no point mutation was found in N gene after high number of passages (Table 1).

3.2. Attenuation

At every 10th passage, the virus was examined by RT-PCR for the presence of virus. 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 1×10^{7} EID_{50}/0.1 mL for passage 65 of IBV 2575/98 and $1 \times 10^{7.4}$ EID_{50}/0.1 mL

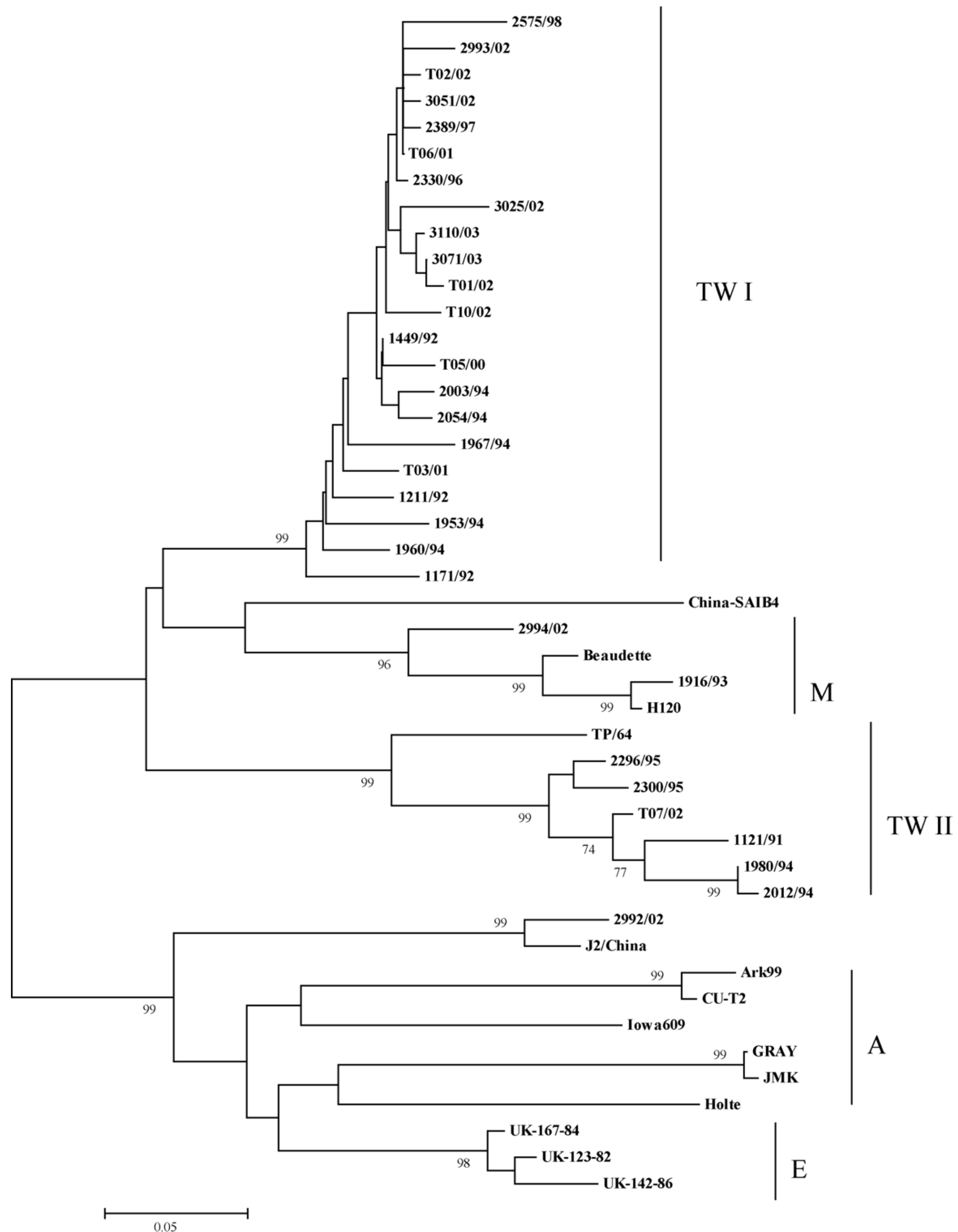

Fig. 1. Phylogenetic tree on nucleotide sequences of rC2U–rC3L regions of the S1 gene of Taiwanese infectious bronchitis viruses (IBV) strains and published data. Interior branch values represent the percent occurrence of the clade per 3000 bootstrap replicates. TW I = Taiwan group I, TW II = Taiwan group II, A = American group, E = Europe group, M = Massachusetts group.

for passage 74 of IBV 2575/98, $1 \times 10^{9.7}$ EID_{50}/0.1 mL for passage 4 and passage 76 of IBV 2296/95.

In the pretest (data was not shown), 1-day-old birds given 1×10^{7} 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.

Table 1
Differences of the deduced amino acid sequences of S1 glycoprotein gene of infectious bronchitis viruses (IBV) before and after high numbers of passages[a]

IBV	Passage number	Position	
		56	94
2575/98	5	P	A
	74	T	S

IBV	Passage number	Position					
		47	56	63	117	131	161
2296/95	7	N	L	S	S	H	S
	76	Y	F	P	A	Y	C

[a] No point mutation in N gene was found after high numbers of passages.

3.3. Safety

At least 10 7-day-old SPF chicks were given $1 \times 10^{4.5}$ EID_{50}/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 $\log_{10}EID_{50}$, in group 2, it was greater than 7 $\log_{10}EID_{50}$, and in group 3, it was 7.4 $\log_{10}EID_{50}$. 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 $\log_{10}EID_{50}$, in group 2, it was 7 $\log_{10}EID_{50}$, and in group 3, it was 9.7 $\log_{10}EID_{50}$. 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).

Table 2
Infectious bronchitis virus (IBV) detection by reverse transcriptase-polymerase chain reaction (RT-PCR)

Group[a]	RT-PCR[c]	Virus isolation
2575/98	0/10[b]	1/10
Control	9/10	8/10
2296/95	0/13[b]	1/13
Control	14/14	8/14

[a] 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 $10^{3.5}$ EID_{50}/0.1 mL of pathogenic IBV 2993/02 or passage 4 of IBV 2296/95. All of the birds were killed and necropsied at 7 days postchallenge.
[b] Significantly different from the control, $P < 0.01$.
[c] At necropsy, the kidneys and tracheas were collected for virus detection by reverse transcriptase polymerase chain reaction (RT-PCR) and for virus isolation.

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.

Table 3
Neutralization index (NI) for antibody titre test[a]

Serum	$\log_{10} EID_{50}$[c]	NI[d]
2575/98[b]	<3	>4.4
Control[b]	>7	<0.4
2296/95[b]	<3	>6.7
Control[b]	7	2.7

[a] Titre calculated by method of Reed and Muench [6].
[b] Sera 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).
[c] EID_{50} = median embryo infective dose.
[d] NI = 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.

4. Discussion

According to the phylogenetic tree (Fig. 1), we know that various differences between Taiwanese strains and foreign strains exist. The phylogenetic tree also shows that most local strains are in Taiwanese clusters, developing attenuated vaccines from local strains is necessary for IBV control in Taiwan. Though live attenuated vaccines reverts to pathogenicity, and there is a potential for the formation of variant viruses by recombinant with field strains, but these vaccines induce good immunity responses [5,11], and fortunately no recombination was found between H120 and the Taiwanese strains in the S1 gene [12]. 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 and/or N gene. Another possibility is that the attenuation might be related to many factors, and not limited to specific loci in the S1 subunit and/or N gene. However, since the S1 subunit is known to contain regions related to viral neutralizing, serotype-specific, and haemagglutination-inhibiting antibodies [14,19], 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 [22].

In the Standard Requirements for Animal Drugs [4], 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, because there is a potential for the formation of variant viruses by recombinant with field strains. In this study we detected IBV in homogenates in the birds inoculated with passage 74 of IBV 2575/98, but no IBV was detected in 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). Since this vaccine was developed for commercial interests in controlling IBV in Taiwan, the next steps will be to determine its efficacy in the field and the effective minimal dose.

Acknowledgements

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

References

[1] Boots AMH, Benaissa-Trouw BJ, Hesselink W, Rijke E, Schrier C, Hensen EJ. Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein. Vaccine 1992;10:119–24.

[2] Box PG, Beresford AV, Robert B. Protection of laying hens against infectious bronchitis with inactivated emulsion vaccines. Vet Rec 1980;106:264–8.

[3] Arvidson Y, Tannock GA, Zerbes M. Efficacy of Australian vaccines against recent isolates of avian infectious bronchitis viruses. Aust Vet J 1991;68:211–2.

[4] Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan. Standard Requirements for Animal Drugs. Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, ExecutiveYuan, Taipei, 2001.

[5] Cavanagh D. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol 2003;32:567–82.

[6] Cavanagh D, Davis PJ, Darbyshire JH, Peters RW. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralising or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J Gen Virol 1986;67:1435–42.

[7] Cavanagh D, Ellis MM, Cook JKA. Relationship between variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection. Avian Pathol 1997;26:63–74.

[8] Cavanagh D, Naqi SA. Infectious bronchitis. In: Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE, editors. Diseases of poultry. 11th ed. Ames, Iowa: Iowa State University Press; 2003. p. 101–19.

[9] Cook JKA, Chesher J, Baxendale W, Greenwood N, Huggins MB, Orbell SJ. Protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus. Avian Pathol 2001;30: 423–6.

[10] EI-Houadfi M, Jones RC. Isolation of avian infectious bronchitis viruses in Morocco, including an enterotropic variant. Vet Rec 1985; 116:445.

[11] Gelb Jr J, Perkins BE, Rosenberger JK, Allen PH. Serologic and cross-protection studies with several infectious bronchitis virus isolates from Delmarva-reared broiler chickens. Avian Dis 1981;25: 655–66.

[12] Huang YP, Lee HC, Cheng M, Wang CH. S1 and N Gene Analysis of avian infectious bronchitis viruses in Taiwan. Avian Dis 2004;48:581–9.

[13] Ignjatovic J, Galli L. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch Virol 1994;138:117–34.

[14] Koch G, Hartog L, Kant A, van Roozelaar DJ. Antigenic domains on the peplomer protein of avian infectious bronchitis virus; correlation with biological functions. J Gen Virol 1990;74:1929–35.

[15] Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al., SARS Working Group. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003;348:1953–66.

[16] Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17:1244–5.

[17] Lu YS, Shieh HK, Tsai HJ, Lin DF, Lee YL. The incidence and virus isolation of infectious bronchitis in chickens in Taiwan. J Chin Soc Vet Sci 1993;19:119–29.

[18] Muneer MA, Newman JA, Halvorson DA, Sivanandan V, Coon CN. Effects of avian infectious bronchitis virus (Arkansas strain) on vaccinated laying chickens. Avian Dis 1987;31:820–8.

[19] Niesters H, Bleumink PN, Osterhaus A, Horzinek MC, van der Zeijst BAM. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology 1989;161:511–9.

[20] Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, et al., SARS Study Group. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 2003;361:1319–25.

[21] Reed LJ, Muench H. A simple method of estimating fifty percent end points. Am J Hyg 1938;27:493–6.

[22] Seo SH, Collisson EW. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J Virol 1997;71:5173–7.

[23] Tseng ZM. Analysis of nucleotide and preparation of inactivated vaccine of infectious bronchitis virus isolated in Taiwan. In: Master thesis, National Chung-Hsing University, Taichung, Taiwan, 1999:13.

[24] Tseng CC, Li NZ, Yao CH, Wang CH. Isolation and adaptation of infectious bronchitis virus in Taiwan from 1993 to 1995. J Chin Soc Vet Sci 1996;22:113–20.

[25] Wang CH, Huang YC. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch Virol 2000;145:291–300.

[26] Wang CH, Tsai CT. Genetic grouping for the isolates of infectious bronchitis virus in Taiwan. Arch Virol 1996;141:1677–8.

[27] Wang L, Junker D, Hock L, Ebiary E, Collison EW. Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. Virus Res 1994;34:327–38.