Recombinant Expression of Bornavirus P24 Protein for Enzyme-Linked Immunosorbent Assay Development

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ABSTRACT

BDV is a neurotropic enveloped RNA virus that induces persistent neurologic disease in a wide host range, including several vertebrate species and humans. The BDV genome encodes six proteins but the P24 protein was identified at higher rates than other proteins in BDV-infected tissues. In this study, BDV-P24 protein was constructed and subcloned into expression plasmid pET22. Recombinant protein expression was confirmed by SDS-PAGE and western blotting. P24 protein was injected into rabbits with the aim of polyclonal antibody production and immunization. ELISA is a fast, cost-effective, and highly sensitive technique with a lower probability of contamination compared to other diagnostic methods. ELISA was performed to evaluate infection in laboratory rabbits and retrospective infection was examined in 50 rabbits. The obtained results in this study indicated that ELISA based on P24 protein has a high potential to detect BDV infection.

Abbreviations

BDV: Borna disease virus
RNA: Ribonucleic acid
N: Nucleoprotein

P: Phosphoprotein
M: Matrix protein
G: Glycoprotein

Introduction

*A*ccording to the definition of the World Health Organization, any infectious disease common between vertebrate animals and humans is classified as a zoonosis [1]. Zoonosis currently accounts for approximately 70% of emerging diseases [2] and causes 2.7 million human deaths worldwide annually [3]. The zoonotic pathogens are highly important due to the following reasons; the pathogen itself is compatible with other human hosts and is capable of causing resistant human-to-human infection without requiring to seed from the animal reservoir [4]. Early detection of the pathogens common between humans and animals through increasing laboratory capacity is a vital step toward the control and prevention of zoonosis [5, 6].

BDV is a highly neurotropic agent in mammalian species, such as horses, sheep, rabbits, rats, mice, guinea pigs, dogs, and cattle [7, 8]. More than 20 various genotypes from the Borna virus were extracted from different hosts, including humans, which creates a potential danger of sharing these viruses between humans and animals [9]. Selective tropism was exhibited by BDV in the nerve cells of the limbic system, especially the cortex and hippocampus, two primitive structures that control many behavioral and cognitive functions [10]. Clinical symptoms entail unusual behavior, sensorial changes, or miss of movement performance. In advanced steps, somnolence, lethargy, stupor, ataxia, and paralysis are observed. Death usually happens four weeks after the initial clinical symptoms [11]. Numerous neuropsychiatric entities have been indicated to be related to the potential markers of BDV infection in humans [12]. Several other studies have presented human cases of BDV infection in different psychiatric disorders, namely schizophrenia, learning problems, emotional disorders, and autism [13-15]. Some BDV-related antigens, antibodies, and RNAs were further detected in people with neurotropic symptoms, such as Parkinson's disease, chronic fatigue syndrome, Guillain-Barre syndrome, viral encephalitis, and multiple sclerosis [16]. Bornavirus was recently detected as the root reason for fatal human encephalitis following organ transplantation procedures, which remarkably enhanced the importance of this disease in humans [17].

BDV, as a negative-stranded RNA virus, has been enveloped by helical capsid [18]. BDV genome has six open reading frames, namely N, P, M, G, X, and L [19]. Molecular biological analysis has indicated that the P40/38 nucleoprotein, p10 protein, and P24 phosphoprotein are expressed continuously in the patient's cells [20]. The P24 protein has been identified at a higher ratio of P40 in the host tissue [21]. Techniques used for detecting BDV infection were indirect immunofluorescence with infected cells, western immunoblot, nested RT-PCR, real-time PCR, and ELISA [12]. Each of these methods faced problems; for example, nested PCR and real-time PCR cannot detect previous infection and there is a possibility of contamination, resulting in false positive results [22, 23]. The establishment of ELISA is facilitated by expressing and purifying large quantities of BDV recombinant antigens for the identification of BDV antibodies in biological samples [24]. ELISA for detecting BDV infection based on different BDV antigens has been performed in several vertebrate species, but so far this method based on P24 protein has not been investigated in rabbits [25, 26]. This study investigated the expression of P24 recombinant protein as well as the production of polyclonal antibodies against it in order to develop an ELISA for detecting BDV infection in laboratory rabbits.

Results

Borna-P24 Expression and Purification

The *pET22b-Borna-P24* construct was transformed to *E. coli* BL21. Expression of Borna-P24 protein was induced through IPTG for 16 hours. The product of recombinant Borna-P24 protein expression was observed in SDS-PAGE. The weight range of this protein was about 25 KDa (Figure 1A). These results were validated by western blotting through anti-His-tag antibodies (Figure 1B).

Investigation of Immunized Rabbit Serum Activity by ELISA

To evaluate the immunogenicity of the recombinant protein, Borna-P24 was injected into one rabbit in four immunizations, and then, ELISA was performed to assess the immune response. The Borna-P24 protein was coated on the well and various dilutions of immunized rabbit serum were added. According to the results of the fourth injection, the antibody titer was enhanced (Figure 2). Therefore, the recombinant Borna-P24 protein had immunogenicity

Recombinant Bornavirus P24 Protein for ELISA Assay Development

to induce rabbit immune response.

Investigation of Selected Rabbit's Serum Activity by ELISA

A total of 50 New Zealand white rabbits were selected from a herd of laboratories, and their previous borna virus infection was assessed by ELISA. After collecting a peripheral blood sample from the marginal vein, the serum was isolated. In this experiment, recombinant Borna-P24 protein was used as an antigen. The result of ELISA was negative for the P24-Borna virus antibody (Figure 3). These results were similar to the negative control and no antibodies were detected in the sera of the tested rabbits.

Discussion

BDV, as a neurotropic, enveloped, negative-stranded RNA virus is responsible for the severe infections of neuron cells [27]. Evidence indicates that BDV has a wide host range that spans several vertebrate species and humans [28, 29]. In previous studies, the P40 and P24 antibodies of the bornavirus were detected in the blood of patients with mental disorders [30]. The P40 values were higher in the chronic stage, whereas the P24 values increased in resistant infections [31].

In this study, for monitoring previous infections, we selected the P24 protein and produced polyclonal antibodies against the P24 recombinant protein for developing ELISA. The expression and purification of P24 protein were performed in large amounts. The recombinant protein was utilized for rabbit immunization. Polyclonal antibody generation in immunized rabbits was confirmed by ELISA. In addition, ELISA was performed in the selected laboratory rabbit herd, and no antibodies were detected. The results were obtained with high reproducibility.

Previous studies have proposed different methods for diagnosing BDV infection in diseased hosts. It is well known that whatever methodology is employed must be sensitive enough to diagnose the disease. Moreover, it is important to avoid the possibility of contamination and false positive results. In the past, some studies suggested diagnostic methods based on nested PCR [21, 22]. This method has inherent problems, such as the risk of contamination during the process and the inability to quantify the result. Other studies have suggested real-time PCR as an optimal way to diagnose BDV infection [23, 32, 33]. In this method, despite quantifying the results, there is still a risk of sample contamination and receiving false positive results. In addition, it should be noted that the mentioned techniques require a significant investment for the required machines. Furthermore, in all types of PCR, only an active infection is identified
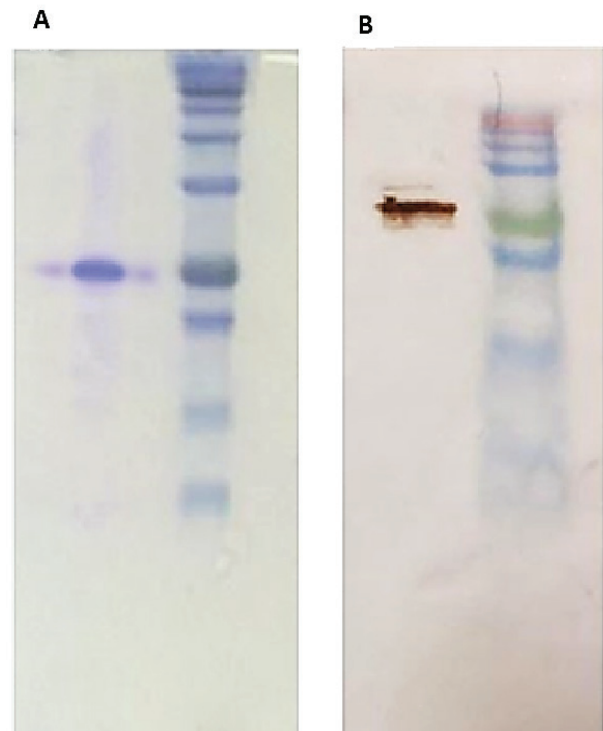

Figure 1.
Recombinant BDV P24 protein expression was induced with IPTG and evaluated by 12% SDS-PAGE (A) and western blotting (B). The MW of the marker lanes was from bottom 11, 17, 20, 25 kDa.
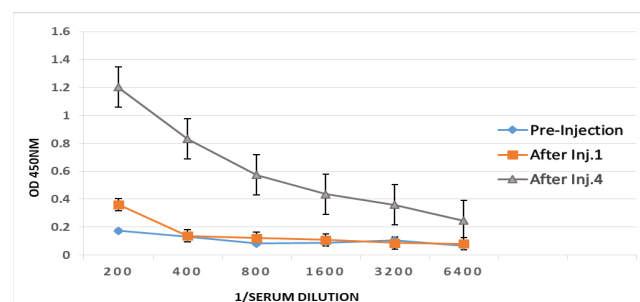

Figure 2.
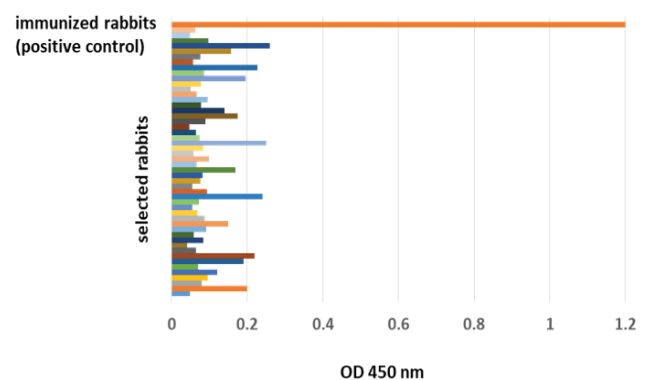Rabbit immune response monitoring by ELISA.

Figure 3.
Comparison of ELISA results of 50 selected rabbits from a laboratory herd and one rabbit immunized with Borna P24 Protein (positive control).

and no previous infection is detected. Today, ELISA is an optimal and applied method in medical diagnostic laboratories. It is low-cost and allows one to check many serum samples with minimum facilities in a few hours. In previous studies, P40 protein was used as an antigen in ELISA [34, 35]. According to other reports, the P24 protein has a higher diagnostic value than other components [16, 21, 36]. Therefore, the P24 protein is the preferred antigen in ELISA.

Conclusions

The results of our study indicated that ELISA based on P24 protein has a high potential for diagnosing BDV infection. These results were obtained from a study on one immunized rabbit and 50 selected rabbits.

Materials & Methods

Gene Construction, Expression, and Protein Purification

BDV *P24* gene sequence with a length of 615 bp subcloned to the expression plasmid *pET22* in frame with His-tag was purchased from Biomatik (Canada) and named *pET22b-Borna-P24*. This sequence is located between two restriction sites *NdeI* at the 5´ end and *XhoI* at the 3´ end. The construct was transformed into *E. coli* BL21 using heat-shock method and one colony was grown in 500 mL Luria-Bertani broth medium and expression of *P24* protein was induced through 0.05 mM concentration of IPTG for 16 hours. Bacterial sediment was collected by centrifugation for protein purification and suspension in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, pH 8.0). Eventually, bacteria were lysed by sonication method. After loading the lysate on the Ni-NTA resin (Qiagen, Germany) column, washing buffer was used to prepare the resin (8 M urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 adjust to pH 6.0) and borna P24 protein was separated using the elution buffer (8 M urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 adjusted to pH 4.3). Purified protein was dialyzed against PBS, and then, lyophilized and stored in -20°C. Finally, Protein concentration was determined with BCA method.

SDS-PAGE and Western Blot

To evaluate the expression of recombinant protein, 12% SDS-PAGE was used. Protein bands in polyacrylamide gel were stained by Coomassie blue. The expressed proteins in the gel were then transmitted to the nitrocellulose membrane for western blotting. The membrane was placed in 3% casein-blocking buffer overnight at 4°C and then, washed with PBS three times. The membrane was incubated with 1/1000 anti-His tag antibody as a primary antibody and then with 1/2000 anti-rabbit HRP-conjugated as a secondary antibody. The membrane was stained with 3, 3´-diaminobenzidine solution.

Production of P24 Polyclonal Antibody

A New Zealand female rabbit was used for immunization. Fifty micrograms of Borna-P24 recombinant protein were injected subcutaneously into the rabbit. Immunization was boosted four times at two-week intervals. A recombinant protein with complete Freund's adjuvant was used in the initial injection and incomplete adjuvant in others. Rabbits were bled from the marginal ear vein. Finally, specific polyclonal antibody titration was performed by ELISA. An amount of 1 μg/mL of recombinant Borna-P24 protein was coated in each well of the ELISA plate and was placed overnight at 4°C. Then, the plate was blocked by 3% skimmed milk and placed at room temperature for up to 1 h. PBS was used to wash the wells five times and 100 μL of serially diluted rabbit serum (1/100 to 1/3200) was added to each well and placed at room temperature for 1 h. With washing intervals, 100 μL of 1/2000 anti-rabbit HRP-conjugated was added and then, the plate was placed at room temperature for 1 h. One hundred microliter of 3, 3´, 5, 5´-tetramethylbenzidine substrate was added to all wells, and the plate was incubated at room temperature for 10 min in the dark. Finally, the reaction was stopped by adding 100 μL of stop buffer (H_2SO_4, 2N) into all wells. Optical density was read at the wavelength of 450 nm by a UV/Vis spectrophotometer (Epoch, BioTek, USA).

Investigation of Serum Titers Against Borna-P24 in Rabbits Herd

Fifty New Zealand rabbits were selected at the laboratory animal center of the Pasteur Institute of Iran (Karaj, Iran). After blood sampling and isolation of serum, ELISA was performed with 1/1000 serum dilution. Rabbit serum immunized with Borna-P24 was used as a positive control.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Recombinant Bornavirus P24 Protein for ELISA Assay Development

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