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Recombinant VP1 protein of duck hepatitis virus 1 expressed in *Pichia pastoris* and its immunogenicity in ducks

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Summary. – The VP1 gene of duck hepatitis virus type 1 (DHV-1) strain VJ09 was amplified by reverse transcription PCR from the liver of a duckling with clinical symptoms of viral hepatitis. The resulting VP1 cDNA was 720 bp in length and encoded a 240-amino-acid protein. In VP1 gene-based phylogenetic analysis, the VJ09 strain grouped with DHV-1 genotype C. The VP1 gene was inserted into the expression vector pPICZαA and expressed in *Pichia pastoris*. The expressed VP1 protein was purified and identified by western blot analysis. To evaluate the recombinant VP1's immunogenic potential in ducklings, the antibodies raised in the immunized ducklings were titrated by ELISA, and lymphocyte proliferation and virus neutralization assays were performed. The results show that the recombinant VP1 protein induced a significant immune response in ducklings and this could be a candidate for the development of a subunit vaccine against DHV-1 genotype C.

Keywords: duck hepatitis virus 1; genotype C; VP1 protein; Pichia pastoris; duck; immunogenicity

Introduction

Duck hepatitis is a highly fatal, contagious, and rapidly spreading viral infection of young ducklings, characterized primarily by hepatitis (Woolcock, 2003). It was first observed in Long Island, New York, in 1945 (Levine and Hofstad, 1945). The major pathologic change in infected ducklings is hepatitis (Levine and Fabricant, 1950). So far, three different viruses, duck hepatitis virus (DHV) types 1, 2 and 3, have been associated with these symptoms; all three viruses were originally classified as picornaviruses (Toth, 1970; Haider and Calnek, 1979). The more common and internationally widespread of the DHV types is duck hepatitis virus type 1 (DHV-1), which belongs to the new genus *Avihepatovirus* of the family *Picornaviridae.* DHV-1 causes a highly lethal acute infection in ducklings under 6 weeks of age, especially in those under 3 weeks of age, but does not affect older birds. The full genomes of DHV-1 strains have been sequenced and reported (Kim *et al*., 2006, 2007; Ding and Zhang, 2007; Tseng and Tsai, 2007a,b). The genome of DHV-1 is a single-stranded, polyadenylated, positive-sense RNA of approximately 7,800 nucleotides bound to a virus-encoded protein ($3B^{VPg}$). The single long open reading frame of the genome encodes a polyprotein of 2,249 amino acids, which is processed by viral proteases into mature viral polypeptides. The P1 region of the polyprotein produces the capsid proteins VP0, VP3, and VP1. The P2 and P3 regions give rise to nonstructural proteins, with P2 encompassing four protein motifs, 2A1, 2A2, 2B, and 2C (Kim *et al*., 2007; Tseng and Tsai, 2007b). In a recent study, Wang *et al.* (2008b) constructed phylogenetic trees from the nucleotide sequences of VP1, VP0, VP3 and partial 3D regions of 42 DHV-1 strains and demonstrated the existence of three distinct genetic

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Abbreviations: AOX1 = alcohol oxidase 1; CAV-9 = coxsackievirus A9; DHV = duck hepatitis virus; DHV-1 = duck hepatitis virus type 1; FMDV = foot-and-mouth disease virus

groups; the authors proposed that the three genetic groups should be termed DHV-1 types A, B, and C.

The major capsid protein VP1 is the most external and dominant of all picornaviral surface proteins and contains most of the motifs known to interact with cellular receptors and neutralizing monoclonal antibodies (Rossmann *et al*., 1985; Oberste *et al*., 2003). As such, VP1 has important biological functions in foot-and-mouth disease virus (FMDV) infection: it has B and T cell epitopes (Bittle *et al*., 1982; Parry *et al*., 1990; Collen *et al*., 1991; van Lierop *et al*., 1992) and is a major immunogenic antigen. The yeast *Pichia pastoris* is a widely used, efficient expression system for a wide variety of heterologous genes (Ning *et al*., 2005; Hu *et al*., 2006; Niu *et al*., 2008). *P. pastoris* offers three key advantages: (1) it has a tightly regulated alcohol oxidase 1 (AOX1) gene promoter capable of being repressed by glucose and glycerol and induced by methanol; (2) it cost-effectively yields a high cell density; and (3) its subcellular organelles, such as the endoplasmic reticulum and Golgi apparatus, allow for post-translational modifications such as folding, disulfide bridge formation, and glycosylation (Cereghino and Cregg, 2000; Cregg *et al*., 2000).

In this report, we describe the expression of a recombinant DHV-1 VP1 protein in *P. pastoris* and evaluate the protein's immunogenic potential in ducklings. To our knowledge, this is the first report of recombinant DHV-1 VP1 production in yeast cells.

Materials and Methods

Virus and antiserum. DHV-1 strain VJ09 was isolated in 2006 in Guangdong, China, from a 1-week-old duckling that displayed the typical clinical symptoms of duck viral hepatitis infection. To isolate the virus, the liver homogenate of the infected duckling was inoculated into the allantoic cavities of 10-day-old duck embryos. Embryos that died within 24 to 72 hr postinoculation and their allantoic fluids and allantoic membranes were harvested, homogenized, and clarified by centrifugation at 12,000 rpm for 10 min. The resulting supernatant was stored at -20°C. Hyperimmune serum against DHV-1 (VJ09 isolate) was produced in 4-week-old SPF chickens that were immunized intramuscularly with live DHV-1 (VJ09 isolate) at a dose of $1.5 \times 10^{8.3}$ ELD_{50} per bird twice with a 1-week interval; the chickens were bled for serum collection at 5 weeks of age. The antiserum was inactivated at 56°C for 30 min.

Plasmids, cells, and reagents. pPICZαA, *P. pastoris* wild-type strain X-33 (Mut^+), and *E. coli* strain DH5α were obtained from Invitrogen (Carlsbad, CA, USA). All restriction enzymes and rTaq polymerase were purchased from TakaRa Biotechnology (Dalian, China). RPMI 1640 medium and FBS were from Gibco (New York, NY, USA), and MTT and HRP-conjugated goat anti-duck IgG (H+L) from KPL (Gaithersburg, MD, USA).

RT-PCR. Total RNA was extracted from liver tissues using TRIZOL (Invitrogen). The RT-PCR primers for amplifying the DHV-1 VP1 gene were designed based on the complete genome sequence of DHV-1 strain C80 (GenBank Acc. No. DQ864514). The primers were 5'-CCAGAATTCGGTGATTCTAACCAG-3' (forward primer), which contains an *Eco*RI site (underlined), and 5'-GTTTCTAGATTCAATTTCCAG-3' (reverse primer), which contains an *Xba*I site (underlined). Reverse transcription was performed according to Invitrogen's instructions. The PCR consisted of 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified PCR products were cloned into the pMD-18T vector. The nucleotide sequence of the VP1 gene for DHV-1 isolate VJ09 was submitted to GenBank (Acc. No. EU289393). Phylogenetic trees were analyzed using DNAStar software.

Construction of recombinant expression vector and expression of DHV-1 VP1 in P. pastoris. The amplified DHV-1 VP1 gene fragment was ligated into pPICZαA, and the resulting recombinant expression vector was designated pPICZαA-VP1. Expression of recombinant proteins in *P. pastoris* X-33 was performed as previously described (Niu *et al*., 2008). Briefly, pPICZαA-VP1 was linearized with *Sac*I and transfected into competent *P. pastoris* X-33 cells by electroporation. All zeocin-resistant colonies were grown on YPDS plates. Mut^+ strains were obtained from MDH plates, and the gene inserts were verified by PCR. Transformed yeast cells harboring the expression plasmid X-33/pPICZαA-VP1 were cultured for about 20 hr in a shaking flask containing 50 ml of BMGY medium until reaching an A_{600} of 2.0–6.0. Cells were harvested by centrifugation at 2000 x g for 5 min at room temperature, and resuspended to an A_{600} of 1.0 in BMMY medium to induce recombinant protein expression. Methanol was added every 24 hr to the final concentration of 1.0% v/v to maintain induction. *P. pastoris* X-33/pPICZαA was used as a negative control. Various concentrations of methanol and culture medium pH were tested to obtain the optimal conditions for VP1 expression: 1.0% methanol, pH 6.0. The expressed VP1 protein was analyzed by 12% SDS-PAGE and identified by western blot analysis.

Purification of DHV-1 VP1. One hundred milliliters of the X-33/pPICZαA-VP1 cell culture medium described above was dialyzed overnight against 20 mmol/l PBS (pH 7.4) containing 300 mmol/l NaCl (buffer A). VP1 was purified from the resulting supernatant using a Ni-affinity chromatography column (HiTrap Chelating HP 5 ml × 1 column; Amersham Bioscience, Fairfield, CT, USA) according to the manufacturer's instructions. Briefly, the column was pre-equilibrated with buffer A, the dialyzed culture supernatant was loaded onto the column, and bound proteins were eluted with a gradient of 0.0–0.5 mol/l imidazole in buffer A. The eluted proteins were analyzed by SDS-PAGE. The fractions containing His-tagged VP1 were pooled, and dialyzed overnight against a solution containing 50 mmol/l potassium phosphate, 50 mmol/l NaCl, and 50% glycerol, pH 8.0. The protein concentrations of the samples were determined by the Bradford assay with BSA as a standard.

Immunization of ducklings. A set of 120 healthy 10-day-old ducklings was divided into four equally sized groups. Each animal was im-

munized with either 50 ppmol/l VP1 protein, 50 ppmol/l VP1 protein plus the adjuvant bursin, or an inactivated DHV-1 vaccine (DHV-1, VJ09 isolate, $10^{7.2}$ $TCID_{50}$/100 µl). One group was immunized with PBS as a control. Each immunogen was delivered subcutaneously three times at 2-week intervals in 0.1 ml of PBS. Blood was collected from five ducklings per group at 7, 21, and 35 days after inoculation. Serum was separated from whole blood, and serum antibody titers were measured. Ducklings were sacrificed to obtain spleens, from which single-cell suspensions were made to assay for lymphocyte proliferation.

ELISA of IgG antibodies. ELISA was performed as previously described (van Houten *et al.*, 2006). Briefly, 96-well microtiter plates were coated overnight with 10 µg/ml recombinant DHV-1 VP1, blocked with 1% BSA for 2 hr at 37°C, and then incubated for 2 hr at 37°C with duckling sera diluted at log intervals (1:10–$1:10^5$). Next, the plates were incubated for 1 hr at 37°C with 50 µl of 1:1000 diluted HRP-conjugated goat anti-duck IgG. TMB substrate was added, and the reaction was stopped by the addition of 2N H_2SO_4.

Lymphocyte proliferation assay. Immunogenicity was assayed by the *in vitro* proliferation ability of immunized splenocytes. Splenocytes recovered from sacrificed ducklings were incubated in 96-well flat-bottomed microtiter plates (100 µl per well of 4×10^6 cells/ml in RPMI 1640) and stimulated by the addition of 10 µg of DHV-1 VP1 protein. Five replicate wells per sample were prepared along with control wells without the VP1 protein. The cultures were incubated at 37°C and 5% (v/v) CO_2 for 2 days and then assayed by a standard MTT-based method with absorbance measurement at 570 nm (Takeuchi *et al.*, 1991)

Virus neutralization assay. On days 7, 21, and 35 after the prime immunization, serum neutralization tests were performed on duck embryonic fibroblasts by the constant virus–variable serum method (Sandhu *et al.*, 1992). Diluted ($1:2^1$–$1:2^6$) duckling sera were heat-inactivated at 56°C for 30 min and then incubated with 100 $TCID_{50}$ of DHV-1 (VJ09 isolate) at 37°C for 1 hr. The development of CPE was observed, and virus neutralization titers were calculated. The reciprocal value of the highest serum dilution giving at least 50% neutralization was taken as the virus neutralization titer.

Statistical analysis. Data were expressed as mean ± standard error. Bonferroni correction multiple comparison tests were used to establish any differences among the four experimental groups (three immunized groups and one control group). Differences between means were considered significant at $p \leq 0.05$.

Results

Expression of recombinant DHV-1 VP1 protein in P. pastoris and its purification

The VP1 gene of DHV-1 isolate VJ09 was amplified by RT-PCR and sequenced. The sequencing result confirmed that the gene contains 720 nucleotides and encodes a 240-amino-acid protein. Multiple alignment analysis showed that the most variable region is located in the C-terminus of VP1 (Fig. 1). To express the VP1 protein, the VP1 gene was amplified and fused in-frame to the α factor secretion signal of the yeast expression vector pPICZαA. After verification of correct insertion, the selected plasmid was transfected into *P. pastoris*. The presence of the recombinant DHV-1 VP1 gene in the transformants was confirmed by genomic PCR with 5' and 3' AOX primers. Positive clones yielded a DNA product of approximately 1,370 bp, which corresponds to the predicted size of the fusion gene (data not shown). Five *P. pastoris* transformants were randomly selected for small-

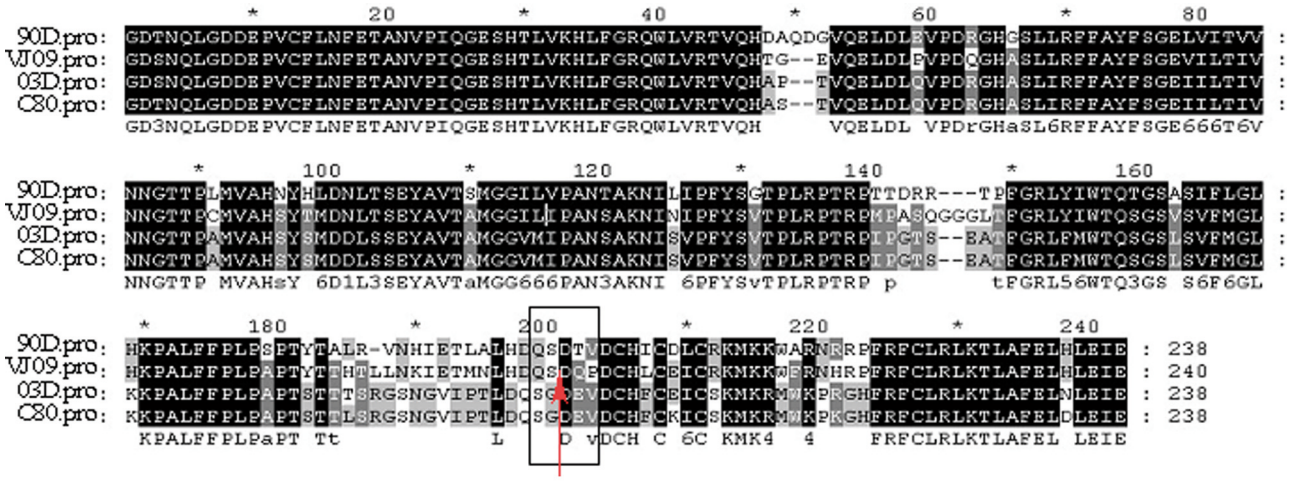

Fig. 1
Multiple alignment of amino acid sequence of VP1 protein of DHV-1 strains
Multiple alignment dendrogram was inferred from the complete VP1 sequences by the DNAstar computer program. Red arrows indicate SD motif.

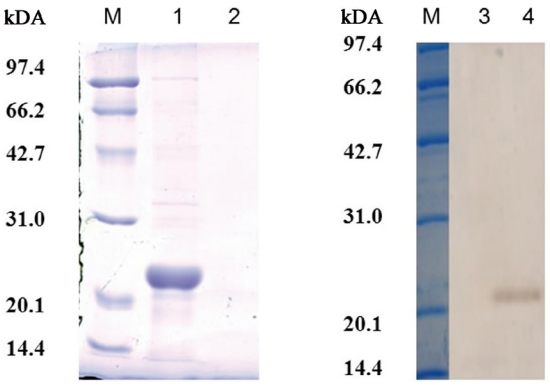

Fig. 2
SDS-PAGE (1–2) and Western blot analysis (3–4) of recombinant DHV-1 VP1 protein expressed in *P. pastoris*
Expression plasmid pPICZαA-VP1 containing VP1 gene (lanes 1, 4), empty expression plasmid pPICZαA, negative control (lanes 2, 3); protein size marker (lane M).

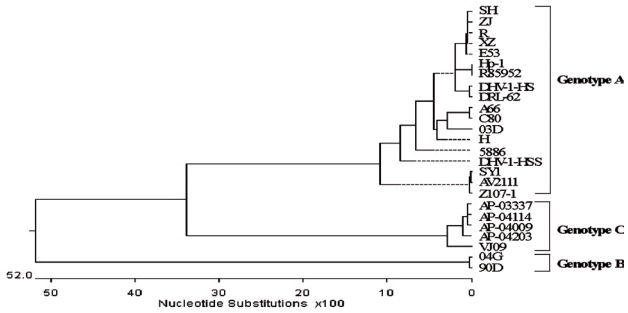

Fig. 3
VP1-based phylogenetic tree of DHV-1 strains
The phylogenetic tree was constructed for the complete VP1 sequence from the multiple alignments by maximum likelihood framework using the DNAstar software.

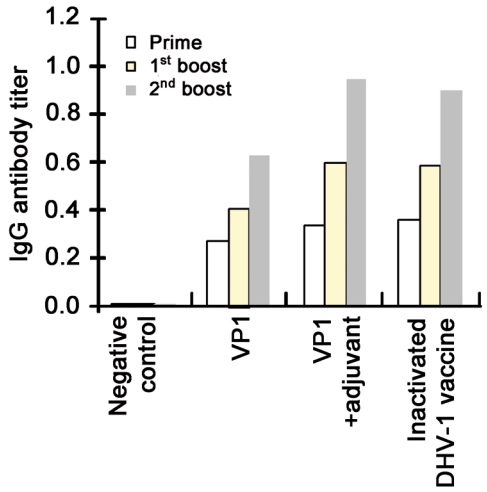

Fig. 4
Titers of IgG antibodies induced in ducklings by vaccination
Vaccination was carried out on days 0 (prime), 14 (1st boost) and 28 (2nd boost) and sera were collected for titration in ELISA 7 days later.

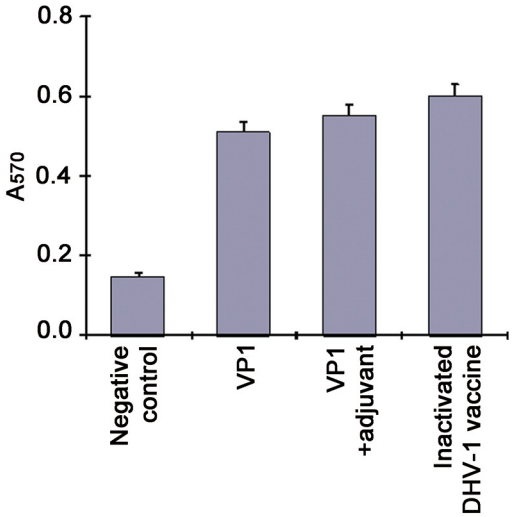

Fig. 5
Lymphocyte proliferation response induced in ducklings by vaccination
Data were expressed as the mean ± standard error. Statistically significant differences $p \leq 0.05$.

scale expression, and their culture media were collected and subjected to SDS-PAGE analysis. All transformants gave a major band with a M_r of approximately 25,000 (Fig. 2). The recombinant VP1 protein was purified by Ni-affinity chromatography. To confirm the identity of the secreted product, western blot analysis was performed using hyperimmune sera against the DHV-1 VJ09 isolate. The result indicated that the expressed and secreted VP1 protein had the specific antigenicity of DHV-1 (Fig. 2). The VP1-based phylogenetic tree of DHV-1 strains revealed that the VJ09 isolate grouped with DHV-1 genotype C (Fig. 3).

Immunogenicity of recombinant DHV-1 VP1 protein in ducklings

Next, we examined whether specific antibodies can be produced in ducklings immunized with the recombinant DHV-1 VP1 protein. The production of DHV-1 VP1-specific IgG antibodies in each of the immunized animal groups was monitored by ELISA. DHV-1 VP1-specific antibody responses were induced following the first vaccination and increased following the second boost vaccination (Fig. 4). The antibody titer of ducklings immunized with DHV-1

Table 1. Virus-neutralization titers of sera of immunized duckling

Immunization	Titer		
	prime	1st boost	2nd boost
VP1	2	8	32
VP1+ adjuvant	8	64	128
Inactived DHV-1 vaccine	4	32	64
Negative control	–	–	–

VP1 plus the adjuvant bursin was significantly higher than that of ducklings immunized with VP1 alone. Ducklings immunized with VP1 plus adjuvant produced almost the same antibody titer as ducklings immunized with the DHV-1 vaccine.

The level of lymphocyte proliferation varied among the animal groups (Fig. 5). The highest level occurred in the group immunized with the DHV-1 vaccine. VP1 plus adjuvant produced the second highest level, which was significantly higher than that observed in VP1-alone group. Little lymphocyte proliferation was induced in the PBS control group.

On days 7, 21, and 35 after the prime immunization, serum samples were taken from the ducklings and tested for DHV-1-neutralizing ability by the virus neutralization assay. The serum virus neutralization titer was higher for ducklings immunized with the inactivated DHV-1 vaccine than for ducklings immunized with VP1 alone (Table 1). However, immunization with VP1 combined with bursin produced the highest DHV-1-neutralizing antibody titer. The virus neutralization titer of the VP1-alone group was 8 after the second vaccination and increased following the third vaccination. These results show that the VP1 protein significantly increased the production of DHV-1-neutralizing antibodies.

Discussion

In this study, we isolated the VJ09 strain of DHV-1 in Guangdong, China, from a duckling. The VP1 sequencing result confirmed that the VP1 gene of the VJ09 isolate contains 720 nucleotides and encodes a 240-amino-acid protein. Multiple alignment analysis revealed the C-terminal region of the VP1 protein to be the most highly diverse region among different DHV-1 strains, suggesting that the VP1 C-terminus may represent an important immunogenic site. In addition, we identified a novel feature in the VP1 of the VJ09 isolate: that it lacks an RGD motif. The RGD motif is a consensus sequence in picornaviruses that mediates many cell-cell and microbe-host interactions (Joki-Korpela *et al.*, 2000; Boonyakiat *et al.*, 2001; Ito *et al.*, 2004). The RGD motif is involved in viral attachment through binding to the cellular integrin receptors. For coxsackievirus A9 (CAV-9), specific interactions between the RGD motif of the capsid protein VP1 and the $\alpha_v\beta_3$ integrin are involved in virus binding and entry into green monkey kidney cells and some other cell lines (Roivainen *et al.*, 1996). The RGD-recognizing $\alpha_v\beta_3$ integrin is known as the vitronectin receptor. The mutation of the RGD motif into SGD or RGSD would affect the interaction of the virion with the cellular receptor (Fry *et al.*, 2005; Rieder *et al.*, 2005). In this study, we did not find the consensus sequence RGD in the VP1 of the DHV-1 VJ09 isolate. However, an SD motif is present at amino acids 198–199 of the VP1, indicating that VJ09 DHV-1 may not bind to cellular integrin receptors through the RGD motif as do FMDV, CAV-9, and enterovirus 9 (EV-9).

Wang *et al.* (2008b) proposed that the genetic groups of DHV-1 be termed DHV-1 genotypes A, B, and C. The report also shows that strains of genotype A have circulated widely in China in recent years. Both genotypes A and B were found in Taiwan (Tseng and Tsai, 2007a,b; Tseng *et al.*, 2007), and genotypes A and C were isolated in South Korea (Kim *et al.*, 2006, 2007). In this study, analysis of VP1 genome alignments for 25 strains of DHV-1 revealed genomic patterns dividing the genomes into three genotypes. Genotype A consists of the deposited ATCC (American Type Culture Collection) strains DRL-62 and R85952; two Korean isolates, DHV-1-HS and DHV-1-HSS; three Taiwan strains, 5886, H, and 04G; and 11 recent mainland China isolates, C80, AV21111, SH, Z107-1, XZ, SY1, ZJ, A66, E53, R, and HP-1. Genotype B contains two Taiwan strains, 90D and 04G, isolated in 1990 and 2004, respectively. Genotype C consists of four South Korean isolates from 2003 and 2004 and the VJ09 strain analyzed in the present study, which was isolated in China in 2006. The above information suggests that different types of DHV-1 were co-circulating in China. Therefore, we think that the emergence and prevalence of genotype C DHV-1 may be the main reason for outbreaks of duck viral hepatitis in China in many ducklings already vaccinated with the traditional DHV-1 vaccine.

To investigate the immunogenicity of the VP1 of the VJ09 isolate, we used an efficient *P. pastoris* expression system to produce the recombinant VP1 protein and then evaluated the protein's potential to induce immune responses in ducklings. A yeast-based heterologous expression system was chosen for two reasons. First, it combines the advantages of prokaryotic expression systems (high expression levels, easy scale-up, and inexpensive growth medium) and eukaryotic expression systems (capacity to carry out most post-translational modifications). Second, it prevents the incomplete refolding of fusion proteins, which can substantially reduce immunogenicity (Yi *et al.*, 2004; Su *et al.*, 2007).

Subunit vaccines generally require the addition of an adjuvant to be effective. A previous study showed that bursin as an adjuvant is a potent immune response enhancer in mice immunized with a Japanese encephalitis virus subu-

nit vaccine and thus represents a promising adjuvant for vaccination (Wang *et al.,* 2008a). In the present study, we used bursin as an adjuvant for the DHV-1 VP1 subunit vaccine to enhance the induction of immune responses in ducklings upon vaccination with DHV-1 VP1 as an antigen. We evaluated the immunogenic potential of this antigen in ducklings through ELISA titration of the raised antibodies and lymphocyte proliferation and virus neutralization assays. Our results show that the VP1 protein elicited high level of antibodies and cellular immune response and thus could serve as a candidate for the development of a subunit vaccine against DHV-1 genotype C.

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