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Vaccination against Very Virulent Infectious Bursal Disease Virus Using Recombinant T4 Bacteriophage Displaying Viral Protein VP2

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Abstract In order to develop a desirable inexpensive, effective and safe vaccine against the very virulent infectious bursal disease virus (vvIBDV), we tried to take advantage of the emerging T4 bacteriophage surface protein display system. The major immunogen protein VP2 from the vvIBDV strain HK46 was fused to the nonessential T4 phage surface capsid protein, a small outer capsid (SOC) protein, resulting in the 49 kDa SOC-VP2 fusion protein, which was verified by sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blot. Immunoelectromicroscopy showed that the recombinant VP2 protein was successfully displayed on the surface of the T4 phage. The recombinant VP2 protein is antigenic and showed reactivities to various monoclonal antibodies (mAbs) against IBDV, whereas the wild-type phage T4 could not react to any mAb. In addition, the recombinant VP2 protein is immunogenic and elicited specific antibodies in immunized specific pathogen free (SPF) chickens. More significantly, immunization of SPF chickens with the recombinant T4-VP2 phage protected them from infection by the vvIBDV strain HK46. When challenged with the vvIBDV strain HK46 at a dose of 100 of 50% lethal dose (LD_{50}) per chicken 4 weeks after the booster was given, the group vaccinated with the T4-VP2 recombinant phage showed no clinical signs of disease or death, whereas the unvaccinated group and the group vaccinated with the wild-type T4 phage exhibited 100% clinical signs of disease and bursal damages, and 30%–40% mortality. Collectively, the data herein showed that the T4-displayed VP2 protein might be an inexpensive, effective and safe vaccine candidate against vvIBDV.

Key words infectious bursal disease virus; VP2; T4 bacteriophage; vaccination

Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family, the genome of which consists of two segments (A and B) of double-stranded RNA [1]. Segment B (approximately 2.8 kb) encodes VP1, a 90 kDa protein with RNA polymerase and capping enzyme activities [2]. Segment A (approximately 3.2 kb) encodes a polyprotein that can be cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 [3]. IBDV particles have non-enveloped, icoasahedral capsids with a diameter of approximately 60 nm. The structure of the virus is based on the T=13 lattice and the capsid subunits are predominantly trimer clustered [4]. VP2 and VP3 form the outer and inner capsid of the virus, respectively [4,5]. The antigenic site responsible for the induction of neutralizing antibodies is highly conformation-dependent and located on VP2 [6].

IBDV multiplies rapidly in developing B lymphocytes in the bursa of Fabricius, and causes infectious bursal disease (IBD) in chickens, as well as other diseases, by immunosuppression [7,8]. Control of IBD in young chickens had been primarily achieved by vaccination with a live attenuated strain of IBDV at the age of 0 to 5 weeks or by transferring high levels of maternal antibody induced by the administration of live and killed IBD vaccines to breeder

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hens [8]. However, since the late 1980s, the emergence of very virulent IBDV (vvIBDV) has rendered IBD even more difficult to control [9–12]. vvIBDV showed antigenicity similar to the classical strains [10], and can break through high levels of maternal antibody and cause 60%–100% mortality in specific pathogen free (SPF) chickens. More challengingly, vaccines against vvIBDV will induce pathogenic effects [13]. Recently, *in vivo* studies showed that vvIBDV adapted to chicken embryo cell cultures using site-directed mutagenesis and a reverse genetics approach was partially attenuated [14]. It is apparent that the risk of reversion of the partially attenuated vvIBDV to wild-type vvIBDV limits its application as a potential live vaccine.

A subunit vaccine overcomes the risk of reversion. As the main host protective antigen harboring most of the neutralization sites, VP2 has been used for the development of subunit vaccines in the following schemes.

(1) Immunization of chickens with the expression vector that *in vivo* expresses VP2 continuously, for example, avian herpesvirus vector [15], fowlpox virus vector [16], fowl adenovirus vector [17], Marek's disease virus vector [18] and Semliki Forest virus vector [19]. However, this method is costly and complicated.

(2) Expression of VP2 as the recombinant protein in systems including *Escherichia coli* [20], yeast [21] and insect cells [22]. However, the recombinant VP2 expressed in *E. coli* failed to provide full protection upon challenge [20]. Although the VP2 expressed in yeast and insect cells proved to be effective, the cost is also very high.

(3) Production of virus-like particles of IBDV. Virus-like particles resemble the native viral capsids structurally and immunologically and elicit excellent antibody responses [23], but the cost of production is very high.

The T4 bacteriophage surface protein display system enables the fusion of foreign proteins with the C terminal of small outer capsid (SOC) proteins and the N terminal of highly antigenic outer capsid (HOC) proteins, so that foreign proteins can be displayed on the surface of the T4 phage [24]. In addition to advantages possessed by other phage display systems, this system features large capacity, high copy numbers of interesting antigens, and assemblage of the T4 phage inside the host cell without recourse to a secretory pathway. The system has been applied to display several peptides, including a 271-amino acid heavy and light chain fused IgG anti-egg white lysozyme antibody [24], the CD4 receptor of a 183-amino acid HIV-1 [25] and a 36-amino acid PorA peptide from *Neisseria meningitides* [26]. However, it has been a challenge to display any protein or polypeptide with more than 400 amino acid residues.

HK46 is a strain of vvIBDV isolated from southern China [10]. We have reported the full-length cDNA sequence of the vvIBDV HK46 strain [10,27].

In this study, VP2 of the vvIBDV HK46 strain consisting of 441 amino acids was successfully expressed using the T4 phage surface protein display system. The recombinant VP2 maintains its immunogenicity and antigenicity, and renders full protection of SPF chickens against the challenge of the vvIBDV HK 46 strain.

Materials and Methods

Plasmids, bacterial strains, T4 phage and IBDV

The integrative plasmid pR and phage T4-Z1 were kindly provided by Dr. Zhaojun REN (Department of Biological Chemistry, School of Medicine, University of Maryland, Baltimore, USA) [24] and maintained at the Animal Genetic Engineering Laboratory (South China Agricultural University, Guangzhou, China). Phage T4-Z1 is a lysozyme-dependent *SOC* gene-deleted mutant of the T4 phage. The integrative plasmid pR contains the *SOC* gene, part of the lysozyme gene upstream of *SOC*, and part of the *denV* gene downstream of *SOC*. The partial lysozyme gene and *denV* were used for homologous recombination with T4-Z1. Foreign genes were inserted into the 3′ terminus of the *SOC* gene at the *Eco*RI site. The HK46 strain was propagated in 5-week old SPF chickens.

Amplification of *VP2* cDNA

Segment A of the HK46 strain has been cloned into the plasmid pAlter-FA [27]. Primers VP2-S/VP2-A were employed to amplify the cDNA of the *VP2* gene by polymerase chain reaction (PCR) from the plasmid pAlter-FA. The primer VP2-S (5′-TGAA*GAATTC*TATGACGAACCTGCAA-3′) was synthesized according to nucleotide 131–145 of segment A and contained an *Eco*RI restriction site in italic. The primer VP2-A (5′-ATTT*GAATTC*CTA**TAG**TGCCCGAATTATGTCCTT-3′) was synthesized according to nucleotide 1463–1480 of segment A and contained an *Eco*RI restriction site in italic and a TAG termination codon in bold. The length of the amplified *VP2* cDNA was expected to be 1350 bp.

Construction and amplification of the recombinant plasmid

According to Ren *et al.* [24], the *VP2* cDNA was digested by *Eco*RI and ligated to a CIAP-treated pR plasmid by T4 DNA ligase, resulting in the recombinant

plasmid pR-VP2, in which the *VP2* cDNA was fused to the 3′ terminus of the *SOC* gene to produce the fusion protein SOC-VP2, with a molecular weight of approximately 49 kDa. The recombinant plasmid pR-VP2 was directly used to transform DH5α using the $CaCl_2$ procedure. Then 10 single ampicillin-resistant colonies were picked and cultured in Lauria broth with ampicillin (LA) medium at 37 ºC for 3 h, and the positive transformants were screened by PCR and designated as E-VP2. In addition, primers SOC-S (5′-GAATCATATGGCTAGTCTCGCGG-3′)/VP2-A were used to amplify the *SOC-VP2* fusion fragment with a length of approximately 1600 bp. The recombinant plasmid was isolated from the positive transformants using the ENZA plasmid miniprep kit (Omega Bio-Tek, Doraville, USA). The DNA sequence of *VP2* was verified by direct sequencing.

Preparation of recombinant T4-VP2

Ten microliters of *E. coli* E-VP2 was inoculated into 500 ml of CM medium (10 mg/ml trypone, 5 mg/ml NaCl) [25] containing 2 μg/ml ampicillin, and incubated at 37 ºC until A_{600}=0.5. Then 50 μl T4-Z1 was added into the culture and incubated at 200 *g* and 35 ºC until the appearance of bacterial fragments. The top layer of CM medium stored at 4 ºC was melted in a microwave oven and warmed to approximately 45 ºC. Then 2 ml of the medium without lysozyme was poured into a sterilized test tube containing 600 μl culture medium. The mixture was blended uniformly in a vortex device, and immediately poured onto a solidified Petri agar plate at 10 μl/plate. The agar plate was incubated overnight at 37 ºC.

If homologous recombination occurred between the plasmid pR-VP2 and T4-Z1, the *VP2* cDNA would be integrated into the genome of T4-Z1, resulting in plaques in the media without lysozyme. Plaques were picked with sterilized toothpicks and inoculated onto the bottom layer of the CM media in a rosette pattern. After incubation at 37 ºC overnight, the rosettes had grown well. They were excised with a sterilized blade and immersed in phosphate-buffered saline (PBS) for 6 h.

Recombinant phages were screened by PCR. Using primers VP2-S/VP2-A, the *VP2* fragment was amplified from the immersion fluid of the positive rosette-shaped plaques. The verified recombinant phage containing the *VP2* cDNA was designated as T4-VP2.

SDS-PAGE and Western blot analysis of recombinant VP2 protein

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out when the phage concentration reached 10^{10} phages/ml. After SDS-PAGE analysis, the recombinant VP2 was verified by Western blot with the monoclonal antibody (mAb) R63 raised against the vaccine of the D78 strain (ATCC VR-2047) by Snyder *et al*. [28].

Immunoelectronmicroscopy of T4 and T4-VP2 phages

Wild-type T4 and recombinant T4-VP2 phages were separately mixed with mAb R63 and incubated at 37 ºC for 30 min before direct smears. After negative staining with phosphotungstic acid, the specimens were observed under a JEM-1010 model transmission electron microscope (JEOL, Tokyo, Japan).

Antigen capture enzyme-linked immunosorbent assay (AC-ELISA) detection of VP2 expression on T4-VP2

Four mAbs (3-1, 9-6, 17-82 and 39A) raised against the virulent strain 002/73 of IBDV by Fahey *et al.* [29], three mAbs (B29, R63 and R69) raised against the vaccine of the D78 strain by Snyder *et al.* [28] and chicken sera against the HK46 strain of vvIBDV were used in AC-ELISA to examine their immunoreactivities to the recombinant phage T4-VP2, wild-type phage T4, and the HK46 and D78 strains. The AC-ELISA procedure was performed as reported previously [27].

Vaccination of chickens with T4-VP2

Thirty-day old white leghorn chickens were randomly divided into three groups, each with 10 chickens. The chickens were raised in isolators with sterilized water and feed. Group 1 was inoculated subcutaneously with T4-VP2 phage oil emulsion vaccine at day 14 postnatal, and given a booster with the same vaccine intramuscularly at day 28 postnatal. Group 2 and group 3 were immunized with wild-type T4 phage and saline oil emulsion vaccines respectively. Each immunizing dose with phages for one chicken was about 2×10^9 phages. Another group of 8 chickens was the negative control, in which birds were not vaccinated or challenged. Blood was collected and allowed to coagulate naturally. The serum was separated by the conventional method and stored at –20 ºC. A commercial IBD ELISA kit (IDEXX, Westbrook, USA) was used to assess the IBD antibody in the sera. At day 56 postnatal, each group was infected by the HK46 strain of vvIBDV at a dose of 100 of 50% lethal dose (LD_{50}) per chicken. The number of sick and dead birds was recorded 7 d post-infection. Each living bird in every group was weighed and killed. The bursa of each chicken was also weighed. The body weight/bursa weight was used to

calculate the B/B value. The bursa index accounted for the ratio of the B/B value of the testing group and that of the control group.

Results

Integration of HK46 *VP2* cDNA into T4-Z1 phage

The *VP2* cDNA was approximately 1350 bp in length. Primers VP2-S and VP2-A, corresponding to the 5′ end and the 3′ end of the *VP2* cDNA, respectively, were employed to amplify the *VP2* cDNA from a plasmid containing segment A of the vvIBDV strain HK46. In order to facilitate the subsequent cloning into the integrative plasmid, an *Eco*RI restriction site was incorporated into each primer. The amplified *VP2* cDNA was cloned into the integrative plasmid pR to form pR-VP2, which was amplified in bacteria.

To express *VP2* in the T4-VP2 phage, correct orientation within the pR-VP2 was needed. The orientation of the *VP2* cDNA within the pR-VP2 was verified by either PCR or direct sequencing (data not shown). It is noted that for the verification by PCR, one primer SOC-S corresponding to the 5′ terminus sequence of the *SOC* gene of T4 phage was employed to amplify the SOC-VP2 fusion gene together with the other primer VP2-A corresponding to the 3′ terminus sequence of *VP2* cDNA. If the correct PCR product (1600 bp in our case) was obtained, it strongly suggested or even verified that the integrated *VP2* had the correct orientation.

The integration of the *VP2* cDNA in pR-VP2 into the T4-Z1 phage through homologous recombination was done following the procedure described in " Materials and Methods". The resultant T4-VP2 recombinant phage was selected and verified by either PCR or direct sequencing (data not shown).

VP2 expression in T4-VP2 recombinant phage

T4-VP2 recombinant phages and T4-Z1 mutant-type phages were enriched by dialysis and subjected to SDS-PAGE analysis. In our experiments, the titer of the phages reached over 10^{10} phages/ml. In the T4-VP2 phage, *VP2* is expressed as part of the SOC-VP2 fusion protein with a molecular weight of approximately 49 kDa. On the electrophoretogram, an extra band of approximately 49 kDa was visible in the T4-VP2 sample. Following SDS-PAGE, the proteins were transferred to a nitrocellulose membrane, which was subjected to Western blot with IBDV monoclonal antibody R63 against VP2. The band of approximately 49 kDa was specifically recognized by the R63 antibody (**Fig. 1**). It demonstrated that VP2 could be expressed in the T4 phage display system, even though the size of VP2 was larger than those peptides or proteins previously expressed.

VP2 expression on the surface of T4-VP2 recombinant phage

Reactivities of various mAbs and chicken sera to IBDV and phages are shown in **Table 1**. The D78 strain of IBDV

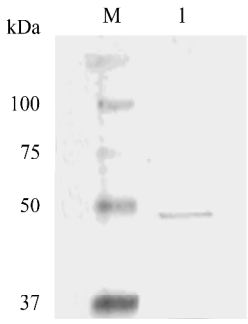

Fig. 1 Western blot analysis of recombinant phage T4-VP2
M, protein molecular weight marker; 1, recombinant phage T4-VP2.

Table 1 Reactivities of various antibodies to infectious bursal disease virus (IBDV) and phages

Antigen	Antibody							
	B29	B69	R63	3-1	9-6	17-82	39A	Anti-IBDV
IBDV D78 [a]	+	+	+	–	+	+	+	+
IBDV HK46 [b]	+	–	+	+	+	+	+	+
Recombinant phage T4-VP2	+	–	+	+	+	+	+	+
Wild-type phage T4	–	–	–	–	–	–	–	–

[a] supernatants from infected chicken embryonic fibroblasts were used as the source of antigen; [b] bursal homogenates from infected specific pathogen free chickens were used as the source of antigen.

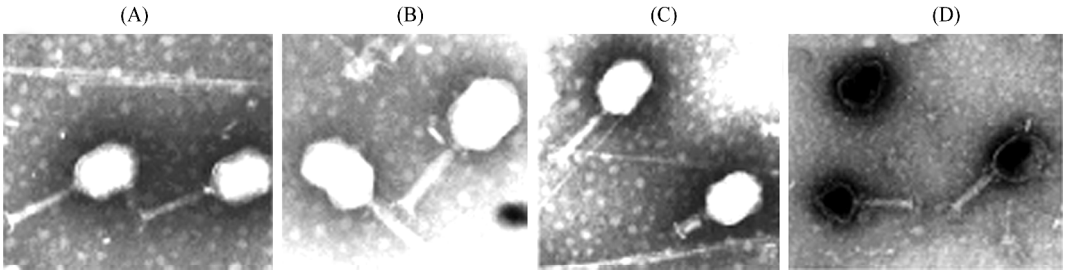

Fig. 2 Electron micrographs of wild-type T4 and recombinant T4-VP2 phages

(A) Wild-type T4 phage. (B) Wild-type T4 phage reacted with mAb R63. (C) T4-VP2 recombinant phage. (D) T4-VP2 recombinant phage reacted with mAb R63.

reacted to anti-sera and mAbs B29, B69, R63, 9-6, 17-82 and 39A, but not mAb 3-1. The HK46 strain of vvIBDV and recombinant phage T4-VP2, in which VP2 from the HK46 strain was expressed on the head of the phage, showed the same reactivities to anti-sera and various mAbs. Both of them showed reactivities to mAbs B29, R63, 3-1, 9-6, 17-82 and 39A, whereas the wild-type phage T4 could not react to any antibody against IBDV. The ELISA data demonstrated that the VP2 expressed on the surface of the T4-VP2 phages was antigenic.

Immunoelectronmicroscopy was employed to directly show whether the VP2 protein was expressed on the surface of the T4-VP2 recombinant phages. Under the transmission electron microscope, both the wild-type and T4-VP2 recombinant phages presented typical morphology, the heads and tails were distinctly visible in white (**Fig. 2**). When the T4-VP2 recombinant phages were stained with mAb R63, only the heads were positively stained by the antibody R63, as indicated by the increased electron density, which showed that VP2 was expressed on the surface of the T4-VP2 recombinant phages.

T4-VP2 recombinant phage as vaccine against vvIBDV

We tested whether the surface-expressed VP2 could be used as an effective vaccine against the HK46 strain of vvIBDV. White leghorn chickens were divided into three groups (10 chickens/group), immunized and boosted with T4-VP2 recombinant phage (Group 1), T4 wild-type phage (Group 2) or saline (Group 3). In another negative control group, chickens were not immunized or challenged.

The specific antibody of IBDV was detected with a commercial ELISA kit and quantified using A_{630}. A_{630} for the negative control serum is represented as N, A_{630} value for positive control sera as P, A_{630} value for the sera from any group as S, and the IBDV antibody level as S/P:

$$S/P=(S-N)/(P-N)$$

The IBDV antibody levels for the three groups over six consecutive weeks are summarized in **Table 2**.

As can be seen from **Table 2**, only Group 1 immunized with the T4-VP2 recombinant phage vaccine showed IBDV-specific antibodies. At the end of these experiments, when the chickens were 8 weeks old, the T4-VP2 vaccine elicited the specific antibodies of IBDV in about 90% of the positive control in the sera. As expected, the chickens in Group 2, immunized with the T4 wild-type phage, showed no IBDV-specific antibodies, and the same result was seen in Group 3.

To investigate whether the specific antibodies of IBDV induced by T4-VP2 were effective to protect the immunized chickens from infection by the HK46 strain of vvIBDV, all chickens from the three groups were

Table 2 IBDV antibody levels in specific pathogen free chickens after immunization with T4 phages

Group	Chicken age (week)					
	3	4	5	6	7	8
Group 1	0.1733±0.0689	0.3833±0.0371	0.4833±0.0393	0.5367±0.0233	0.7067±0.0384	0.9000±0.0173
Group 2	0.1123±0.0359	0.1247±0.0231	0.1447±0.0285	0.2022±0.0317	0.2578±0.0463	0.2365±0.0236
Group 3	0.1006±0.0231	0.1533±0.0145	0.1967±0.0296	0.1833±0.0176	0.2733±0.0318	0.2300±0.0569

Group 1, chickens were immunized with T4-VP2 recombinant phage; Group 2, chickens were immunized with T4 wild-type phage; Group 3, chickens were given saline. Specific antibody of IBDV in the sera was detected by ELISA, and expressed as relative values to the positive control. Data were expressed in mean±SD (n=10).

challenged with the HK46 strain at a dose of 100 of LD_{50} per bird 4 weeks after the booster was given. The health of all the challenged chickens was recorded 7 d post-infection and the results are summarized in **Table 3**. No chicken from the group vaccinated with T4-VP2 showed clinical signs of IBD. In contrast, all chickens from the Group 2, immunized with wild-type T4 phage, and Group 3, given saline, showed clinical signs of IBD. Four chickens in Group 2 and three in Group 3 died within the recording period. The apparently healthy chickens from the group immunized with the T4-VP2 showed variable signs of temporary bursa damages that were soon fully repaired. At day 7 post-infection with the HK46 strain, the bursa indexes of chickens immunized with T4-VP2, the wild-type T4 phage and saline were 0.93, 0.72 and 0.78 respectively (**Table 4**).

Table 3 Protective effect of immunization with recombinant phages to challenge against infectious bursal disease virus in specific pathogen free chickens

Group	Chicken (*n*)		
	Infected	Sick	Dead
Group 1	10	0	0
Group 2	10	6	4
Group 3	10	7	3

Group 1, immunized with recombinant phages; Group 2, immunized with wild-type phages; Group 3, given saline.

Table 4 Bursa indexes of chickens 7 d post-challenge with very virulent infectious bursal disease virus strain HK46

Group	Bursa versus body (g/kg) [a]	Bursa index
Group 1	2.58±0.24	–
Group 2	2.01±0.22	0.78
Group 3	1.85±0.14	0.72
Group 4	2.39±0.20	0.93

Group 1, negative control without vaccination, no challenge (*n*=8); Group 2, control without vaccination, challenged with HK46 strain (*n*=7); Group 3, immunized with wild-type T4 phage, challenged with HK46 strain (*n*=6); Group 4, immunized with T4-VP2 recombinant phage, challenged with HK46 strain (*n*=10). [a] data are represented as mean±SD.

Discussion

We have demonstrated here that the VP2 protein expressed on the surfaces of T4 phages maintained enough requisite conformational epitopes to elicit high humoral responses in immunized chickens and, more importantly, to effectively protect the immunized chickens from infection by vvIBDV.

The development of an inexpensive, effective and safe vaccine against vvIBDV has been an ongoing effort. When vvIBDV first appeared, the effective vaccines were partially attenuated vvIBDV. However, they carry the intrinsic hazard of being pathogenic due to incomplete inactivation of the virulent virus, or due to a reversal to virulence of the attenuated viruses. To overcome the shortcomings of whole virus-based vaccines, a variety of subunit vaccines based on VP2 have been reported [14–23].

VP2 is known to be the main host protective antigen. The antigenic epitopes located within VP2 are highly conformation-dependent and able to elicit serologically-specific neutralizing antibodies. In addition, the mature VP2 protein in the vvIBDV particle contains more than one glycoside by post-translational glycosylation. It has been assumed that proper glycosylation is required for maintaining the conformational epitopes of VP2. It is natural that most of the efforts on developing VP2-based vaccines have been focused on eukaryotic expression systems, for example, formation of virus-like particles in insect cells [23,30], expression of recombinant VP2 proteins in yeast [21] or insect cells [22], and expression of VP2 *in vivo* using different expression vectors [15–19]. However, none of these eukaryotic expression systems are practically applicable in developing countries because of their complexity and the high costs of production and administration. Nonetheless, it was reported that expression of VP2 together with VP3 and VP4 in *E. coli* produced viral particles. Even though the viral particles from *E. coli* only provided partial protection against infection by vvIBDV, it strongly suggested that certain conformational epitopes on VP2 proteins were properly maintained in the viral particles.

The T4 phage surface protein display system is able to display heterologous proteins as fusion proteins with its own SOC and HOC proteins on the surface of the phage particles. In consideration of the spatial limits for the display of foreign proteins on the surfaces of T4 particles, the sizes of the diversified peptides or proteins displayed are relatively small [24–26]. It is possible to suspect that a large foreign protein may destroy the configuration of the endogenous surface proteins, resulting in no production of T4 viral particles.

Our results have shown that the VP2 protein of 441 amino acids from the HK46 strain was successfully expressed on the surface of the T4 phages as the VP2-SOC fusion protein. Although the largest size of the for-

eign protein that could be displayed on the surface of T4 phages is not known, the expression of VP2 clearly demonstrated that the T4 display system has great elasticity in the accommodation of large size proteins. Our work opens the door for displaying large proteins on the T4 phages.

As discussed earlier, VP2 proteins undergo glycosylation during maturation. VP2 from the HK46 strain has three potential glycosylation sites. Even though VP2 proteins displayed on the T4 phages lack any glycosylation, they not only elicit a high level of specific antibodies for IBDV, but also provide effective protection against the challenge of the HK46 strain. We do not have data to compare the configuration of VP2 displayed on T4 phages with its configuration on native particles, but we have every reason to believe that the displayed VP2 proteins maintain their conformational epitopes. It is plausible that the maintenance of conformational epitopes may be due to the spatial constraints imposed on VP2 by SOC.

The VP2 displayed on the T4 phages as vaccines conferred full protection to the immunized birds. All the birds immunized with the T4-VP2 were free of clinical signs of disease or death. It is noted that an anatomical investigation revealed temporary bursal damages in the immunized birds. The bursal damages were repaired spontaneously shortly after infection. This system will be a useful tool in studying the molecular mechanisms that explain how vvIBDV causes bursal damages, clinical signs of disease or death.

In summary, we have developed an effective and safe VP2-based subunit vaccine against vvIBDV. The employment of the T4 display system provides various advantages, including inexpensive production, convenient storage and transportation, and easy administration. This inexpensive, effective and safe vaccine is particularly applicable to developing countries.

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