Expression and Immunological Studies of Classical Swine Fever Virus Glycoprotein E2 in the Bi-Cistronic Baculovirus/Larvae Expression System

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To develop an economical, easy technique for producing recombinant E2 glycoprotein (rE2) of classical swine fever virus (CSFV) as a candidate immunogen, a bi-cistronic baculovirus/larvae expression vector was constructed using p10 promoter, an internal ribosome entry site, and the gfp gene. Trichoplusia ni larvae were successfully infected with the occluded recombinant baculovirus via feed, and the characteristics of rE2 were confirmed by immunoblot and glycosylation stain. rE2 at a concentration of 0.6–0.8 mg/ml without degradation was obtained from hemolymphs of infected larvae that emitted high levels of green fluorescence. Immunization assays indicated that mice and piglets immunized with rE2-containing hemolymph elicited high titers of anti-CSFV E2 antibodies with virus-neutralizing activity. This is the first study to indicate that baculovirus/T. ni larvae-expressed rE2 can be served as a vaccine candidate. This system provides an economical alternative for the production of vaccine components in the veterinary industry.

Key words: baculovirus; bi-cistronic; classical swine fever virus (CSFV) E2 glycoprotein; green fluorescent protein (GFP); Trichoplusia ni larvae

Classical swine fever (CSF) is a highly contagious, economically important disease of domestic and wild pigs that it is classified by the Office International des Epizooties (OIE) as a disease requiring immediate notification. Even in recent years, epidemics of CSF have been reported in regions of Asia, Latin America, Eastern Europe, and the former USSR.1,2 Classical swine fever virus (CSFV), the causative agent of this disease, is an enveloped, positive-strand RNA virus belonging to the genus Pestivirus in the family Flaviviridae. The envelope of CSFV contains three glycoproteins, Erns, E1, and E2.2 Among these, E2 is the most immuno-genic, and is a virulence determinant.3 Post-translational modification (N-linked glycosylation) is required for the immunogenicity of E2.2,4 Recombinant CSFV E2 glycoprotein (rE2) has been produced in a variety of expression systems, including bacteria, plants, adenovirus, and a baculovirus/insect cell system.3,5–7 However, the bacteria-expressed rE2 was not an effective immunogen because the protein must be in the correct conformation.8 In contrast, baculovirus-expressed rE2 has been found to be a potent immunogen, and two subunit vaccines, BAYOVAC6 CSF Marker and Porcilis8 Pestivirus, have been developed for commercial use.9

Cost is a very important criterion for vaccines used in veterinary medicine, especially in developing countries.9 Although high yields of rE2 have been obtained from baculovirus/insect cell expression systems on a laboratory scale, culture of insect cells in bioreactors for mass production is both difficult and costly.10 As an alternative, using insect larvae as a “mini-bioreactor” can generate satisfactory levels of recombinant protein, facilitate proper post-translational modification, and dramatically reduce the cost of production, because no sterile conditions, culture instruments, or media are required during the production process.11 During the last decade, a variety of recombinant proteins have been expressed using insect larvae, including enzymes, antigens, and cytokines.12,13 However, the characteristics of CSFV E2 expression in larvae were still unknown.

The aim of this study was to obtain high levels of immunogenic rE2, where production is easy and requires less cost. A reliable baculovirus expression system was developed that proved capable of orally infecting larvae of Trichoplusia ni and led to the production of substantial levels of rE2 in larval hemolymph. To monitor rE2 expression in larvae, the gene of green fluorescent protein (GFP) was also incorporated, expressed under the control of the internal 4

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Abbreviations: AcNPV, Autographa californica nuclear polyhedrosis virus; CSFV, classical swine fever virus; GFP, green fluorescent protein; hpi, hours post infection; IRES, internal ribosome entry site; MOI, multiplicity of infections; PBS, phosphate buffered saline; PIBs, polyhedral inclusion bodies
ribosome entry site (IRES) of *Rhopalosiphum padi* virus (RhPV). The recombinant protein had physical and antigenic characteristics of the native E2 glycoprotein and induced significant levels of neutralizing antibody in mice and piglets. Results indicate that the bi-cistronic baculovirus/larvae expression system represents an easy and economical way to produce vaccine components for use in the veterinary medicine.

### Materials and Methods

**Insect cell lines and larvae.** The vectors, viruses, cell lines, and insects used in this study are listed in Table 1. S9 cells were maintained at 26°C in TNM-FH (Trichoplusia ni modified formula Hank’s) basal medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum. T. ni larvae were reared in a non-sterile climatic chamber at 26 ± 1°C and fed an artificial insect diet, as described by O’Reilly et al.\(^{(1)}\)

**Construction of recombinant transfer vector.** To construct a bi-cistronic transfer vector, pAcP\(_{10}\)-MCS-IR-GFP, a DNA fragment having multiple cloning sites (MCS) was obtained from plasmid p Bluescript SK by PCR with primers MCS-F (5′-GCTCCACCG-3′) and MCS-R (5′-ATCGAATTCCTGCAGCCCGG-3′). The MCS fragment was introduced into the RgIII and EcoRI restriction sites of transfer vector pAcUW21. Subsequently, the IRES-GFP-poly(A) DNA fragment was obtained from the plasmid pBacIR-GFP\(^{(14)}\) with EcoRI digestion, and was cloned into the transfer vector. The protein concentrations of samples were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Five μg of each sample was subjected to SDS–PAGE, under reducing conditions (1% glycerol, 0.4% SDS, 12.5 mM Tris–HCl, 4% -mercaptoethanol, pH 6.6) or non-reducing conditions (1% glycerol, 0.4% SDS, 12.5 mM Tris–HCl, pH 6.6), and transferred to PVDF membranes (Millipore). Two primary antibodies were used to identify rE2 by Western blot: porcine anti-CSFV polyclonal antibody (pAb; 1:5,000 dilution) and WH303 mAb specific to CSFV E2 (1:1,000 dilution). The secondary antibodies were goat anti-porcine and goat anti-mouse IgG-HRP-conjugate (1:5,000 dilution; Zymed, San Francisco, CA). The protein bands were visualized by the ECL chemiluminescence system (PerkinElmer, Waltham, MA). The intensity of rE2 in the hemolymph extract was quantified using an immunofluorescence microscope equipped with FITC and rhodamine filters.

**Glycoprotein staining assay.** Glycosylation stain was performed by the method described by Sehgal et al.\(^{(16)}\) First, the rE2 in the

### Table 1. Vectors, Viruses, Cell Lines, and Insects Used in This Study

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK</td>
<td>Cloning vector containing multiple cloning sites; Amp(^{\prime}); 3 kb</td>
<td>Fermentas</td>
</tr>
<tr>
<td>pBacIR-GFP</td>
<td>BlueBac transfer vector containing RhPV IRES fragment and gfp gene; Amp(^{\prime}); 7.7 kb</td>
<td>13</td>
</tr>
<tr>
<td>pAcUW21</td>
<td>Baculovirus transfer vector containing P(_{10}) promoter, the entire polyhedrin gene, Co/E1 ori, fl ori, and Amp(^{\prime}); 9.2 kb</td>
<td>Pharamingen</td>
</tr>
<tr>
<td>pAcP(_{10})-MCS-IR-GFP</td>
<td>pAcUW21::MCS::IRES::gfp; 10.8 kb</td>
<td>This study</td>
</tr>
<tr>
<td>AcNPV</td>
<td>Wild-type strain of <em>Autographa californica</em> nuclear polyhedrosis virus</td>
<td>TACTRI*</td>
</tr>
<tr>
<td>vAcP(_{10})-E2</td>
<td>Recombinant baculovirus containing P(_{10})::CSFV E2::IRES::gfp cassette and entire polyhedrin gene</td>
<td>TACTRI*</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPLB-S9-AE (S9)</td>
<td>The insect cell line was used to replicate baculovirus</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PK-15</td>
<td>The porcine kidney cell line can be used to replicate CSFV</td>
<td>ATCC(^{(8)}) CCL-33</td>
</tr>
<tr>
<td>Insect larvae</td>
<td></td>
<td></td>
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<tr>
<td>Trichoplusia ni</td>
<td>The cabbage looper is a member of the moth family Noctuidae</td>
<td>TACTRI</td>
</tr>
</tbody>
</table>

\(^{1}\)TACTRI: Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council for Agriculture

\(^{8}\)ATCC, American Type Culture Collection

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\(^{(1)}\) O’Reilly et al.

\(^{(10)}\) Tong et al.

\(^{(11)}\) Sehgal et al.
Expression and Immunological Studies of CSFV E2 in Larvae

hemolymph of the infected larvae at 72 hpi was purified using a commercial immunoprecipitation kit (Pierce, Rockford, IL) with WH303 mAb following the manufacturer’s instructions. Subsequently, samples were subjected to SDS–PAGE, followed by Western blotting with 5 μg/ml of concanavalin A-labeled peroxidase (ConA-HRP; Sigma) in PBS containing 1 mM MnCl₂, 1 mM CaCl₂, and 1 mM MgCl₂ for 16 h at 25 °C. Finally, the membrane was washed with PBST and developed in 3,3’,5,5’-Tetramethylbenzidine (TMB) liquid substrate for 5 min. Each sample was subjected to SDS–PAGE, transferred to PVDF membranes, and identified as rE2 by WH303 mAb.

Protease assay. The hemolymphs of larvae infected with 1 × 10⁵ PIUs of vAcP₁₀⁻E2 or wild-type AcNPV were extracted at 96 hpi. Larval hemolymph without infection was used as control. Ten mg of papain (Sigma) was dissolved in 1 ml of distilled water as positive control. A cysteine protease assay at neutral pH was performed as described by Hiyoshi et al. First, the sample (83 μl) was added to 583 μl of 0.2% azocasein (Sigma) in sodium phosphate buffer (50 mM, pH 7.2). The mixture was incubated at 37 °C for 4 h, followed by the addition of 27 μl of 100% TCA (Trichloroacetic acid) to stop the reaction. Subsequently, the mixtures were centrifuged at 14,000 rpm for 5 min, and 333 μl of 9 M urea was added to the supernatant before measurement of the absorbance at 405 nm. One unit of cysteine protease activity was defined as the amount of enzyme capable of increasing the absorbance by 1 at 405 nm within 1 h.

Protease sensitivity of rE2. Cysteine protease inhibitor leupeptin (Sigma) was dissolved in absolute ethanol at a final concentration of 10 μM, and was used to pre-treat AcNPV-infected hemolymph for 1 h. Five μg of purified rE2 in 20 μl of sodium phosphate buffer was added to 20 μl of the hemolymph of the AcNPV-infected larvae, the hemolymph of uninfected larvae, leupeptin-treated hemolymph, and 10 μg of papain. Ethanol at a final concentration of 1% was used as vehicle control for the agents. The mixture was incubated at 37 °C for 2 h, followed by the addition of 10 μl of sample buffer (5×), and boiling for 5 min. Each sample was subjected to SDS–PAGE, transferred to PVDF membranes, and identified as rE2 by WH303 mAb.

Immunization of mice. SPF male BALB/c mice at 6 weeks of age were purchased from The National Experimental Animal Center (Taipei, Taiwan). The mice were cared for and handled following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (permit no. 98-09). Two groups of mice (n = 5) were immunized intraperitoneally with rE2-containing hemolymph at either 8 or 16 μg of rE2 mixed with Freund’s complete adjuvant (Sigma) at a final volume of 200 μl. As negative control, five mice were injected with 100 μg of hemolymph extract from the AcNPV-infected larvae. Immunization was performed 3 times (one prime and two boosts) at a 2-week interval. Blood samples were collected from each animal prior to vaccination and at 13 d after immunization. Sera were isolated, and incubated at 56 °C for 30 min to inactivate the complement. These vaccination experiments were carried out on at least two separate occasions.

Immunization of piglets. Cross-breed piglets 6 weeks of age were purchased from a non-vaccinated, CSFV-free farm. They were cared for and handled following the protocol approved by the IACUC of National Chung Hsing University (permit no. 98-09). Nine piglets were randomly allotted to a rE2 vaccine group (n = 5) and a control group (n = 4). The piglets were immunized intramuscularly twice at a 3-week interval. One dose of vaccine consisted of hemolymph extract containing 50 μg of rE2 (the rE2 vaccine group) or 200 μg of crude extract from AcNPV-infected Sf9 cells (the control group) emulsified with the adjuvant Montanide ISA563 (Seppic, Paris, France) at a 1:1 ratio. Blood samples were collected from each animal prior to vaccination and at 21 d after immunization. Sera were isolated, and incubated at 56 °C for 30 min to inactivate the complement.

Measurement of anti-E2 antibodies. To evaluate the serological responses of the vaccinated animals, a commercially available CSFV E2 antibody test kit (IDEXX Laboratories, Westbrook, Maine) was used following the manufacturer’s instructions. The optical densities of the samples (OD₄⁵₀) and controls were measured at a wavelength of 450 nm using an ELISA microplate reader (Dynex Technologies, Chantilly, VA). The degree of CSFV E2 specific antibodies in the serum was calculated as follows: E2-blocking percentage = (N − S)/N × 100, where N is the mean OD₄₀₀ of the negative control and S is the mean OD₄₀₀ of the test sample. The test samples are regarded as serologically positive if the blocking percentage ≥ 40%.

Virus neutralization test. Titers of neutralizing antibody in the serum samples were determined as described by Lin et al. Replicates of serially 2-fold dilutions of heat-inactivated (30 min, 56 °C) serum samples (50 μl) were mixed with the 200 TCID₅₀ CSFV LPC strain (50 μl) in a microtitration plate, and incubated at 37 °C for 1 h. After the addition of 1 × 10⁴ porcine kidney (PK-15) cells (100 μl) and incubation at 37 °C for 72 h, an indirect immunofluorescence assay was performed to detect CSFV antigen in the cytoplasm. mAb WH303 was used as the first antibody and a FITC-conjugated goat anti-mouse IgG (Invitrogen) as the second antibody. Cells were observed under a fluorescence microscope, and the ability of test sera to neutralize infectivity of CSFV was determined. Neutralization titers were expressed as the reciprocal of the highest serum dilution that prevented viral growth in 50% of two replica wells. If the titer was <1/8, the test sample was classified as absence of virus neutralizing ability.

Statistical analysis. Statistical analysis was performed by Student’s t-test. Results are expressed as the mean ± standard error of the mean (SEM).

Results

Generation of bi-cistronic recombinant baculovirus. A bi-cistronic transfer plasmid, pAcP₁₀-MCS-IR-GFP, was developed having nine restriction sites after the P₁₀ promoter and before the IRES-GFP sequence so that the gene of interest could be cloned into the first cistron of the vector. Subsequently, a cDNA fragment encoding the signal sequence and structural region of CSFV E2 (341 amino acid residues) but lacking the C-terminal transmembrane region (TMR) was cloned into pAcP₁₀-MCS-IR-GFP. Then this chimera gene was engineered into the genome of a polyhedron-positive AcNPV under the control of the P₁₀ promoter. The resulting recombinant baculovirus was named vAcP₁₀-E2 (Fig. 1A).

Expression of rE2 in insect cells and T. ni larvae. To confirm that the recombinant virus vAcP₁₀-E2 would simultaneously express rE2 and GFP, Sf9 cells were infected with vAcP₁₀-E2 at a MOI of 5 for 3 d, and immunofluorescence staining for rE2 was performed. The infected cells were identified based on a phenotype that was positive for both occlusion body production (occ⁺; Fig. 1B) and GFP expression (GFP⁺; Fig. 1C). As Fig. 1D indicates, the infected cells exhibiting occ⁺ / GFP⁺ phenotype also revealed the red fluorescence, confirming the expression of CSFV rE2.

To test further whether rE2 could be expressed and secreted in the T. ni larvae, fourth-instar larvae were infected orally with 1 × 10⁵ PIUs of vAcP₁₀-E2 and secretion of rE2 into the hemolymph was analyzed by Western blot using porcine antibodies specific to CSFV E2. Larvae infected with the wild-type AcNPV served as negative control. As Fig. 2A indicates, the non-reducing form of rE2 was identified as a broad band of approximately 96 kDa in the hemolymph of the vAcP₁₀-E2-infected larvae, in agreement with the size predicted for the homodimer, a correct conformation of CSFV E2 glycoprotein. In contrast, no protein bands were detected in the control larvae. Moreover, the
accumulation of rE2 in the supernatant of the infected Sf9 cells was more than that in the cell lysate, suggesting that the putative signal sequence efficiently channels rE2 into a secretion pathway of insect cells.

To determine whether the larvae-expressed rE2 was glycosylated, it was purified by immunoprecipitation and probed with ConA-HRPO, a highly specific carbohydrate-binding molecule. The monomer of purified rE2 was identified as an approximately 48-kDa glycosylated protein (Fig. 2B, lane 1). As a positive control, the heavy chain of mouse IgG showed a distinct band of 50 kDa (lane 2). Considered together, these findings confirm that rE2 was successfully secreted into the larval hemolymph, and was expressed as a glycoprotein with correct conformation.

Time course of GFP and rE2 expression in T. ni larvae

Fourth-instar larvae were fed 1 x 10^5 PIBs of vAcP_{10}-E2 or AcNPV (WT). A. Photographs were taken at different times post-infection using an inverted fluorescence microscope at magnification 20 x at the same exposure time (10 ms). hpi, hours post infection. Scale bar, 1 mm. B. Expression of rE2 in each larva at different time points was quantified using purified rE2 as standard in Western blot analysis with WH303 mAb. The immunoblots shown are representative of at least two independent experiments. C. The average amount of 48-kDa rE2 at each time point was obtained from the hemolymph of 10 infected larvae.

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Time course of GFP and rE2 expression in T. ni larvae

The kinetics of GFP expression in the larvae infected with 1 x 10^5 PIBs of vAcP_{10}-E2 were examined. As Fig. 3A indicates, faint green fluorescence was first observed at 60 hpi. At 72–96 hpi, most of vAcP_{10}-E2-infected larvae emitted high levels of fluorescence that
were not observed in the control larvae infected with AcNPV. Therefore, a clear and simple distinction was achieved between infected and uninfected larvae. It was also observed that there was a gradual decrease in fluorescence intensity, from 96 hpi to 108 hpi.

Hemolymphs of the GFP+ larvae were harvested from 60 hpi to 108 hpi. rE2 expression in every larva was examined by Western blot using WH303 mAb specific to CSFV E2. A representative immunoblot is shown in Fig. 3B. The accumulation of rE2 in the larval hemolymph was time-dependent. After infection for 72 h, a high level of rE2 expression was detected in every larval hemolymph. However, several immunoreactive bands smaller than 48 kDa were also identified, but not observed in the AcNPV-infected larvae, suggesting partial proteolytic processing of rE2. The yield of 48-kDa rE2 in the larva was quantified by comparing it with the intensity of purified rE2 (Fig. 3B). As Fig. 3C indicates, the expression level of rE2 increased dramatically, and reached a plateau at 72–84 hpi. The maximal expression of rE2 monomer in the hemolymph was in a range of 600–800 μg/ml and constituted about 10.4% of total soluble protein, but the amount of rE2 monomer rapidly declined, from 84 to 108 hpi.

Protease activity assay and protease sensitivity of rE2
To confirm that AcNPV infection would enhance protease activity in the larval hemolymph, cysteine protease activities were measured. The protease activities of the hemolymphs infected with the wild-type AcNPV and vAcP10-E2 at 96 hpi were 0.93 and 0.97 U/ml respectively (Fig. 4A). In contrast, the protease activity of normal, uninfected larval hemolymph was only 0.06 U/ml. The results suggest that high levels of cysteine protease were present in the baculovirus-infected hemolymph.

To determine further whether the secreted rE2 was degraded by protease, a protease sensitivity test for rE2 was performed. As Fig. 4B indicates, the purified rE2 was still intact after treatment with normal hemolymph (lane 1). Notably, rE2 was degraded to minor immunoreactive bands by the hemolymph of the AcNPV-infected larvae (lane 2). In contrast, the level of rE2 degradation was obviously lower in the AcNPV-infected hemolymph (lane 1). Notably, rE2 was degraded to minor immunoreactive bands by the hemolymph of the AcNPV-infected larvae (lane 2). In contrast, the level of rE2 degradation was obviously lower in the AcNPV-infected hemolymph that was pre-treated with leupeptin (lane 5). These findings confirm that the secreted rE2 was indeed degraded to minor immunoreactive bands by the protease of AcNPV-infected hemolymph.

Immunization efficacy of larvae-derived rE2 in the mice
Hemolymph extracts containing 8 or 16 μg of rE2 were used to immunize the mice 3 times at a 2-week interval. CSFV E2-specific humoral responses were assessed using a commercial ELISA kit. As Fig. 5 indicates, the E2-blocking percentages increased to 42.9% in the mice immunized with 8 μg of rE2 after the second boost, and the blocking percentages of the mice immunized with 16 μg of rE2 rose to 49.1% after the first boost, and reached 59% after the second boost, indicating the presence of high levels of anti-CSFV antibodies in the sera (p < 0.01). In contrast, the blocking percentages for the control group remained lower than 10% after the second boost.
Induction of Anti-E2 Antibodies and CSFV Neutralizing

Fig. 6. Induction of Anti-E2 Antibodies and CSFV Neutralizing Antibodies in Immunized Piglets. Five piglets immunized with rE2-containing hemolymph twice at a 3-week interval. Four piglets immunized with crude extract from AcNPV-infected Sf9 cells served as negative control. A, The level of E2-specific antibodies in the sera was measured with a CSFV antibody test kit. The test samples were regarded as serologically positive when the blocking percentage was ≥40%. B, Sera neutralizing antibody titers (SN titers) were expressed as the reciprocal of the highest dilution that caused complete neutralization.

Immunization efficacy of larvae-derived rE2 in pigs

Five 6-week-old piglets were immunized with hemolymph extracts containing 50 μg of rE2 twice at a 3-week interval, and four control piglets were immunized with lysate of AcNPV-infected Sf9 cells served as negative control. A, The level of E2-specific antibodies in the sera was measured with a CSFV antibody test kit. The test samples were regarded as serologically positive when the blocking percentage was ≥40%. B, Sera neutralizing antibody titers (SN titers) were expressed as the reciprocal of the highest dilution that caused complete neutralization.

Discussion

Applications of subunit marker vaccines have been found to be a promising strategy for the control and eradication of the CSF, but using cell cultures for mass production of CSFV rE2 is costly. To replace this method, a bi-cistronic baculovirus/larvae system expressing CSFV rE2 was developed in this study. The baculovirus construct vAcP10–E2 synchronously infected hundreds of T. ni larvae when delivered via feed instead of intracuticular injection. This is the first study to demonstrate that CSFV rE2, which is expressed in larvae, is highly effective in inducing a specific antibody response in mice and piglets.

We found that the rE2 was secreted into the larval hemolymph and expressed as a glycoprotein with correct conformation. It has been demonstrated that the secreted forms of recombinant glycoprotein present in the hemolymph of infected larvae were correctly processed and folded, but we found high levels of cysteine protease activity in the hemolymph of larvae infected with AcNPV at 96 hpi, and that this resulted in the degradation of the secreted rE2. This agrees with previous studies indicating that baculovirus can produce a papain-type cysteine protease to degrade the expressed recombinant protein in larvae. To avoid rapid degradation of the recombinant protein by baculovirus-derived proteases, an appropriate time point to collect the infected larvae is critical. However, it is difficult to distinguish the degree of infection and/or the level of protein expression based merely on the larval appearance.

To remove these obstacles, a bi-cistronic expression vector was developed that allowed vAcP10–E2-infected larvae with GFP phenotypic to be easily discriminated from uninfected larvae. The level of expression and degradation of rE2 appeared to correlate directly with the fluorescence intensity emitted from infected larvae (Fig. 3), suggesting that the gfp gene can serve as a real-time marker for the co-expression of the recombinant protein in larvae. By fluorescence intensity, the maximal yield of rE2 without degradation in the hemolymph at 72 hpi was about 600–800 μg/ml, much higher than the yield from other expression systems.

We found that mice immunized with rE2-containing hemolymph developed higher titers of anti-CSFV E2 antibodies than the negative controls after booster vaccinations, as reported by Ferrer et al. However, the most convincing evidence of the immunogenicity of CSFV E2 was obtained from the vaccination experiments with pigs. Determination of the immunization efficacy of larva-derived rE2 in piglets was done in this study. The ELISA results showed that the anti-E2 antibodies rose significantly but not over 40% in rE2-vaccinated pigs after primary immunization (Fig. 6A). Notably, all the rE2-vaccinated piglets were serologically positive after the boost vaccination. Many studies indicate that the presence of virus-neutralizing antibodies is a crucial step in blocking CSFV infection. In order to confirm the ELISA results with a functional assay of antibodies, virus neutralization assay was performed. We found that the levels of anti-E2 antibodies in the rE2-vaccinated pigs were not high enough to neutralize CSFV post first vaccination, because their SN titers were less than 1:8 (Fig. 6B).
However, the rE2-vaccinated piglets developed high titers of virus-neutralizing antibodies, ranging from 1:76 to 1:256, after boost vaccination. This is consistent with the results of ELISA. Recently, Lin et al.\(^1\) suggested that SN titers of about 1:32 confer effective protective immunity against CSFV infection. They found that piglets vaccinated with 100 µg of yeast-expressed rE2 twice at 3-week intervals showed a completely protective immune response against CSFV challenge. Similarly, Terzel\(\acute{c}\) et al.\(^2\) found that piglets immunized with the commercial BAYOVAC\(\textsuperscript{6}\) CSF Marker vaccine showed SN titers ranging from 1:8 to 1:32, and all survived CSFV challenge. Collectively, our results indicate that the immune response elicited by the candidate without further purification. This system also provides a simple distinction between infected and uninfected larvae and reduces the degradation of the target protein.

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References