Cloning of the Bovine Immunodeficiency Virus gag Gene and Development of a Recombinant-Protein-Based Enzyme-Linked Immunosorbent Assay†

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Bovine immunodeficiency virus (BIV) is a lentivirus that causes lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness, and emaciation (20, 21, 24). Studies have shown that BIV resembles human immunodeficiency virus (HIV) type 1 and other lentiviruses, e.g., equine infectious anemia virus and feline immunodeficiency virus, in its structural, genetic, antigenic, and biological properties (5, 6, 16). Although BIV has been characterized in detail molecularly (5, 6, 7, 11, 15), little is known about the biology of naturally occurring BIV infection. There has been no confirmed evidence of association of BIV infection with a naturally occurring disease condition. However, BIV infection was reported to be associated with decreased lymphocytic blastogenic response (12) and milk production (13). A possible immune dysfunction in BIV-infected calves, with a drop in CD4/CD8 ratio and a decrease in antibody response to virus vaccines, was also reported (27). Progress in the development of rapid tests for diagnosis of BIV in cattle has been slow because of the inability to generate adequate amounts of BIV antigens required for seroepidemiological studies. BIV can be propagated well only in primary bovine cell cultures, and there is a lack of availability of continuous cell lines expressing high levels of BIV. The yields of native viral protein from primary bovine cell culture are quite low (24), and those proteins show a high background reactivity with negative control sera in serological tests (25). Thus, there is a need for recombinant BIV antigens to facilitate studying the epidemiology and prevalence of BIV antibodies.

The BIV genome has been cloned, and the complete nucleotide sequence has been determined (5). Among the structural proteins predicted by the nucleotide and amino acid sequences of BIV is the core protein encoded by the gag gene. The Gag precursor of BIV has been shown to have a molecular mass of 53 kDa (17), and, by analogy to HIV cleavage products, it is predicted to be processed into three smaller proteins, p17, p26, and p15 (10, 14), which are the matrix, capsid, and nucleocapsid proteins, respectively. Immune sera from calves experimentally infected with purified BIV detected p26 proteins in immunoblot analysis (25). This suggested that p26 is the major core, or capsid, protein of BIV. Thus, the gag gene product is an important viral antigen that induces a strong immune response in infected cattle. Recently, a purified recombinant BIV Gag protein was used in an immunoblot assay to detect BIV antibodies in field bovine serum samples (29). The method proved to be sensitive and specific by comparing the results with PCR (29). However, the immunoblotting is tedious and time-consuming. In addition, the recombinant Gag protein was expressed in pATH10 system as a TrpE fusion protein (3). The TrpE fusion protein accounts for more than 50% of the recombinant protein. Since calves are exposed to a variety of bacterial infections, the large proportion of fusion protein gives high background reactivity. Therefore, the fusion proteins may not be suitable to be used as an enzyme-linked immunosorbent assay (ELISA) antigen. Furthermore, the expressed level of Gag fusion protein in the pATH10 is relatively low, and it is difficult to purify required amounts of the specific protein. The present study was undertaken to express recombinant Gag protein in higher quantities and to develop an ELISA for the diagnosis of BIV infection.

MATERIALS AND METHODS

Construction of prokaryotic BIV expression plasmids. For expression of BIV in Escherichia coli, the pATH gag-3 clone, containing 781 bp of the gag coding region from BIV was cloned into an expression vector, pQE32, which expressed high levels of recombinant protein from Escherichia coli. The ELISA was standardized by a checkerboard titration against known BIV-positive and -negative sera from cattle and a monoclonal antibody to the Gag protein. A total of 139 cattle serum samples, from the diagnostic laboratory at Kansas State University, Manhattan, and from the Dairy Station, Louisiana State University, Baton Rouge, were compared by ELISA and immunoblot assays for the detection of BIV-specific antibodies. Of 26 cattle sera samples which tested positive using the immunoblot assay, 23 were positive by ELISA, thus establishing a strong correlation between the two tests. The sensitivity and specificity of ELISA relative to immunoblotting were 0.88 and 0.93, respectively. ELISA proved to be as specific as immunoblotting but was much less time-consuming and easier to perform.

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region, was used as a PCR template to generate a 751-bp gag product (Fig. 1). The 5’ primer (GAGATCCAGCGGCAAGCTGATAAGGAA) corresponded to nucleotides (nt) 644 to 664 in the BIV genome, with a BamHI site added at the beginning (underlined). The 3’ primer, AAGCTTATCCCACTACCTACATCG, corresponded to nt 1375 to 1395 in the BIV genome, with a HindIII site added (underlined). PCR amplification with these primers left the BamHI and HindIII restriction sites at the ends of the product. pQE32, a modified version of pDest55RBSII, a prokaryotic expression vector that uses the T5 promoter and two HindIII restriction sites enabled the cloning of the gag gene. The pQE32 vector was replicated in a bacterial strain, E. coli M15, which expresses a high level of the cloned gene under control of the lac repressor, permitting induction of recombinant protein expression by isopropyl-D-thiogalactoside (IPTG).

Expression and purification of recombinant Gag protein. The construct was maintained in E. coli M15, and expression of the Gag protein was induced with 2 mM IPTG for 4 h. E. coli strain M15 transformed with a pQE32 expression vector containing the BIV gag gene, or transformed with plasmid pQE32 alone as a negative control, was grown in 1.5 ml of Luria-Bertani broth containing both ampicillin and kanamycin overnight at 37°C. The cultures were then inoculated with fresh Luria-Bertani broth (1:4 dilution) and incubated for 30 min at 37°C. IPTG was added to a final concentration of 2 mM to induce protein expression. After 4 h of induction, cells were harvested by centrifugation at 4,000 x g for 15 min and lysed by sonication in lysis buffer B (8 M urea, 0.1 M NaH2PO4, 0.01 M, KCl, 10 mM Tris [pH 8.3], 2.5 mM MgCl2) and then incubated with an equal volume of extraction buffer B (10 mM Tris [pH 8.3], 2.5 mM MgCl2, 1% NP-40, 120 μg of protease K per ml) at 60°C for 1 h. DNA was then extracted with a phenol-chloroform solution, followed by precipitation with isopropanol. After washing with 70% alcohol and air drying, DNA was suspended in distilled water and the DNA concentration was determined. DNAs from bovine blood samples and BIV-positive and BIV-negative control DNAs were amplified by PCR using a pair of primers designed to target a 242-bp highly conserved BIV gag gene.

RESULTS

Cloning, sequencing, and expression in E. coli of BIV Gag protein. One 751-bp gag gene, corresponding to nt 644 to 1395 of the BIV genome, was made by PCR amplification (Fig. 1) and subcloned into the prokaryotic expression vector pQE32, designated pQE32-gag, for the production of Gag protein. pQE32-gag was sequenced across the fusion junction to ensure preservation of the reading frame. The sequence results (data not shown) indicated that the recombinant 29-kDa Gag protein contains only 13 amino acids from the bacterial part, which accounts for 5% of the total protein. After electrophoresis and Coomassie blue staining, a distinct 29-kDa Gag-specific band was observed in the bacterial lysates containing pQE32-gag after IPTG induction (Fig. 2a, lane 2). The observed molecular mass was in agreement with the predicted molecular mass of 29 kDa from the construct. The protein was purified byning Ni-NTA columns and is shown as one band in SDS-PAGE (Fig. 2a, lane 3). The total cellular protein from a clone containing the plasmid, pQE32, served as the negative control (Fig. 2a, lane 1). The recombinant protein constituted approximately 25% of the total protein after IPTG induction. The final yield of the fusion protein was about 1,500 μg/ml of bacterial culture (data not shown).

Immunoblot detection of Gag proteins. In order to test the reactivities of the expressed Gag protein, immunoblot analysis was carried out using a monoclonal antibody against Gag pro-
to other bovine viruses, e.g., BVDV and bovine leukemia virus (BLV), did not react with the BIV Gag protein (Fig. 3, lanes 6 and 7). Representative BIV-positive samples (lanes 8 and 9) and BIV-negative samples (lanes 10 and 11) were also included. The minor band below the 29 kDa in lane 2 may result from the degradation product of Gag protein.

**Optimization of ELISA using recombinant Gag protein.** Preliminary titration experiments were conducted to determine optimum reagent concentrations and Immulon plates using specific monoclonal antibodies and known BIV-positive and -negative bovine sera. The most optimal and consistent results were obtained when 0.4 μg of Gag protein was used to coat each well of the Immulon I plate. Sample concentrations were determined by standard checkerboard titration, using 0, 1:50, 1:100, and 1:200 dilutions, with the 1:50 dilution found to be optimal based on the best signal-to-noise ratio. Samples were run in triplicate; the greatest outlier OD reading was discarded, and the remaining two values were averaged to give an adjusted OD reading for each sample. This was done for each positive and negative control for each run. The greatest outlier OD reading being discarded was determined based on the coefficient of variation (CV). If the CV for the OD reading in each sample was greater than 5%, the OD reading was discarded. If all the three CVs for each sample were within 5%, the OD reading with the largest CV was discarded. Signal-to-positive ratios for each sample were determined according to the following formula used in the Kansas State University diagnostic laboratory (26a): (adjusted ODsample - adjusted ODnegative)/(adjusted ODpositive - adjusted ODnegative).

In order to establish a positive cutoff point, the adjusted ODS of the negative controls were averaged and compared with the average adjusted ODS of all positive controls, according to the formula [(average adjusted ODnegative)/(average adjusted ODpositive - average adjusted ODnegative)] × 2, which gave a cutoff of 0.76 with a 95% lower confidence limit. Samples were judged to be positive if the sample signal-to-positive ratio was equal or greater than 0.76. Using this cutoff value, the ELISA results for 139 samples were obtained. The comparison of the results with those obtained by immunoblotting are summarized in Table 1. A total of 139 samples from Kansas State University and Louisiana State University were tested by both ELISA and immunoblotting. The number of positive samples as determined by both tests was 23, and the number of negative samples by both tests was 105. Three samples were positive by immunoblotting but negative by ELISA, while eight samples positive by ELISA were negative by immunoblotting. All BIV-positive samples by immunoblotting were confirmed by PCR (data not shown). Using standard formulas (23) to determine sensitivity and specificity, immunoblot results were treated as the true status. Sensitivity and specificity using immunoblot as the true status can be calculated according to the following equations: sensitivity = A/(A + C) and specificity = D/(B + D), where A is the number of samples ELISA positive and immunoblot positive, B is the number ELISA positive and immunoblot negative, C is the number ELISA negative and immunoblot positive, and D is the number ELISA negative and immunoblot negative. With values for A, B, C, and D of 23, 8, 3, and 105, respectively, the sensitivity equals 88% and the specificity equals 93%. A statistical analysis was performed on the data sets using McNemar’s method. McNemar’s test asked whether the results of the ELISA and immunoblotting were different, and no significant difference was found at the P level of 0.10. In conclusion, the two methods agreed with each other.
Molecular mass standards in kilodaltons are given for markers at the left. The arrow indicates a BIV-specific band.

FIG. 3. Immunoblot analysis using BIV Gag protein as the antigen and probing with serum from cattle naturally infected with BIV (1:100) (lane 1), serum from cattle experimentally infected with BIV (1:100) (lane 2), sera from one normal calf and two non-BIV-infected calves (1:20) (lanes 3, 4, and 5), BVDV- and BLV-positive sera (1:20) (lanes 6 and 7), representative tested BIV-positive serum samples (1:100) (lanes 8 and 9), and tested BIV-negative serum samples (lanes 10 and 11). Molecular mass standards in kilodaltons are given for markers at the left. The arrow indicates a BIV-specific band.

DISCUSSION

BIV infection is prevalent worldwide, and seropositive cattle have been identified in many countries (1, 4, 7, 8, 9, 13, 22). Although there were no reports on direct associations of BIV infection and naturally occurring disease among cattle, there were also no large-scale surveys on the prevalence and distribution of BIV among cattle. The lack of large surveys for BIV was due partly to the difficulty in obtaining large amounts of BIV antigen for the test. In order to develop a rapid diagnostic test for BIV antibodies in bovine serum, we cloned the p26 Gag protein from BIV strain R29 and developed an ELISA using the recombinant Gag protein as an antigen for detection of BIV antibody.

As a potential diagnostic tool, a recombinant-protein-based ELISA for BIV infection offers many advantages over other methods. Using recombinant Gag protein as an antigen for diagnosis of BIV will eliminate the use of native viral protein, which is difficult to produce. High levels of protein expression are attainable in a prokaryotic expression system contributing to the purity of the final antigen preparation, thus improving the specificity of the immunoassay. The recombinant Gag protein expressed in this study proved to be a very sensitive and specific reagent for screening for BIV infection. The BIV-specific nature of this reactivity is confirmed by the absence of reactivity seen in immune sera from animals infected with other bovine viruses, such as BVDV and BLV.

The Gag protein was chosen as the antigen for a variety of reasons. BIV Gag protein is highly immunogenic and contains sequences that are conserved among the lentiviruses. It has been demonstrated that Gag protein elicits an early, strong, and long-lasting immune response in cattle (25, 26). Antibodies to BIV Gag protein can be detected as early as 2 weeks after BIV infection and can last for at least 2 years. The recombinant Gag protein expressed in E. coli presents epitopes similar to those of the native viral protein and could be recognized specifically by sera from BIV-infected and -immunized animals. Based on sequence analysis of the BIV gag coding region, there are no obvious glycosylated sites (5). Therefore, it is unlikely that the mature Gag protein is glycosylated in vivo.

The gag gene from BIV has been cloned into a baculovirus expression system (17), but the protein expressed had a relatively low yield. Recently, the gag gene was expressed as a TrpE fusion protein in E. coli (3), and the protein yield was still relatively low. The bacterial part of the fusion protein in the TrpE system accounts for 50% of the total which might create problems for ELISA. The Gag protein expressed here was shown to be expressed at very high level, and the protein was purified to be near homogeneity as evidenced by a single band in Coomassie blue-stained gels (Fig. 2). Furthermore, the bacterial fusion part only accounts for 5% of the total protein, which greatly increased the specificity of the recombinant Gag protein used as an antigen in immunoassay.

Western blotting was the most widely used method to evaluate serological evidence of BIV in cattle serum samples (29). The Western blot assay was considered to be sensitive and reliable because its results were confirmed by PCR (29), and the Western blot method was chosen as the classic reference test in diagnosis of HIV infection (18). In this study, an ELISA method was developed for detection of BIV antibody in serum samples and its results were compared with those obtained by Western blotting. Ten known BIV-positive and -negative serum samples were tested for BIV antibodies using the two methods, which gave consistent results. However, there were some discrepancies for the field serum samples tested using the two methods (Table 1). There were eight ELISA-positive samples which were Western blot negative and three ELISA-negative samples which were Western blot positive. Those eight positive samples were considered weakly positive samples by ELISA because they had signal cutoff values of around 0.76 to 0.78, which was near the lower limit for positive samples (0.76). At the 90% confidence limit, those samples were considered to be negative. The three ELISA-negative samples had signal cutoff values of around 0.73, which were near the upper limit for the negative sample. At the 99% confidence limit, those samples were considered to be positive. All 11 samples were considered to be indeterminate by ELISA. These differences could be due in part to differences in test sensitivity. Generally, ELISA is more sensitive than Western blotting because it needs a relatively smaller amount of antigen in testing. The
differences may be caused by the presence of other closely related retrovirus antibodies in field serum samples which may interfere with the BIV test. Since BIV is closely related to other retroviruses, antibodies against other retroviruses in serum samples might give false-positive results in Western blot and ELISA tests. A PCR test could overcome this problem by using unique primers designed for BIV. Since isolation of BIV from samples is very difficult, there is no way to confirm the true status of BIV infection. Thus, a combination of three assays, i.e., ELISA, Western blotting, and PCR, was necessary in order to confirm the indeterminate ELISA profiles. According to the recommendations of the World Health Organization, a combination of three tests is recommended for diagnostic testing for HIV (2). In HIV testing, a combination of three assays was reported to be necessary to avoid false-positive results (2).

In conclusion, the expressed protein has been used successfully as an antigen for ELISA. The ELISA established here is shown to be as specific as immunoblotting, and it has the advantage of being less time-consuming and thus more economical. Therefore, it provides a better means than the currently available serological tests to study BIV antibody status in cattle herds.

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**TABLE 1. ELISA and immunoblotting results**

| Sample | ELISA result | Immunoblot result | Sample | ELISA result | Immunoblot result | Sample | ELISA result | Immunoblot result |
|--------|--------------|--------------------|--------|--------------|--------------------|--------|--------------|--------------------|
| 1      | –            | –                  | 48     | +            | +                  | 95     | –            | –                  |
| 2      | –            | –                  | 49     | –            | –                  | 96     | –            | –                  |
| 3      | +            | +                  | 50     | –            | –                  | 97     | +            | +                  |
| 4      | –            | –                  | 51     | –            | –                  | 98     | –            | –                  |
| 5      | –            | –                  | 52     | +            | +                  | 99     | –            | –                  |
| 6      | –            | –                  | 53     | –            | –                  | 100    | –            | –                  |
| 7      | –            | –                  | 54     | +            | –                  | 101    | +            | +                  |
| 8      | –            | –                  | 55     | +            | +                  | 102    | +            | +                  |
| 9      | –            | –                  | 56     | +            | –                  | 103    | –            | –                  |
| 10     | –            | –                  | 57     | –            | –                  | 104    | –            | –                  |
| 11     | +            | –                  | 58     | –            | –                  | 105    | –            | –                  |
| 12     | –            | –                  | 59     | –            | –                  | 106    | +            | +                  |
| 13     | –            | –                  | 60     | –            | –                  | 107    | +            | +                  |
| 14     | –            | –                  | 61     | –            | –                  | 108    | +            | +                  |
| 15     | –            | –                  | 62     | +            | +                  | 109    | –            | –                  |
| 16     | +            | +                  | 63     | +            | –                  | 110    | –            | –                  |
| 17     | +            | +                  | 64     | +            | +                  | 111    | +            | +                  |
| 18     | –            | –                  | 65     | –            | –                  | 112    | –            | –                  |
| 19     | –            | –                  | 66     | –            | –                  | 113    | –            | –                  |
| 20     | –            | –                  | 67     | +            | +                  | 114    | –            | –                  |
| 21     | –            | –                  | 68     | –            | –                  | 115    | –            | –                  |
| 22     | –            | –                  | 69     | +            | +                  | 116    | –            | –                  |
| 23     | –            | –                  | 70     | +            | –                  | 117    | +            | –                  |
| 24     | –            | –                  | 71     | –            | –                  | 118    | +            | +                  |
| 25     | –            | –                  | 72     | –            | –                  | 119    | +            | +                  |
| 26     | –            | –                  | 73     | –            | –                  | 120    | –            | –                  |
| 27     | –            | –                  | 74     | –            | –                  | 121    | –            | –                  |
| 28     | –            | –                  | 75     | +            | –                  | 122    | –            | –                  |
| 29     | –            | –                  | 76     | –            | –                  | 123    | –            | –                  |
| 30     | –            | –                  | 77     | –            | –                  | 124    | –            | –                  |
| 31     | –            | –                  | 78     | –            | –                  | 125    | –            | –                  |
| 32     | –            | –                  | 79     | –            | –                  | 126    | –            | –                  |
| 33     | –            | –                  | 80     | –            | –                  | 127    | –            | –                  |
| 34     | –            | –                  | 81     | –            | –                  | 128    | –            | –                  |
| 35     | –            | –                  | 82     | +            | –                  | 129    | –            | –                  |
| 36     | –            | –                  | 83     | –            | –                  | 130    | –            | –                  |
| 37     | –            | –                  | 84     | –            | –                  | 131    | –            | –                  |
| 38     | +            | +                  | 85     | –            | –                  | 132    | –            | –                  |
| 39     | –            | –                  | 86     | –            | –                  | 133    | +            | +                  |
| 40     | –            | –                  | 87     | –            | –                  | 134    | –            | –                  |
| 41     | –            | –                  | 88     | –            | –                  | 135    | –            | –                  |
| 42     | –            | –                  | 89     | –            | –                  | 136    | –            | –                  |
| 43     | +            | +                  | 90     | –            | –                  | 137    | –            | –                  |
| 44     | –            | +                  | 91     | +            | +                  | 138    | –            | –                  |
| 45     | –            | –                  | 92     | +            | –                  | 139    | –            | –                  |
| 46     | –            | –                  | 93     | –            | +                  |        |               |                    |
| 47     | –            | –                  | 94     | –            | –                  |        |               |                    |

* Samples 1 to 40 were from Kansas State University, and samples 41 to 139 were from Louisiana State University.
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