1995

Viral variation of the bovine immunodeficiency-like virus

David Lee Suarez
Iowa State University

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Viral variation of the bovine immunodeficiency-like virus

by

David Lee Suarez

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Microbiology, Immunology and Preventive Medicine
Major: Veterinary Microbiology

Approved:
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In Charge of Major Work
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For the Major Department
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For the Graduate College

Iowa State University
Ames, Iowa
1995
Dedicated to Samuel Grayson Suarez
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CHAPTER 1. GENERAL INTRODUCTION

The bovine immunodeficiency-like virus (BIV) was discovered over 25 years ago, but the impact that this virus has on the cattle population remains unknown. This lack of understanding of the pathogenesis of BIV may be the result of several different things. First is the possibility that BIV has a very slow onset of disease. With most other lentiviruses, the disease outcome occurs many months or years after the individual is infected with the virus. Two examples of lentiviruses that have a long period before clinical disease are the human immunodeficiency virus (HIV) and the Maedi-Visna virus. The second possibility is that BIV may cause an immune deficiency type disease that allows a variety of other pathogenic agents to cause disease. This results in a variety of disease manifestations that are difficult to trace back to the original causative agent. Examples of this include HIV and the feline immunodeficiency virus (FIV). Both agents cause a primary immunodeficiency that allows many secondary pathogens to cause clinical illness. Having a variety of secondary disease conditions can obscure the original pathogen, and this may be occurring with BIV. Third, our current animal husbandry methods may be minimizing the effect of BIV infection. If BIV only causes disease after a prolonged incubation period, cattle may be culled from the herd before BIV causes disease. Fourth, perhaps the proper experimental methods are not being used to study this virus. Evidence from other viruses show a selection of the virus when it is cultured in vitro. With HIV, a loss of diversity is observed in the virus that is recovered by cell culture techniques (Kusumi et al., 1992). Attempts to culture the virus from naturally infected animals may be attenuating the virus, so that when cattle are
experimentally inoculated with cell culture adapted virus, it may be an attenuated virus that is not characteristic of what is observed in the field. Fifth, the isolates of BIV that are being used for experimental study are attenuated. Until recently, the only isolate that was available for study was the R29 isolate. Most of the scientific studies with BIV have used the R29 isolate, R29-derived isolates or reagents based on the R29 isolate. This reliance on R29 may have skewed our view of what BIV may cause in natural infections. Finally, the possibility that BIV is well adapted to its host and causes no clinical disease must be recognized as a possibility. For example, spumaviruses, another type of retrovirus, appear to be widespread in cattle, cat, simian and human populations, but cause no apparent disease. It remains a difficult puzzle to determine the role BIV may have in cattle disease.

I believe that BIV is likely a disease causing agent, based on comparative studies with other lentiviruses. It causes a persistent infection in cattle and it appears to be widespread in the cattle population. The molecular biology of the virus shows many similarities with other lentiviruses including the presence of regulatory genes and methods of transactivation. My research, at least partially, involves the study of BIV as a potential pathogen. Of the six different possibilities that were addressed above as reasons why the role of BIV in disease maybe unclear, I am concerned primarily with points four and five. This includes the possibility that R29 may not represent BIV isolates that are present in naturally infected animals and that an in vitro selection factor may be involved in attenuating BIV isolates recovered in cell culture.

Examination of different viral isolates can be accomplished by a variety of different methods. Biologic differences, antigenic differences and sequence
differences are all methods of examining viral variation. Examining nucleotide sequence may be the most probing since biological and antigenic differences are the result of nucleotide changes. However, an examination by several of these criteria provides the most complete comparisons between different isolates of the same virus.

Other lentiviruses have been shown to occur as quasispecies, where almost every virion has a different nucleotide sequence when compared to each other. The quasispecies state is thought to occur mainly by the low fidelity of the reverse transcriptase enzyme that allows a high rate of error introduction into the transcription process. Thus allowing for the generation of diversity. Different quasispecies are then positively or negatively selected for and particular variants become more or less prevalent. These facts point out the value of studying both different field isolates of BIV as well as examining different variants of the R29 isolate of BIV.

In this thesis, R29-derived isolates and other field isolates are compared both for biological differences as well as nucleotide differences, with the intent of evaluating the potential role of BIV in disease. The first step in examining diversity is to obtain diverse isolates for study. BIV is very difficult to culture from naturally infected animals and only two additional isolates, other than R29, were isolated for biological comparisons. PCR amplification of different isolates is used to demonstrate some diversity among BIV isolates, and cloning and sequencing of PCR product is also used for sequence comparisons. For the purposes of examining the nucleotide sequences, selected areas of the lentiviral genome will be used for examination. In other lentiviruses the reverse transcriptase gene has been shown to be a highly conserved part of the genome.
The surface envelope gene has been described as having regions that are highly divergent in nucleotide sequence. Therefore in the work presented here, these two areas of the BIV genome are examined to assess the amount of sequence diversity of BIV. Evidence of biological variation in cell culture and after experimental inoculations are also examined for the cultured isolates.

**Dissertation Organization**

An alternative dissertation format was used for this dissertation, and it includes three papers that have been published, accepted for publication, or are in preparation for submission to scientific journals. Chapter 3, "Isolation and characterization of new wild-type isolates of bovine lentivirus", has been published in the *Journal of Virology*. Chapter 4, "Improved early and long-term detection of bovine lentivirus by a nested polymerase chain reaction test in experimentally infected calves", has been accepted for publication by the *American Journal of Veterinary Research*. Chapter 5, "Identification of hypervariable and conserved regions in the surface envelope gene in the bovine lentivirus", is submitted to *Virology*. For all three papers, I am the primary author and principal investigator. The dissertation also contains a general introduction, literature review, general conclusions and references cited in the literature review. The literature review encompasses parts of a review article, for which I was the primary author, entitled "Bovine lentivirus (BIV): Diagnosis, prevalence, and pathogenesis", that was published in the Proceedings of the 98th Annual Meeting of the United States Animal Health Association.
CHAPTER 2. LITERATURE REVIEW

Introduction

The lentiviruses that are known or suspected to cause disease in their natural hosts are listed in Table 1. The bovine immunodeficiency-like virus (BIV) remains a suspected pathogen, both by comparisons with other lentiviruses and by observations of experimental studies done with the virus. Table 1 divides the lentiviruses into the immune-mediated type diseases at the top, the immunodeficiency type diseases at the bottom, with BIV in between. If BIV does cause disease, it is unclear in which of the two groups of viruses BIV will belong.

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<td></td>
<td>Caprine arthritis encephalitis virus</td>
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<td></td>
<td>Maedi-Visna virus</td>
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<tr>
<td></td>
<td>Jembrana disease virus</td>
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<tr>
<td><strong>Bovine immunodeficiency-like virus</strong></td>
<td></td>
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<tr>
<td></td>
<td>Human immunodeficiency virus Type 1</td>
</tr>
<tr>
<td></td>
<td>Human immunodeficiency virus Type 2</td>
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<tr>
<td></td>
<td>Non-human primate lentiviruses</td>
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<td></td>
<td>Feline immunodeficiency virus</td>
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</table>

BIV is one of three different retroviruses that are known to infect cattle in the United States, and they include the bovine leukemia virus (BLV), the bovine syncytial virus (BSV) and the bovine immunodeficiency-like virus (BIV). A fourth retrovirus that infects cattle is the Jembrana disease virus (JDV), and it causes
an acute disease with high mortality in *Bos javanicus* cattle. JDV can infect *Bos taurus* and *Bos indicus* cattle, but it only causes a sub-clinical infection (Kertayadnya, et al., 1993). JDV is thought to be a lentivirus that is more closely related to BIV than to other lentiviruses, but it is thought to be restricted in distribution to Indonesia. The relationship of JDV and BIV appears to be analogous to the infection of humans by two lentiviruses, HIV-1 and HIV-2. This literature review will report what is known about BIV, and it will highlight some deficient areas of BIV research.

**Pathogenesis**

In examining the known pathogenesis of BIV, it is important to understand the experimental models that were used to gain this information. Four distinct categories of BIV inoculum have been used in experimental studies with cattle, with each inoculum giving unique results. Three of these inocula were derived from the original isolate of BIV and can be divided into low passage R29 (Van Der Maaten et al., 1972), bovine viral diarrhea virus (BVDV) contaminated high passage R29 (Carpenter et al., 1992; Flaming et al., 1993; Van Der Maaten et al., 1990; Whetstone et al., 1990), and BVDV-free high passage R29 (Flaming et al., 1993; Suarez et al., 1993). The fourth category of inoculum is the recently described Florida isolates (Suarez et al., 1993).

R29 was originally isolated from an eight-year-old dairy cow that had a persistent lymphocytosis, progressive weakness, and emaciation. Necropsy data showed a generalized hyperplasia of the lymph nodes and a mild perivascular cuffing in the brain (Van Der Maaten et al., 1972). Virus was isolated on fetal bovine spleen cell cultures and this inoculum was given to a group of colostrum-deprived calves. The principal observations from this initial inoculation of a low-
passage isolate was a transient mononuclear cell increase early in infection (10-20 days post-inoculation [p.i.]), a second mononuclear cell increase later in infection (60-150 days p.i.), and clinical evidence of enlargement of subcutaneous lymphatic nodules (Van Der Maaten et al., 1972).

The R29 isolates of today have undergone significant changes in the intervening twenty years. For example, the R29-1203 isolate has been passed through fetal bovine spleen cell cultures, canine thymocyte cell line, Madin-Darby bovine Kidney cell line, and is currently being maintained in fetal bovine lung cell cultures, and must be considered highly tissue culture adapted (Black, 1989; Bouillant et al., 1989; Van Der Maaten et al., 1972; Whetstone et al., 1991). The R29-derived isolates were also contaminated with a noncytopathic strain of BVDV during in vitro passage, and although BVDV-free isolates are available today, numerous animal studies were conducted with BVDV-contaminated isolates (Carpenter et al., 1992; Flaming et al., 1993; Van Der Maaten et al., 1990; Whetstone et al., 1990). Evidence of attenuation of the currently available R29 isolates is the loss of the transient mononuclear cell increase early and late after infection (Carpenter et al., 1992; Flaming et al., 1993, Van Der Maaten et al., 1990). R29-derived isolates still appear to generate a lymphoproliferative response, but it is not as great as that originally described (Carpenter et al., 1992). The recently described Florida isolates, when examined in experimental inoculations, had some of the same observed clinical signs as the original BIV R29 isolate, including a transient mononuclear cell increase early in infection and a lymphoproliferative response, composed of a mild follicular hyperplasia. The late mononuclear cell increase was not observed in the very limited number of animals followed for long periods of time (Suarez et al., 1993). Further
experimental studies with the FL112 isolate have revealed that the early mononuclear cell increase observed in experimental inoculations was predominantly B cells (Suarez et al., 1993).\(^a\) No large changes were observed in other cell populations during short-term infection with this isolate.\(^a\)

Two infectious molecular clones of R29, R29-127 and R29-106, have been made and sequenced (Braun et al., 1988; Garvey et al., 1990). R29-127 and R29-106 are very similar except for an 87 nucleotide base pair deletion in the 5' end of the surface envelope (SU) gene of R29-106 (Garvey et al., 1990). All of the R29-derived isolates sequenced or examined by PCR have a R29-106 phenotype, with the 87 b.p. deletion. The R29-127 size genome was present in very low numbers, as measured in a Southern Blot analysis of the culture that it was cloned from (Braun et al., 1988). No evidence is available to support that the R29-127 size genome is still present in current viral stocks. This size difference in the envelope gene is further confounded by the observation that the Florida isolates and all other natural field isolates studied have a larger SU gene size than R29-127.\(^b\) It appears that the R29 SU gene no longer represents the SU gene observed in BIV isolates that are out in the field. While R29 and its derivatives have been very valuable in the study of BIV, caution must be used when trying to interpret what a natural infection of BIV might cause in the way of


clinical signs or possible disease as compared with what has been reported using R29. A further caution needs to be made for the BVDV contaminated isolates that were used in some studies, since BVDV is a known immunosuppressive agent early after infection, causing fever and lymphopenia (Potgieter, 1992).

BIV causes a persistent infection in cattle with a detectable antibody response occurring typically two to four weeks post-inoculation (p.i.). Virus can be detected in peripheral blood by PCR as early as 3 days p.i. and it can continue to be detected for up to 3 years p.i. (Suarez et al., 1995). Virus can be isolated early after experimental inoculation, from 3 to 7 days p.i. (Whetstone et al., 1990; Suarez et al., 1993; Suarez et al., 1995). Antibody response peaks early in infection, and in many experimentally infected animals antibody levels to p26 and gp110 decline, sometimes to undetectable levels (Whetstone et al., 1990; Onuma et al., 1992; Suarez et al., 1995).

Several studies have examined the potential role of BIV as an immunosuppressive agent (Flaming et al., 1993; Martin et al., 1991; Onuma et al., 1992). BIV has not been demonstrated to cause a clinically apparent immunosuppression (i.e., greater susceptibility to infections with atypical pathogens) in experimentally inoculated cattle. Several reports have described specific dysfunctions of neutrophils, monocytes, and humoral response, but no clear trend has developed between different research groups (Table 2). For example, Martin (1991) described a decreased lymphocyte blastogenesis response 6 months p.i. (Martin et al., 1991). Flaming (1993) using a larger

sample size, demonstrated a slight increase in lymphocyte blastogenesis response at 4-5 months p.i. and between 19 and 27 months p.i. (Flaming et al., 1993). Again the use of only R29-derived isolates, some contaminated with BVDV virus, have confounded some of these reports. Small sample sizes have also made analysis of the data difficult. The Martin study had two principals, the Onuma study had three principals, and the Flaming study had three to eight principals in each study group (Martin et al., 1991; Onuma et al., 1992; Flaming et al., 1993). To date, no convincing evidence for immunosuppression by BIV has been demonstrated, and therefore, the use of the name bovine immunodeficiency virus is probably misleading. However, further examination of the role of BIV as an immunosuppressive agent is warranted because of its similarities to other immunosuppressive viruses.

Table 2  Immune Studies on BIV Infected Cattle

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<thead>
<tr>
<th>Study</th>
<th>BIV Isolate</th>
<th>Cattle #</th>
<th>Reported Immune Dysfunctions</th>
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<tr>
<td>Martin, 1991</td>
<td>R29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>Decreased lymphocyte blastogenesis response</td>
</tr>
<tr>
<td>Onuma, 1992</td>
<td>R29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>Several monocyte functions decreased, slight delay in humoral response to mouse serum antigen</td>
</tr>
<tr>
<td>Flaming, 1993</td>
<td>R29</td>
<td>3</td>
<td>None described</td>
</tr>
<tr>
<td>Flaming, 1993</td>
<td>R29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>Increased lymphocyte blastogenesis; decreased neutrophil ADCC&lt;sup&gt;c&lt;/sup&gt; response</td>
</tr>
<tr>
<td>Flaming, 1993</td>
<td>R29 and R29-106&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td>Decreased neutrophil ADCC and iodination response; increased lymphocyte blastogenesis</td>
</tr>
</tbody>
</table>

- a BVDV status of inoculum unknown
- b BVDV contaminated inoculum
- c Antibody dependent cell mediated cytotoxicity
BIV has also been implicated as causing encephalitis, specifically causing perivascular cuffing in the brain. This histopathologic lesion is not pathognomonic of BIV infection and it has been associated with several other disease agents. The most commonly cited reason for believing BIV causes encephalitis is the data from the necropsy report of cow R29 (Van Der Maaten et al., 1972). Other unsupported observations for brain lesions have been cited in review papers (Gonda et al., 1994). Anecdotal and other unpublished data suggest a potential role of BIV in causing perivascular cuffing in the brain, but no data at this time support an active BIV infection in the brain or evidence of clinically detectable encephalitis of infected cattle.

BIV has often been cited as causing a lymphadenopathy (Gonda et al., 1994; Gonda et al., 1987). Experimental inoculations do not support this clinical sign as being a common aspect of BIV infections. BIV has been reported to have a lymphoproliferative capacity in its ability to cause a transient mononuclear cell increase after infection and by the common observation of mild follicular hyperplasia in animals experimentally infected with BIV (Carpenter et al., 1992; Suarez et al., 1993; Van Der Maaten et al., 1972). In the initial experimental inoculation study by Van Der Maaten (1972), infected cattle were described as having palpably enlarged subcutaneous lymph nodes (Van Der Maaten et al., 1990). However, a generalized peripheral lymphadenopathy was not described in this study or any other study of experimentally infected animals, and it is very much in doubt if lymphadenopathy is a clinically salient feature of BIV infection.

The role of BIV as a disease agent is still debatable. One serological study has suggested a role of decreased milk production in BIV-infected animals (McNab et al., 1994). Other than in experimental studies, no other studies have
been reported that looked at objective parameters when comparing cattle infected with BIV. Serological studies must be carefully interpreted since the BIV infection may only be associated with another agent that is responsible for decreased milk production or some other clinical parameter. Results obtained using the attenuated R29 isolate may be masking the real disease role of BIV in natural infections. The question of whether BIV causes or does not cause disease is no closer to being answered today than it was 25 years ago. BIV elicits a characteristic response in cattle and it is assumed to cause a lifelong infection. Because most other lentiviruses cause disease, it is tempting to want to believe that BIV also causes disease. More research is needed to objectively examine the potential role of BIV in disease.

**Surrogate Models**

Because of the difficulty and expense of working with cattle, especially for long term infections, a smaller model of BIV infection has been investigated. Experimental studies with mice, rats, guinea pigs, rabbits, goats and sheep have been described (Van Der Maaten et al., 1990; Gonda et al., 1990; Whetstone et al., 1991; Van Der Maaten et al., 1992; Pifat et al., 1992; Hirai et al., 1994; Smith et al., 1994; Jacobs et al., 1994). BIV has only been shown to productively infect rabbits and sheep, as evidenced by a serologic response to BIV and PCR detection of provirus in blood from sheep and rabbits, and isolation of virus at necropsy from rabbits (Van Der Maaten et al., 1992; Hirai et al., 1994; Jacobs et al., 1994; Pifat et al., 1994, Smith et al., 1994). A single report of a natural infection with BIV has been reported in a sheep, based on an antibody response to the virus (Smith 1993).
Sheep have been experimentally inoculated with R29-derived isolates of BIV (Van Der Maaten et al., 1990; Smith et al., 1994; Jacobs et al., 1994). Most of the inoculated sheep seroconverted to the virus, but virus could not be reisolated in cell culture from any of the inoculated sheep (Van Der Maaten et al., 1990; Smith et al., 1994; Jacobs et al., 1994). A nested PCR test was used in one study to document that provirus was present in peripheral blood from some of the sheep (Jacobs et al., 1994). No alterations in humoral immunity or cell-mediated functions tests have been observed (Smith et al., 1994). Experimentally infected sheep, when compared to control sheep, were observed to have altered numbers of neutrophils, eosinophils, and several lymphocyte subset populations (Jacobs, 1994). BIV does not appear to replicate as well in sheep as it does in cattle, and clinical disease has not been observed.

Rabbits were experimentally inoculated with R29-derived isolates of BIV (Van Der Maaten et al., 1992; Pifat et al., 1992; Hirai et al., 1994). Recovery of BIV by cocultivation of rabbit spleen cells with cells sensitive to BIV replication were described in all three studies (Van Der Maaten et al., 1992; Pifat et al., 1992; Hirai et al., 1994). BIV was also recovered from several other organs in one study at necropsy (Pifat et al., 1992). PCR was also used to detect BIV proviral DNA from peripheral blood mononuclear cells and tissues (Pifat et al., 1992; Hirai et al., 1994). Immunohistochemical staining in one study demonstrated BIV antigen in mononuclear cells from the spleen (Pifat et al., 1992). Humoral immune response was decreased to some antigens and moderate lymphoid hyperplasia was observed in some of the BIV infected rabbits (Hirai et al., 1994). BIV does not appear to replicate as well in rabbits as it does
in cattle, and no clinical disease has been observed. Alternative models remain of limited value in the study of the pathogenesis of BIV in cattle.

**Molecular Biology**

BIV was originally thought to be in the same group of viruses as Visna-Maedi virus on the basis of morphology of the virion, cytopathic effect in cell culture and the lymphocytic response observed in calves after experimental inoculation (Van Der Maaten et al., 1972). Electron microscopy studies of BIV in fetal bovine spleen cell cultures, demonstrated the extracellular virion had a diameter of 80 to 130 nanometers, the nucleocapsid formed during budding, and extracellular forms had an electron dense core (Van Der Maaten et al., 1972; Boothe et al., 1974). BIV was also shown to have reverse transcriptase activity with a preference for Mg$^{2+}$ (Georgiades et al., 1977). Additional studies with BIV showed an antigenic relationship of the p26 putative capsid protein (CA) with gag proteins of HIV, SIV and EIAV by homologous and heterologous radioimmunoassay, and considerable nucleotide homology of the reverse transcriptase gene with other lentiviruses (Gonda et al., 1987). These were the initial studies that provided evidence that BIV was a retrovirus in the lentivirus subfamily, and further research has expanded on this initial information.

With the sequencing of two infectious molecular clones of BIV, R29-127 and R29-106, the genomic organization of BIV was determined. BIV has the characteristic retroviral organization of long terminal repeat (LTR)- gag-pol-env-LTR (Garvey et al., 1990). The long terminal repeat (LTR) of the BIV genome has been described as having the TAR region important in the transactivation of this virus. Sequence analysis showed several other transcription factor binding sites, including NF-kB, AP1, AP4, and Sp1 binding sites. These binding sites can
act as promoters for viral transcription when cellular proteins bind to them. These promoter elements may increase transcription of the viral genome after the cell has become activated, and therefore they may be important in the replication of the virus.

The first large open reading frame of the BIV genome is *gag*. The *gag* open reading frame contains three major structural proteins, the matrix protein (MA), capsid protein (CA), and the nucleocapsid protein (NC), that are analogous to proteins observed in other lentiviruses (Battles et al., 1992; Tobin et al., 1994). Three smaller proteins, identified as p2L, p3 and P2, have unknown functions (Tobin et al., 1994). The amino terminal, carboxy terminal and partial to complete amino acid sequences of these proteins have been determined, and the purified proteins have been used as antigens in protein immunoblots. The MA and CA proteins migrated near their expected molecular weight, but the NC protein migrated slower than would have been predicted (Tobin et al., 1994). All three proteins could be recognized by some experimentally infected and naturally infected cattle serum (Whetstone et al., 1990; Tobin et al., 1994; Battles et al., 1992). Proteins in the *gag* gene section are proteolytically cleaved from a *gag* precursor protein (pPr53* gag*) and from a *gag-pol* polyprotein precursor protein (pPr170* gag-pol*) (Battles et al., 1992). These precursor proteins have been detected immunologically in protein immunoblot studies (Battles et al., 1992), and are cleaved into at least 6 different proteins that are present in the mature virion (Tobin et al., 1994).

The *gag* gene or portions of the *gag* gene have been cloned and expressed in both *E. coli* and Baculovirus expression systems (Atkinson et al., 1992; Rasmussen et al., 1990). The expressed proteins from both methods were
recognized by serum from experimentally and naturally infected cattle (Atkinson et al., 1992; Rasmussen et al., 1990). The baculovirus expressed \textit{gag} protein has been used to screen cattle naturally infected with BIV in an ELISA format (Cockerell et al., 1992; Coats et al., 1994). The \textit{E. coli} expressed \textit{gag} protein, \textit{gag3}, was used to inoculate mice to make monoclonal antibodies to the \textit{gag} protein. Two monoclonal antibodies were characterized and shown to recognize the p26 CA protein of BIV (Wannemuehler et al., 1993).

The second large open reading frame of the BIV genome is the \textit{pol} gene. This includes the reverse transcriptase (RT) enzyme, whose activity was one criteria for inclusion in the retrovirus family (Georgiades et al., 1977; Kashanchi et al., 1991). The p72 protein, the putative RT enzyme, has been detected with sera from experimentally and naturally infected cattle (Whetstone et al., 1991). The remainder of information about the \textit{pol} gene is based on analysis of nucleotide sequence. The \textit{pol} gene is thought to be translated as a \textit{pol} precursor that is part of the \textit{PR170gag-pol} precursor (Garvey et al., 1990). The \textit{pol} proteins are thought to be translated after a ribosomal frameshifting event at a poly-A site at the beginning of the \textit{pol} gene (Garvey et al., 1990; Battles et al., 1992). Three separate proteins are thought to be made by the cleaving of the \textit{pol} polyprotein into a protease (PR), RT, and endonuclease/integrase proteins (Garvey et al., 1990). The predicted RT gene of BIV has been compared to other lentiviral RT genes, and highly conserved motifs have been observed (Gelman et al., 1992). Phylogenetic tree analysis showed that BIV is a distinct member of the lentivirus family (Gonda et al., 1994). Sequence comparison of R29-derived isolates of BIV and the recently described Florida isolates of BIV have shown greater than 92% sequence homology (Suarez et al., 1993).
The third large open reading frame is the *env* gene, which encodes the surface envelope gene (SU) and the transmembrane gene (TM) (Garvey et al., 1990). A 3.8 kb RNA transcript has been identified that encodes the *env* proteins (Oberste et al., 1991). The start site for the SU gene is thought to originate from the second ATG start site. Several studies further suggest this to be the case, but it has not been proven (Rasmussen et al., 1992; Oberste et al., 1991; Chen, P. et al., 1994). The SU and TM proteins are thought to be proteolytically cleaved at a RKPR motif, and this proteolytic cleavage region is similar to that observed in other lentiviruses (Garvey et al., 1990). Both proteins are glycosylated and can be detected with immune serum on a protein immunoblot (Whetstone et al., 1991). Recombinant proteins have been made in *E. coli* and baculovirus expression systems that react with BIV immune serum (Chen, P. et al., 1994; Rasmussen et al., 1992). Three molecular clones of R29 have been made and sequenced, including R29-127, R29-106, and R29-ND (Garvey et al., 1990; Nadin-Davis, Genbank). All three *env* genes have high sequence similarity, with the only large difference being a 87 b.p. deletion of the 5' end of the SU gene in R29-106 and R29-ND. The larger R29-127 has been used as the prototype for BIV, even though it was present in only very low levels in the cell culture from which the molecular clone was made (Garvey et al., 1990).

Complex retroviruses are defined as having regulatory proteins important in the replication life cycle of the virus. Two BIV proteins, tat and rev, have been shown experimentally to be of some importance in the regulation of the virus, and will be discussed in greater detail. Another regulatory protein, vif, has been identified based on similarities with other lentiviral vif proteins, but little experimental data has been reported about this protein (Oberste et al., 1992;
Oberste et al., 1991). It is thought to be transcribed from the central region of the BIV genome, overlapping the 3' end of the polymerase gene (Oberste et al., 1992). A 4.1 kb transcript was identified that may encode the vif gene, but this could not be confirmed (Oberste et al., 1991). Three additional proteins, predicted or reported as unpublished observations, include Tmx, Vpw and Vpy (Garvey et al., 1990; Gonda et al., 1994). All the regulatory proteins are produced by alternative splicing of mRNA from the central region of the BIV genome and a region downstream of the TM gene (Oberste et al., 1991; Gonda et al., 1994).

A transactivation protein (tat) was predicted based on sequence similarities with the HIV tat gene (Garvey et al., 1990). A transactivating gene was shown to be present in BIV infected cells by using a chloramphenicol acetyl transferase (CAT) reporter system that could increase transcription up to 7 fold over controls (Pallansch et al., 1992). The tat transcript was shown to be a spliced RNA transcript of 1.4 kb (Oberste et al., 1991). Two exons, both from the env reading frame, form the tat transcript, but the tat protein is believed to be transcribed from the first exon only (Liu et al., 1992). The tat protein is thought to have a nuclear localization motif as well as an arginine rich region that binds to an RNA transcript (Liu et al., 1992; Chen, L. et al., 1994). The BIV transactivating response element (TAR) was shown to be in the BIV LTR region downstream of the transcription start site, and is predicted to form an RNA stem loop structure that is recognized and bound by the tat protein (Carpenter et al., 1993). An arginine-rich region of the tat protein was shown to bind to the BIV TAR element with high affinity and specificity. This binding does not require any
cellular proteins for this interaction, and therefore it is different from HIV tat binding to the HIV TAR element (Chen, L. et al., 1994).

The BIV rev protein is also thought to be a spliced transcript with two coding exons, both overlapping the env gene (Garvey et al., 1990; Oberste et al., 1991). A 1.7 kb RNA transcript was described that is thought to be the rev transcript (Oberste et al., 1991). The rev gene is phosphorylated and was shown to localize in the nucleus after translation (Oberste et al., 1993). The rev gene regulates the production of both gag protein as well as env protein (Oberste et al., 1993). BIV rev probably plays an important role in the regulation of the BIV life cycle, and it is important in the production of virions from the infected cell (Oberste et al., 1993).

**Diagnosis**

Isolation of BIV from field samples has been very difficult at best. Numerous attempts to culture the virus have resulted in failure. Currently, only two published reports have documented the successful culturing of BIV from naturally infected cattle, allowing three different isolates to be available for study (Van Der Maaten et al., 1972; Suarez et al., 1993). These isolations were made from cocultivation of primary fetal bovine spleen or lung cell cultures with blood buffy coat cells from infected cattle. However, this same technique has proven to be unsuccessful in other attempts at isolation from known naturally and experimentally infected animals. The prominent cytopathic effect (CPE) that has been described for BIV in cell culture is syncytial formation after multiple blind passages. However, it has taken up to 8 blind passages in cell culture before CPE is observed, which results in a 3- to 6-week lag time before infection with BIV becomes detectable in clinical samples (Van Der Maaten et al., 1972; Suarez et
al., 1993). The use of a reverse transcriptase assay, a syncytial assay, and immunoperoxidase assay have also been used to monitor for BIV infection of cell cultures (Gonda et al., 1987; Onuma et al., 1990; Horzinek et al., 1991; Kashanchi et al., 1991; Geng et al., 1992; Nadin-Davis et al., 1993; Wannemuehler et al., 1993; Jacobs et al., 1994). Contamination of cell cultures with more aggressive viruses, like bovine herpesviruses and bovine syncytial virus, is common (Van Der Maaten et al., 1972; Suarez et al., 1993). Experimental testing with R29-derived isolates has demonstrated that BIV does not grow well on established cell culture lines and it is best propagated and isolated on primary fetal bovine cell cultures, based on antigen production as measured by agar gel immunodiffusion and radial immunodiffusion tests (Whetstone et al., 1991). The optimum conditions for culturing wild-type virus, however, may still remain to be identified. There have been reports that BIV has been grown on heterologous species cell cultures such as a canine thymocyte cell line (Bouillant et al., 1989). These cell cultures were not compared to the fetal bovine primary cell cultures either in sensitivity for virus recovery or in the production and processing of viral proteins. Currently available techniques in virus isolation are still too unreliable to be of routine diagnostic value.

Methods reported for the serodiagnosis of BIV include: immunofluorescence (Amborski et al., 1989; Black et al., 1989; Whetstone et al., 1990; Horzinek et al., 1991; Muluneh, 1994), Western immunoblot (Whetstone et al., 1991; Forman et al., 1992; Jacobs et al., 1992; McNab et al., 1994; Hirai et al., 1994), enzyme-linked immunosorbent assay (ELISA) with whole virus antigens (Onuma et al., 1992) or recombinant antigens (Cockerell et al., 1992; St. Cyr Coats et al., 1994), and a focus immunoassay (Muluneh, 1994).
Immunofluorescence, usually performed as an indirect fluorescent antibody assay (IFA), is a sensitive and specific test. However, there are multiple limitations to using this assay. The test has to be carefully controlled to assure that all of the reagents used, including cell cultures, reference sera and conjugates, are free of bovine viral diarrhea virus (BVDV). The pattern of cytoplasmic fluorescence produced with both BIV and BVDV are indistinguishable. Since BVDV is a common virus among cattle, as well as a common contaminant in cell culture, it could account for a high false positive rate of diagnosis. Sera from field cases, especially from older animals that have been exposed to multiple vaccinations and bacterial and viral antigens, tend to have a high, nonspecific background that interferes with the interpretation of the test. In order to avoid this, sera must be diluted, often times so much so that specific antibodies can no longer be detected, accounting for false negatives or a no test. Lastly, reading IFA accurately demands highly skilled personnel and expensive, specialized equipment.

The BIV Western immunoblot is a highly specific and sensitive test that can detect the presence of viral polypeptide-specific antibodies. Since antibody reactivity is directed to specific viral polypeptides of known molecular size, results can be visualized with a high degree of confidence. Although the Western immunoblot is a very good assay, it is too cumbersome to be used for processing large numbers of samples. A second drawback to the test is that it is difficult to prepare antigen that has sufficient amounts of env glycoprotein to give a reliable test. Different cell cultures express viral glycoproteins at varying levels, but even under optimal conditions only a small percentage of total viral protein is env glycoprotein (Layne et al., 1992). This is compounded by the fact that the
glycoprotein is fragile and easily lost during preparation if care is not taken to minimize that loss. The two major polypeptides that are diagnostic for BIV are the major core p26 \textit{gag} and the gp110 \textit{env} (Whetstone et al., 1991). If viral antigen preparations are not optimized for glycoprotein, then Western immunoblot reactivities are limited to p26. This could be somewhat averted by using recombinantly prepared \textit{gag} and \textit{env} proteins (Rasmussen et al., 1990; Rasmussen et al., 1992; Wannemuehler et al., 1993). We know from experimentally inoculated cattle, that reactivity to p26 commonly diminishes or falls below detectable levels\(^d\) (Whetstone et al., 1992; Suarez et al., 1995). Even recombinant antigens cannot help if antibody levels in the animal have fallen below detectable levels.

A third type of serological test is the ELISA. Antigens for the ELISA can be prepared from either whole virus or recombinantly-derived material. Although this test is easily used with large numbers of samples, the specificity is usually confirmed by a Western immunoblot assay. ELISAs are often highly sensitive tests, but because of nonspecific reactions from bovine serum samples due to extraneous proteins in the antigen preparation, false positives are common. The use of recombinant \textit{gag} antigens have been reported (Cockerell et al., 1992; St. Cyr Coats et al., 1994). However, this test has never been described in detail and validation methods have not been published. Complete correlation between ELISA and Western immunoblot assays is probably not possible because there are basic differences in the two tests, but the Western immunoblot test is

considered more specific because antibody to specific viral proteins can be compared.

With all of these assays it must be remembered that each type of test detects antibodies to different types of antigens. Comparisons of the different serodiagnostic tests have not been made. With experience borrowed from HIV, the ELISA test can be a very sensitive test for lentiviruses, but a confirmatory test with high specificity in the form of a Western immunoblot is required to prevent false positives. The IFA, because of the difficulties in interpretation, has become less prevalent as a routine diagnostic test for this virus. Our lab primarily uses the Western immunoblot assay for final determination as a serodiagnostic test. As with HIV, it is advisable to use more than one type of test to confirm the serological status of a sample.

A hot start single reaction PCR (Nadin-Davis et al., 1993) and a nested PCR (Suarez et al., 1995) have both been described for the detection of BIV-infected cattle. Both of these tests are used to detect proviral DNA. Both techniques rely upon the use of multiple primer sets to amplify different areas of the BIV genome to overcome the potential of the primers missing amplification of variant virus strains. The nested PCR test was shown to be more sensitive in other retroviral systems because of the greater number of cycles involved. This is an important feature since lentivirus-infected animals are thought to usually have a very low number of infected cells in peripheral blood. Both techniques use a Southern blot hybridization assay for confirmation of PCR product, but only the single reaction PCR test requires the Southern blot test for maximal sensitivity of the test. Direct comparisons between these two tests have not been made, and it is not possible to tell if one has a clear advantage over the other.
Nested PCR can detect BIV both early after infection, before virus specific antibodies appear and before virus can be isolated, and later in infection when antibodies fall below detectable levels and virus is not easily recovered (Suarez et al., 1995). Neither PCR test was used to examine large numbers of naturally infected animals. These tests have a great potential, but currently they are still untested in the field. Even a successful PCR test, however, does not lend itself to processing large numbers of samples and quality control assurances must be high to prevent false positives.

**Prevalence**

The prevalence of BIV infection in United States cattle herds is unknown and with existing diagnostic technologies, we can only estimate the true prevalence. Various serologic studies have been reported that show that BIV is widely distributed, being present in the United States, Canada, Australia, New Zealand, Netherlands, Germany, and other foreign countries (Table 3) (Amborski et al., 1989; Black et al., 1989; Van Der Maaten et al., 1990; Horzinek et al., 1991; Horner et al., 1991; Cockerell et al., 1992; Forman et al., 1992; Jacobs et al., 1992; McNab et al., 1994; St. Cyr Coats et al., 1994; Muluneh, 1994).

The three largest seroprevalence studies, using samples randomly selected from available sera from across three countries, detected antibody levels of 1.4% to 5.5% (Black, 1989; Horzinek et al., 1991; McNab et al., 1994). McNab's (1994) study of Canadian cattle also reported a herd infection rate of 18.1% (McNab et al., 1994). Other studies of individual herds have shown a higher incidence of infection using a recombinant ELISA system (Cockerell et al., 1992; St. Cyr Coats et al., 1994).
Table 3  BIV Serologic Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th># Cattle</th>
<th>% Positive</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amborski et al., 1989</td>
<td>IFA</td>
<td>235</td>
<td>2%</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>Black, 1989</td>
<td>IFA</td>
<td>1997</td>
<td>4%</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>Van Der Maaten, 1990</td>
<td>WB</td>
<td>85</td>
<td>25.8%</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>and Whetstone, 1990</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horzinek et al., 1991</td>
<td>IFA &amp; WB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>957</td>
<td>1.4%</td>
<td>Netherlands</td>
</tr>
<tr>
<td>Horner, 1991</td>
<td>WB</td>
<td>93</td>
<td>12%</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Cockerell et al., 1992</td>
<td>ELISA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95</td>
<td>21%</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>Forman et al., 1992</td>
<td>ELISA&lt;sup&gt;c&lt;/sup&gt; &amp; WB</td>
<td>120</td>
<td>7-17%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Australia</td>
</tr>
<tr>
<td>Jacobs et al., 1992</td>
<td>WB</td>
<td>39</td>
<td>23%</td>
<td>Canada</td>
</tr>
<tr>
<td>McNab et al., 1994</td>
<td>WB</td>
<td>928</td>
<td>5.5%</td>
<td>Canada</td>
</tr>
<tr>
<td>St Cyr Coats et al., 1994</td>
<td>ELISA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>221</td>
<td>50%</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>Muluneh, 1994</td>
<td>IFA &amp; cell</td>
<td>380</td>
<td>6.6%</td>
<td>Germany</td>
</tr>
</tbody>
</table>

Indirect Flourescent Antibody test (IFA), Western Blot (WB), Enzyme-Linked Immunosorbent Assay (ELISA)

- Screened with IFA, and confirmed with WB
- Recombinant <i>gag</i> antigen ELISA
- Whole virus antigen ELISA
- Overall seropositive rate was not given

Detailed comparisons of the different serologic detection methods have not been made. Only one study has reported comparisons between two different BIV serologic detection methods, the IFA and Western immunoblot tests. These tests, when comparing the same serum samples, showed a 6.7% positive rate by IFA, and when WB was used as a confirmation test, only 1.4% positive. When selected IFA negative samples were tested by WB, some samples were found to
be positive (Horzinek et al., 1991). In this study the two methods did not correlate well with each other, and with no "gold" standard for the detection of BIV infection, it is not clear which is the better technique.

With the various studies that have been completed, a statement of seroprevalence of BIV-infected animals cannot be made. From the available data, the number of infected cattle in an individual herd may be very high, but the overall prevalence in the United States is probably much lower. It has been hypothesized that the rate of infection may be much higher in Southern states for a variety of potential reasons, but it is not possible to conclusively make this claim with the available data. The largest survey of cattle, by Black (1989), was almost exclusively of cattle from Southern states, and showed a lower overall incidence of antibody detection as compared to McNab's (1994) Canadian study, which appears to contradict the hypothesis of a higher seroprevalence in the South (Black, 1989; McNab et al., 1994). The studies did use different detection methods (IFA and Western immunoblot) which makes direct comparison of the two studies difficult. The incidence of BIV infection in dairy cattle has also been thought to be higher than in beef cattle, but again the published data does not support this idea (Amborski et al., 1989). The available data does support the hypothesis that BIV is widely distributed, and suggests that it is probably present in every country with a large cattle population.

Public Health Concerns

The relatedness of BIV to HIV has caused some concern about the potential public health implications of this virus in our food supply. Several studies have examined the ability of BIV to grow in human cell lines. BIV was shown to grow in cultures derived from malignant bone marrow cells (Georgiades
et al., 1978). However, other studies using primary human and embryonic cell cultures showed no evidence of active infection (Kashanchi et al., 1991; Georgiades et al., 1978). No evidence of an active infection of humans has been reported, even after accidental inoculation with the virus (Whetstone, et al., 1992). Some sera from BIV infected cattle can recognize some HIV proteins on Western immunoblot tests, and some high-titered sera from HIV infected patients can recognize BIV proteins on Western immunoblot tests (Jacobs et al., 1992). The role that this cross-reaction may play in the persistently indeterminate response of humans to the HIV Western blot assay was examined with no evidence that response to BIV antigens was the source of the indeterminate reaction (Whetstone et al., 1993). Current evidence suggests no role for BIV as being a public health risk (Brownlie, 1994).

**Viral Variation**

Variation of BIV in vivo or in vitro has not been extensively studied. However, retroviruses, and lentiviruses in particular, are known to be highly variable in nucleotide sequence, with substitutions, frameshift mutations, deletions, insertions, and recombinations being described (Temin, 1993). This sequence diversity affects how the virus functions and interacts with its host. The source of the variation for retroviruses, and lentiviruses in particular, will be described as well as how this affects the distribution of mutations in the viral genome.

Retroviruses are RNA viruses that go through a DNA intermediate stage during their life cycle. Reverse transcription, transcription of DNA from an RNA template, is one of the most important sources of retrovirus variation that has been described. The retrovirus virion consists usually of two copies of the
positive sense single-stranded RNA viral genome, RT and a host-derived tRNA that is used as a primer. Upon entering a permissive cell, the virion uncoats to release the RNA genome, RT enzyme, and the tRNA. The tRNA binds to the primer binding site on one of the RNA molecules and initiates first strand synthesis by the RT enzyme. The negative sense DNA strand is made from the RNA template first, the RNA template is digested by ribonuclease H and the RT uses the negative sense DNA as the template to synthesize the complementary positive sense DNA to form double stranded DNA. The double stranded DNA is integrated into the host cell genome, and the integrated provirus is the template when virion RNA is produced to complete the life cycle (Dahlberg, 1988; Williams, et al. 1992). Reverse transcription has two steps where errors are most commonly introduced. First, the making of the negative-sense strand of DNA from the positive sense RNA found in the virion. Second, the use of this negative-sense strand of DNA as a template for replication of the positive-sense strand of DNA (Williams, et al., 1992; Temin, 1993). The RT enzyme is particularly error prone, because like other RNA dependent transcriptases it lacks 3'-5' exonuclease activity. 3'-5' exonuclease activity is an important error correcting mechanism built into many DNA dependent DNA polymerases. The first step of reverse transcription is thought to be the most error prone, since no host error correcting mechanisms are available. If, during the transcription of the positive-sense DNA strand, an error is made when the double stranded DNA is integrated into the host genome, the host's DNA error correction mechanisms are available to correct the error. The host, however, cannot distinguish which is the correct strand of DNA and the error has a 50% chance of being corrected to the original state. Another place for errors to be introduced is when full length viral
genome is transcribed by host DNA dependent RNA polymerases. These enzymes also lack 3'-5' exonuclease activity and can introduce errors at appreciable rates (Williams, et al., 1992; Temin, 1993).

The RTs found in different lentiviruses are similar, but they do have quantitative differences in fidelity. For example, spleen necrosis virus has a higher error rate than bovine leukemia virus in similar forward mutation assays (Mansky et al., 1994). The fidelity of the HIV RT was measured to be one-third that of other retroviruses studied in an in vitro system (Takeuchi et al., 1988).

The template can also affect the type and rate of mutations. Homooligomeric runs often cause frameshift mutations. Poly-A runs were described as having particularly high error rates (Bebenek et al., 1989; Williams et al., 1990; Burns et al., 1994). Particular substitutions were observed more often, including the guanine to adenine substitution. This substitution is sequence oriented with the NGAN sequence being the most likely to have the guanine to adenine mutation (Bebenek et al., 1989; Vartanian et al., 1991). Strand jumping by RT from a small repeat to another on the same strand or on another strand was also postulated as a means to insert or delete regions of sequence (Temin, 1993).

Recombination was shown to have a role in the variation of retroviruses (Vartanian et al., 1991; Stuhlmann et al., 1992; Kellam et al., 1995), with both heterologous and homologous recombination demonstrated in different retroviral systems. Recombination can occur in the virion between the two RNA genomes that are copackaged, usually during the reverse transcription step. The packaging of heterologous RNA genome is also an important component for recombination to occur. In one retroviral experimental system, recombination occurred at about $10^{-4}$ per virus replication cycle (Stuhlmann et al., 1992).
Different recombinants were observed in HIV that had effects on the evolution of the virus (Kellam et al., 1995; Vartanian et al., 1991).

The sources of retroviral variation have been documented, but the importance of this variation has not been addressed. While variation occurs essentially randomly throughout the retroviral genome, specific areas of the retroviral genome may have high variability and other areas may have low variability. For example, five regions of the surface envelope gene of HIV were described as hypervariable (Modrow et al., 1987). A hypervariable region is defined as having much higher sequence variation than the rest of the gene. Relatively conserved regions were also found in the SU gene of HIV. The RT gene was one of the most conserved regions of the HIV genome (Gelman et al., 1992). The difference in variability between these two regions was not that one gene was more mutable than the other gene, but that one protein could tolerate greater variability than the other protein and still function properly. Mutations that give the recipient a selective advantage in its environment have a greater chance of being replicated. Selection of variants by its environment drives the apparent concentration of mutations to particular regions of the genome.

The RT gene, even as one of the most conserved regions of the viral genome, is under a constant selection pressure from the environment. The selection process is most apparent when antiviral drugs targeted to the RT enzyme are given to the patient or added to culture medium in vitro. It has been well characterized that HIV will become resistant to AZT, a nucleoside inhibitor of HIV, usually in less than a year in the patient (Kilby et al., 1994; Kellam et al., 1995). Resistance to AZT occurs much faster than in a year, but AZT resistant mutants require a time period to become the predominant virus type in the
The patient has a decreasing clinical response to the treatment over time as the antiviral drugs become less effective at inhibiting viral replication. HIV was shown to be resilient in that the RT enzyme became resistant to a number of antiviral drugs and still remained active in the life cycle of the virus.

The surface envelope gene has well described hypervariable genes that have been shown to be important in many aspects of the lentivirus lifecycle. One selection factor on these hypervariable regions is the host immune response. The hypervariable regions are predicted to be and have been demonstrated to be surface epitopes of the SU gene. With HIV, the third hypervariable region is important in the virus's binding to the hosts CD4 receptor (Levy, 1993). Antibody to the V3 region of the SU gene can block this binding and neutralize the virus. However, viral mutation allows selection of viral variants with altered V3 regions allowing it to avoid antibody binding and escape neutralization. Escape from the antibody response is also thought to play an important role in disease pathogenesis of equine infectious anemia virus infection of horses (Salinovich et al., 1986). The virus causes an acute infection of horses, and the horse mounts an immune response and recovers. However, the virus is not eliminated and variants arise that evade the host immune response ultimately causing a reoccurrence of clinical symptoms (Salinovich et al., 1986). Immune response is not the only selection factor that directs the location of mutations, but it is one aspect that has been observed in several lentivirus systems.

Sources of viral variation have been described and some of the selection pressures that affect this variation have been described, but another term, quasispecies, needs to be introduced. Most RNA viruses, because of their high variability, are thought to occur in a quasiviral state, where literally every virion is
different from every other virion (Nowak et al., 1991; Williams et al., 1992; Duarte et al., 1994). For HIV, the rate of mutation occurrence has been estimated at about 1 in every 10,000 bases transcribed (Takeuchi et al., 1988; Ji et al., 1994). The virus is about 8.5 kilobases in size, so after every round of HIV replication a mutation will likely occur that makes it unique in the population. As described earlier, many of these mutants will be selected for or selected against. Eventually, the most fit type of virion in the population becomes predominant. Many other less common variants remain in the population of virus. These less common variants can quickly become the predominant isolate if conditions of the host change or additional factors are introduced. An earlier example was the administration of antiviral drugs to a patient, providing a strong selection pressure for the appearance of variants that are resistant to the drug. These drug resistant variants may already be present in the host, albeit at very low levels, and because of selection pressure from the antiviral drug, they become the predominant type variant in the patient. The quasisviral theory of almost unlimited variants of a virus existing in a patient has been observed in many different RNA virus systems, and plays an important role in the evolution of the virus.
CHAPTER 3. ISOLATION AND CHARACTERIZATION OF NEW WILD-TYPE ISOLATES OF BOVINE LENTIVIRUS

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Abstract

Two new isolates of bovine lentivirus, also known as bovine immunodeficiency-like virus (BIV), were obtained from a seropositive cattle herd in Florida. This is the first report of new isolates of BIV since the original BIV strain, R29, was isolated in 1969. The two new BIV isolates were derived from blood buffy coat cells cocultivated in vitro with fetal bovine lung (FBL) cell cultures. The new isolates differed in vitro from the original R29 isolate in replication and syncytial formation in FBL cells. Both new isolates were confirmed as BIV by immunofluorescence, Western blot, and polymerase chain reaction (PCR). Sequence analyses of the PCR pol gene product showed a 92.6% and 93.6% homology to the published nucleotide sequence of BIV R29-

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127, a molecular clone derived from BIV R29. Each of the new BIV isolates was inoculated into two calves, and virus was recovered between 5 and 10 days post inoculation (PI) with BIV seroconversion between 10 and 21 days PI. Virus has been recoverable and antibody detectable for at least four months PI. Two calves developed a transient, elevated mononuclear cell count, similar to what was reported for BIV R29 in the original experimental calf inoculations. No other clinical abnormalities have been observed.

The original isolation of the bovine immunodeficiency-like virus (BIV) was made in 1969 from an eight year old dairy cow from Louisiana that had a persistent lymphocytosis and was becoming progressively emaciated. At the time of necropsy, this cow had clinical and histopathological lesions including perivascular cuffing in some vessels in the brain and enlarged lymph nodes and hemal nodes (28). The viral isolate from this dairy cow, designated R29, was originally described as a Visna-like virus because of similarities to the ovine lentivirus. With the finding that the human immunodeficiency virus (HIV-1) was a lentivirus, renewed interest in the bovine Visna-like virus resulted in its molecular characterization that conclusively placed BIV in the lentivirus family as a unique member of the group (4, 10, 11).

BIV causes a persistent infection in cattle and serological data indicate that it may have a world-wide prevalence (2, 3, 13, 14, 29, 30). BIV shares a similar genomic organization with other lentiviruses including the pol, gag, env and tat genes (10, 24). Antigenically, antisera to BIV p26 cross reacts with p24
of HIV-1, and antisera to equine infectious anemia virus (EIAV) cross reacts with p26 and p24 of BIV (11, 31). Two infectious molecular clones of BIV R29, BIV R29-127 and BIV R29-106 have been sequenced, showing that the virus is a unique member of the lentivirus family (4, 10). BIV R29-106, however, did not replicate well in cattle after experimental inoculation (6).

All molecular characterizations and animal inoculation studies on BIV have been completed using the original BIV R29 isolate, which has been passaged extensively in vitro or frozen for over 20 years, and it may be attenuated (6, 8, 10). The R29 isolate has also become contaminated with a noncytopathic strain of bovine viral diarrhea virus (BVDV). The original experimental inoculations of BIV R29 in colostrum deprived calves induced a mild lymphocytosis and enlargement of peripheral subcutaneous lymph nodes, with no overt clinical signs of disease (28). In recent studies, using either BIV R29-1203 inoculant free of BVDV, or R29 inoculant in BVDV vaccinated cattle, or BIV R29-106 molecular clone inoculant, the original experimental observations of leukocytosis and lymph node enlargement have not been observed, suggesting that the virus may have become attenuated (6, 10). Attenuation of lentiviruses in cell cultures has been described in detail for EIAV (5, 25). However, the virulence of one EIAV isolate was increased with rapid animal passage (25). Similar experiments with BIV have not resulted in a change in the course of infection. The BIV R29-106 molecular clone, which is infectious for cell cultures in vitro, was not sustained by animal passage through whole blood transfer (6, 10).

Several studies with BIV have explored the possibility that this virus may cause immune system alterations, similar to HIV-1 or feline immunodeficiency virus (FIV). However, because the original BIV isolate, R29, has become
contaminated with a non-cytopathic strain of BVDV, an RNA virus that can cause immune suppression in the bovine, interpretation of data acquired from cattle inoculated with this isolate becomes complicated (6, 21). Studies examining immune function of BIV in vivo have demonstrated either mild or no immunosuppression based on lymphocyte blastogenesis tests, neutrophil function tests, mononuclear subset analysis, and histopathological changes (6, 8, 21). Available research results have not shown that BIV is a disease causing agent in cattle in experimental inoculations. These reports were all based on studies of cattle infected for less than 27 months (6, 8, 21, 28, 29, 30). Thus, the possibility that BIV has a long incubation period before causing disease or its ability to act as a cofactor in disease can not been ruled out (6, 29).

In this report we describe in vitro and in vivo characterization of two new wild-type isolates of BIV which were isolated from two cows in a seropositive dairy herd in Florida. A BVDV free BIV isolate of R29, BIV R29-1203, was also used in this study as a positive control to help contrast the differences of the new isolates to R29 derived isolates. The R29-1203 isolate was prepared by vaccinating a BIV/BVDV negative calf with an autogenous BVDV vaccine prepared (Dr. S. R. Bolin, National Animal Disease Center, Ames, IA) from a clone of the non-cytopathic BVDV isolated from BIV R29 cell cultures. After the calf developed neutralizing antibodies to BVDV, it was inoculated with BIV R29. BIV was recovered from blood buffy coat cells, tested both in vitro by cell culture methods and in vivo by calf inoculation, found to be free of BVDV, and designated BIV R29-1203.

In vitro characterization of BIV isolates. A Florida dairy herd with BIV seropositive cattle was identified by Western blot assay and blood samples in
EDTA tubes were taken for virus isolation attempts (31). The blood samples were centrifuged, and the buffy coat cells were removed for coculture with fetal bovine lung (FBL) cells cultured in an Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum, polybrene (4μg/ml), gentamicin (50μg/ml), and 0.02% L-glutamine. These cultures were blind passaged and/or cultured with fresh FBL cell cultures until the cytopathic effects of syncytial formation and cell lysis were observed. Evidence of viral replication by syncytial formation was observed in two cultures, FL491 and FL112, after the 4th blind passage in culture. The two cows from which the isolates were derived were seropositive for BIV on Western blot assay, seropositive for bovine leukemia virus (BLV) on AGID and seronegative for bovine syncytial virus (BSV) on agar gel immunodiffusion (AGID) (19,22, 31). Continued culturing of FL491 and FL112 gave increased syncytial formation with limited cell lysis. The new isolates showed differences in replication characteristics in vitro as compared to BIV R29, including higher cocultivation ratios, longer incubation times between passage, and differences in syncytial appearance typified by a decreased number of nuclei per syncytia, greater granularity and slower lysis of the syncytia as compared to BIV R29 and BIV R29-derived isolates. Syncytial nuclei from the new wild-type isolates often formed a circular pattern in culture which was not commonly observed in BIV R29-derived cultures. The BIV R29-1203 isolate demonstrated higher cytopathogenicity in cell culture than did the new isolates. The FL491 and FL112 wild-type isolates had similar replication patterns in vitro with smaller syncytia and less lytic effect on the cell cultures. The replication characteristics of the different BIV isolates were maintained in relation to their parent strain after animal inoculation.
The new wild-type isolates of BIV have biological differences in comparison to R29 in vitro, and in vitro characteristics of other lentiviruses have been shown to correlate with the virulence in vivo (7). Specifically, the more cytopathogenic lentiviral strains in culture are associated with greater in vivo virulence in HIV-1 and greater lymphoproliferative change in the ovine lentivirus, Maedi-Visna (7, 12, 16, 17). Different regions of the viral genomes have been associated with these in vitro and in vivo characteristics (9,12, 23,32). Biological variation should not be unexpected among BIV isolates, and these differences are probably controlled by several different areas of the genome.

FL491 and FL112 were BIV positive by immunofluorescence assay using a BIV-specific antiserum and a fluorescein-conjugated rabbit antibovine immunoglobulin G, but both isolates were indirect immunofluorescence assay negative for BLV and BSV by the same technique (30). Western blot assays, using viral antigen prepared as previously described (31), from each of the isolates with positive reference control sera were positive for BIV, negative for BLV, and negative for BSV. The AGID tests, using positive reference sera for BLV and BSV, were negative (data not shown) (19,22). DNA samples for PCR reactions were prepared from BIV infected FBL cell cultures using proteinase K followed by phenol/ chloroform extraction following standard procedures (20). One ug template DNA, 20 pmoles of primer, 1.25 units Taq polymerase (Cetus Corp., Norwalk, CT) in a 50 ul reaction were used in a standard PCR reaction mixture with first cycle conditions at 94°C for 2 min, 51°C for 15 sec, 72°C for 2 min, followed by 30 cycles of 94°C for 45 sec, 51°C for 15 sec and 72°C for 1 min and a final extension step at 72°C for 10 min. The pol gene (+) sense primer 5' ATG CTA ATG GAT TTT AGG OA 3' and the (-) sense primer 5' CAT
CCT TGT GGT AGA ACA TT 3' amplified, from BIV R29-1203, FL491, and FL112 infected cell cultures, a 242 base pair product (FIG. 1), the size predicted from published sequence data and previously reported work (10, 15). Southern blot analyses using the BIV pol gene specific probe confirmed the identity of the PCR products (20). The PCR product was then directly sequenced by purifying the product with a Centricon 100 microconcentrator and using 40 ng of DNA with Taq polymerase in a dye terminator sequencing system on an automated sequencer (ABI Applied Biochemistry 373A DNA Sequencer, performed at the Iowa State University Nucleic Acid Center, Ames, IA). Nucleotide sequences were analyzed for homology using the Wilbur-Lipman methods (DNASTAR program Align). The 212 base pair sequenced PCR products were compared to the published sequence for the putative reverse transcriptase gene segment of BIV R29-derived molecular clone BIV R29-127 (10). The BIV R29-1203 isolate had a 99.6% nucleotide sequence homology with the published sequence, with only a single, silent nucleotide substitution. The BIV FL491 and FL112 isolates had 93.6% and 92.6% nucleotide sequence homology, respectively, to the published BIV sequence, and a 96% nucleotide sequence homology when compared to each other. All sequenced products maintained the same open reading frame for a segment of the putative reverse transcriptase gene, and no insertions or deletions were seen in the sequenced product. The predicted amino acid homology for BIV FL491 and FL112 was 93.8% and 92.3%, respectively.

The reverse transcriptase region of the genome is highly conserved among other lentiviruses and it is expected that BIV is similar. Comparison of the pol gene region of the BIV R29-1203 isolate, which has been through an animal
Figure 1  Products of PCR amplification (left) of a 242 base pair segment of the pol gene from BIV R29-1203 (lanes 1), BIV FL491 (lanes 2) and BIV FL112 (lanes 3) and Southern blot hybridization (right) of those products using a $^{32}$P-labeled pol gene probe. Negative (lanes 4) and positive (lanes 5) PCR controls and molecular size ladder are also shown.
passage and over thirty-five passages in cell culture, to the published sequence for BIV R29-127, a molecular clone of BIV R29, demonstrates that BIV R29-1203 has only a single, silent nucleotide change from the published sequence. This suggests that this segment of the reverse transcriptase region may be highly conserved in BIV and provides a valuable guidepost in comparing new isolates. The new Florida isolates have a 7-8% nucleotide sequence divergence in the conserved pol segment when compared to the Louisiana BIV R29-127, demonstrating the divergence of these isolates from R29. The new isolates have only a 4% nucleotide sequence divergence between them suggesting that they are more closely related field isolates. Comparisons of different HIV-1, Visna and FIV isolates show that the diversity of nucleotide sequences of isolates generally is greatest when they are from geographically distinct populations (1, 18, 26, 27). In comparison with these three lentivirus groups, the new Florida BIV isolates appear to be distinct from the Louisiana isolate, but are not as divergent as what can be seen with other geographically distinct lentivirus isolates. Sequencing of other BIV isolates will help to determine if the Florida isolates are distinct or continuums of the same BIV population.

**In vivo characterization of BIV isolates.** Four calves, negative for antibodies to BIV, BLV, and BSV were experimentally inoculated with the FL491 and FL112 isolate. Each calf received BIV infected FBL cells and culture fluids intravenously in the jugular vein and subcutaneously in the area around the jugular vein as follows: BIV FL491 (Jersey calf 1268 and Holstein calf 848), and BIV FL112 (Jersey calves 1275 and 1269). Calves were observed for clinical symptoms and blood samples were taken at 3, 5, 7, 10, 14, 21, and 28 days post inoculation (PI) for virus isolation, serology, and leukocyte (WBC) differential
counts. Thereafter, the calves were sampled weekly for serologic and WBC response and every two to four weeks for virus isolation attempts.

All four calves developed BIV specific antibody responses as determined by Western blot analysis. Results of western blots of serum samples from calf 1275 from preinoculation, PI day 35, and PI day 91, and from calf 1268 preinoculation, PI day 35 and PI day 105, are shown (Fig. 2). Calves 1268 and 848, inoculated with BIV FL491, developed a BIV specific serological response starting at PI day 10 and 20, and virus was recovered from samples starting on PI day 10 and 11, respectively. Calves 1275 and 1269, inoculated with BIV FL112, both developed a BIV specific serological response starting at PI day 14, and virus was isolated from samples by PI day 5 and 7, respectively. Viral antigens, prepared from virus recovered from experimentally inoculated calves 1268 and 1275, were used in Western blot (Fig. 2) and were positive with BIV reference sera. Western blot antigens were also tested with both BLV and BSV reference sera and were negative (data not shown).

The WBC response for calves 1275 and 1269, inoculated with FL112, showed a transient leukocytosis consisting mainly of mononuclear cells (Fig. 3). The WBC response for calves 1268 and 848, inoculated with FL491, was slightly increased with an increased percentage of mononuclear cells, but remained within the normal range (Fig. 3). The calves had an expected prescapular lymph node enlargement on the side that the inoculation materials were injected, but showed no other clinical symptoms.

Preinoculation sera from all calves were negative for neutralizing antibodies to BVDV, BLV and BSV, except for calf 848 which had neutralizing
Figure 2  (Top) Western blot assay reacting BIV reference serum with viral antigens prepared from BIV R29-1203 (lane 1), BIV FL491 (lane 2), an isolate of FL491 from calf 1268 (lane 3), BIV FL112 (lane 4), and an isolate of FL112 from calf 1275 (lane 5). Molecular size markers are indicated on the left.  (Bottom) Western blot assays of sera from calf 1268, inoculated with BIV FL491, from preinoculation (lane 1), day 35 PI (lane 2) and day 105 PI (lane 3) and calf 1275, inoculated with BIV FL112, from preinoculation (lane 4), day 35 PI (lane 5) and day 91 PI (lane 6). Molecular size markers are shown on the left. The 20Kd band, seen in pre and post inoculation serum samples from both calves, is not BIV-specific.
FIGURE 3  Comparison of mononuclear and polymophonuclear cell counts in two calves that were inoculated with BIV FL112 (calves 1275 and 1269) and BIV FL491 (calves 1268 and 848). Calves 1275 and 1269 showed the greatest mononuclear cell increase, similar to the WBC increase reported in the original experimental inoculations with BIV R29 (31). No changes in polymophonuclear cell population were observed for either calf.
antibody to BVDV. After inoculation with BIV, the three BVDV seronegative calves remained negative for virus neutralizing antibodies to BVDV. AGID tests for antibodies to BLV and BSV also remained negative on multiple samples from all calves taken from the 7th through the 14th weeks PI.

The new isolates in experimental inoculations caused a leukocytosis similar to what was described in the original experimental inoculations with BIV (28). More cattle need to be inoculated to confirm the initial observation that these new isolates cause leukocytosis, to rule out the possibility of biological variation among cattle. These studies may provide further insight into the question of possible attenuation of the BIV R29 isolate and verify that BIV does cause a leukocytosis. Results from the current study, as well as from future studies performed with these new wild-type isolates, should provide a clearer picture of the true effect of experimental inoculation with BIV.

In conclusion, this study provides information on the biological and genetic characteristics of BIV and provides new BIV isolates for experimental study. Since it is possible that the original BIV R29 isolate was attenuated through multiple cell culture passages, these new, low passage, contaminant free, isolates will allow for measurements of possible immune suppression in vivo that may more accurately reflect a natural infection. The new isolates of BIV are important to continue investigations of the pathogenesis of BIV infections and to elucidate the potential role of BIV as a disease agent or as a cofactor in disease. The new isolates increase the potential value of BIV as an animal model for lentivirus infections.
Acknowledgements

NOTE: GenBank accession numbers for sequences are: L06524 (BIV FL112); L06525 (BIV FL491); L06526 (BIV R29-1203).

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References


CHAPTER 4. IMPROVED EARLY AND LONG-TERM DETECTION OF BOVINE LENTIVIRUS BY A NESTED POLYMERASE CHAIN REACTION TEST IN EXPERIMENTALLY INFECTED CALVES

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Abstract

A nested polymerase chain reaction (PCR) test was developed to examine infection with the bovine lentivirus, bovine immunodeficiency-like virus (BIV), in cattle. Primers were designed to amplify 2 separate regions of the pol and env segments of the BIV genome. Two calves were experimentally infected with an isolate derived from the original strain of BIV, R29, or with a recent field isolate, FL491. Serial blood samples were collected and examined by virus isolation, protein immunoblot, and nested PCR. The nested PCR test detected BIV infection by 3 days after inoculation, earlier than the other 2 methods, and continued to identify infected cattle 9 to 15.5 months after inoculation, even when results from virus isolation and serology became negative. Nested PCR also detected multiple sized env products in samples obtained later in the infection.

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from the calf that received FL491, giving evidence that viral quasispecies were selected during in vivo replication of the virus. Results indicated that the nested PCR test is more sensitive than virus isolation or serology for the detection of BIV infection in cattle.

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**Introduction**

The bovine lentivirus known as bovine immunodeficiency-like virus (BIV) was first described in 1972.¹ Although BIV has many structural similarities to other lentiviruses, it does not appear to be closely related to any member of that group.² Infection by BIV appears widespread in cattle on the basis of serologic studies.³⁻⁷ Experimental inoculations of sheep, goats⁸ and rabbits⁹ with BIV has resulted in infection, verified by a humoral immune response. Apparent naturally acquired infection in a sheep also has been reported.¹⁰ Isolates of BIV have been shown to cause lymphoproliferative changes, including transient increases in mononuclear cell counts¹¹ and enlargement of subcutaneous lymphatic nodules, in experimentally infected cattle.¹ However, specific disease has not been recognized in acutely infected cattle. In one study a possible link between BIV infection and decreased milk production in BIV infected dairy cows was suggested.⁷ A direct role for BIV in chronic progressive disease or as a cofactor in a specific disease has not been demonstrated.

One of the difficulties in studying BIV is the lack of a test to accurately diagnose infected animals. Virus isolation from naturally infected animals remains difficult and unreliable, and currently only 3 different isolates have been reported.¹,¹¹ Serologic tests, including indirect fluorescent antibody and protein immunoblot assays, have been the most widely used methods for identifying BIV-infected animals.³,⁶,⁷,¹²,¹³ Serious deficiencies, however, are inherent in the
use of these tests. Serologic assays cannot detect BIV-infected cattle early in the infection, before detectable antibodies are present, or often late in the infection, when cattle may lose detectable BIV-specific antibody responses, even though virus can still be recovered. The most widely used serologic test is the protein immunoblot assay, in which pelleted virus is used as the antigen. Antibodies to p26, the major gag protein, and gp110, the surface envelope protein, are the most important in determining whether an animal is seropositive for BIV. In cattle experimentally infected with BIV, antibody to p26 can be detected as early as 14 days after inoculation and antibody concentrations peak 6 to 8 weeks after inoculation. The gp110 response is usually detected after the p26 response, with the peak in this response sometimes observed many months after inoculation.

An extended-cycle PCR test, in which a hot start is used and that directly identifies the BIV genome from infected white blood cells, has been described and a normal 30 cycle PCR test has been used for amplifying BIV-specific DNA from cell culture. For other retroviruses, including bovine leukemia virus, human immunodeficiency virus, and feline immunodeficiency virus, nested PCR tests have been developed to identify virus in experimentally induced and naturally acquired infections, because of the greater sensitivity and specificity of the tests.

Because of problems associated with identification of BIV positive animals by use of virus isolation and serology, the purpose of the study reported here was to develop a direct detection method for BIV infection.

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Materials and Methods

Viruses and animal inoculations- A 3-month-old Jersey calf, calf 1268, was inoculated iv and sc with cell culture supernatant and with cells from fetal bovine lung (FBL) cell cultures infected with FL491, a recently described field isolate of BIV. An 8-week-old Holstein calf, 289, was inoculated similarly with R29-1203, an isolate derived from the original strain of BIV, R29. 302 days after infection, calf 289 was hyperimmunized with pelleted virus from R29-1203 in an aluminum hydroxide adjuvant; the dose was repeated 2 weeks later. Calves were housed in isolation facilities, and had been tested prior to inoculation and found to be free of antibodies to BIV, bovine leukemia virus (BLV), bovine syncytial virus (BSV), and bovine viral diarrhea virus (BVDV).

Virus isolation- Blood samples (10 ml) were collected in EDTA containing tubes and centrifuged at 800 X g for 20 minutes at 4 C, and buffy coat cells were collected for cocultivation with FBL cell cultures in 25 cm² flasks. These cultures were blind passaged and/or cultivated with fresh FBL cell cultures until the cytopathic effects (CPE) of syncytial formation and cell lysis were observed. Cultures were blind passaged 9 times without evidence of CPE before being considered negative. Cultures were grown in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum, polybrene (4 mg/ml), gentamicin (50 mg/ml), and 0.02% L-glutamine. Uninoculated stock cultures of FBL cells and the new isolates were tested and found to be uninfected with bovine viral diarrhea virus and mycoplasmas.

Serology- The R29-1203 virus was used to prepare antigen for protein immunoblot assays to detect BIV antibodies, as previously described. Briefly, virus was obtained from infected culture supernatant by ultracentrifugation
(100,000 X g for 1 hour) through 40% glycerol phosphate-buffered saline solution, pH 7.2. The concentrated virus was suspended in 1 ml of phosphate buffered saline, solubilized in 500 mM NaCl; 50 mM Tris pH, 8.0; 5 mM EDTA; 1% Triton X-100; 50 µM phenylmethylsulfonyl flouride, and protease inhibitors, and then boiled in 2X treatment buffer solution (0.125 M Tris hydrochloride, pH 6.8; 4% sodium dodecyl sulfate; 20% glycerol; 10% 2-mercaptoethanol; 0.0002% bromphenol blue) for 5 minutes. Test sera were diluted 1:10 in a solution of 10 mM Tris and 150 mM NaCl, pH 8.6 and tested by protein immunoblot, as previously described. Antibodies to bovine leukemia virus and bovine syncytial virus were not found by agar gel immunodiffusion assay in pre- and postinoculation sera from both calves. Antibodies to BIV were not found by protein immunoblot assay in preinoculation sera from both calves. Positive and negative reference antisera against BIV, BLV, and BSV were available (NADC, Ames, IA).

Preparation of DNA. Blood samples (10 ml) from test cattle were collected in vacuum tubes with EDTA. Samples were centrifuged, buffy coat cells collected, and remaining RBCs lysed in distilled water followed by equal volumes of 2X Calcium- and Magnesium- free Hank's balanced salt solution. Treatment with distilled water and 2X Hank's balanced salt solution was repeated until all RBCs had been visually lysed and the cell pellet was white. Samples were frozen at -70 C for later use or immediately suspended in buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA) with 1% SDS. Proteinase K was added, at 100 µg/ml of sample and the samples were incubated in a 37 C water bath for 3 to 6 hours. After digestion, samples were extracted with an equal volume of
phenol/chloroform in a density gradient\(^b\) system, and the aqueous layer was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The nucleic acid was precipitated and pelleted, and suspended in 100 μl of molecular-biology-grade water\(^c\); Nucleic acid concentrations were determined by a UV spectrophotometer.

**Polymerase chain reaction** - In each 50 μl reaction, standard PCR buffer and nucleotide conditions, 3 mM MgCl\(_2\), 20 pmoles of each primer (Fig 1), and 1.25 U of Taq polymerase were used in both steps of the nested PCR. The first step primers in the env reaction were P01 and 36; second step primers were P02 and 37. The first step primers for the env reaction were 04 and 06; second step primers were 01 and 45. In the first step, 0.5 μg of sample nucleic acid was used. In the second step, 2 μl from the first step reaction was added to a second reaction mixture and used as the source of DNA. For the env primers, a duplicate PCR reaction was performed with only 4 pmol of primers for the first step of the reaction and other conditions remaining the same.

Cycling conditions for the first step PCR were the same for pol and env primer sets and included, for the first cycle of the first step, 94 C for 2 minutes, 51 C for 15 seconds, and 72 C for 2 minutes followed by 30 cycles of 94 C for 45 seconds, 51 C for 15 seconds and 72 C for 1 minute, with a final extension step of 72 C for 10 minutes. Second step cycling conditions started with 1 cycle of 94 C for 2 minutes, 61 C for 15 seconds, and 72 C for 1 minute, followed by 30 cycles of 94 C for 45 seconds, 61 C for 15 seconds, and 72 for 1 minute, with a final extension step of 72 C for 10 minutes to complete the reaction. Primers to

\(^b\) Phase lock gel 1 Heavy (5'---->3', Inc. Boulder, CO)

\(^c\) Omnisolve, EM Science
Figure 1  Nucleotide positions of the 5' nucleotide of the pol and env nested primers are represented on the bovine immunodeficiency-like virus (BIV) proviral genome, on the basis of R29-127 molecular clone sequence. The outer primers for the pol set are P01 and 36, and the inner nested primers are P02 and 37. The outer primers for the env set are 06 and 04, and the inner nested primers are 01 and 45. The positive control actin primers are Actin1 and Actin2.
the bovine actin gene (Fig 1) were used in a separate, single-step PCR reaction as a positive sample control. Reaction conditions were the same as those used for BIV PCR, and cycling conditions were the same as those used for the second step of the nested PCR (annealing temperature 61 C).

To minimize contamination of samples with PCR product from other reactions, we used a laboratory hood for the preparation of the PCR tests, a second hood in a different room to add the DNA from the first reaction to the second, a third room for the PCR machine, and a fourth room for the analysis of PCR products. Gloves and aerosol-reducing pipette tips were used for all PCR work.

Southern blot hybridization- On a 1.5% agarose gel, 20 μl of the PCR product was electrophoresed and stained with ethidium bromide. Gels were transferred onto a nylon membrane, using standard Southern blot conditions, and DNA was fixed onto the membrane by baking at 80 C for 2 hours under a vacuum. BIV-specific DNA probes used included a PUC-5 plasmid with pol insert (BIV R29-127; nucleotide position, 1801-3770 ), a PUC-12 plasmid with env insert (BIV R29-127; nucleotide position, 5460 -8052 ), and the gene-specific inserts excised from the plasmid vectors. Probes were labeled with an enhanced chemiluminescence direct-labeling system and added at 20 ng/0.125 ml/cm². Prehybridization, hybridization, high stringency washing ( sodium citrate 0.0015M; sodium chloride 0.015M; and 0.4% sodium dodecyl sulfate at 55 C) and detection were performed according to kit protocols. X-ray film was used for detection of the chemiluminescent reaction.

\[ \text{d} \quad \text{ECL, Amersham Intn.} \]

\[ \text{e} \quad \text{Hyperfilm-ECL} \]
Primer design. The primers in this study were designed by use of a number of criteria including matching of primer pairs for similar melting temperatures, balancing GC/AT ratios, and absence of inherent secondary structure. Primers sequences were chosen to target conserved sequences within the viral genome. The rationale used to determine conserved sequences was based on several criteria. Ideally, when sequence data from multiple BIV isolates were available for the region of interest, conserved areas could be readily identified and primers were selected from these regions. However, if only a single sequence was available, the DNA sequence was translated in the putative reading frame and the antigenic regions of the protein were determined by a computer program^.

Primers for the surface env gene of BIV were selected in regions predicted to be poorly antigenic and assumed to be internal regions of the protein. These predicted internal regions have been shown to be a more conserved part of the env region of the genome, compared with that of surface protein amino acids.23 Primers for the reverse transcriptase region of the pol gene were located by use of conserved regions of this gene when comparing several lentiviruses.24

The 3' nucleotide of a primer is the most critical in binding to the template. A mismatch at this codon can greatly decrease the ability of the primer to efficiently amplify the target sequence.25,26,27 The third amino acid codon position is the most commonly changed nucleotide because it is the position where silent mutations most often occur. Therefore, all primers used in the current study were designed so that the 3' nucleotide of the primer was in the first or second amino acid codon position of the putative reading frame for the viral sequence to be amplified (Fig 2).

^ Protean, DNASTAR
Results

Virus isolation first detected BIV in calves 289 and 1268 at 7 and 10 days after inoculation, respectively. Virus was isolated from calf 289 every time that virus isolation was attempted after 7 days. The number of blind passages required before large numbers of syncytia were observed increased over time from 1 on day 14 to 3 on day 291. Calf 1268 had positive results on virus isolation for all samples after 10 days, except on day 197 after inoculation. The number of blind passages before large numbers of syncytia were observed also increased from 3 blind passages on day 21 after inoculation up to 9 blind passages on days 365 and 470.

**Figure 2**

PCR primers were designed to have the 3' nucleotide of the primer coincide with the first or second codon position in the protein reading frame. The 3' nucleotide of primer 37 (capital letters) is located in the second position of the lysine codon (underlined). The 3' nucleotide of primer P02 (capital letters) is located in the first position of the asparagine codon (underlined). Primer 37 is on the positive sense coding strand of the reverse transcriptase gene and primer P02 is on the noncoding strand.
**Figure 3** Protein immunoblot results from serial serum samples from calves 289 (top) and 1268 (bottom), in which viral antigens prepared from BIV R29-1203 were used. Positive control serum from a BIV-infected sheep was used for both blots. Calf 289 was inoculated with BIV R29-1203, and was hyperimmunized twice with concentrated BIV R29-1203 in adjuvant. Calf 1268 was inoculated with BIV FL491. Both calves developed a strong antibody response to p26, BIV gag protein.
Calves 289 and 1268 seroconverted, on the basis of results from protein immunoblot. (Fig 3) Antibody to p26 was first detected in calves 289 and 1268 at 21 and 14 days after inoculation, respectively. In calf 289, antibodies to p26 and other BIV gag proteins peaked at 56 days after inoculation and declined to undetectable levels after 180 days. In contrast, antibodies to gp110 were first detected in calf 289 at 41 days after inoculation, and remained detectable throughout the study period. After hyperimmunization with pelleted BIV in an aluminum hydroxide adjuvant, calf 289 regained a strong antibody response to gag proteins. In calf 1268, antibody to p26 peaked at 28 days after inoculation and then slowly declined to barely detectable amounts by the end of the study period (470 days), with only a questionably detectable antibody response to gp110 observed from days 28 through 56. Both calves responded to several other BIV proteins, including several gag proteins.

With nested PCR, results of BIV testing of preinoculation samples from both calves were negative, but both calves had positive results for BIV at 3 days after inoculation. Calf 289 had positive results, using the pol primers, but negative results with the env primers, at 3 days. Calf 1268 had positive results with env primers, but negative results with the pol primers at 3 days. All samples tested from both calves gave positive results with both sets of primers after 3 days. With the pol primers, predicted product size of 176 base pairs (bp) was observed in all samples. In several samples, a product of approximately 425 bp was observed, which hybridized with the BIV pol plasmid on Southern blot (Fig. 4). Nested PCR was performed with a sample from calf 289 at 291 days after inoculation. A gel stab of the larger 425 bp product was used as the sample for another 30-cycle round of PCR, using a combination of the 4 different pol
primers. The 176 bp product was detected with primer combination PO2 and 37, and a 425 bp product was detected with primers 37 and 36. Product was not observed with the other primer combinations.

Predicted product size for the env primers, on the basis of the published sequence for BIV infective molecular clone R29-106, was 385 bp.28 In calf 289, analysis of all samples revealed the predicted product size. For calf 1268, a single, larger product was observed early in infection, but later 3 separate bands that hybridized to the env probe were observed (Fig 5). A larger band, predicted to be the size of a product amplified by use of primers 06 and 04, also was detected on several days and hybridized with the env plasmid. Differences were not observed when the plasmid with insert or the excised insert alone were used for detection of the PCR product. Hybridization was not detected when selected blots were hybridized with the opposite plasmid probe (eg, env plasmid probe with pol PCR blot).

Discussion

Except for very early after infection, virus isolation from the cattle in this study appeared to be a sensitive test to detect BIV. Culturing virus from both cattle became increasingly more difficult, however, as time after inoculation increased. One difficulty with the use of virus isolation to identify animals infected with BIV is that more than 2 months of blind passages may be required before results are positive, thus requiring a long delay before results for a sample can be considered negative. Another major difficulty is that isolation of virus from naturally infected cattle is difficult and has only been reported in a few animals.1,11 Numerous attempts to culture virus from other cattle identified as being seropositive or PCR positive for BIV have been unsuccessful in our
Figure 4  Polymerase chain reaction (PCR) amplification of pol gene (left) from serial blood buffy coat samples from calf 289. The predicted product size, 176 base pairs, was observed in every sample, starting at 3 days after inoculation. Polymerase chain reaction products were confirmed by Southern blot hybridization (right) using a BIV pol plasmid probe. A 425 base-pair band of hybridization observed in several lanes resulted from amplification of BIV DNA from primers of the first and second reactions. A control without DNA was included to test for product contamination, and all results were negative.

Figure 5  Polymerase chain reaction amplification of the surface envelope gene (left) from serial buffy coat samples from calf 1268. From the earliest samples, only a single 440-base-pair product was observed, but later 2 additional BIV env bands at 500 and 580 base-pairs became apparent. All 3 bands hybridized with a BIV env plasmid probe on Southern blot hybridization (right). The 1,700-base-pair, BIV-specific band observed in some lanes resulted from amplification from primers of the first and second reaction.
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experience. The FBL cell cultures that we used have been the most sensitive cell type identified for culturing BIV R29, but they may not be adequate for isolating BIV from many naturally infected animals. Virus isolation remains a limited tool for examining animals naturally infected with BIV.

Serologic tests have been the primary means of identifying animals infected with BIV, but recent evidence suggests that this method may not be detecting all infected animals. In our study, we used the protein immunoblot test with whole pelleted virus antigen to detect BIV-specific antibodies in infected cattle. We observed decline or loss of detectable antibody to the 2 most diagnostically important proteins of BIV: p26, the major nucleocapsid $gag$ protein, and gp110, the surface $env$ protein. In this study, calf 289 lost detectable antibody to p26 in just 6 months after inoculation. If serology were the only method used to test for BIV, this calf would have been considered uninfected during the first 2 weeks after inoculation, before antibodies could be detected, and infection status would be only questionable after 6 months. When this calf was hyperimmunized with BIV at 302 days after the original inoculation, it produced a strong antibody response to p26 antigen, suggesting that functional B cells that could respond to this antigen had not been lost. After day 42, a weak antibody response to gp110, which persisted throughout the experiment, was detected in calf 289. This difference in response to $gag$ and $env$ proteins has been described for BIV and other lentiviruses.

By use of a protein immunoblot with whole virus antigen, we detected an antibody response to $gag$ p26, but were never able to detect a unequivocal

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response to env glycoproteins in calf 1268. For both calves the response to
gp110 was less than for p26. Several explanations for this difference are
possible. First, antigenic differences of the env proteins of the isolates used for
detection and those that were used to inoculate the cattle were different. The
surface envelope gene of HIV has been described to have high sequence
divergence among different isolates that would cause epitope differences that
could affect antibody detection. Second, the whole virus antigen preparation
may have less env protein compared with gag protein. For HIV, 100 times more
gag p24 than env gp120 is predicted to be present in whole cell preparations.
Because BIV is also a lentivirus, gp110 is anticipated to be present in less
amounts than those of p26 in the intact virus. Third, lentivirus env glycoproteins
are reportedly fragile and easily sheared off during the process of
ultracentrifugation through a glycerol cushion, this has been reported with BLV.
Fourth, glycoproteins are observed as smears on blots because of variable
amounts of glycosylation of each molecule. This glycosylation effectively
decreases the concentration of antigen available for detection, and a single sharp
band cannot be observed. The inability to confidently detect antibodies to gp110
in the cattle raises some concern about the use of protein immunoblot to detect
gp110 in clinical samples. Calf 1268, like calf 289, also had a decrease in
antibodies to p26. Although the test was never considered negative during the
study period, antibodies may have continued to decrease to undetectable level.

The nested PCR test was designed to decrease the possibility of false-
negative and false positive results. False negative results were decreased by
having 2 sets of primers to different areas of the BIV genome, pol and env, used
in 2 separate PCR tests. This design decreased the possibility that a single test
yielding a false-negative result would result in an animal infected with BIV being considered uninfected. Use of multiple primer pairs also decreased the possibility of sequence variation causing false-negative results. We have observed large sequence divergence, especially in the env region, among different isolates of BIV. When using a DNA polymerase without 3'→5' exonuclease activity, a single base pair mismatch at the 3' nucleotide of the primer may result in a negative PCR amplification. Other multiple nucleotide mismatches between primer and template also can result in poor or no amplification of the target sequence. Therefore, the first step of the nested PCR reaction was performed at a lower annealing temperature, to allow for binding of primer to template even in the presence of a few base pair mismatches. The second step of the reaction was performed at higher temperatures, to provide the specificity required in the reaction. Having 2 sets of primers allowed for some variation in the target with 1 set of primers, but still allowed amplification by the other set of primers. The primers were designed to be in conserved areas of the viral genome, on the basis of computer predictions, reported sequences of BIV, and conserved sequence among lentiviruses.

False negative results also were decreased by evaluating multiple samples. Multiple samples are important when the target is present at the limits of detection for the test. Although nested PCR has been shown to be highly sensitive, another lentivirus study has revealed that virus amounts in blood may be low during some periods of the infection. Therefore, sufficient target may be present in 1 sample, but not in the next. Finally, a sample may be considered to have negative results because it is not properly prepared for efficient PCR amplification. For example, high salt content in the sample may decrease
amplification by the polymerase. A positive sample control, using primers to the actin gene, was evaluated on all samples with negative results, to assure that the DNA was suitable for PCR amplification. False-positive results are a real danger when using PCR, because of the enormous power for amplification by this procedure, and established laboratory techniques were used to decrease this possibility. This includes PCR setup, amplification, and product handling were performed in separate rooms, PCR consumables were autoclaved before use, and all pipet tips used had filters to prevent aerosolization of reagents. The PCR product was further confirmed by Southern blot hybridization with plasmid probes to rule out non-specific product.

In this study, PCR results were similar for both cattle, with positive results for pol and env primers detected after the fifth day after inoculation. Day 3 samples gave positive results with only the pol gene primers for calf 289 and with the env gene primers for calf 1268. Virus amounts in these early samples may have been at the limit of detection for the test, with 1 sample having sufficient target for a positive amplification and the next sample being deficient in target.

In the pol test, 2 products were observed in some samples. An additional PCR amplification revealed that the larger product was the result of amplification of target by carry over primer from the first step reaction to the second step reaction. Results of the env test also had similar higher molecular size bands predicted by various primer interactions. Decreasing the amount of primer in the first reaction from 20 pmol to 4 pmol decreased the number and the intensity of these additional bands.

Different sized env target products were detected between calves 1268 and 289. Target DNA amplified from calf 289 was of the size predicted on the
basis of the published sequence for BIV R29-106. Product from calf 1268 was approximately 150 bp larger at early sample dates, but this was the size predicted from sequencing of the surface envelope gene of the FL491 isolate. In samples obtained from calf 1268 later in the infection, a second and third distinct band were observed above the originally predicted band. Neither band decreased in intensity when the env primers from the first reaction were decreased in a confirmatory test. All three bands hybridized to the BIV-specific plasmid probes. The highest band is the size that would be predicted by amplification of primers 04 and 01, and may represent a reaction of primers from the first reaction with the second reaction that was observed with the pol primers. The middle band is approximately 50 bp larger than the predicted size fragment of FL491, and it cannot be explained by interaction with the primers in this reaction. We hypothesize that these products represented different sized quasispecies detected in this calf. Sequence variation was observed in the surface env region from the original BIV R29 isolate, with an 87-bp difference between R29-127 and R29-106. Both of the Florida isolates were larger than the original R29 isolate in this region of the BIV genome, with FL112 being larger than FL491, the BIV isolate used in this study. The larger quasispecies of FL491 that was detected in later samples from calf 1268 may have been present in the original inoculum. As the virus replicated in vivo, these genotypes were sufficiently amplified for detection by the nested PCR test in vitro.

In this study, the nested PCR test was more sensitive than was serology or virus isolation for the detection of BIV in experimentally infected calves. The BIV infection was detected earlier with PCR than with the other 2 methods, and detection of BIV was constant throughout the course of infection. Whether
nested PCR with use of the primers described here will have the same sensitivity in detecting naturally infected animals is unknown. The nested PCR test will be a valuable tool in examining the pathogenesis of BIV in experimentally infected animals.

Acknowledgements

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References


20. Malmquist WA, VanDerMaaten MJ, Boothe AD. Isolation, immunodiffusion, immunofluorescence and electron microscopy of a syncytial


CHAPTER 5. IDENTIFICATION OF HYPERVERSAL AND CONSERVED REGIONS IN THE SURFACE ENVELOPE GENE IN THE BOVINE LENTIVIRUS

Submitted to Virology

David L. Suarez\textsuperscript{1,2} and Cecelia A. Whetstone\textsuperscript{1,3}

Abstract

The surface envelope (SU) of nine different isolates of the bovine lentivirus (BIV) were compared for nucleotide and deduced amino acid (aa) sequence diversity. Analyses were done both on isolates derived from the original reference strain, R29, and on field isolates of BIV. Six conserved and seven hypervariable regions were identified. Many of the hypervariable regions were located in areas predicted to be on the surface of the SU protein. The SU gene comparison among all isolates showed up to a 50% aa sequence divergence. When a conserved region of the reverse transcriptase gene was compared among 8 of the isolates, there was less than 12% aa sequence divergence. When comparing all isolates, the greatest size differences in the SU gene are observed in the 2nd hypervariable region (V2) with up to 104 aa difference.

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between the largest and smallest variant. R29-106, an infectious molecular clone of the original isolate of BIV, has an 87 bp deletion in V2 as compared with prototype isolate R29-127. All R29-derived isolates sequenced for this study had a SU gene size similar to R29-106. The four field isolates sequenced for this study had SU genes larger than R29-127. R29-derived isolates may not be representative of BIV currently present in United States cattle.

**Introduction**

The bovine lentivirus, also known as bovine immunodeficiency-like virus (BIV), was first isolated in 1969 from an 8 year dairy cow (Van Der Maaten et al, 1972). This original isolate, R29, was the only BIV isolate available for study until recently, when two new isolates, FL491 and FL112, were cultured from a Florida dairy herd (Suarez et al., 1993). BIV remains difficult to isolate in cell culture, and these three isolates are the only cultured isolates widely available for study. BIV is known to cause a persistent infection in cattle and it appears to be widespread in the U.S. cattle population as well as abroad (Suarez et al, 1994). However, the role of BIV in disease still remains unclear. Although several conditions are associated with BIV infected cattle, including encephalitis, lymphadenopathy, and immunodeficiency (Gonda et al., 1994), these conditions have not been reported in experimentally infected cattle. A serological survey of Canadian dairy cattle demonstrated a statistical link between BIV infection and lower milk yields (McNab et al., 1994). Because lentiviruses typically produce chronic and diverse disease, continued research into a possible role of BIV in disease is merited.
The high mutation rate of lentiviruses as a group is well documented and is thought to play a role in their pathogenesis (Temin, 1993). Lentiviruses are thought to occur as a quasispecies, where almost every virion is different, due to point mutations, insertions and deletions spread throughout the genome (Temin, 1993). These mutations are often concentrated in certain regions of the genome, even though they probably arise randomly across the genome because of both the low fidelity of the reverse transcriptase enzyme and recombinational events. Specific areas have such high sequence diversity that they were described as hypervariable regions. The surface envelope (SU) gene of several lentiviruses, including the human immunodeficiency virus (HIV) (Starcich et al., 1986; Modrow et al., 1987), feline immunodeficiency virus (FIV) (Rigby, et al., 1993; Pancino, et al., 1993) and the simian immunodeficiency virus (Burns et al., 1991), have multiple hypervariable regions. Hypervariable regions are often predicted to be on the surface of the SU protein, suggesting that they may be important in helping the virus evade host immune response (Salinovich et al., 1986; Levy, 1993). Hypervariable regions were also shown to be important in several other viral characteristics including cell tropism, virulence, growth characteristics, and syncytium-forming ability (Stamatatos et al., 1993; Sullivan et al., 1993).

The SU gene of BIV has been studied for potential splicing sites and immunoreaction. The env gene of BIV is thought to be translated from the second initiation codon in the open reading frame of the env region (Garvey et al, 1990). Studies done using BIV env either from infected cell culture or from a baculovirus expression system support the hypothesis of initiation at the second potential start site (Rasmussen et al, 1992 Oberste et al., 1993). The env gene encodes both the SU gene and the transmembrane envelope (TM) gene that are
thought to be proteolytically cleaved at a RKPR motif. This proteolytic cleavage region is similar to that observed in other lentiviruses (HIV) (Garvey et al., 1990). Recombinant proteins starting from this second initiation codon demonstrated immunoreaction with serum from BIV infected animals (Rasmussen et al, 1992).

Sequence variability was previously described for a small region of the RT gene of R29-derived isolates and the Florida isolates, with up to 7% sequence divergence being observed (Suarez et al., 1993). Sequence variability also was observed in the SU gene of R29 derived infectious molecular clones, R29-106 and R29-127. The major difference between the molecular clones was an 87 b.p. deletion in the 5' end of the SU gene of R29-106 as compared to the prototype sequence of R29-127 (Garvey et al., 1990). Variability in size in the 5' region of the SU gene also was observed with a nested PCR test of the SU gene between a R29-derived isolate, R29-289, and a Florida isolate, FL491 (Suarez et al., 1995).

The purpose of this study is to further define the SU gene of BIV and identify both conserved and hypervariable regions of the gene. Analyses of nucleotide and amino acid sequence divergence, size polymorphisms, and conservation of cysteine and N-linked glycosylation sites are made. Data are compared with sequence from a region of the reverse transcriptase (RT) gene, a region of the lentiviral genome considered to be highly conserved. High passage R29-derived isolates are compared to more recent BIV isolates for size differences in the SU gene and the possible implications on experimental inoculation studies are discussed.
Table 1  
BIV Isolates Used in this Study

<table>
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<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Cell Cultures Used for in vitro Propagation</th>
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<td>R29-1203</td>
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<td>Epithelial tracheal primary cell line</td>
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<td>R29-106</td>
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<tr>
<td>R29-ND</td>
<td>Molecular clone of R29$^C$</td>
<td>Canine thymocyte cell line</td>
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</table>

$^a$ Sequence derived from PCR amplified DNA from naturally infected animal  
$^b$ Not applicable  
$^c$ Sequenced obtained from GeneBank

Methods

Virus Isolates  
Virus isolates used in this study are presented in Table 1. Two infectious molecular clones, R29-106 and R29-107, have been previously sequenced and the results published (Garvey et al., 1991). An additional molecular clone of R29, R29-ND, has also been sequenced and the sequence is available from Genbank. All three clones were cloned from integrated provirus from cell culture infected with the R29 isolate. R29-106 and R29-107 were both...
cloned from the same bovine epithelial tracheal cell culture (Braun et al., 1988). R29-ND was cloned from persistently infected canine thymocyte cells (Bouillant et al., 1989). The R29-1078 virus isolate was recovered 33 months after the cow 1078 was experimentally infected with blood from a cow infected with a R29-derived isolate, 1481 (Van Der Maaten et al., 1990). The R29-1203 isolate was previously described as a BVDV-free isolate of BIV (Suarez et al., 1993), and this isolate was recovered from an inoculated calf six weeks after experimental exposure. The R29-1203 isolate was the inoculum used to infect bullock 289, and the R29-289 isolate was recovered 291 days post-inoculation (Suarez et al., 1995). Two isolates, FL491 and FL112, were cultured from a dairy herd from Florida (Suarez et al., 1993). The R29-1078, R29-1203, R29-289, FL491, and FL112 isolates were all recovered and maintained in fetal bovine lung primary cell cultures. The OK 14 and OK 40 plasmid clones were derived from the same sample of blood from a cow naturally infected with BIV from a herd in Oklahoma. The blood sample from the Oklahoma cow was collected in vacuum tubes with EDTA by a clinician at the Oklahoma State Veterinary College. The sample was sent by next day delivery to the National Animal Disease Center, and the WBC's were isolated and the DNA was extracted according to previously described methods (Suarez et al., 1995). The Oklahoma BIV isolate has remained unculturable.

**PCR and Sequencing** Proviral DNA provided DNA from the isolates grown in cell culture was PCR amplified and sequenced. The cells were proteinase K digested in TNE, phenol/ chloroform extracted, ethanol precipitated and quantitated as previously described (Suarez et al., 1995). For the SU gene
sequence, 0.5 µg of template DNA, 20 pmol of primers 93 and 06 (Table 2), 2.5 units Taq polymerase, 3 mM MgCl₂, and standard PCR buffer were used in a 100 ul reaction mixture. The PCR cycling conditions were: 120 sec. at 94 C, 15 sec. at 61 C, 120 sec. at 72 C for 1 cycle; 45 sec. at 94 C, 15 sec. at 61 C, 60 sec at 72 C for 30 cycles; and a final extension step of 72 C for 10 min. For RT gene sequence, one microgram of template DNA, 20 pmol of primers 93 and 06 (Table 2), 2.5 units Taq polymerase, 3 mM MgCl₂, and standard PCR buffer were used in a 100 ul reaction mixture. The PCR cycling conditions were: 120 sec. at 94 C, 15 sec. at 56 C, 120 sec at 72 C for 1 cycle; 45 sec. at 94 C, 15 sec. at 56 C, 60 sec at 72 C for 30 cycles; and a final extension step of 72 C for 10 min.

The RT gene for the OK sample, an uncultured isolate, was sequenced from PCR-amplified DNA using a modification of the above procedure. The sample was run for 40 cycles instead of 30 cycles with the conditions described above. Multiple reaction tubes with the same template were prepared and the PCR product was purified for sequencing by repeated washings in a Microcon 100 (Amicon) with molecular biology grade water (Omnisolve, EM Science). The PCR product was sequenced by a dye terminator sequencing system (ABI Applied Biochemistry 373A DNA sequencer; performed at the Iowa State University Nucleic Acid Center). Specific primers spread throughout the SU gene were used to initiate the sequencing reaction (Table 2).

**Cloning**  
The SU gene from the Oklahoma isolate was PCR amplified, cloned into a plasmid vector, and sequenced. A nested PCR reaction was employed to generate enough PCR product for cloning. The first step PCR conditions used 0.5 µg of WBC derived DNA, 20 pmol of primers 93 and 14
Table 2  Oligonucleotide primers used for PCR amplification and DNA sequencing. Nucleotide positions were based on the R29-127 molecular clone map of the BIV genome (Garvey et al, 1991). Primers on the top stand represent positive sense primers and bottom strand represent negative sense primers. When two sets of primers are represented at the same position, the primers are very similar, except that one of the primers has a 12-b.p. 5' extension for use in cloning of the SU gene.

<table>
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(Table 2), 1.25 units Taq polymerase, 3 mM Mg\textsuperscript{2+}Cl\textsubscript{2}, and standard PCR buffer were used in a 50 ul reaction mixture. The PCR cycling conditions were: 120 sec. at 94 C, 15 sec. at 51 C, 120 sec at 72 C for 1 cycle; 45 sec. at 94 C, 15 sec. at 51 C, 60 sec at 72 C for 30 cycles; and a final extension step of 72 C for 10 min. Two ul of the reaction mixture was used as the template for the second step reaction with 20 pmol of dUTP modified primers, 808 and 42 (Table 2), 1.25 units Taq polymerase, 3 mM Mg\textsuperscript{2+}Cl\textsubscript{2}, and standard PCR buffer were used in a 50 ul reaction mixture. The PCR cycling conditions were: 120 sec. at 94 C, 15 sec. at 51 C, 120 sec at 72 C for 1 cycle; and 45 sec. at 94 C, 15 sec. at 51 C, 60 sec at 72 C for 30 cycles and a final extension step of 72 C for 10 min. The PCR product was used with a ligation independant cloning system (Cloneamp, Life Technologies, Inc.) according to manufacturer's instructions. E. coli cells (Max Efficiency DH5a, Life Technologies, Inc.) were transformed according to manufacturer's instructions. The recombinant bacteria were screened with a PCR-based procedure. Plated bacterial colonies were picked and grown overnight in 3 ml of LB broth. One ul of the broth was used as template for the PCR reaction. The 1 ul of broth was added to 32 ul of molecular biology grade water (Omnisolve, EM Science) in a reaction tube and the tube was heated and cooled to release the plasmid DNA for PCR. The conditions to release plasmid for PCR were: 30 sec at 65 C, 30 sec at 14 C, 90 sec at 65 C, 180 sec at 97 C, 60 sec at 14 C, 180 sec at 65 C, 60 sec at 97 C, 60 sec at 65 C and 10 min at 80 C for 1 cycle. Buffer, 20 pmol of primers 01 and 45 (Table 2), MgCl\textsubscript{3}mM, dNTPs, and 1.25 Taq polymerase units were added after the bacteria were processed with the following cycling conditions: 120 sec. at 94 C, 15 sec. at 61 C, 90 sec at 72 C for 1 cycle; 45 sec. at 94 C, 15 sec. at 61 C, 60 sec at 72 C for 30
cycles; and a final extension step of 72 C for 10 min. The PCR products were analysed in agarose gel with ethidium bromide staining. Positive clones were grown overnight in LB broth and the plasmid was purified using a commercial plasmid isolation kit (Perfect Prep, 5 Prime-3 Prime).

**Data Analyses**  DNA sequence analysis was performed using the program SeqMan (DNASTAR). Sequence alignments were performed with the program Megalign (DNASTAR) using the clustal method. Rooted phylogenetic tree analyses were also performed with the Megalign program. Antigenic indexes were calculated using the Emini method with the computer program Protean (DNASTAR).

**Results**

A total of five RT gene and seven SU gene sequences from different sources were examined. An additional three RT and SU sequences were available from Genbank and were included in the analyses. The sequences derived from R29, which included five RT and six SU sequences, were first analyzed separately because of the small amount of sequence divergence that was observed. The RT gene showed very little sequence divergence among the different isolates, and only a single amino acid difference was observed in the R29-ND sequence. Greater sequence divergence was observed in the SU gene. Up to 5% sequence divergence was observed among the R29-derived isolates. Phylogenetic tree analyses of amino acid homology and sequence homology divided the isolates into two groups (Fig. 1). The first group included R29-127, R29-106, and R29-ND, and the second group included R29-1203, R29-289, R29-1078. Nine amino acid changes, spread throughout the SU gene, separated the
**R29-derived Nucleotide Comparison**

**R29-derived Amino Acid Comparison**

**Figure 1** Phylogenetic tree analyses of the SU gene of R29-derived isolates of BIV based on the Clustal method (Megalign, DNASTAR). The top tree is based on nucleotide comparisons and the bottom strand is based on amino acid sequence comparison.
two groups. An 87 nucleotide base pair (29 a.a.) size difference was observed between R29-127 and all other R29-derived isolates.

A second sequence comparison included R29-127, R29-289, FL491, FL112, and OK. For the RT gene sequence, a single sequence represents the OK sample, because this sample was uniform enough to derive sequence directly from PCR amplification of the RT gene. The OK SU gene, however, was more complex and was cloned. Two different OK clones, OK 14 and OK 40, were included in the SU gene analyses.

Among the isolates, the RT gene region had up to 10% divergence in nucleotide sequence homology and up to 11% amino acid sequence divergence (Fig. 2). No size variation was observed in any of the RT gene regions examined. The sequence variation appeared to be spread throughout the RT gene segment, and no areas of either high variability or conservation were observed.

Six sequences were used for the main analysis of the SU gene. The OK clones were amplified with a primer that overlaps the first 18 b.p. of the SU gene, and this part of the sequence was inferred by the successful PCR reaction. The sequences R29-127 and R29-289, FL491, FL112, OK 14 and OK 40. Comparison of a.a. sequence showed diversity as high as 42% among the different isolates (Fig. 2). The variation was not randomly distributed throughout the genome, and both conserved and hypervariable regions were identified. A conserved region was defined as a region larger than 12 a.a. that had less than 10% sequence divergence from the consensus. A hypervariable region was defined as a region larger than 12 aa with greater than 30% sequence
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**Figure 2**  (Top) Percent nucleotide (above diagonal) and amino acid (below diagonal) sequence similarities of the RT gene. (Bottom) Percent nucleotide (above diagonal) and amino acid (below diagonal) sequence similarities of the SU gene.
divergence from the consensus. A total of six conserved regions and seven hypervariable regions were defined (Fig. 3). They are referred to in order as C1, C2, C3, etc. for the conserved regions and V1, V2, V3, etc. for the hypervariable regions similar to the convention used in other lentiviruses. Five potential N-glycosylation sites were conserved among all 6 isolates (Fig. 3). The average number of potential N-linked glycosylation sites for each isolate was 14.6, with R29-289 and OK-14 having the least at 13 and FL112 having the most at 17. Nine cysteine residues were conserved among the six isolates (Fig. 3). The average number of cysteine residues for each isolate was 13.7, with a low of 12 for R29-289 and a high of 15 for FL112 and OK 14. Conservation of cysteine residues was 66%.

Large size differences in the SU gene were observed among the different isolates. 104 a.a. (312 nucleotide b.p.) separated the smallest and largest SU genes. Previously described size differences were observed between R29-127 and R29-106 (Garvey et al., 1990). All R29-derived sequences examined in this study had a R29-106 size genome. SU gene sequences of the four other isolates were larger than the R29 isolates, and each differed in size from each other. The greatest size differences were in hypervariable regions V2, V4, and V7, with the V2 region showing the most variability among all six isolates, exhibiting size polymorphisms of up to 76 a.a. Other smaller insertions and deletions were spread throughout the SU gene, with most being in the defined hypervariable regions.

Antigenic indexes for the SU protein were calculated and compared to the defined hypervariable regions. A close relationship between hypervariable regions and predicted antigenic regions were observed. Specifically, in the V2
Figure 3  Amino acid sequences of the SU gene of six BIV isolates.

Alignment of the sequences was done with the assistance of Megalign (DNASTAR). Numbering of the sequences is given as the total a.a. at the ends of the sequence. R29-127 is used as the prototype sequence. For the other isolates, dots indicate identity with R29-127, dashes indicate the absence of an amino acid. Potential N-glycosylation sites present in all isolates are shaded. Symbols: conserved cysteine residues *; conserved regions •• ; hypervariable regions ••••• ; ovals indicate each of the nine amino acid differences that separated the culture isolates and the animal-associated isolates of R29.
region, with its great size variation, a corresponding widening of the predicted antigenic region was seen among the different isolates (Fig. 4).

**Discussion**

As anticipated, a high degree of variation was observed in the SU gene among the different isolates. The RT gene was conserved, a finding consistent with observations of other lentiviruses (Greene et al., 1993; Levy, 1993).

The SU and RT genes, representing a total of seven isolates of BIV, were sequenced for this paper and an additional three isolates' sequences were available from previously reported data and from Genbank. Six of the sequences were very similar because they were derived from the same isolate of BIV, R29. R29 was originally isolated in 1969 on fetal bovine spleen cells (Van Der Maaten et al., 1972). Originally, when this low passage isolate was used for experimental inoculations of calves, it did not cause clinical disease, but rather a lymphoproliferative response characterized by an increased blood mononuclear cell count and enlarged subcutaneous lymphatic nodules (Van Der Maaten et al., 1972). The R29 isolate was eventually frozen and not actively studied until after the cause of AIDS was shown to be a lentivirus. The R29 isolates currently available have been propagated in several different cell culture types. The infectious molecular clones of R29, R29-106 and R29-127, were cultured in and cloned from bovine epithelial tracheal cells (Braun et al., 1989), the R29-ND isolate was propagated in canine thymocyte cells (Bouillant et al., 1989), and the R29-1203 isolate was propagated in canine thymocyte cells, Black 20 bovine kidney cells and fetal bovine lung cells (Suarez et al., 1994). The R29 isolate no longer causes the same response in experimentally infected cattle as that originally described (Van Der Maaten et al., 1972; Carpenter et al., 1992),
Figure 4  Antigenic indexes of the SU gene sequences of three isolates of BIV using the Protean computer program (DNASTAR). The three bars under the graphs represent the three hypervariable regions with the greatest size polymorphisms.
possibly because it has been extensively passaged in a number of different cell culture types.

Phylogenetic tree analyses comparing both SU nucleotide and amino acid sequence show that the R29 isolates cluster into two groups, the cell culture group and the animal inoculated groups. Three isolates, R29-106, R29-127 and R29-ND were all isolates that were not passaged through animals before being sequenced. The remaining three isolates had all been passaged through cattle at least once before being sequenced. R29-289 and R29-1078 had been through two animal passages before being sequenced. Based on phylogenetic tree analysis, R29-289 was clustered as close to R29-1078 as to R29-1203 which is surprising, since R29-1203 was used to inoculate the calf from which R29-289 was isolated. A total of 9 amino acid changes completely separated the cell culture group and the animal inoculated group of isolates. These changes were spread throughout the SU gene and were in conserved and hypervariable gene regions (Fig. 3). We conclude that the SU gene of BIV is subject to selection pressure from both in vivo and in vitro passage. These potentially attenuated viruses have been the only isolates of BIV available for study until 1993.

SU gene analysis, focusing on the isolates R29-127, R29-289, FL491, FL112, OK 20 and OK40, showed that these isolates also separated into three different groups based on their origin (data not shown). The R29-derived isolates were the most closely related, even though one was cell culture derived and the other was obtained from an inoculated animal. Both Florida isolates came from the same dairy herd and they were more closely related to each other than to other isolates. The OK uncultured isolates came from the same cow and were
isolated from WBCs by PCR amplification and cloning of the SU product. Two clones were included in the analyses because they demonstrated a high degree of sequence divergence. The six isolates, when aligned to each other, demonstrate regions of the SU gene that are either conserved or hypervariable. Conserved and hypervariable gene regions have been observed in other lentiviruses, and have been shown to be important in a number of virus functions.

The different hypervariable regions were named V1-V7, similar to other lentiviruses. Large size differences were observed between the different isolates, especially in the V2 region. An 87 b.p. nucleotide difference was observed in the R29-derived infectious molecular clones that were originally sequenced. This size difference was the only large size difference between these otherwise very similar clones. The R29-106 infectious molecular clone size appeared to predominate in the cell culture from which these two molecular clones were made, since the R29-127 size molecular clones could not be detected in restriction enzyme mapping with Southern Blots (Garvey et al., 1990). The 4 additional R29-derived isolates also had the R29-106 size SU gene. It is unknown if any R29-127 sized virus is still present in uncloned viral stocks.

The V2 region varied by 72 amino acids when comparing R29-289 and OK40. The SU gene of OK40 was a total of 104 amino acids or 20% larger than R29-289. Size polymorphisms of the SU gene have also been observed in other lentiviruses, with greater variation observed in specific hypervariable regions. (Kusumi et al., 1992; Ball et al., 1994, Rigby et al., 1993; Simmonds et al., 1990) Although size polymorphisms are common among lentiviruses, the size variation of the V2 region of BIV, at 74 a.a. between the largest and smallest isolates, is higher than that observed in other lentiviral systems. The SU genes of R29-
derived isolates are considerably smaller than more recent field types. The Florida isolates are of intermediate size in this collection. It is unknown if the size differences observed are related to virus pathogenicity or in the ease of virus isolation. The R29-derived isolates, with the smallest SU gene, are the easiest to recover in experimentally infected animals (Suarez et al., 1995) and appear non-pathogenic, not even causing the early mononuclear cell increase that was observed in earlier inoculations (Carpenter, 1992; Flaming et al., 1993). The Florida isolates, with an intermediate recovery rate from infected animals, cause a mononuclear cell increase early in infection (Suarez et al., 1993; Suarez et al., 1995). The OK isolate has remained unculturable and has not been characterized in experimental infections. Caution must be used in the analyses and use of data from experiments that have used R29-derived isolates, because they may not be typical of what is observed in naturally acquired BIV infections.

The experimental evidence for hypervariable regions in the SU gene of BIV is consistent with observations of other lentiviruses. Hypervariable regions were consistently located in regions that were predicted to be antigenic, and, therefore, on the surface of the protein (Modrow et al., 1987). The surface epitopes that are often contained in these hypervariable regions may provide a selective advantage to the virus by having high antigenic variation that helps it evade the host immune response. This appears to be an important mechanism for some lentiviruses such as equine infectious anemia virus (Salinovich et al., 1986). Alternatively, these hypervariable regions may be structural scaffolding that can tolerate high sequence diversity, similar to that observed on the "rims of the canyon" of human rhinoviruses (Chapman et al., 1993). BIV is thought to have a generally random mutation process, but it does maintain both conserved
genes, such as the RT gene, and conserved regions of variable genes, such as the SU gene. Therefore, a selection pressure must be present that allows mutations to be maintained in the viral genome. However, with the lack of knowledge about the role of BIV in disease, it is unclear if these differences are important in the pathogenesis of this virus. Continued research into the pathogenesis of BIV and the role that viral variation may have on this virus are warranted.

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References


CHAPTER 6. GENERAL CONCLUSIONS

The state of knowledge about BIV is heavily weighted to what has been determined with R29 or its derivatives, because for over twenty years the R29 or R29-derived isolates were the only isolates available for study. However, looking at other viral systems, it is well known that the virus isolate used for experimental studies will affect the results observed. For example, the equine infectious anemia virus, another lentivirus, has both attenuated and virulent isolates described. Adaptation of growth of EIAV to certain cell lines promotes an attenuated form of the virus (Carpenter et al., 1989). Other differences in pathogenesis of different isolates have also been described for the Maedi-Visna virus (Lairmore et al., 1987). These factors illustrate the importance of examining new BIV isolates and exploring the possibility that R29-derived isolates may not represent BIV isolates found currently in the field.

To compare R29-derived isolates to other isolates, new isolates needed to be cultured. BIV, however, is a difficult virus to culture in presently described cell lines. However, two new isolates, FL112 and FL491, were cultured from BIV seropositive cows from a Florida herd using essentially the same methods that were described for culturing the R29 isolate. The Florida isolates were more closely related to each other than to R29, based on sequence comparisons of the RT gene, but differed in biological activity. The FL112 isolate had a greater mononuclear cell increase after experimental inoculation than did the FL491 isolate. This early mononuclear cell increase had also been described for the R29 isolate of BIV after the initial experimental studies, but in later experimental studies this transient mononuclear cell increase was apparently lost (Van Der
Maaten, et al., 1972; Carpenter et al, 1992). Further differences observed between the Florida isolates and the R29-derived isolates in cell culture included differences in syncytial forming capacity in cell culture, and the ease of recovery in cell culture of R29-derived isolates compared to the Florida isolates from experimentally infected cattle. It is unknown if these biologic differences reflect the viral replication rate of the different isolates or the tissue culture adapted nature of the R29-derived isolates. Most, if not all, of the remaining R29-derived isolates have been passed through at least two different cell lines and, therefore, must be considered highly tissue culture adapted.

The next step in examining for viral variation was to develop tools to look for variation. The nested PCR test was developed as a test to identify infected cattle and as a tool to sequence parts of the BIV genome. For these reasons the PCR test was developed to examine both conserved and variable regions of the BIV genome. The reverse transcriptase (RT) gene was used for the conserved region and the surface envelope (SU) gene was used as the variable region. The nested PCR test was also compared to the protein immunoblot test and virus isolation as methods for detecting cattle experimentally infected with BIV. The nested PCR test was more sensitive than the other two methods. A loss of antibody to the p26 gag protein was observed with the protein immunoblot test in both cattle tested. This loss of antibody has been observed before (Whetstone et al., 1990; Onuma et al, 1992), and it has the potential to be important diagnostically, because current serologic tests rely heavily on the presence of

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antibody to p26 for detection of BIV infection. An additional unexpected finding was observed in the PCR results from calf 1268. When examining the env PCR product, a detectable shift in the size of the BIV population was observed. The nested PCR env primers were directed to amplify a 5' region of the SU gene, and in calf 1268 these primers amplified only a single size early in infection, but later during infection a larger BIV band representing a larger genotype was detected that became the predominant size product observed. An apparent selection process for a larger variant appeared to be going on in vivo.

Direct comparisons of sequences from different BIV isolates has allowed a more detailed analysis of differences between isolates. A total of five isolates from cell culture, three sequences available from Genbank, and direct PCR amplification and/or cloning of BIV from an Oklahoma cow were used in the sequence analyses. Six of the sequences were from R29-derived samples. When these six samples were analyzed separately, the isolates could be differentiated into two groups. The difference in the two groups correlated with whether the isolates had been through a recent animal passage. R29-1078, R29-1203, and R29-289 all were isolates that had been cultured recently from experimentally inoculated cattle, and they had nine amino acid changes from R29-127 in common. This analysis provided evidence of an in vitro vs. in vivo adaptation process of the different isolates. The R29-derived isolates that our group is currently using have a size similar to R29-106 and not to R29-127, and the Florida and Oklahoma isolates have a larger SU gene than R29-127. When the SU gene of all the isolates were examined, high sequence diversity was observed. Similar to what had been observed in other lentiviral systems, the variation was concentrated into hypervariable and conserved regions. The
mapping of the different regions of the SU gene of BIV should be valuable for future experimental studies and aid in the design of new diagnostic reagents. The size difference of R29-106 and R29-127 is located in the second hypervariable (V2) region of the SU gene. The V2 region also had the greatest size variation among all the isolates studied.

At this time it is not possible to link the biological differences with the actual sequence differences, however the data supports several different hypotheses concerning BIV. The first hypothesis is that R29 derivatives are attenuated. R29-derived isolates no longer cause the same activity in cattle that was originally described (Van Der Maaten et al., 1972; Carpenter et al., 1992). Also, a definitive pattern of sequence differences appears to have been selected for by prolonged cell culture as opposed to animal passage. Both of these characteristics suggest that current R29-derived isolates are attenuated similar to what has been described for EIAV (Orrego et al., 1982; Carpenter et al., 1989). The R29-derived isolates that our group has for experimental inoculations are substantially smaller than the Florida isolates and the uncultured naturally infected isolates. This provides circumstantial evidence that R29-derived BIV isolates do not represent the BIV isolates found in the field. These factors when considered together suggest that R29-derived isolates are attenuated, and caution must be used when interpreting their use in pathogenesis studies.

A related hypothesis is that the original R29 that was isolated in cell culture predominantly had an SU gene of the same size, or larger, as R29-127, and that by continued passage in culture the R29-106-sized SU gene became predominant. It was observed when these two infectious molecular clones were made that the cell culture contained virus that was predominantly R29-106 in size
The loss of the R29-127-sized genome may account for the loss of the mononuclear cell increase during the early part of the infection process. The newer Florida isolates also cause a transient mononuclear cell increase early in infection. The hypothesis that the smaller genome size is selected for in cell culture can be tested with reagents available today, but the proportion of R29-127 sized and R29-106-sized virus in cow R29 or the original cell cultures will probably never be known. The additional hypothesis that the size of the V2 region plays a role in the transient mononuclear cell increase in cattle can also be tested with additional animal experiments using BIV mutants constructed with wild-type SU gene on a R29 background.

The hypothesis that the original R29 isolate was predominantly R29-127 sized and the smaller variant was selected by cell culturing has already been stated. We have evidence, in cow 1268, that a larger variant of FL491 was apparently selected for in vivo. Additional evidence of selection of the larger genotype in vivo was the OK isolate. This isolate has not been cultured and when the SU gene was examined in vivo it had the largest SU gene that has been sequenced. These data support the idea of selection of small genotypes in cell culture and selection of larger genotypes in vivo.

The last hypothesis is that the BIV lentiviral system may be unique as to the amount of size variation that is observed between different isolates and quasispecies in the same animal. Size variation is commonly observed in other lentiviruses, but not to the extent that was described with BIV. The V2 region in particular has large size variations, and the possible implications as to the biological role that this difference may have has been described. The identification of conserved and hypervariable regions of the BIV genome will also
provide a general framework for associating additional biological features with specific sequences and provide additional information for use in diagnostic tests.

This dissertation looks at viral variation of the BIV genome and provides support for a number of different hypotheses about BIV. This report includes the isolation and characterization of two new isolates of BIV, but additional isolates of BIV need to be cultured to have a more complete understanding of BIV. Developing a cell culture system that allows for routine isolation of field isolates of BIV, therefore is needed. The role of BIV in cattle disease still remains unclear; however, this report does provide evidence that the R29-derived isolates may not be the best isolates to use to examine the disease potential of BIV. Additional pathogenesis studies with the Florida isolates and future isolates is recommended.
REFERENCES


