Application of Equine Infectious Anemia Virus Core Proteins Produced in a Baculovirus Expression System to Serological Diagnosis

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Abstract: Equine infectious anemia virus (EIAV) core proteins were obtained from a baculovirus expression system. Recombinant baculoviruses (rBVs) highly expressed the Gag precursor and p26 antigens in an rBV-infected SF21 cell culture supernatant. Enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) were conducted using the expressed proteins to detect antibodies from experimentally infected horses. The expressed antigens showed low background levels, high specificity and sensitivity in ELISA and AGID. The results of the serological tests using the expressed antigens were identical to those using a manufactured trial antigen. rBVs containing gag and p26 genes were found to express high quality and large quantities of Gag and p26 antigens, respectively. The antigens were quite useful for detecting anti-EIAV antibodies from virus-infected horses.

Key words: Equine infectious anemia virus, Gag and p26 proteins, Protein expression, Recombinant baculovirus

Equine infectious anemia (EIA) is an important disease in horses characterized by viral persistence, immunologically mediated lesions and variable clinical courses (10, 13, 14, 19). Studies on viral structural proteins have indicated that equine infectious anemia virus (EIAV) are formed by two virus-encoded glycosylated proteins, gp90 and gp45 (21), and four virus-specific non-glycosylated core proteins designated as p15, p26, p11 and p9 (2, 7, 8, 19). These core proteins are produced by proteolytic cleavage of the 55 kilodalton (kDa) precursor polypeptide, Pr55vir, encoded by the EIAV gag gene (11, 27). The major EIAV core protein is a group-specific antigen, p26, which constitutes about 40% of total virion protein.

A reliable serologic diagnostic method for the detection of serum antibody, AGID, was developed by Coggins and Norcross (3). AGID is the most common means for diagnosing disease since horses infected with various immunological distinct strains of EIAV possess the precipitating antibody against the major group-specific anti-
Materials and Methods

Viruses and cells. The P337-V70 strain of EIAV was propagated in horse leukocyte cell cultures as described previously (14). The Autographa californica nuclear polyhedrosis virus (AcNPV) and Spodoptera frugiperda Sf21 cells were kindly supplied by Dr. M. Matsuura, National Institute of Health. BacPAK6 virus was purchased from Clontech, U.S.A. Insect cells were propagated at 28°C in TC 100 insect medium supplemented with 10% fetal calf serum, and applied to serum-free growth medium (Sf-900 II SFM; GIBCO BRL, U.S.A.) for suspension cultures.

Experimentally infected horses. Two thoroughbred horses aged 11 and 13 years old were inoculated subcutaneously with 10^3 TCID_{50} of the P337-V70 strain. Body temperature was recorded twice daily, and serum samples were taken periodically.

Primer design and polymerase chain reaction (PCR) amplification. The primers for PCR amplification of the gag and p26 genes were prepared based on the sequence of the EIAV Wyoming strain (11). The primer sequences for the total gag and p26 genes are shown in Table 1. Primers for the p26 gene were designed to include the Bam HI site and initiation codon (ATG) for p26-F, and Bam HI and duplicated termination codon (TTATTA) for p26-R on the 5’ termini to the p26 gene coding sequence (Table 1). PCR amplification using these primers was performed for 25 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

Construction of rBVs. The amplified gag PCR product was purified by Magic PCR Preps (Promega, U.S.A.) and directly ligated into the Sma I-digested and dephosphorylated baculovirus transfer vector pAcYM1. The construct thus obtained was introduced into Escherichia coli, XL1-Blue. After determining insert size and orientation by agarose gel electrophoresis with suitable endonuclease digestion, a recombinant plasmid containing the desired insert was propagated in E. coli and purified by Magic Midi Preps (Promega, U.S.A.). The purified recombinant plasmid DNA was co-transfected with Bsu 36I-digested BacPAK6 DNA (Clontech, U.S.A.) by lipofectin (GIBCO BRL, U.S.A.) according to the method of King and Possee (12).

The method for producing rBV containing the p26 gene was the same as that of the gag gene except for digestion with Bam HI and ligation with the Bam HI-digested and dephosphorylated pAcYM1 vector. After transfection, rBVs were harvested and purified by three rounds of plaque formation in Sf21 cells.

Preparation of the expressed antigens. Suspension cultures of insect cells were inoculated with rBVs at 10 plaque-forming units per cell and harvested daily. Cell lysates and supernatants were evaluated by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) and ELISA to determine optimal conditions. The infectious fluids were harvested at 3 days post-infection (pi) and centrifuged at 12,500 × g for 30 min. The supernatants were concentrated 30 times by 70% ammonium sulfate precipitation. Following dialysis with 0.01 m phosphate-buffered saline (pH 7.2), the solutions were centrifuged at 10,000 × g for 30 min. The supernatants were filtrated by ultrafree-CL 100,000 (Millipore, U.S.A.), and low molecular materials were eliminated by ultrafree-CL 30,000 for Gag protein and ultrafree-CL 10,000 for p26 protein.

ELISA procedure. ELISA was performed using the Gag and p26 proteins as antigens, as described by a manufacture trial kit supplier (Nisseiken, Japan) (29). Optimal concentrations of antigens and reagent dilution were determined by the checker-board technique using reference horse sera in an ELISA manufacture trial kit.

AGID test. To assess accuracy and applicability of the proteins, purified Gag and p26 proteins were examined by AGID as described by Coggins and Norcross (3).

Results

Construction of rBVs

PCR products not only of the gag but also the p26

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td><strong>gag</strong></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>GTA AGA TGG GAG ACC CTT TG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GTA GGA GGC CTT TTC TCT AG</td>
</tr>
<tr>
<td><strong>p26</strong></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>CGG ATC CGA TGC CAA TCA TGA TAG ATG GGG CTG GAA ACA G</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CGG ATC CGT TAT TAA AGT GCT TTT GCC AAC AAC ATC ATC TTC TG</td>
</tr>
</tbody>
</table>

Bam HI: start codon
Bam HI: Stop codon

Table 1. Primer sequences

Underlining indicates restriction enzyme sites within primers.
gene were faithfully amplified using the primer sets according to the sequence of the Wyoming strain (11). After plaque purification, rBVs expressing the Gag precursor and p26 proteins were identified by SDS-PAGE. The proteins were detected as 26 and 55 kDa bands in the gel by staining with Coomassie brilliant blue (Fig. 1A, lanes 2 and 3, respectively), although control BacPAK6 virus infected and non-infected cell lysates showed no bands in the corresponding area on the gel (Fig. 1A, lanes 4 and 5).

To determine whether 55 and 26 kDa proteins are expressed in the gag and p26 genes of rBVs, Western blot analysis was performed after separation by SDS-PAGE using horse anti-EIAV serum. Only 26 and 55 kDa protein bands indicated specific reactions with the horse anti-serum (Fig. 1B, lanes 3 and 4). No specific reaction could be seen in the control lanes (Fig. 1B, lanes 1 and 2). The purified virion showed the 26 kDa major band that reacted with anti-serum by Western blotting (Fig. 1B, lane 5).

**Production of Gag and p26 Proteins**

To obtain large amounts of expressed proteins, the production kinetics of the proteins in Sf21 cells with serum-free medium were studied. Maximum production in the suspension culture of Sf21 cells was observed at 2 to 3 days pi by ELISA for either protein (Fig. 2). The expressed proteins were harvested at 3 days pi and used for further experiments. After the concentration and purification steps explained in “Materials and Methods,” approximately 2 mg Gag and 12 mg p26 proteins

![Fig. 1](image1.png)

**Fig. 1.** A: SDS-PAGE for analysis of expressed Gag and p26 proteins in Sf21 cells by staining with Coomassie brilliant blue. Lane 1, molecular weight standards; lanes 2 and 3, extracts from Sf21 cells infected with rBVs containing p26 and gag genes, respectively; lanes 4 and 5, extracts from Sf21 cells infected with BacPAK6 virus and non-infected Sf21 cells, respectively; and lane 6, EIAV proteins purified by sucrose gradient. Arrows on the right indicate positions of the 55 and 26 kDa bands expressed by rBVs. B: Specific identification of expressed Gag and p26 proteins by Western blot analysis. Lanes 1 and 2, extracts from Sf21 cells infected with BacPAK6 virus and non-infected Sf21 cells, respectively; lanes 3 and 4, extracts from Sf21 cells infected with the rBVs containing p26 and gag genes, respectively; and lane 5, purified p26 protein from EIA virions by sucrose gradient. Arrows on the left indicate positions of the expressed Gag (55 kDa) and p26 (26 kDa) proteins specifically reacted with EIAV-positive horse serum.

![Fig. 2](image2.png)

**Fig. 2.** Production kinetics of Gag and p26 proteins expressed in Sf21 cell-culture supernatant infected with rBVs, Gag (■) and p26 (○).

![Fig. 3](image3.png)

**Fig. 3.** Determination of specificity of expressed Gag and p26 proteins in AGID. Wells 1 and 4, expressed p26 antigen; wells 2 and 5, disrupted EIAV antigen from a commercial AGID kit; wells 3 and 6, expressed Gag antigen; the central well (S), reference EIAV-positive serum in the kit.
were obtained from 1,000 ml of rBV-infected Sf21 cell culture supernatants.

Comparison of Expressed Proteins with Manufactured Trial Antigen by ELISA

To evaluate the applicability of expressed Gag and p26 proteins as antigens for ELISA, the purified proteins were compared with a manufactured trial antigen. Optimal concentrations of expressed Gag and p26 proteins as coating antigens were determined as 31 ng/well and 25 ng/well, respectively, using the reference horse sera in the kit. The mean OD of the expressed proteins with the reference horse sera coincided with that of the manufactured trial p26 antigen (data not shown).

AGID Using Gag and p26 Proteins

To determine the specificity of Gag and p26 proteins in AGID, the antigens were compared using an EIAV AGID manufacture trial kit (Nisseiken, Japan). Optimal dilution of the expressed antigens was determined using the reference horse serum and compared with the commercial antigen. Both proteins produced clear precipitation lines between the reference positive serum and the manufactured trial antigen (Fig. 3).

Detection of Serum Antibody from Experimentally Infected Horses

Two horses were experimentally inoculated with the virulent P337-V70 strain of EIAV. Both horses developed typical persistent infection and pyrexia recurred at afebrile intervals during 106 days of observation. Serum samples were collected periodically before and after inoculation of the virus. The results are shown in Fig. 4. ELISA antibody was detected at 18 days pi (third day after first febrile, Fig. 4, A and B) in both horses. The ELISA antibody reached its highest level at 22 days pi in horse No. 1 and at 25 days pi in horse No. 2, decreased gradually from 35 to 50 days in horse No. 1 and 36 to 57

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Fig. 4. A: Course of body temperature and antibody response of horse No. 1 inoculated with the P337-V70 strain using expressed Gag and p26 proteins. B: Course of body temperature and antibody response of horse No. 2. Figure on top shows body temperature curve, figures in the middle and below show antibody titers detected by ELISA and AGID using the expressed Gag (■) and p26 (○) antigens compared with manufactured trial ELISA and commercial AGID antigens (▲).
days in horse No. 2, and rose again during observation. The expressed proteins and manufactured trial antigen could detect the ELISA antibody in the horses to essentially the same degree.

The same serum samples were used in AGID with expressed Gag and p26 proteins serving as antigens. The AGID antibody was first detected at 22 days pi in horse No.1 and 18 days in horse No. 2, and was present throughout the observation period in both horses. The sensitivity of AGID using the expressed antigens was thus shown to be basically the same as that of the manufactured trial antigen (Fig. 4, A and B).

Discussion

The major core protein (p26) of EIAV is a product of the viral gag gene (27), and represents about 40% of the virion mass (19) with group and interspecies antigenic determinants (6, 16, 18). This protein appears highly conserved among EIAV isolates (24) and is widely used for diagnosis of the disease (1, 3, 30). The antigen has been obtained from persistently virus-infected cell culture supernatant, and much infectious fluid is needed owing to poor virus yields.

This study used the baculovirus expression system to obtain a large amount of the antigen for serological diagnosis. rBVs containing gag and p26 genes highly expressed the Gag precursor and p26 antigens in Sf21 cells with serum-free medium. The expressed proteins in the supernatant of rBV-infected cells were purified by ammonium sulfate precipitation and ultrafiltration. This method is very simple and protein purification is quite easy as compared to PAGE for Gag precursor protein expressed by bacteria (1). The Gag precursor protein of lentiviruses expressed in rBV-infected insect cells is targeted to the plasma membrane and accumulates in virus-like particles budding from the cell surface (4, 5, 22). This particle formation is dependent on myristylation of the N-terminal glycine. Royer et al (23) reported the truncated form of N-myristylated rBV-expressing human immunodeficiency virus type 1 Gag protein to consist of membrane-enveloped, core-like particles and nonparticulate soluble proteins, whereas non-N-myristylated Gag protein was released only in soluble form. These observations agree well with our results. Electron microscopic observation of Gag rBV-infected insect cells demonstrated the formation and release of virus-like particles from the cells. No particle formation was observed in p26 rBV-infected cells (data not shown).

To evaluate the applicability of expressed Gag and p26 proteins to serological diagnosis, the proteins were used as ELISA and AGID antigens. ELISA indicated low background levels, high specificity and sensitivity, and results identical to those with the manufactured trial antigen. Sera obtained periodically from the two infected horses were detected positive at 18 days pi, just after the first febrile episode. The ELISA titer rose gradually and remained high until the end of the experiment in both horses. Although expressed Gag protein showed a relatively low OD among the antigens used, ELISA titers using the expressed p26 showed high levels and good coincidence with those using the manufacture trial kit (Fig. 4, A and B). The results of AGID also showed close agreement for the expressed and manufactured trial antigens.

The Gag and p26 proteins expressed should be quite useful for detecting EIAV antibodies in virus-infected horse sera in AGID and ELISA. However, these tests have several disadvantages such as horse sera showing false-positive reactions with antigens from virus-infected cell cultures. A nonspecific reaction in AGID may be caused by the repeated injection of bovine serum components in a vaccine (29). As the rBV-expressed proteins contain no bovine serum components, it should be possible to obtain much higher quality EIAV core proteins. Thus, it is necessary to test horse sera showing false-positive reactions for field use of the rBV-expressed proteins.

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References