An Analysis of *Bovine immunodeficiency virus* and *Jembrana disease virus* Infections in *Bos javanicus*

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BSc (Hons)

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Abstract

Two closely related bovine lentiviruses have been described, *Jembrana disease virus* (JDV) and *Bovine immunodeficiency virus* (BIV), that produce very different clinical manifestations in infected cattle. JDV causes an acute disease with a case fatality rate of about 21% in *Bos javanicus* (Bali cattle) and is endemic in the cattle population of parts of Indonesia. BIV produces a subclinical infection in *Bos taurus* and buffalo and serological evidence has shown that this virus has a worldwide distribution, possibly including Indonesia.

Attempts were made to confirm a previous report that BIV was present in the *B. javanicus* population in Indonesia. BIV proviral DNA was not detected in any of the animals although JDV proviral DNA was detected in 12 of 171 animals, only one of which was seropositive.

To define the kinetics of BIV infection in *B. javanicus* and determine the optimal time for sampling to detect BIV infection, 13 animals were experimentally infected with the R29 strain of BIV. No clinical effects were detected but proviral DNA was detected from 4-60 days post-infection (dpi) with peak titres 20 days dpi, and a transient viraemia from 4 to 14 dpi. An antibody response to TM was detected 12 dpi but an anti-capsid (CA) antibody response was detected in one animal only and not until 34 dpi. The results indicated that detection of BIV in infected Bali cattle using PCR would have a greater chance of success soon after infection and prior to the onset of a CA antibody response.

To determine the effect of BIV infection on subsequent JDV infection in *B. javanicus*, 15 cattle were infected with BIV-R29 and 9 of these were subsequently infected 42 days later with JDV. The response to BIV was typical of that observed
previously but BIV infection did not markedly modify the response to subsequent infection with JDV. In response to JDV infection, all cattle previously infected with BIV still developed an acute disease process typical of Jembrana disease. The results suggested that despite the close genetic and antigenic relationship between BIV and JDV, BIV infection does not confer protection against subsequent JDV infection.

The close antigenic relationship between BIV and JDV is a problem in the development of specific serological tests and immunosurveillance of JDV infection. To develop reagents capable of differentiating between antibody to BIV and JDV infections, peptide mapping was used to define linear B cell epitopes on the matrix (MA), CA and surface unit (SU) proteins of JDV. Short overlapping peptides that spanned these regions were synthesised and used in an ELISA format to screen their reactivity with a panel of bovine sera from animals experimentally infected with JDV_{Tab87}, JDV_{Pul01} or BIV-R29. Peptides representing potential immunoreactive epitopes were identified that appeared to offer promise in the development of JDV-specific serological tests and need to be tested further with a panel of sera taken from naturally infected cattle.
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

..............................

Tegan Josephine McNab.
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To Bong for just being you.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
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<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BFL</td>
<td>Bovine foetal lung</td>
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<td>BIV</td>
<td>Bovine immunodeficiency virus</td>
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<tr>
<td>BVDV</td>
<td>Bovine viral diarrhoea virus</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CAEV</td>
<td>Caprine arthritis encephalitis virus</td>
</tr>
<tr>
<td>CE</td>
<td>Cell equivalents</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C (beta) chemokine receptor 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C (alpha) chemokine receptor 4</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxynucleotide Triphosphate</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>dpi</td>
<td>Days post-infection</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EIAV</td>
<td>Equine infectious anaemia virus</td>
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<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
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<td>Feline immunodeficiency virus</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>Horse radish peroxidase</td>
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<td>HTLV-1</td>
<td>Human T-cell lymphotropic virus type 1</td>
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<tr>
<td>ID</td>
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</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactive</td>
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<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>JDV</td>
<td>Jembrana disease virus</td>
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<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>M-tropic</td>
<td>Macrophage tropic</td>
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<tr>
<td>VMV</td>
<td>Visna maedi virus</td>
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<tr>
<td>MHR</td>
<td>Major homology region</td>
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<tr>
<td>MA</td>
<td>Matrix</td>
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<td>NC</td>
<td>Nucleocapsid</td>
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<tr>
<td>Nef</td>
<td>Negative factor</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate-buffered saline-Tween 20</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pi</td>
<td>Post-infection</td>
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<td>PR</td>
<td>Protease</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of virion proteins</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>SIV</td>
<td><em>Simian immunodeficiency virus</em></td>
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<td>SRLV</td>
<td>Small ruminant lentivirus</td>
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<tr>
<td>SU</td>
<td>Surface unit</td>
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<tr>
<td>T-tropic</td>
<td>T-lymphocyte-tropic</td>
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<tr>
<td>TCID$_{50}$</td>
<td>Median tissue culture infective dose</td>
</tr>
<tr>
<td>Tat</td>
<td><em>Trans</em>-activator of transcription protein</td>
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<tr>
<td>TM</td>
<td>Transmembrane glycoprotein</td>
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<tr>
<td>U3</td>
<td>3' Untranslated region</td>
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<td>U5</td>
<td>5' Untranslated region</td>
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<tr>
<td>Vif</td>
<td>Viral infectivity protein</td>
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<tr>
<td>VL</td>
<td>Viral load</td>
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<tr>
<td>Vpr</td>
<td>Viral protein R</td>
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<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Vpx</td>
<td>Viral protein X</td>
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<tr>
<td>WIB</td>
<td>Western immunoblotting</td>
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<tr>
<td>YT</td>
<td>Yeast tryptone broth</td>
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Publication and International Conference Presentations

Publications arising from this thesis


Manuscripts submitted for publication


Oral presentations

“Bovine immunodeficiency virus infection fails to provide protection against subsequent Jembrana disease virus infection” Presented at the European Society for Veterinary Virology Conference in Budapest, Hungary 2009.

Poster presentations

Chapter 1: Introduction

Two bovine lentiviruses have been described, *Jembrana disease virus* (JDV) and *Bovine immunodeficiency virus* (BIV). The 2 viruses, although genetically and antigenically related, have been reported to have very different pathogenic effects in cattle. JDV causes a severe, acute disease in Bali cattle (*Bos javanicus*) and a mild disease or subclinical infection in other breeds of cattle, including *B. taurus* (Soeharsono et al., 1990). The disease in Bali cattle is acute and associated with a marked febrile response, very high titres of infectious virus in the blood and a case fatality rate of about 21% (Soesanto et al., 1990). BIV infection is generally not associated with significant clinical changes in *B. taurus* breeds of cattle, although they have been reported: one study found an association between BIV and decreased milk yield in dairy cattle (McNab et al., 1994) and another associated BIV infection with marked weight loss and concurrent infections, suggesting immunosuppression (Snider et al., 2003b). JDV appears to have a limited geographic distribution and is restricted to Indonesia where Bali cattle are found (Hartaningsih et al., 1993), while serological surveys have provided evidence for a worldwide distribution of BIV in both cattle and buffalo (*Bubalus bubalis*) (Gonzalez et al., 2008; McNab et al., 1994; Meas et al., 1998; Meas et al., 2000a; Meas et al., 2000b; Suarez et al., 1993). There is also serological evidence of infection with a related non-pathogenic BIV-like virus in Bali cattle in Indonesia on the island of Sulawesi and also in Bali (Barboni et al., 2001; Desport et al., 2005). Although infection with a BIV-like virus has been suspected in Bali cattle in Indonesia, this has not been confirmed. There is no evidence of the nature of BIV infection in this cattle species and what effect BIV infection might have on subsequent JDV infection.
This thesis describes the development of a serological diagnostic assay to differentiate JDV and BIV infection, the effects of BIV in Bali cattle and the interaction between BIV and JDV infections in Bali cattle. As a background to these investigations, a review of the literature relating to JDV and the other lentiviruses has been undertaken and is reported in Chapter 2. The review includes the key features of the various lentiviruses, comparing their genome arrangement, replication and host immune responses to infection. It also includes the various techniques that have been described for diagnosis of lentivirus infections, methods of differentiating between closely-related lentivirus infections and the effects of infection with multiple strains of closely related lentiviruses.

Chapter 3 describes an attempt to detect JDV and BIV in Bali cattle on the island of Bali. Two quantitative PCR (qPCR) assays were developed to detect JDV and BIV proviral DNA within peripheral blood mononuclear cells (PBMC) of naturally infected animals.

Despite serological evidence for the presence of BIV in the Bali cattle population on the island of Bali, the investigations reported in Chapter 3 failed to detect evidence of BIV in the cattle that were sampled. Investigations were therefore undertaken to determine the susceptibility of Bali cattle to BIV infection in an effort to better understand the nature of the infection in this species, and these investigations are reported in Chapter 4. Nineteen cattle were experimentally infected with the R29 strain of BIV and monitored for up to 65 days after infection. The presence of virus in plasma and other tissues, the presence of proviral DNA in the PBMC and other tissues, and the immune response to virus antigens was examined and is reported.
The possibility that BIV infection of Bali cattle might modify the effect of subsequent JDV infection was also investigated and these results are presented in Chapter 5. These studies were undertaken to determine what might happen on the island of Sulawesi, where BIV infection is suspected to occur in Bali cattle, if JDV were to spread through the Bali cattle population of that island. If prior BIV infection were to inhibit subsequent JDV infection, it was hypothesised that this might form the basis of a method of vaccination for the control of Jembrana disease.

Due to the presence of cross-reactive epitopes between JDV and BIV proteins, current serological assays are not capable of discriminating between antibody to the 2 bovine lentiviruses. During the studies undertaken and reported in Chapters 3, 4 and 5, the difficulty of distinguishing antibody to BIV and JDV made it difficult to monitor the serological response to the individual virus infections. An attempt was therefore made to develop a peptide antigen capable of differentiating between antibody to the 2 viruses. Overlapping virus peptides were synthesized and used in an enzyme linked immunosorbent assay (ELISA) format with serum samples taken from JDV and BIV infected cattle to determine their reactivity to the peptides. The results are reported in Chapter 6.

A general discussion of the results obtained and reported in the thesis is presented in Chapter 7.
Chapter 2: Literature Review

This review covers the general biological features of the retroviruses and lentiviruses, in particular the lentiviruses that cause disease in animals. Also reviewed are the various immune responses to animal lentivirus infection (including cross-reactive epitopes) and the phenomenon of lentivirus superinfection resistance.

The nomenclature used in this thesis for viral genes and proteins is that suggested by (Fauquet, 2005) where gene names are in lower case and italicised, eg. env, and where the abbreviations for the encoded proteins have the initial letter in uppercase and are not italicised, eg. Env and TM.

Retroviruses

Physiochemical characteristics of retroviruses

The key feature of the family Retroviridae is their mode of replication, involving reverse transcription of the virion RNA into linear double-stranded DNA (Baltimore, 1970; Temin et al., 1970), and the integration of this double-stranded proviral DNA into the genome of the cell. This reverses the normal flow of genetic information from DNA to RNA, hence the name retrovirus (Coffin, 1997).

Retrovirus taxonomy

Many viruses have been classified within the family Retroviridae with a significant proportion of them associated with disease (Coffin, 1997). On the basis of evolutionary relatedness, retroviruses are separated into 7 genera: Lentivirus, Spumavirus, Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus and Epsilonretrovirus (Buchschacher, 2001). The lentiviruses and spumaviruses are distinct from the other 5 genera in that they do not have oncogenic potential. Some of the well known oncogenic retroviruses include Rous sarcoma virus, Human T-
lymphotropic virus (HTLV-1) and Mouse mammary tumour virus. Less well understood and researched are the spumaviruses which cause no known disease, of which *Human spumavirus* is the type species (Coffin, 1997; Goff, 2001). The lentiviruses have been studied extensively since the discovery of *Human immunodeficiency virus* (HIV).

**Lentiviruses**

Most lentivirus infections are characterised by a long asymptomatic period before the onset of chronic clinical disease with a slow but inevitable progression to death (Campbell et al., 1998). Examples of lentiviruses inducing this type of chronic infection include *Visna maedi virus* (VMV), HIV and *Caprine arthritis encephalitis virus* (CAEV). Some, however, induce a rapidly progressive acute disease, including JDV, *Equine infectious anaemia virus* (EIAV) and the *Simian immunodeficiency virus* (SIV) SIV<sub>smm-PBj</sub>. This section will review features of lentiviruses that are common to the majority of members of the genus, including genome structure and organisation, their mode of replication and common clinical features of infection.

**General structure of lentiviruses**

Lentiviruses are roughly spherical in shape with an average virion diameter of approximately 100 nm including the surrounding bilayered lipoprotein envelope (Fauquet, 2005). They are sensitive to heat, detergent and formaldehyde (Goff, 2001). The envelope contains 2 types of surface projections, the surface unit (SU) and transmembrane (TM) glycoproteins (Fauquet, 2005; Wagner, 1999). The internal virion is composed of the distinctively cone-shaped capsid (CA) which surrounds the nucleocapsid (NC) and contains protease (PR), integrase (IN) and reverse transcriptase (RT) enzymes (Figure 2.1). The RNA genome is located within the nucleocapsid (Goff, 2001).
The lentivirus genome

Lentiviruses have 2 identical linear, positive-sense, single-stranded RNA genomes which range in size from 7-10 kb (Goff, 2001; Peterlin, 1995). Reverse transcription takes place from one strand at a time. There are 3 major open reading frames (ORF) in each strand that transcribe and translate 3 polyproteins which are then cleaved by proteases into approximately 8 proteins. The 3 polyproteins are Gag, Pol and Env (Figure 2.2). The gag ORF produces the structural proteins MA, CA and NC. The pol ORF encodes the intravirion enzymes: RT responsible for copying the single-stranded RNA genome into the double-stranded DNA, IN which is required to incorporate the double stranded DNA into the host genome forming the provirus, and PR, required to cleave the encoded polyproteins into smaller proteins (Miller et al., 2000; Tobin et al., 1994). The env gene produces the 2 envelope proteins, SU and TM, which play a vital role in receptor recognition and entry of the virus into the cell (Fauquet, 2005; Wagner, 1999).
In addition to the obligatory \textit{gag}, \textit{pol} and \textit{env} ORF common to every retrovirus, lentiviruses also have a number of accessory genes (Table 2.1), including \textit{rev}, \textit{tat}, \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef} (Fauquet, 2005), which modulate the replication of the virus and probably contribute to clinical latency and pathogenic mechanisms (Clements et al., 1996). Rev plays an essential role in the replication cycle of all lentiviruses as it facilitates the export from the nucleus of unspliced RNAs whose translated products are later utilized in virus replication (Malim et al., 1989). Like Rev, Tat is produced early in the replication cycle and plays a role in the expression of viral transcripts from a promoter within the long terminal repeat (LTR) (Miller et al., 2000). HIV Tat has also been shown to modulate the expression of cellular genes and has numerous other roles in viral replication and pathogenesis (Chen et al., 2000). FIV is the only lentivirus without the \textit{tat} ORF but ORF2 encoded by \textit{orf2} is predicted to have Tat-like activity and to act via cellular transcription factors during the expression of viral transcripts from the promoter within the viral LTR (Chatterji et al., 2002; Miller et al., 2000; Miyazawa et al., 1994) but requires additional elements within the LTR, unlike other lentivirus Tat proteins (Chatterji et al., 2002). It has been proposed that VMV and CAEV lack a \textit{tat} ORF and that the ORF that is designated \textit{tat} in these viruses (named \textit{tat} because of its similar position in the genome to the \textit{tat} ORF in the primate lentiviruses) instead encodes a Vpr-like accessory protein (Villet et al., 2003).
Figure 2.2. Genome organisation of lentiviruses. The location of the structural and accessory genes are indicated, orientated 5’ to 3’. Each virus has 3 major open reading frames, gag, pol and env, which transcribe and translate 3 polyproteins. Image from Craigo et al. (2010).
The vif ORF is transcribed and translated to Vif (viral infectivity factor), thought to aid in the infectivity and spread of virus, although its mechanism of action is still unclear (Clements et al., 1996; Miller et al., 2000). Vif has recently been implicated in protecting virions against the actions of Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) proteins. APOBEC3G and APOBEC3F are cytidine deaminases which are packaged into HIV-1 virions and result in the production of non-infectious virions due to the hypermutation of HIV proviral DNA. Vif protects the virus against lethal incorporation of the APOBEC proteins by marking them for ubiquitin-dependent degradation (Goila-Gaur et al., 2008, Romani et al., 2009).

Vpr encodes Vpr (viral protein R) which, in HIV-1, mediates the nuclear import of viral RT complexes in non-dividing cells and alters the cell cycle and proliferation status of the infected host cell. In HIV-2 and SIVsm, Vpr inhibits cell cycle progression while Vpx (encoded by vpx) is responsible for the nuclear import of the viral RT complex (Fletcher et al., 1996; Stivahtis et al., 1997). The vpu ORF encodes Vpu (viral protein U) that enhances the efficiency of virion production and induces rapid degradation of CD4 (Maldarelli et al., 1993). The nef ORF encodes Nef (negative factor) whose function is to decrease the expression of CD4 on T-cells; it is thought to have this effect by increasing the rate of endocytosis of CD4 on the cell surface which would ultimately prevent re-infection of cells that already harbour the virus (Benson et al., 1993; Goff, 2001; Peterlin, 1995). The accessory ORF s2 is unique to EIAV and its role in virus replication is unclear but it is thought to play a part in replication and virulence of EIAV (Li et al., 1992; Nilsen et al., 1996).
Table 2.1. Accessory genes of each lentivirus, presence indicated by √ (Chakrabarti et al., 1987; Chatterji et al., 2002; Clements et al., 1996; Dewhurst et al., 1990; Freed, 2001; Li et al., 1992; Miller et al., 2000; Nilsen et al., 1996; Stivahtis et al., 1997; Tobin et al., 1994). SIVsm has a gene encoding the Vpx protein, other SIVs lack this gene (Stivahtis et al., 1997). The presence of tat within the VMV and CAEV genomes has been debated (Villet et al., 2003).

<table>
<thead>
<tr>
<th></th>
<th>HIV-1</th>
<th>HIV-2</th>
<th>SIV</th>
<th>FIV</th>
<th>VMV</th>
<th>CAEV</th>
<th>EIAV</th>
<th>BIV</th>
<th>JDV</th>
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**Lentivirus replication**

Infection of a cell by a lentivirus commences at the surface of the host cell (Figure 2.3). The SU interacts with specific receptors on the target cell. For example, HIV-1 SU interacts with the CD4 receptor located on the surface of T-lymphocytes and monocytes/macrophages. The interaction brings about a conformational change in the TM glycoprotein and ultimately leads to the fusion of the viral envelope with the
membrane of the target cell (Clapham et al., 2002; Clements et al., 1996). The virus then moves into the cytoplasm where it is partially uncoated and virion enzymes encoded by pol are released. The virion enzymes RT and RNase H copy the viral RNA within the partially uncoated virion, generating a double-stranded DNA copy of the viral RNA genome, referred to as the provirus. The provirus forms a complex with a number of viral proteins including IN, MA, NC, RT, and possibly others, to form the pre-integration complex, which translocates to the nucleus. Integrase then catalyses the insertion of the linear, double-stranded viral DNA into the host cell chromosome (Freed, 2001). At this point, the proviral DNA may remain integrated and the cell can remain latently infected, or a productive infection may result. It remains unclear as to what causes a cell to become productively infected, although it is thought that the presence of specific transcription factors present in mature cells may stimulate transcription of viral genes. For example, it has been proposed that the binding of the transcription factor NF-κB, which is found in activated T-lymphocytes, to the HIV-1 LTR, is important for transcriptional activation in vitro of HIV-1 in T-cell lines (Clements et al., 1996; Wagner, 1999). Others have proposed that defective HIV-1 particles preferentially activate CD4+ T-cells which render them permissive for HIV replication and help to drive HIV pathogenesis (Finzi et al., 2006).

Once transcription has been activated, expression of viral mRNA begins when RNA polymerase II binds to the U3 region of the 5’ LTR and transcription then proceeds towards the 3’ end of the provirus and into the host DNA. The RNA is cleaved and polyadenylated at the R-U5 border of the 3’ LTR (Figure 2.4), which yields a complete unspliced viral genomic RNA suitable for incorporation into the virion particle. A portion of the RNA produced at this level is then spliced by the cellular
splicing machinery to give rise to one or more subgenomic RNAs. Both the unspliced and spliced RNAs are exported from the nucleus for translation (Goff, 2001).

Once in the cytoplasm, the RNAs transcribed from *gag*, *pol* and *env* are translated by ribosomes into polyproteins (large precursor protein molecules). In HIV, the *gag* ORF encodes a polyprotein precursor of 55 kDa, designated Pr55\textsuperscript{Gag}, that is cleaved by viral PR to produce MA (p17), CA (p24), NC (p7) and p6. The *pol*-encoded enzymes are initially synthesised as part of a large polyprotein precursor, Pr160\textsuperscript{GagPol} that is cleaved by the virus encoded protease into PR, RT and IN. The Env precursor, gp160, is cleaved by cellular protease into SU (gp120) and TM (gp41) (Egberink et al., 1992; Freed, 2001).

Gag and Gag-Pol precursors assemble beneath the plasma membrane and incorporate viral genomic RNA during the process of budding, while SU and TM glycoproteins are also inserted into the viral envelope at this stage. After the virion has been released, it matures when PR cleaves Gag precursors into their functional subunits. The mature virion is then able to infect other cells (Tobin et al., 1994).
Figure 2.3. Schematic illustration of the lentivirus replication cycle depicting the major events in the replication cycle. Image from Ganser-Pornillos et al. (2008).

Figure 2.4. Schematic illustration of the changes to lentivirus long terminal repeats during the change from viral RNA to proviral DNA to viral mRNA (transcript). Illustrations are orientated 5’ to 3’. The positions of the R (repeat), U3 and U5 regions are shown. Viral mRNA is expressed when RNA polymerase II binds to the U3 region of the 5’ LTR. Image from Coffin et al. (1997).
Common characteristics of lentiviruses

Members of the genus *Lentivirus* share a number of features. They all replicate in non-dividing terminally differentiated cells, have the ability to integrate their genomes into the chromosomal DNA of non-dividing infected cells and they are highly species-specific in terms of their ability to cause disease. Common clinical features of infection shared by the majority of lentiviruses include long asymptomatic incubation periods before the onset of a usually chronic disease, persistence of virus infection in the face of vigorous immune responses including neutralising antibody and cytotoxic T-lymphocytes (CTL), multi-organ disease and replication in cells of the immune system and brain (Clements et al., 1996; Mealey et al., 2004; Trautwein, 1992). It is often the immune response of the host against the infected cells that results in the wide range of disease symptoms observed (Gonda, 1992).

The ability of the viruses to persist in the infected host is, at least in part, associated with the capacity of lentiviruses to exhibit a wide array of genetic and antigenic variations, particularly in *env*. Variations are produced in response to biological and immunological selective pressures as the virus (usually) successfully avoids clearance by defence mechanisms (Leroux et al., 2001; Mealey et al., 2004).
Primate lentiviruses

HIV and SIV cause disease in primates and it is the study of these lentiviruses that has resulted in most of our knowledge of the molecular biology and mechanisms of disease associated with lentivirus infections. This section will review HIV and SIV with a particular focus on their genome organisation, cell tropism, clinical features of diseases they induce, and species specificity.

**Human immunodeficiency virus**

Arguably the most studied virus in the world today, HIV was first identified in 1983 as the agent responsible for an acquired immunodeficiency syndrome (AIDS) which led to opportunistic infections and eventually death (Barre-Sinoussi et al., 1983; Gallo et al., 1984). It has assumed very significant pandemic proportions and it is estimated that about 33 million people are infected with the virus (UNAIDS, 2008).

Based on epidemiological and genetic studies, HIV isolates form two clusters, HIV-1 and HIV-2, which are distinguished by variations primarily in env. They also show differences in transmission rate and pathogenicity (Levy, 2009). HIV-1 is the type species of the genus *Lentivirus* and although the published literature describing aspects of the molecular biology of HIV-1 and the associated infection is very large, a brief overview only will be given in this review.

Many lentiviruses evade the host immune system via their genetic variability (Lopez et al., 2006). Phylogenetic analyses of HIV-1 isolates from around the world indicate that more than 10 major groups of distinct genetic subtypes or clades of HIV-1 can be distinguished. Clades are differentiated by genomic differences in env of 15% or greater (Barker, 1995). The distribution of the clades tends to have a geographic basis: Subtype C (clade C) infections are most commonly found in south Africa, India, Ethiopia and east Africa. Subtype A clades are found in eastern Europe,
central Asia, west, east and central Africa while subtype B is present in the Americas, western Europe and east Asia. Subtype D is present in north Africa, the middle east, east and central Africa (Hemelaar et al., 2006).

In HIV the major determinants of cell tropism are the cell surface receptors used to gain entry into the cell. HIV-1 uses the CD4 receptor and a co-receptor to enter the host cell (Sattentau et al., 1988). The main cells that have the 2 receptors needed for HIV-1 to gain entry are the CD4+ T-helper subset of lymphocytes, the CD4+ cells of macrophage lineage and some dendritic cells (Clapham et al., 2002; Freed, 2001).

Major co-receptors for HIV-1 include the 2 chemokine receptors CCR5 (expressed on macrophages) and CXCR4 (expressed on T-cells) but other co-receptors are used (Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996). CCR5-utilising HIV-1 variants dominate the early phases of HIV-1 infection while CXCR4-utilising HIV-1 variants dominate the latter phases of HIV-1 infection, and the switch from CCR5-utilising to CXCR4-utilising is associated with accelerated disease progression (Ito et al., 2003).

HIV-1 will establish infection in both humans and chimpanzees but will only cause disease in humans, with a few rare exceptions (Freed, 2001, Keele et al., 2009).

There are 3 phases to the course of HIV infections. In the initial phase, there is a period of rapid virus replication associated with influenza-like symptoms, commencing about 2 weeks post infection (pi) and lasting for 2-3 weeks. Subsequently, there is a variable asymptomatic period of weeks to years during which lymphadenopathy can develop. In the third phase, the destruction of the T-cell population causes the onset of AIDS, seen in about 30% of infected people within 5-7 years and later in others (Campbell et al., 1998). The loss of T-cells results in the body being unable to overcome opportunistic infections, and the immune system
finally fails, which without anti-viral therapy will ultimately lead to death (Wagner, 1999). The loss of CD4+ T-cells and the changes in HIV RNA levels are shown in Figure 2.5. Key clinical manifestations of HIV-1 infection are immunodeficiency, lymphadenopathy, opportunistic infections, encephalopathy, emaciation, Kaposi's sarcoma and other cancers (Gonda, 1992).

![Figure 2.5. The progression from infection with HIV to death in a victim. The patient was infected at or near week 0. The diagram illustrates the levels of HIV RNA, the gradual decline in CD4+ T-cells and the onset of clinical signs. Image from Pantaleo et al. (1995).](image)

**Simian immunodeficiency viruses**

Simian lentiviruses have been identified in several species of non-human primates by epidemiological studies using serological assays and virus isolation. Each SIV is named with a subscript that denotes the species from which the virus was first isolated, for example those from sooty mangabeys as SIV$_{smm}$, from macaques as SIV$_{mac}$ and from chimpanzees as SIV$_{cpz}$ (VandeWoude et al., 2006). In their natural host to which they have adapted they do not normally cause disease but when they infect species in which they do not normally occur, disease frequently results. These
viruses share many features in common with HIV, particularly nucleotide homology, genome organisation, size (approximately 9 kb in length), receptor usage as well as the clinical manifestations of disease they induce in non-natural hosts (Clements et al., 1996; Sattentau et al., 1988).

All SIV have a tropism for CD4+ macrophages or T-cells. Macrophage-tropic (M-tropic) SIV can efficiently replicate in macrophages, primary CD4+ T-cells and a variety of T-cell lines whereas T-cell-tropic (T-tropic) SIV replicate in primary CD4+ T-cells and in T-cell lines but not macrophages (Edinger et al., 1999). Both M- and T-tropic SIV strains use CCR5 to gain entry into CD4+ cells (Edinger et al., 1997). The locations of viral replication are thought to be responsible for the varying clinical manifestations of SIV infection: replication in cells of the monocyte/macrophage lineage results in disease manifestations in the central nervous system and lungs; replication in lymphocytes results in a loss of CD4+ lymphocytes which in turn results in immunodeficiency and increased susceptibility to opportunistic infections (Sharma et al., 1992).

While the majority of SIV cause disease after a relatively long period of infection, there are strains which cause disease after only a short incubation period, such as SIV$_{smmPBj14}$. This strain causes an acute disease and death within 6 to 10 days after intravenous inoculation into pig-tailed macaques and rapidly replicates to high titres (Fultz et al., 1994, Tao et al., 1995). The lethal nature of this phenotype is possibly associated with insertions within the V1 region of $env$ and within the NF-$\kappa$B enhancer element in the LTR, which enhances transcription and replication kinetics (Tao et al., 1995).
SIV that originate in African primate hosts are thought to be relatively ancient viruses that result in non-pathogenic infections in their native hosts. A similar situation is seen with lentivirus infections of non-domestic cats (Hahn et al., 2000; Terwee et al., 2005).

**Non-primate lentiviruses**

*Feline immunodeficiency virus*

FIV is associated with acquired immunodeficiency in cats and was first isolated in California (Pedersen et al., 1987). Since its initial isolation, similarities in the disease syndrome induced in cats to that of HIV in humans have created considerable interest in the virus as a potential animal model for HIV infection (Dandekar et al., 1992; Dua et al., 1994; Gardner et al., 1989; Olmsted et al., 1989). The virus infects several species of *Felidae*, including the domestic cat *Felis cattus*, throughout the world but particularly in Europe, East Asia, Australasia and North America (Brown et al., 1994; Duarte et al., 2006; Little et al., 2009; Olmsted et al., 1992). Circumstantial evidence suggests that the most efficient mode of virus transmission is horizontally by biting (Yamamoto et al., 1989). Other studies have shown that it can be transmitted through semen and vertically during pregnancy (Jordan et al., 1998; Wasmoen et al., 1992).

FIV has a broader target cell range than HIV and replicates in both CD4+ and CD8+ lymphocytes, macrophages and immunoglobulin G-positive lymphocytes (Beebe et al., 1994; Brown et al., 1991; English et al., 1993). The primary receptor of FIV is CD134, a T-cell-activation antigen and co-stimulatory molecule (Shimojima et al., 2004). Known co-receptors include CXCR4 (Willett et al., 1997). The progression from infection to immunosuppression to death in domestic cats is well characterised and mirrors HIV-1 infection in humans. There is a transient acute stage of the illness
a few weeks pi that is characterised by fever and lymphadenopathy. The next phase is asymptomatic, lasting up to 5 years. The third and final phase of infection is characterised by a gradual decline in CD4+ T-lymphocytes leading to immunodeficiency. This stage, like AIDS in HIV-infected humans, is characterised by the occurrence of opportunistic infections, neurological disorders and tumours of various aetiologies, resulting in death usually within a few months (Dean et al., 1996; Egberink et al., 1992; Ryan et al., 2003; Sauter et al., 2001).

Analogous to SIV\textsubscript{smnP Bj14} inoculation into pig-tailed macaques, FIV-C\textsubscript{PGamma} also causes an acute disease with a high case fatality rate. Mortalities range from 50 to 100% in kittens ≤12 weeks of age after a short incubation period following intravenous inoculation However, in contrast to SIV\textsubscript{smnP Bj14}, acute phase virulence was induced by acute-phase virus passage (Diehl et al., 1995) and the severe and rapidly progressive disease could not be induced in young adult cats (Pedersen et al., 2001).
**FIV in non-domestic feline species**

Serological surveys of a number of non-domestic feline species have revealed at least 17 species with FIV-like virus infection (Brown et al., 1994; Olmsted et al., 1989; VandeWoude et al., 2006; VandeWoude et al., 2002). Isolates from pumas (also referred to as cougar, mountain lion and panther; *Puma concolor*), lions (*Panthera leo*), Pallas cat (*Felis manul*) and bobcats (*Lynx rufus*) have been genetically characterised (Poss et al., 2006; Poss et al., 2008), and these isolates are distinct from each other and are related to, but distinct from FIV of the domestic cat (Olmsted et al., 1992). The standard nomenclature for designation of strains originating from different species is to append genus and species identifiers for the feline species as a subscript to FIV. For example FIV isolated from a lion is referred to as FIV_ple and domestic cat as FIV_fca (Brown et al., 1994; VandeWoude et al., 2006).

The available evidence indicates these viruses have been endemic within cat families for a long period (Biek et al., 2006). Although the clinical effects of FIV from non-domestic cat populations have not been well studied, it appears that infections due to these viruses do not cause widespread disease (Biek, 2006; Brown et al., 1994; VandeWoude et al., 2002; VandeWoude et al., 2003). The asymptomatic nature of infection is most probably because of a long evolutionary association between virus and host (Biek, 2006). Signs of neurologic disease have been reported in captive lions (Brennan et al., 2006) but this may be attributed to the animals outliving their “normal” life span. FIV_pco from pumas is able to establish a productive infection in domestic cats but does not cause T-cell dysregulation (unlike FIV_fca) or clinical signs and is able to be cleared from PBMC rapidly (Terwee et al., 2005; VandeWoude et al., 2003). These cats also generate humoral and cell-mediated immune responses
reactive against both FIV and non-domestic cat isolates of FIV (VandeWoude et al., 2003).

**Equine infectious anaemia virus**

EIAV, the cause of a chronic relapsing or intermittent anaemia in horses, was the first lentivirus to be identified and the first non-plant virus to be discovered (Leroux et al., 2004; Ligné, 1843; Vallée, 1904). The virus causes a persistent infection in horses and closely related equids, such as donkeys and mules (O'Rourke et al., 1988; Spyrou et al., 2003). It is transmitted mechanically mainly by blood-feeding biting arthropods such as tabanids, or iatrogenically on contaminated needles, but contact infection can also occur (Hawkins et al., 1976; Kemen et al., 1978; Li et al., 2003; Williams et al., 1981). Because of its transmission by arthropods, the infection is most common in geographic areas with long vector seasons (Issel et al., 1988).

EIAV is an exclusively tissue macrophage-tropic lentivirus which utilises the equine lentivirus receptor-1 to gain entry into macrophages (Sellon et al., 1992; Zhang et al., 2005). The virus causes a relapsing disease characterised by periods of depression, fever, diarrhoea, anaemia, thrombocytopenia and haemorrhaging due to severe depletion of platelets, which are associated with a high level viraemia (Hammond et al., 1997; Harrold et al., 2000; O'Rourke et al., 1988; Oaks et al., 1998). The disease cycles begin approximately 1-3 weeks pi, last 3-5 days and occur at irregular intervals of weeks to months. The periodic disease cycles occur for 8-12 months pi, after which infected horses normally progress to a subclinical infection lasting for the life of the infected animal; these persistently infected animals serve as a source of infection for other animals (Hammond et al., 1997; Issel et al., 1982; Montelaro et al., 1984; Oaks et al., 1998; Payne et al., 1998; Salinovich et al., 1986). Between the clinical episodes of disease, viral loads are greatly reduced (of the order of 4- to 733-
fold) and viral transcription within macrophages is restricted (Oaks et al., 1998). In response to immune pressure, rapid genomic variations occur during replication which results in altered glycoprotein structures and antigenic changes. Variants have been detected within 28 days and this variation is responsible for the cyclical nature of the disease, new antigenic variants having a replication advantage as they are not susceptible to pre-existing immunity (Ball et al., 1992; Montelaro et al., 1984; Payne et al., 1984; Salinovich et al., 1986).

**Small ruminant lentiviruses**

It was initially thought that VMV and CAEV were specific for sheep and goats, respectively, but recent evidence has shown that VMV and CAEV are capable of infecting both sheep and goats as well as some small ruminant species living in the wild (Guiguen et al., 2000; Leroux et al., 1997; Narayan et al., 1980; Pisoni et al., 2005; Ravazzolo et al., 2006; Shah et al., 2004). As a result, VMV and CAEV are now collectively referred to as small ruminant lentiviruses (SRLV). The 2 viruses share many similarities at both the genomic and antigenic level, particularly in *gag* and *pol* (Brinkhof et al., 2007; Jolly et al., 1989; Pasick, 1998; Pyper et al., 1986; Zanoni, 1998), and there is antigenic cross-reactivity between their CA proteins (Clements et al., 1980). Some SRLV, however, show significant differences in their LTR, *env* and regulatory genes (Jolly et al., 1989; Pyper et al., 1986; Zanoni, 1998). They also differ phenotypically, with the prototypic Icelandic VMV strains inducing syncytia and lysis of infected cell cultures whereas the prototypic CAEV-Cork strain induces syncytia with a persistent but non-lytic infection of cell cultures (Narayan et al., 1980; Pisoni et al., 2007; Querat et al., 1984).
Visna maedi virus

VMV is a natural pathogen of sheep (Zhang et al., 2000; Zink et al., 1987) and has been isolated from the majority of sheep-rearing areas of the world, excluding Australia and New Zealand (Dawson, 1988). VMV was initially reported as a cause of progressive pneumonia of sheep in South Africa in 1915, then in Montana in the 1920's, then in sheep in Iceland in the 1950’s (Dawson, 1988; Jolly et al., 1989; Sigurdsson et al., 1952; Sigurdsson et al., 1957). The virus name is derived from the Icelandic language where “maedi” can be translated as “dyspnoea” (difficult breathing due to pneumonia) and “visna” as “fading away” (due to a demyelinating leukoencephalomyelitis), representing the 2 forms of the disease (Pepin et al., 1998).

The main route in which the virus is transmitted is thought to be via ingestion of infected colostrum and/or milk or via the respiratory tract, involving direct inhalation of infected respiratory secretions, including the inhalation of infected alveolar macrophages (McNeilly et al., 2008; Pepin et al., 1998; Preziuso et al., 2004). Monocytes, lung tissue macrophages and spleen tissue macrophages (but not T-lymphocytes) are the major cell targets in vivo for VMV replication and viral expression is greatly increased when the monocytes mature into macrophages (Gendelman et al., 1986). In PBMC populations, dendritic cells are the most permissive for viral replication (Gorrell et al., 1992; Zhang et al., 2000).

Clinical disease can take months to years to develop (Nilsen et al., 1996) and consists of a multi-system disease characterised by a chronic infiltration and proliferation of mononuclear cells in the lungs (chronic interstitial pneumonia, maedi) and the central nervous system (affected by a chronic and progressive, paralytic disease characterised by inflammatory and demyelinating lesions in the CNS leading to wasting and paralysis, visna) (Narayan et al., 1980). Organs less commonly affected...
include the joints (arthritis) and mammary glands (Bird et al., 1993; Deng et al., 1986; Gorrell et al., 1992; Zink et al., 1987).

**Caprine arthritis encephalitis virus**

CAEV causes an economically significant disease in infected goats, particularly in Europe, the Americas, Asia and Australia (Campbell et al., 1998; Herrmann et al., 2003b; Mselli-Lakhal et al., 2007; Zanoni, 1998). The main route of virus transmission is via colostrum but horizontal spread is also achieved via infected secretions if there is direct contact between infected and susceptible animals (Ravazzolo et al., 2006) or from doe to foetus either prior to or during the birth process (East, 1993).

The virus, like VMV, infects cells of the monocyte/macrophage lineage and dendritic cells and virus expression is activated during maturation of monocytes to macrophages (Narayan et al., 1983). It does not infect lymphocytes and so does not directly result in immunosuppression, unlike SIV and HIV. Infection results in a chronic inflammatory disease affecting the joints, central nervous system, lungs and mammary glands, and depending on the organs affected, can result in emaciation, respiratory distress, mastitis, paralysis or arthritis (Dawson, 1988; Herrmann et al., 2003b; Narayan et al., 1983) (Ravazzolo et al., 2006). Up to 40% of infected goats develop chronic arthritis (Cheevers et al., 1997).

**Bovine lentiviruses**

Currently there are 2 lentiviruses known to infect cattle, BIV and JDV. The first bovine lentivirus discovered was BIV (Tobin et al., 1994; Van der Maaten et al., 1972) while JDV has more recently been identified as a lentivirus (Chadwick et al., 1995b).
While the clinical and pathological syndromes associated with the 2 bovine lentiviruses are markedly different, the viruses share a very close genetic and antigenic relationship (Chadwick et al., 1995b). These are shown in Table 2.2.

Table 2.2. Comparison of putative protein products of JDV$_{Tab87}$ and BIV127.

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</table>

$^a$Alternate forms of tat and rev generated by utilisation of alternative splice donor site. Data sourced from Chadwick et al. (1995b).
**Bovine immunodeficiency virus**

BIV is a naturally-occurring lentivirus found in cattle and based on serological surveys, infections occur worldwide (Campbell et al., 1998; McNab et al., 1994; Meas et al., 2000a; Suarez et al., 1995). The virus may be transmitted horizontally by iatrogenic routes, blood-sucking arthropods, via natural or artificial insemination or via milk from infected cows (Egberink et al., 1992; Meas et al., 1998; Meas et al., 2000a). BIV is best known as a virus infection of *B. taurus* but New Zealand white rabbits (*Oryctolagus cuniculus*) and sheep have been infected experimentally, resulting in a persistent infection (Gonda, 1992; Jacobs et al., 1994; Pifat et al., 1992).

**Genome structure**

The first isolation of BIV was from a cow in Louisiana and this isolate, designated R29 (Van der Maaten et al., 1972), has since been cloned (Braun et al., 1988) and sequenced (Garvey et al., 1990). BIV-R29 is antigenically and genetically stable during long-term, persistent infection (Carpenter et al., 2000).

The BIV proviral genome is 8.96 kb in size and consists of the 3 main ORF *gag*, *pol* and *env* and 6 accessory genes *rev*, *tat*, *vif*, *tmx*, *vpw* and *vpy* (Figure 2.2). The functions of *vif*, *tmx*, *vpw* and *vpy* in BIV are yet to be described (Egberink et al., 1992; Gonda et al., 1987; Miller et al., 2000; Tobin et al., 1994). The BIV Gag polyprotein that is translated is processed into a number of proteins, the 3 major proteins found in all lentiviruses (MA, CA and NC) and 3 smaller proteins (p2L, p3 and p2) (Tobin et al., 1994).
**Cell tropism and clinical signs associated with disease**

Two studies have indicated that BIV may be pantropic. An initial *in vivo* study with BIV isolate FL112 found proviral DNA in CD3+, CD4+ and CD8+ cells, B cells, monocytes and WC1 cells in both the acute and chronic stages of infection (Whetstone et al., 1997). A subsequent study involving the R29 isolate detected proviral DNA and infectious virus within CD2+ (located on CD4+ and CD8+ T-cells), WC1+ (located on γδ T-cells), mature B cells and monocytes during the early stages of infection (Heaton et al., 1998). However, it is not clear from either study whether these cell types were productively infected, as only proviral DNA was detected. BIV-R29 is able to productively infect primary cultures of embryonic bovine spleen and lung, Madin-Darby bovine kidney cells and embryonic rabbit epidermal cells producing syncytia, cell lysis and infectious virus (Ferens et al., 2007; Gonda, 1992; Heaton et al., 1998; Pifat et al., 1992; Suarez et al., 1993; Whetstone et al., 1990; Zhang et al., 1997a; Zhang et al., 1997b).

BIV is most efficiently transmitted via infected blood and cell-free and cell-associated tissue culture-derived virus (Pifat et al., 1992). The re-use of contaminated needles during vaccination and blood collection, communal sharing of colostrum fed to calves and the failure to cleanse contaminated instruments, have also been suggested to play a role in the spread of BIV (Gonda, 1992). Inoculation usually results in a subclinical infection without significant clinical effects (Flaming et al., 1993) but clinical signs have been reported and include a transient leucocytosis, lymphoid hyperplasia, lymph node enlargement, wasting and in some cases immune suppression (Carpenter et al., 1992; Egberink et al., 1992; Zhang et al., 1997b). The impact of BIV infection to the overall health of herds has not been established (Tobin et al., 1994), although there is one Canadian study which suggested BIV infection
had a significant effect on milk yield (McNab et al., 1994). Other studies have suggested that persistent BIV infection plays a role in reducing functional immune competence as shown by the frequent development of concurrent infections in BIV-infected animals (Meas et al., 2000a; Snider et al., 2003a; Zhang et al., 1997b). It would seem, however, that a direct role for BIV in chronic progressive disease or as a cofactor in a specific disease is unlikely (Jacobs et al., 1994; Suarez et al., 1995).

**Jembrana disease virus**

In contrast to BIV, JDV causes a significant acute disease process in Bali cattle (*Bos javanicus*) in Indonesia (Dharma, 1997; Kertayadnya, 1997). The first reported outbreak of Jembrana disease occurred in Sangkargung, a village in the Jembrana district of the island of Bali in Indonesia (Figure 2.6) in December 1964. The disease only affected Bali cattle and while reference is sometimes made to an effect on buffalo (*Bubalus bubalis*) this was anecdotal and has not been confirmed. The disease spread through all districts of Bali by August 1965 with an estimated 20,000 to 70,000 deaths. The disease then disappeared but subsequent smaller outbreaks were detected in 1972 in the Tabanan district and in 1981 in the Karangasem district of Bali (Ramachandran, 1996; Soeharsono, 1997b).
Figure 2.6. (A) The location of Bali in relation to other Indonesian islands (D.F.A.T., 2008). Districts of Bali province, adapted from Streetdirectory.com (2009). Jembrana disease was first reported in the district of Jembrana. Antibodies to JDV have since been detected in Sumatra, Java and Kalimantan. Clinical disease and virus have also been detected in Sumatra and Kalimantan.
Early investigators proposed *Rinderpest virus* and then a rickettsia as probable causes of Jembrana disease (Soeharsono, 1997b). A viral aetiology was subsequently demonstrated, a conclusion based on filtration studies that suggested the infectious agent in blood was too small for a rickettsia (Ramachandran, 1996). Based on its estimated size of 50-100 nm, the probable presence of a lipid-containing envelope, electron microscopic observations and reverse transcriptase activity, the agent was then considered as a probable member of the family *Retroviridae* (Kertayadnya, 1997; Wilcox et al., 1992) and was subsequently identified, based on genome structure and genomic nucleotide sequence analysis, as a lentivirus (Chadwick et al., 1995b).

**Significance of Jembrana disease**

The case fatality rate resulting from experimentally induced Jembrana disease is approximately 21% (Desport et al., 2009a). During the original outbreak commencing in 1964, it was retrospectively estimated that 60% of the Bali cattle and buffalo population on Bali island were affected in the following 12 months, of which 98.9% died (Ramachandran, 1996); this high prevalence and high case fatality rate is unlikely and the evidence is obscure as at the time there were no veterinary services on the island. The effect of JDV is widespread throughout Bali, as a consequence of the important role that Bali cattle play in society. They are a source of employment and help to sustain and generate profit in the agricultural system via the provision of draught power and manure which in turn helps to improve soil fertility (Wiryosuhanto, 1997).

**Transmission and distribution of Jembrana disease**

The probable modes of transmission of JDV include mechanical and contact transmission. Virus can be mechanically transferred from infected cattle to
uninfected cattle by iatrogenic means or by arthropods. Examples of iatrogenic routes include multi-use needles during vaccination programs, as has been shown to occur in EIAV (Li et al., 2003). Arthropod transmission is thought to be limited to the acute viraemic episode when there is a high titre of virus in blood of affected cattle, but it requires reasonably close contact between animals and the restriction of movement of infected animals by quarantine has helped to reduce the spread of the virus. The likelihood of contact transmission is supported by the detection of the virus in secretions such as saliva, urine and milk and the ability to reproduce the disease by conjunctival and oral inoculation of the virus (Hartaningsih et al., 1993; Soeharsono et al., 1995b).

The disease has now been detected in 4 Indonesian islands: Bali, Kalimantan (Indonesian Borneo), West Sumatra and East Java (Figure 2.6). There are no reports of JDV infection of Bali cattle in any other area of Indonesia and clinical Jembrana disease has not been reported in other cattle types which has led to the belief that the disease is specific to Bali cattle (Hartaningsih et al., 1993; Wilcox et al., 1995). However, other cattle types and buffalo can be infected experimentally and become infected under field conditions (Soeharsono et al., 1995a) although they do not seem to develop clinical disease. *Bos taurus, B. indicus*, crossbred Bali cattle (*B. javanicus* x *B. indicus*) and buffalo can be infected and develop viraemia (Soeharsono et al., 1990; Soeharsono et al., 1995a). The clinical changes and lesions that occur in these cattle types are consistent with those observed in Bali cattle, but they are much milder and would be difficult to detect under field conditions.
The JDV genome

The genome of JDV is 7732 bp in length and is the smallest of all lentivirus genomes. It contains the 3 major ORF typical of all retroviruses, *gag*, *pol* and *env*, as well as accessory genes, *vif*, *tat*, *rev* and *tmx* (Figure 2.2) (Chadwick et al., 1995b; Chen et al., 1999; Nilsen et al., 1996).

JDV is a unique lentivirus in that it is genetically stable, and there is a high level of nucleotide conservation in *gag*, *pol* and *env* sequences taken from isolates throughout Indonesia. *Gag* sequences in isolates from Bali and Sumatra had 97 to 100%, nucleotide identity. *Env* sequences were also unexpectedly conserved with 96-99% nucleotide identity, and 95 to 99% amino acid identity. The largest divergence was seen in an isolate from South Kalimantan with only 88% identity to that of the original JDV_{Ttab87} isolate (Desport et al., 2007).

This high level of nucleotide conservation is similar to the LTR regions of EIAV that have been reported to be highly conserved during persistent infection of horses (Maury et al., 2005; Reis et al., 2003). It is in contrast to other lentiviruses such as HIV-1 (Balfe et al., 1990; Lamers et al., 1993; Wolfs et al., 1990), FIV (Brown et al., 1994; Duarte et al., 2006) and CAEV (Valas et al., 2000) which exhibit greater levels of genetic variation, particularly in envelope glycoprotein regions, in sequences obtained from within one individual (Lamers et al., 1993; Simmonds et al., 1990), between individuals and between different geographic locations (Balfe et al., 1990; Maki et al., 1992).
**Cell tropism and clinical signs associated with Jembrana disease**

It has been suggested that during the early stages of JDV infection, initial rounds of virus replication occur in lymphoid tissue before a rapid and widespread dissemination of virus to other tissues during the second phase of replication (Chadwick et al., 1995a). JDV has a tropism for mature IgG-containing cells (Desport et al., 2009a) and JDV-infected cells are found within spleen, lymphoid tissues, bone marrow, lung, liver and kidney, as shown by *in situ* hybridisation techniques for the detection of JDV genomic RNA (Chadwick et al., 1998).

JDV is not a typical lentivirus in that it causes an acute disease syndrome after a short incubation period (Chadwick et al., 1995a). The incubation period before the onset of clinical signs is 4-12 days and the clinical signs continue for 5-12 days (Soeharsono, 1997a). Major clinical signs include a febrile response, lethargy, anorexia and enlargement of superficial lymph nodes. Less frequently observed clinical signs include erosions of oral mucous membranes, hypersalivation, nasal discharge, diarrhoea and blood in the faeces. JDV has also been implicated in the suppression of the humoral immune response in infected cattle, perhaps not surprising as the histopathological changes reflect a disease primarily affecting the lymphoid system (Dharma et al., 1994; Wareing et al., 1999). The major haematological changes associated with JDV infection include leucopenia, lymphopenia, eosinopenia and a slight neutropenia. In addition to these changes, a mild thrombocytopenia, a normocytic normochromic anaemia, elevated blood urea concentrations and reduced total plasma protein are also observed during the acute disease (Soesanto et al., 1990).
Immune response to infection with a lentivirus

The immune response to lentiviruses frequently takes longer to develop than in many other virus types. For example, ponies infected with EIAV do not seroconvert to envelope glycoproteins and CA until 3 weeks pi, with Env antibodies being predominant. However, it requires 6-8 months for the EIAV humoral immune response to fully mature into a high avidity, conformational epitope-specific response (Hammond et al., 1997). The EIAV-specific CTL activity develops 3-4 weeks pi and this correlates with the resolution of the primary viraemia (Hammond et al., 1997). Studies involving the depletion of CD8+ lymphocytes in rhesus monkeys subsequently infected with SIVmac support the role of these cells in controlling viraemia (Schmitz et al., 1999) as do other studies of natural HIV-1 infections in humans (Koup et al., 1994). Neutralising antibody is detected 2-3 months pi (Hammond et al., 1997). In sheep infected with VMV, the seroconversion to CA also occurs about 3 weeks pi (Singh et al., 2006) although serum neutralising antibodies are not detectable until 1-3 months pi (Petursson et al., 1976), slightly earlier than in EIAV. The time course of the immune response to HIV infection is summarised in Figure 2.7 and its relationship to the development of other features of the virus infection are shown in Figure 2.8.
Also of importance in limiting infection with lentiviruses are non-adaptive immune responses. Studies of HIV and FIV have shown that natural killer cells have an important role in controlling acute infection (Howard et al., 2010) while cytidine deaminases such as the previously mentioned APOBEC family of proteins restrict viruses shortly after they have entered the cell by interfering with viral DNA formation (Goila-Gaur et al., 2008, Romani et al., 2009). Other important innate immune responses include TRIM5α which is thought to inhibit HIV-1 transduction in rhesus macaque cells, non-coding RNAs such as micro RNAs and silence inducing RNAs (Strebel et al., 2009), anti-viral cytokines such as interferon and dendritic cells (Williams et al., 2009).
Figure 2.7. Estimated time course for host immune response to acute HIV infection. Image from Levy (2007).

Figure 2.8. Estimated time course for maturation of the host immune response in EIAV-infected animals. Image from Leroux et al. (2004). Env-specific cytotoxic T-cells appear within the first month after infection as do Env- and p26 (CA)-specific IgG. Neutralising antibodies appear after 2-3 months after infection.
Humoral immune response to infection with bovine lentiviruses

A number of assays have been used to detect and quantify the immune response to the bovine lentiviruses, most commonly Western immunoblotting (WIB) and ELISA. Antigen can be prepared using cell culture systems (Burki et al., 1992; Hammond et al., 1997; O'Rourke et al., 1988; Whetstone et al., 1991; Whetter et al., 1990), sucrose-gradient purified plasma from infected animals (Hartaningsih et al., 1994) or recombinant protein production (Bird et al., 1993; Burkala et al., 1999; Burkala et al., 1998). Other techniques have been used to monitor the immune response to bovine lentivirus infections, including immunofluorescent assays (O'Rourke et al., 1988), agar gel immunodiffusion (AGID) (Burki et al., 1992; Coggins et al., 1972; Hartaningsih et al., 1994; Whetter et al., 1990) and radioimmunoprecipitation (Beyer et al., 2001; Morin et al., 2003; O'Rourke et al., 1988). Western immunoblot assays were more sensitive than AGID tests for detection of antibody to BIV (Whetstone et al., 1991) and for JDV an ELISA was also more sensitive than AGID (Hartaningsih et al., 1994).

Experimental infection of Bali cattle with JDV results in the production of antibodies detectable by ELISA and WIB to the CA protein 6-11 weeks pi, although occasionally animals can seroconvert as early as 2 weeks pi (Desport et al., 2009a; Hartaningsih, 1997). The antibody response to JDV peaks 23-33 weeks pi and persists beyond 59 weeks (Hartaningsih et al., 1994). As JDV has not been cultured in vitro, there have been limited investigations of the neutralising antibody response to JDV. There is only a single report available of a neutralising antibody response to JDV, which relied on the infection of cattle to determine virus neutralisation. The evidence from this study suggested that neutralising antibody to JDV developed only after a prolonged period following recovery, and therefore that neutralising antibody
did not seem essential in the recovery from acute Jembrana disease. Antibodies capable of neutralising JDV were detected in cattle 4 and 28 months after cattle had recovered from clinical disease and titres were low, in the range of 1:2 to 1:20 (Hartaningsih et al., 2001).

In experimental BIV infections, antibody develops earlier than in JDV infections. Antibody to the CA protein can be detected as early as 14 days post infection (dpi) with titres peaking 6-8 weeks pi. Multiple studies have confirmed the early CA antibody response and determined that it persists for up to 2.5 years after initial infection (Heaton et al., 1998; Suarez et al., 1995; Whetstone et al., 1990; Whetstone et al., 1991) although there is a single report that found the CA response to BIV infection decreased dramatically by 40 weeks pi (Isaacson et al., 1995). Antibodies capable of neutralising BIV have been detected as early as 17 weeks pi and persist for 44 months pi (Carpenter et al., 2000). Antibodies against BIV TM could be detected 4 weeks pi, peaking 10-30 weeks pi and persisted for at least 50 weeks pi (Scobie et al., 1999); in some cattle they were detected 4 years pi (Isaacson et al., 1995). The antibody response to the SU protein of BIV was reported to take many months to develop (Suarez et al., 1995).

The CA and TM proteins of BIV and JDV contain cross-reactive epitopes which has made serological differentiation between infections with JDV and BIV impossible (Desport et al., 2005; Kertayadnya et al., 1993; Lu et al., 2002). Attempts have been made to develop reagents capable of differentiating between these infections but these have been unsuccessful (Desport et al., 2005).
Assays for the detection of lentivirus infections in ruminants

Small ruminant lentiviruses

Serological methods such as ELISA (using whole virus, recombinant proteins or synthetic peptides as antigens), competitive ELISA (using monoclonal antibodies), AGID, radio-immunoprecipitation and WIB are most commonly performed to detect SRLV infection and there are a number of kits available commercially (Brinkhof et al., 2007; Brodie et al., 1993; de Andres et al., 2005; Herrmann et al., 2003a; Pasick, 1998). It is important to note, however, that no “gold standard” method of diagnosis exists (Brinkhof et al., 2007; de Andres et al., 2005).

Of a number of assays recently evaluated by Brinkhof and van Maanen (2007) for the serodiagnosis of SRLV infections in sheep and goats, the best performing assay was an ELISA. This assay involved the sensitisation of the solid phase with a combination of recombinant VMV CA protein produced in E. coli and a TM derived peptide. The performance of this assay may be due to the simultaneous detection of antibodies against CA and TM. Antibodies to CA are produced early after infection but decline once clinical signs appear and TM antibodies are produced later but persist into the clinical phase. Therefore any assay which combines the detection of antibodies to these 2 proteins could be expected to cover a greater proportion of the infection period (Boshoff et al., 1997; Brinkhof et al., 2007).

A number of supplementary tests are often performed to confirm or resolve indeterminate ELISA results, including AGID, WIB and radioimmunoprecipitation, and these can be supplemented by PCR assays for the detection of proviral DNA or viral RNA in PBMC or plasma (Barlough et al., 1994; Brinkhof et al., 2007; Brodie et al., 1993; de Andres et al., 2005; Wagter et al., 1998; Zanoni et al., 1992).
Large ruminant lentiviruses

Diagnosis of BIV infection plays a crucial role in controlling the spread of infection and the pathogen-free preparation of vaccines prepared using cattle, for example tick fever vaccines (Lew et al., 2004). Assays used for detecting BIV infection include qPCR (Lew et al., 2004), nested PCR, cell culture syncytium assays, WIB assays (Meas et al., 1998; Meas et al., 2000a; Snider et al., 2003b; Suarez et al., 1995; Zhang et al., 1997a), and ELISA using recombinant CA and TM protein antigens (Barboni et al., 2001; Burkala et al., 1999). Techniques to quantify proviral and viral BIV loads in tissues have not been described.

All attempts to culture JDV in vitro have failed and it has not been possible to detect JDV infections using virus isolation techniques (Kempster et al., 2002; Wilcox et al., 1992). Two techniques have been described that enable the detection and quantification of JDV virus load, the JDV gag quantitative Reverse Transcriptase-PCR (qRT-PCR) assay and a JDV p26 antigen capture ELISA (Stewart et al., 2005). The qRT-PCR technique was optimised with plasma samples taken from animals experimentally infected with JDV, and it was found to be robust and sensitive, with an apparent sensitivity 100-fold greater than that of a standard RT-PCR. The capture ELISA was relatively insensitive when compared to the qRT-PCR, although it provided an economical method for monitoring of virus in the absence of more sensitive techniques (Stewart et al., 2005).
Difficulties with serological testing

A number of factors have been reported to be critical in the interpretation of serological assays. In lentivirus infections, one problem encountered is the delayed nature of the antibody response. For example, in donkeys experimentally infected with EIAV, the animals respond weakly and produce antibodies 42 dpi while horses produce antibodies which can be detected as early as 16 dpi (Spyrou et al., 2003). Hence, testing donkeys less than 42 dpi will yield a false-negative result. Problems with delayed antibody responses have also occurred in CAEV (Brinkhof et al., 2007; Rimstad et al., 1993) and JDV infections (Desport et al., 2009a; Hartaningsih et al., 1994). Another problem in lentivirus infections is that antibody titres to viral proteins may fluctuate between positive and negative after infection, though reasons for this are unclear (de Andres et al., 2005).

Problems can occur in the detection of neutralising antibody in non-primate lentivirus infections (Pozzetto et al., 1986; Sahu et al., 1994). Some North American strains of VMV do not appear to induce neutralising antibodies (Zink et al., 1987). In other VMV infections, the time required to develop detectable levels of neutralising antibody after infection may vary considerably, from 12-14 dpi (Bird et al., 1993) through to 1-3 months (Petursson et al., 1976). The titre of neutralising antibody reported in response to lentivirus infections has also varied considerably in different systems: in response to CAEV in goats it was reported to be of low titre and low affinity (Kennedy-Stoskopf et al., 1986; Pisoni et al., 2007) as was the response of horses to EIAV infection (O'Rourke et al., 1988) but the titre of neutralising antibody in sheep in response to VMV infection was reported to be as high as 1:640 (Narayan et al., 1978).
Another important factor which should be considered in lentivirus serological tests is cross-reactive epitopes on viral proteins, which can make serological differentiation of antigenically related viruses difficult. Antigenic cross-reactivity between structural proteins of lentiviruses, particularly the CA protein, is well known (Cheevers et al., 1988; Gnann et al., 1987a). Cross-reactivity has been reported between the CA protein of JDV and BIV (Burkala et al., 1998; Desport et al., 2005; Kertayadnya et al., 1993), between the TM glycoprotein of BIV and JDV (Burkala et al., 1998) and the SU glycoprotein of CAEV and VMV (Gogolewski et al., 1985; Valas et al., 2000), among others.

**Techniques to identify epitopes and distinguish between viral infections**

Peptide mapping is a useful technique to identify specific epitopes that differ between 2 viruses that cross-react antigenically (Valas et al., 2000; Van Regenmortel, 1999b). The reactivity to peptides produced in this manner provides information on the antigenic characteristics of similar viruses infecting an animal, which permits differentiation between the virus infections (Gnann et al., 1987a; Mordasini et al., 2006; Pisoni et al., 2007). Peptide mapping involves the generation of a panel of overlapping peptides which cover the entire amino acid sequence of the protein of interest. These peptides are chemically synthesised and used in immune binding assays, usually in an ELISA format for high throughput, against a panel of reference sera taken from natural and experimental infections (Ball et al., 1992; Bertoni et al., 1994). Synthetic peptides have been used, for example, to identify epitopes that differentiate HIV-1 and HIV-2 infections (Gnann et al., 1987a; Gnann et al., 1987b).

Another technique to identify unique epitopes is phage display (Xiao et al., 2008). This technique is made possible by the expression of peptide libraries on the surface
of filamentous phage particles. The first step in the process involves the insertion of one random oligonucleotide into one phage. The peptide encoded by the oligonucleotide is then expressed within the pIII or pVIII coat protein of the filamentous phage fd or M13 (D'Mello et al., 1999). These random phage display libraries are subsequently screened for reactive epitopes with an antibody of interest. The peptide sequence can be determined after amplifying the DNA from the selected phage by PCR and sequencing techniques (D'Mello et al., 1999; Van Regenmortel, 1999a; Westwood, 2000; Williams, 2000).

Another technique suitable for defining epitopes is the use of protein expression libraries. To construct expression libraries, DNA from a coding sequence of interest is digested with DNase I to generate random DNA fragments with an approximate average of 200 bp in length. These fragments, which can encode peptides, are ligated into vector DNA (for example λgt11) whilst preserving the reading frame of a fusion protein such as β-galactosidase. Ligations are packaged into Escherichia coli and the cloned fragments are expressed as fusion proteins from the recombinant phages. The random libraries are subsequently screened for reactive epitopes with an antibody of interest. The peptide sequence can be determined after amplifying the DNA from the selected phage by PCR and then sequencing this product (Bertoni et al., 2000; Bertoni et al., 1994; Pancino et al., 1993; Van Regenmortel, 1999a).

One traditional method of defining epitopes is the limited fragmentation of proteins by chemical cleavage, using cyanogen bromide or enzymatic digestion. Fragmentation is then followed by immunoblotting of protein fragments (Westwood, 2000). Other methods that have been used include crystallographic analysis of antigen-antibody complexes and the binding of anti-peptide antibodies to either natural or chemically modified proteins (Van Regenmortel, 1999a).
**Lentivirus superinfection**

Dual infection of an animal with 2 viruses can be the result of coinfection or superinfection. Coinfection occurs when 2 viruses infect at or near the same time prior to seroconversion. Superinfection is the infection of an animal (or a cell) by 2 genetically distinct viruses where the infection with one virus precedes infection with the second virus, sometime after seroconversion (Blackard et al., 2002; Gottlieb et al., 2004; Jurriaans et al., 2008).

A natural case of superinfection of goats with CAEV and VMV has recently been documented (Pisoni et al., 2007). Several studies have reported superinfection of HIV-1 infected individuals (Gottlieb et al., 2004; Jurriaans et al., 2008; Piantadosi et al., 2007; Smith et al., 2006). Between 2002 and 2005, 16 cases of HIV-1 superinfection in humans were reported (Smith et al., 2005). Although this number seems small, natural cases of superinfection have generated considerable interest as they challenge the assumption that HIV-1 specific immune responses generated against primary infection are protective against subsequent infection by different strains of the same virus (Allen et al., 2003).

**Benefits of superinfection - superinfection resistance**

Experimental studies have shown that pre-infecting an animal with a relatively less pathogenic virus protects against challenge with a second, more pathogenic lentivirus, a situation referred to as superinfection resistance (Table 2.3). This has been demonstrated in FIV (Terwee et al., 2008; VandeWoude et al., 2002), SIV (Cranage et al., 1998; Nilsson et al., 1998; Stebbings et al., 2004) and heterologous SHIV infections (Sealy et al., 2009).

Domestic species of cats that have been asymptptomatically infected with non-pathogenic lion lentivirus (FIV$_{ple}$) or puma lentivirus (FIV$_{pco}$) have shown resistance
to subsequent challenge with pathogenic FIV (Brown et al., 1994). All cats became infected with the pathogenic FIV but prior exposure to FIV_{ple} or FIV_{pco} ameliorated the normal clinical effects of FIV infection: CD4+ cell depletion was reduced and, in some cases, plasma and PBMC FIV loads were reduced (Terwee et al., 2008; VandeWoude et al., 2002).

Infection with live attenuated SIV_{macC8} of cynomolgus macaques ameliorated the effects of subsequent infection with pathogenic wild-type SIV_{macJ5} (Stebbings et al., 2004), its derivative SIV_{mac220} (Cranage et al., 1998) and SIV_{sm} (Nilsson et al., 1998). This was shown by a significant decrease of cell-associated virus and plasma viral RNA loads (Cranage et al., 1998; Stebbings et al., 2004) and negative virus isolation in a proportion of animals (Nilsson et al., 1998). Indian rhesus macaques infected with SIV_{macC8} also resisted superinfection with the virulence-reverted form of SIV_{macC8} (Sharpe et al., 1997). Likewise, Indian rhesus macaques infected with SIV_{macGX2} were completely protected against challenge with SIV_{mac220} (Sharpe et al., 2004) and macaques infected with SIV_{mac239Δnef} were protected against challenge with SIV_{mac251} (Connor et al., 1998; Daniel et al., 1992).

Superinfection resistance holds promise as a way to ameliorate the effects of lentivirus infection and disease, in effect to act as a potential vaccine. It has been proposed that if the mechanism of protection conferred could be better understood, then a safe and effective HIV vaccine, for example, could be developed (Cranage et al., 1998; Stebbings et al., 2004). The effect of challenge with a non-pathogenic virus on the course of infection with a pathogenic virus also offers an opportunity to examine host-virus and virus-virus interactions and their effect on pathogenicity and resistance to virulent lentivirus infections (VandeWoude et al., 2003).
Mechanisms of superinfection resistance

The mechanisms responsible for protection against superinfection are not well defined. Protection has not been directly correlated with humoral or cellular immune responses including virus neutralising activity (Connor et al., 1998; Cranage et al., 1998; Daniel et al., 1992; Nilsson et al., 1998; Sharpe et al., 1997; Stebbings et al., 2004; Stebbings et al., 2002; VandeWoude et al., 2002) and in some cases protection is not dependent on challenge-driven expansion of immunodominant epitope-specific CD8+ T-cells (Sharpe et al., 2004). While CD8+ T-cells are important for control of primary viraemia, they do not seem to play a central role in protection against superinfection (Stebbings et al., 2005). Non-immunological phenomena such as virus interference or antiviral factors such as CD8 suppression factors induced by defective particles have been suggested as playing a role in superinfection resistance (Cranage et al., 1998; Stebbings et al., 2004; Stebbings et al., 2002). Virus interference has been detected in vitro when cell cultures were infected with a retrovirus and were relatively resistant to infection by a related retrovirus; the phenomenon occurs only when both viruses share the same receptor and results from a restricted penetration into the cell (Corbin et al., 1993).

Adverse consequences of superinfection

While the positive effects of superinfection, namely resistance, have been well described, superinfection or mixed infection of animals with 2 viruses has resulted in potentially adverse effects. Goats infected naturally with both CAEV and VMV were shown to contain chimeric viruses with CAEV-VMV envelope glycoproteins; this was expected to have dramatic effects on the species specificity of the viruses and their capacity to cross species barriers (Pisoni et al., 2007).
Table 2.3. Experimental superinfection studies conducted in a range of lentivirus animal model systems.

<table>
<thead>
<tr>
<th>Model system</th>
<th>Primary virus infection</th>
<th>Secondary pathogenic virus strain</th>
<th>Time between primary and secondary infections (weeks)</th>
<th>Effect on pathogenic virus</th>
<th>Reference</th>
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<tbody>
<tr>
<td>FIV in domestic cat</td>
<td>FIV&lt;sub&gt;pco&lt;/sub&gt;</td>
<td>FIV-C</td>
<td>4</td>
<td>Reduced CD4+ T-cell depletion.</td>
<td>(Terwee et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>FIV&lt;sub&gt;pco&lt;/sub&gt; or FIV&lt;sub&gt;ple&lt;/sub&gt;</td>
<td>FIV-B</td>
<td>27</td>
<td>Reduced CD4+ T-cell depletion, reduced plasma and PBMC FIV load.</td>
<td>(VandeWoude et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>FIV&lt;sub&gt;Petaluma&lt;/sub&gt;</td>
<td>FIV-M2</td>
<td>28</td>
<td>Reduced total viral load, reduced CD4+ T-cell depletion.</td>
<td>(Pistello et al., 1999)</td>
</tr>
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<td>SIV in rhesus macaques</td>
<td>SIV&lt;sub&gt;mac251&lt;/sub&gt; or SIV&lt;sub&gt;smE660&lt;/sub&gt;</td>
<td>Reciprocal infection</td>
<td></td>
<td>Reduction in peak viraemia, amelioration of infection.</td>
<td>(Yeh et al., 2009)</td>
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<td></td>
<td>SIV&lt;sub&gt;macGX2 (nef-disrupted)&lt;/sub&gt;</td>
<td>SIV&lt;sub&gt;mac220&lt;/sub&gt;</td>
<td>89 or 122</td>
<td>Complete resistance (determined by negative virus isolation).</td>
<td>(Sharpe et al., 2004)</td>
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<td>SIV in cynomolgus macaques</td>
<td>SIV&lt;sub&gt;macC8&lt;/sub&gt;</td>
<td>SIV&lt;sub&gt;mac32H/L28&lt;/sub&gt;</td>
<td>3 or 20</td>
<td>Reduction of viral RNA and DNA load.</td>
<td>(Berry et al., 2008)</td>
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<td>Attenuated SIV&lt;sub&gt;macC8&lt;/sub&gt;</td>
<td>SIV&lt;sub&gt;macJ5&lt;/sub&gt;</td>
<td>3</td>
<td>Reduced cell-associated virus loads, reduced plasma virus load.</td>
<td>(Stebbings et al., 2004)</td>
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</table>
Chapter 3: Attempts to detect *Bovine immunodeficiency virus* infection in Bali cattle in Indonesia with a PCR-based assay

Summary

Attempts were made to provide evidence for the occurrence of BIV in cattle in Indonesia. One hundred and seventy one genomic DNA and serum samples were taken from Bali cattle in the Bangli and Tabanan regions on the island of Bali. Genomic DNA samples extracted from PBMC were screened for the presence of BIV or JDV proviral DNA using both real time and conventional PCR methods and direct sequencing of any amplified products to confirm their identity. Serum samples were screened for antibodies against JDV using a range of antigens in a WIB or ELISA format and 21 of the 171 animals were identified as being seropositive by a positive WIB reaction with the p26 CA protein of JDV and at least one other positive serological test. BIV proviral DNA was not detected in any of the cattle but JDV proviral DNA was detected in 12 animals, only one of which was seropositive.
Introduction

Two bovine lentiviruses are suspected to circulate in the Bali cattle population of Indonesia. The presence of *Jembrana disease virus* (JDV) is well documented and both the disease and antibodies to the virus have been detected in cattle on the islands of Bali, Sumatra and Java (Hartaningsih et al., 1993). The disease also now occurs in all Kalimantan provinces in Indonesian Borneo (Hartaningsih, personal communication). Despite the widespread distribution of Bali cattle in the eastern islands of Indonesia, clinical Jembrana disease has not been reported in these areas. However, there are reports of JDV antibody-positive cattle in some of the regions of Indonesia that are free of clinical Jembrana disease, including on the island of Sulawesi (Desport et al., 2005), suggesting the presence of a second non-pathogenic virus that is antigenically related to JDV, possibly BIV. Serological evidence was also presented for the presence of a BIV-like virus in Bali cattle in Bali where JDV is endemic (Barboni et al., 2001) although this has not been confirmed by virus isolation.

The objectives of the investigations reported in this thesis were to attempt confirmation of the presence of non-pathogenic BIV-like viruses in Bali cattle in Bali, to develop methodology for the detection and differentiation of infection by these viruses and JDV, perhaps enabling the determination of the distribution of each virus in Indonesia, and to investigate the interaction between the 2 viruses in infected Bali cattle. Ideally, these investigations required the isolation of the non-pathogenic BIV-like virus that is reputedly present in Indonesia, that would then enable its experimental inoculation into animals not only to determine the effects of these viruses in Bali cattle but also for the production of reagents. Use of the local non-pathogenic virus would eliminate the need to import an
exotic strain of BIV for these investigations. Unsuccessful attempts were made previously to isolate a non-pathogenic bovine lentivirus from cattle in Sulawesi: blood samples were obtained from antibody-positive Bali cattle in Sulawesi, the samples were transported by air to Bali and inoculated into Bali cattle on arrival. The inoculated cattle did not seroconvert to BIV or JDV, suggesting the virus was not present in the inoculum. Further attempts to detect the Sulawesi virus were abandoned due to political unrest in the area from where cattle had been sampled and the expense involved (Hartaningsih, personal communication).

This Chapter describes the results of attempts to confirm the report of Barboni et al. (2001) that Bali cattle on the island of Bali were infected with BIV, and from which attempts to isolate the virus could be made.

**Materials and methods**

*Field samples*

Peripheral blood samples were taken from 171 Bali cattle (*Bos javanicus*) in 2 districts (Bangli and Tabanan) on the island of Bali, Indonesia. They were collected from the jugular vein, into sterile 10 ml vacutainer tubes containing 15% EDTA (BD) for extraction of genomic DNA, and into plain tubes for preparation of serum samples for serological tests.

*Preparation of PBMC genomic DNA*

After collection into vacutainer tubes containing EDTA, the Bangli samples were centrifuged (900 g for 10 min at 4°C) and the buffy coat was subsequently transferred into 10 ml tubes containing 6 ml distilled H₂O, then mixed by inverting 3 times. Three ml of 2X PBS was then added and mixed by inverting 3 times after which tubes were
centrifuged again (250 g for 10 min at 4°C). The resulting supernatant was discarded and
the pellet washed twice with 10 ml PBS before the pellet was gently resuspended in 1 ml
Hank’s medium supplemented with 20% v/v heat inactivated foetal calf serum and 6% v/v
DMSO (Sigma) and stored in 200 µl aliquots at -20°C until required. Genomic DNA was
extracted from PBMC using the FlexiGene DNA Kit (Qiagen) according to the
manufacturer’s instructions and stored at -20°C until used. The concentration of PBMC
genomic DNA in each sample was determined using a spectrophotometer (Nanodrop ND-
1000).

After collection into vacutainer tubes, the Tabanan blood samples were centrifuged (900 g
for 10 min at 4°C) and the buffy coat was collected and mixed with 2 ml of PBS, then
overlayed onto 6 ml Ficoll (Amersham) in a sterile 10 ml tube and centrifuged at 400 g for
20 min at 4°C. The cells at the interphase were collected and washed 3 times in PBS by
mixing with PBS followed by centrifugation (400 g for 20 min at 4°C). The washed cells
were then resuspended with 1 ml PBS and stored at -20°C until required. Genomic DNA
was extracted from the purified PBMC using the QIAamp DNA Mini Kit (Qiagen)
according to the manufacturer’s instructions and the DNA was stored at -20°C until used.
The concentration of PBMC genomic DNA in the samples was determined using a
spectrophotometer.

**Verification of DNA quality**

Extracted DNA samples were initially tested by PCR using gene-specific glyceraldehyde
3-phosphate dehydrogenase (GAPDH) primers to test for integrity of the DNA. If no PCR
product was obtained with these primers then the sample was discarded. The GAPDH
primers used were as described previously (Mohan et al., 2001) and the reaction consisted
of 200 ng of PBMC genomic DNA, 1X PCR buffer, 1.25 mM MgCl₂, 0.2 mM of each dNTP, 0.8 mM of each primer (GAPDH F, CCTTCATTGACCTTCACTACATGGTCTA, GAPDH R, GCTGTAGCCAAAATTCTATTGTGTTACCA; Invitrogen), 0.687 U Taq polymerase, and ultrapure water to a final volume of 25 µl. All reagents were sourced from Fisher Biotec unless otherwise stated. Thermal cycling conditions were 1 cycle of 94°C for 3 min, 35 cycles at 94 °C for 30 s, 60°C for 30 s and 72°C for 45 s, a final extension step of 72°C for 7 min and they were then held at 14°C in a Bio-Rad thermal cycler.

**qPCR for detection of JDV proviral DNA**

The JDV proviral DNA genome copy number was quantified using a DNA plasmid-based standard curve derived using JDV plasmid clone #139 as a template as previously described (Stewart et al., 2005). The JDV sequence within the pT7T3 vector spanned JDV nucleotides 19 to 2881 (U21603). The qPCR assay specifically amplified 118 bp in JDV gag. All reactions consisted of 1X iQ Supermix (100 mM KCl, 40 mM Tris-HCl (pH 8.4), 1.6 mM dNTPs, 50 U/ml of iTaq DNA polymerase, 6 mM MgCl₂, undefined stabilisers, Bio-Rad), 0.6 mM of each primer (gag1f-GGGAGACCCGTCAGATGTGGA, gag1r-TGGGAAGCATGGACACCTTCA; Invitrogen) prepared as described previously (Stewart et al., 2005), 0.1 µM fluorogenic probe (Geneworks), 200 ng of extracted PBMC genomic DNA and made up to a final volume of 10 µl using ultrapure water (Bio-Rad). Thermal cycling conditions were the same as previously described (Stewart et al., 2005), except that the reverse transcriptase step was omitted.
Conventional PCR for detection of JDV proviral DNA

A number of DNA samples which were tested by qPCR but provided results below the cut-off for a positive result but where the results were clearly greater than that for the JDV-negative control DNA, were re-analysed using conventional PCR. The primer pair for detecting JDV proviral DNA by conventional PCR (JDV 1: GCAGCGAGGGCAATTTTGATAGGA, JDV 3: CGGCGTGGTGCCACCCCATG) were located within the gag open reading frame and specifically amplify a 360 bp fragment (Desport et al., 2007). Reactions conditions consisted of 1X buffer, 1 mM MgCl₂, 0.2 mM of each dNTP, 0.88 mM of each primer (Invitrogen), 1.374 U Taq polymerase, 400 ng PBMC genomic DNA and made up to a final volume of 50 µl with ultrapure water. Unless otherwise stated, all reagents were from Fisher Biotec. Thermal cycling conditions were the same as those described (above) for the GAPDH primer pair. Reaction conditions in the second round of amplification, where necessary, were the same as the first except 1 µl of first round PCR product was added into 25 µl reaction volumes.

Sequence analysis of PCR products

Direct DNA sequencing was performed to confirm and compare the proviral DNA detected in the 2 cattle populations. If one band was produced by conventional PCR amplification, the PCR product was purified using a PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. If multiple bands were produced, the PCR product of the correct size was excised from the agarose gel and purified using a Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions. Ten ng of purified product was sequenced using 1 µl of Big Dye Terminator (Applied Biosystems), 1.5 µl of
Big Dye Terminator sequencing buffer (Applied Biosystems), 3.2 pmol of JDV 1 or
JDV 3 primer and made up to a volume of 10 µl using ultrapure water (Fisher Biotec).
The sequencing reaction consisted of a 2 min hold at 96°C and 25 cycles of 96°C for 10 s,
60°C for 30 s and 60°C for 4 mins. The sequencing reaction was purified by ethanol
precipitation according to the protocol supplied by Applied Biosystems. Samples were
then sequenced using an ABI 3730 48 capillary machine at the State Agricultural
Biotechnology Centre, Murdoch University.

Sequences were edited using Chromas Lite and aligned using the ClustalW program
(http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis was performed
using Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/index.cgi) and phylogenetic
trees were edited using MEGA (http://www.megasoftware.net/index.html).

*qPCR for BIV proviral DNA*

All animals were tested for the presence of BIV proviral DNA using a qPCR assay as
described previously (Lew et al., 2004), with the following modifications: 1X iQ
Supermix (Bio-Rad), 100 ng of each primer (BIVF1-
ACAAAAACTACGGGAATACCCTACA, BIVR1-
TCTTTTAGATCTCTGTGGGCTCTTTC; Invitrogen), 0.1 mM of probe (6FAM
CCACAATCCGAGGAGT; Applied Biosystems) and 200 ng of PBMC genomic DNA.
The reaction volume was made up to 10 µl using ultrapure water (Fisher Biotec). The
qPCR assay specifically amplified 73 bp in BIV pol. The limit of quantification of the
assay was 20 copies per reaction (determined by amplification of specific known
quantities of cloned viral DNA).
Serological tests

Serum samples were tested for the presence of antibodies to JDV using ELISA or WIB with a range of antigens. The assays were conducted by Dr J. Lewis (this laboratory) using antigens that included a recombinant JDV p26-his, recombinant fused JDV p26/TM-his, JDV TM peptide and plasma-derived JDV native antigen (kindly provided by N. Hartaningsih). The WIB assay that utilised native JDV antigen derived from the plasma of infected cattle was conducted as described previously by Hartaningsih et al. (1994); results were recorded as positive if there was reactivity with the 26 kDa CA protein. All other ELISA and WIB assays were conducted and interpreted as described previously (Lewis, 2009).

The recombinant JDV p26-his construct expressed full-length JDV\textsubscript{Tab87} CA (Barboni et al., 2001). The construct was kindly supplied by Dr. Margaret Collins, transformed into BL21 (DE3) \textit{E. coli} for protein expression and was purified using Ni-NTA agarose resin in chromatography columns. The fused p26/TM construct was generated in a previously described manner (de Andres et al., 2005; Rosati et al., 2004) whereby JDV capsid was fused directly to the putative TM principle immunodominant domain (PID) epitope, transformed into \textit{E. coli} BL21 and purified as described for the recombinant JDV p26-his. The JDV TM peptide ELISA was prepared as previously described (Ditcham et al., 2009) and encompassed the PID of JDV TM (Barboni et al., 2001).

As there was no “gold standard” test for JDV antibody detection, samples were considered “positive” when a positive result was obtained in WIB using the plasma-derived JDV antigen and at least one other assay was also positive. Antibody to JDV and BIV cannot be differentiated due to the presence of numerous cross-reactive epitopes on the CA.
(Desport et al., 2005; Kertayadnya et al., 1993) and TM proteins (Burkala et al., 1998). Therefore, the use of these antigens in WIB assays will equally detect antibody to both JDV and BIV.

Results

Serology

Twenty one of the 171 cattle from which DNA was also analysed were seropositive for antibody to the p26 CA of JDV by WIB utilising native JDV antigen and at least one other assay (Table 3.1).

PCR

The GAPDH gene was detected by PCR in PBMC genomic DNA samples from 171 cattle and these were deemed to have DNA of sufficient quality to test using qPCR or conventional PCR. These 171 samples were then screened for the presence of JDV proviral DNA by PCR and JDV-specific PCR products were detected in 12 of the 171 PBMC genomic DNA samples tested (Table 3.1). The sequences had between 99 and 100% homology with the reference JDV_{Tab87} strain. The sequences generated are shown in Figure 3.1.

With the exception of one animal (Tabanan Y26), all cattle in which JDV proviral DNA was detected were seronegative (Table 3.1). In the 11 seronegative and PCR positive cattle, 10 (83%) were negative in every serological assay and one (Bangli 35) was positive in one of the 5 serological assays.

BIV proviral DNA was not detected in any of the 171 PBMC genomic DNA samples tested.
Table 3.1. Results of PCR diagnostic assays and serological assays (ELISA and WIB) in 171 samples taken from Bali cattle (*B. javanicus*) in the Bangli and Tabanan region of Bali, Indonesia. The results shown are those from 52 cattle where any assay provided a positive result, and where samples provided negative results in all assays the results are not shown. Italics indicate the sample was positive by both JDV PCR and serology.

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<th>JDV PCR</th>
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<th>CA WIB</th>
<th>Fused CA/TM ELISA</th>
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<td>Fused CA/TM ELISA</td>
<td>Fused CA/TM WIB</td>
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\(^a\) Age data not available for Bangli cattle.

\(^b\) Positive status given where a positive result was obtained in the qPCR or conventional PCR assay and confirmed by direct sequencing.

\(^c\) Positive status given where a positive result was obtained with the JDV plasma-derived antigen WIB and at least one other serological assay.
Figure 3.1. Sequence alignment of *gag* nucleotide sequences from 12 animals with JDV taken from Bangli and Tabanan. Reference sequences JDV Tab87 (accession number U21603), JDV Pul01 (accession number DQ229295) and BIV127 (accession number NC_001413.1) are included for comparison. Sequences were aligned using ClustalW2. "***" indicates all nucleotides are identical in that column.
Discussion

Although JDV proviral DNA was detected in the PBMC of 12 of 171 cattle examined, which included cattle from 2 adjacent districts of Bali, BIV proviral DNA was not detected in any of the animals tested. The result does not provide proof of the absence of BIV in the Bali cattle population of Bali but it also does not provide any support for the serological evidence reported by Barboni et al. (2001) that cattle within Bali are infected not only by JDV but with a second antigenically related but presumably non-pathogenic bovine lentivirus. The methodology used by Barboni et al. (2001) involved the production of recombinant BIV CA and JDV CA and their use in a WIB format or a combination of the recombinant CA with a BIV or JDV TM peptide in an ELISA format. Sera were screened initially using the JDV antigens, then JDV antibody negative sera were screened using the BIV antigens; if the serum reacted in the second set of assays the sample was declared BIV seropositive and JDV seronegative. Given the high level of cross-reactivity between JDV and BIV CA and TM antigens (Desport et al., 2005; Kertayadnya et al., 1993), including the cross-reactivity of the antigens used in the study, these findings should be interpreted with considerable caution. Results from this laboratory (Desport et al., 2005) have shown that the reagents used by Barboni et al. (2001) would not differentiate between JDV and BIV antibody.

The attempt to identify cattle infected with BIV by the detection of proviral DNA was unsuccessful but not surprising. It is difficult to detect BIV in naturally infected cattle and with the exception of the reports describing 3 successful attempts in the USA and Japan (Suarez et al., 1993; Van der Maaten et al., 1972) and one from Japan (Meas et al., 1998), this has not been reported, although there have been limited reports of the detection of
proviral BIV DNA in naturally infected cattle (Lew et al., 2004; Meas et al., 1998; Meas et al., 2000b; Snider et al., 2003b). In the current study, both antibody-positive and antibody-negative cattle were examined, and 2 specific PCR assays were used. Either both assays lacked the sensitivity to detect the level of provirus present in cattle, or maybe BIV-like viruses are present in Bali but were not present in the populations sampled, or possibly BIV-like viruses do not occur in the cattle population of Bali. Modifications to the PCR assays, such as degenerate primers and less stringent PCR reaction conditions, may have assisted in the detection of proviral DNA. However, based on previous experience in this laboratory, alterations such as these have a tendency to produce false positive results. Further investigation of the pathogenesis of BIV infection in Bali cattle is needed to examine the kinetics of virus replication and persistence after infection and the optimal time for sampling.

JDV proviral DNA was detected in 12 animals sampled, suggesting recent infection, even though there was no evidence of clinical Jembrana disease immediately preceding and following the sampling. This is the first reported detection of JDV in clinically normal cattle. The lack of variation between the PCR amplicons suggests a common virus strain was circulating in the cattle, although the lack of variation is also consistent with previous studies reporting a high level of nucleotide conservation in gag sequences of JDV detected in Bali (Desport et al., 2007) and they may not have arisen from a single source. All the 21 JDV-seropositive cattle were 4 years or older indicating minimal transmission of JDV between the cattle in the preceding 4 years, and this is consistent with the lack of reports of Jembrana disease outbreaks in Bali during this period (Hartaningsih, personal communication).
Provisional DNA positive-seronegative animals are rare but not uncommon after natural lentivirus infections, and have been reported in cattle infected with BIV (Meas et al., 2000a), cats infected with FIV (Dandekar et al., 1992) and sheep infected with VMV (Leginoagaiko et al., 2009). These observations, and the detection of JDV provirus in 12 cattle, only one of which was seropositive, highlights the difficulty associated with conclusively detecting natural lentivirus infections. There are a number of possible reasons for the lack of concordance between serological and PCR assays. Firstly, the antibody response to JDV is delayed and has been reported to be detectable only from 11 weeks post infection (Hartaningsih et al., 1994), hence serological assays would only detect antibodies in the period after this time and this is a period of active virus replication (Stewart et al., 2005). The detection of JDV proviral DNA in seronegative animals suggests recent infection with JDV, prior to seroconversion. Secondly, there are a proportion of animals infected with JDV that do not mount an antibody response to the CA antigen (Desport et al., 2009a; Ditcham et al., 2009). These animals are referred to as atypical responders and account for 15% of all animals experimentally infected but it seems unlikely that this would account for the lack of antibody in 11 of the 12 PCR-positive animals detected. Thirdly, JDV proviral DNA levels in the PBMC are hypothesised to persist at very low levels in naturally infected cattle, similar to SRLV infections (de Andres et al., 2005). JDV proviral DNA has been readily detectable in experimentally infected cattle, even many months after infection but is normally detected only with difficulty in naturally infected cattle (Desport, personal communication).

Further studies are required to characterise the response of Bali cattle to inoculation with BIV, including whether the animals can become actively infected and whether the virus
will persist in the animals over time. It will also be of interest to determine if and at what level BIV proviral DNA and viral RNA loads occur. The development of reagents that are able to distinguish between BIV and JDV infections in an ELISA will help in the identification of BIV infections and in Indonesia may clarify discrepancies between genomic and antibody based assay results.
Chapter 4: *Bovine immunodeficiency virus* produces a transient viraemic phase soon after infection in *Bos javanicus*

**Summary**

Infection of Bali cattle in Indonesia with a non-pathogenic bovine lentivirus similar to BIV is suspected but efforts to detect the virus have been unsuccessful. To define the kinetics of BIV infection and seroconversion in Bali cattle and determine the optimal time for sampling for detection of virus in infected cattle, 13 cattle were infected with the R29 strain of BIV and monitored for up to 65 days. No clinical signs were observed in the infected cattle following infection. Proviral DNA was detected in PBMC from 4-60 days with peak titres 20 dpi. There was a transient viraemia from 4 to 14 dpi with a maximum titre of $1 \times 10^4$ genome copies/ml plasma. An antibody response to the TM glycoprotein commenced 12 dpi but an antibody response to the CA protein was detected in one animal only and not until 34 dpi. The results indicated that detection of BIV in infected Bali cattle is similar to *B. taurus* with levels of proviral DNA detectable during the early stage of infection. Based on these results, a CA based serological assay would not identify the majority of infected cattle.
Introduction

Bali cattle are particularly susceptible to JDV and develop an acute disease process soon after infection. The acute disease is characterised by a transient febrile response, enlargement of superficial lymph nodes, high virus titres in the plasma and a number of haematological changes including leucopenia and thrombocytopenia. The case fatality rate is about 21% and recovered animals are resistant to re-challenge with the virus (Desport et al., 2009a; Soeharsono et al., 1990; Soesanto et al., 1990). In contrast, breeds such as *B. taurus* (Fresian cattle) and *B. indicus* (Ongole cattle) develop a mild febrile response but no other clinical signs of disease (Soeharsono et al., 1990).

The effects of BIV in Bali cattle are unknown but the pathogenesis of BIV in *B. taurus* has been investigated by several groups. Many of the studies that were undertaken assumed that the virus would produce effects long after infection, akin to many other lentiviruses, and they therefore followed the infections for long time intervals. In experimentally infected *B. taurus*, the original R29 isolate caused no major clinical signs in the period up to 27 months after infection (Flaming et al., 1993; Zhang et al., 1997b) although subclinical changes in experimentally infected cattle were reported by others. Subclinical changes reported have included lymphocytosis and follicular hyperplasia (Carpenter et al., 1992) and immune suppression at 3-7 weeks post-infection (Zhang et al., 1997b). Other isolates of BIV (FL491 and FL112) were reported to cause a transient increase in PBMC (Suarez et al., 1993). The FL112 isolate caused a transient B-cell lymphocytosis that peaked 14 dpi (Whetstone et al., 1997) and is also reported to cause lymphadenopathy and non-suppurative meningoencephalitis 12 months post-infection (Munro et al., 1998). Serological studies have also reported no major associations with significant clinical
changes but one study based in North America found associations between BIV and decreased milk yield in dairy cattle (McNab et al., 1994) while another reported marked weight loss with frequent and severe concurrent infections (Snider et al., 2003b).

This Chapter reports the experimental infection of Bali cattle with BIV that was conducted with 2 objectives. First, to determine if Bali cattle were more susceptible to infection with BIV than *B. taurus*, similar to their greater susceptibility to JDV than other cattle species (Soeharsono et al., 1995a). Second, to determine the kinetics of virus replication and persistence of BIV and the development of the antibody response after infection, so as to provide insights into the optimal periods in which to sample naturally infected cattle to detect the virus. The experiments were conducted with the R29 strain of BIV as attempts to detect BIV in Indonesian cattle were not successful (Chapter 3).

**Materials and methods**

**Animals**

Nineteen cattle were obtained from Nusa Penida, an island adjacent to Bali where Jembrana disease has never been detected and the cattle have been consistently negative for antibody to JDV and BIV. The cattle were housed indoors as previously described (Soeharsono et al., 1990). Six weeks prior to infection with BIV the cattle were vaccinated against *Bovine viral diarrhoea virus* using Pestigard (Pfizer) twice at 0 and 4 weeks apart as the FBL cell culture was contaminated with BVDV. BVDV is a common contaminate of BIV cell cultures and foetal bovine serum (Makoschey et al., 2003).
**Virus**

BIV-R29 was obtained from J Brownlie and M Collins, Royal Veterinary College, UK, passaged in primary bovine foetal lung (BFL) cell cultures grown in RPMI medium (Invitrogen) supplemented with 10% foetal bovine serum (Thermo Scientific) and antibiotics in 75 cm\(^2\) flasks (Nunc). For inoculation into cattle, infected cells exhibiting marked syncytium formation were scraped from the surface of flasks 24 h after infection and the cells suspended in RPMI medium. The titre of the virus was retrospectively estimated by titration in BFL cell cultures and the total inoculum administered to each animal determined to be \(1.38 \times 10^6\) 50% tissue culture infectious doses (TCID\(_{50}\)).

**Experimental infection and sampling of cattle**

The infectious inoculum was divided into 2 equal amounts, one administered intravenously and the other subcutaneously. Seven virus infected cattle (CB169-CB175) were monitored for 42 days after infection and 6 (CB177-CB182) for 65 days after infection. An additional 6 cattle (CB183-CB189) were inoculated with an equivalent volume of uninfected BFL cells in RPMI medium and monitored for 65 days as controls.

Animals were observed daily for the development of clinical signs of disease. Rectal temperatures were measured daily for the duration of the study. Blood samples were obtained as required by venipuncture of the jugular vein and used for determination of total leukocyte counts, extraction of DNA from PBMC, extraction of RNA from plasma and serum for serological tests.

The 6 cattle monitored for 65 days were killed and a complete post-mortem examination conducted. Tissue samples (retropharyngeal and prescapular lymph
nodes, spleen, bone marrow, kidney, lung and thymus) were collected into RNAlater (Ambion) for DNA and RNA extraction.

**Extraction of DNA and RNA from tissues**

Total DNA was extracted and purified from tissue samples using a DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instructions.

Total RNA was extracted and purified from tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Disruption and homogenisation was performed using the TissueLyser (Qiagen) and an optional on-column DNase digestion step using an RNase-Free DNase Set (Qiagen) was included to remove any contaminating genomic DNA.

A Ficoll-Paque™ Plus (GE Healthcare) gradient was used to purify PBMC from heparinised blood according to the manufacturer’s directions. Genomic DNA was subsequently extracted from the cells using a QIAamp DNA Mini Kit (Qiagen).

Viral RNA was extracted from plasma using the QIAamp Viral RNA Extraction Kit (Qiagen) as recommended by the manufacturer.

**Quantitation of BIV proviral DNA**

Extracted DNA samples were initially tested by PCR using gene-specific (GAPDH) primers to test for sample integrity. If no product was obtained then the sample was not used in the analysis. The GAPDH primers used were as described in Chapter 3.

Proviral DNA loads were determined with GAPDH-positive samples by a qPCR assay as described in Chapter 3.

BIV Proviral DNA loads were normalised to GAPDH copy number according to previously published methods (Terwee et al., 2008). Briefly, the number of cell equivalents (CE) in 200 ng of PBMC genomic DNA or 200 ng of tissue DNA was
determined by qPCR using GAPDH-specific primers. All reactions consisted of 1X iQ SYBR® Green Supermix (Bio-Rad), 400 µM of each primer (GAPDH156F: GGTGATGCTGGTGCTGAGTA, GAPDH342R: GGCACTGCTGACAATTTGA; Invitrogen), 200 ng of extracted DNA and were made up to a final volume of 10 µl using ultrapure water (Fisher Biotec). Thermal cycling conditions were 1 cycle of 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s and a final extension step of 72°C for 10 min. The DNA-based plasmid standard was generated using the Mohan et al. (2001) GAPDH primers. The BIV proviral DNA copy number generated using the protocol from Lew et al. (2004) was then expressed as the number of BIV proviral DNA copies/100 000 cell equivalents (CE). The number of CE was determined using the GAPDH qPCR protocol.

When DNA yields from PBMC were less than 50 ng/ul and insufficient for qPCR, samples were tested by conventional PCR using primers (Heaton BIV F, 5’ CCCCAGGTCCCCATCAACATCC and Heaton BIV R, 5’ GTCTTCCCCCATCCGTAACATCTCC) as previously described (Heaton et al., 1998).

Quantification of BIV RNA
Viral RNA loads were determined using qRT-PCR with primers and probes as described for the qPCR for provirus except that 2 µl of viral RNA, 0.2 µl iScript Reverse Transcriptase for One-Step RT-PCR (Bio-Rad) and 1X iScript RT-PCR reaction mix for probes was included per reaction and a 10 min reverse transcription incubation step at 50°C was included at the start of the reaction. To determine viral loads within the tissues, the above protocol was followed except 200 ng of tissue RNA was included in each reaction instead of 2 µl of viral RNA. Results were expressed in BIV viral RNA genome copies/µg of total RNA.
Enzyme linked immunosorbent assays

The BIV TM antibody response was determined by ELISA with 1:25 dilutions of sera and a peptide antigen as previously described (Barboni et al., 2001; Ditcham et al., 2009), except that the 36 amino acid TM peptide RVSYLEYVEEIRKQVFFGCKPHGRYCHFDGFPEEV (Proteomics International) of BIV-R29 was converted from linear to cyclic form (TM<sup>c</sup>) as described previously (Scobie et al., 1999). BIV hyperimmune serum was included as a positive control and serum from uninfected cattle was used as a negative control in every plate.

The CA antibody response was determined by ELISA using a recombinant protein antigen. To produce the CA protein, full length BIV CA protein was cloned into the pTrcHisB vector and transformed into JM109 chemically competent E. coli. Bacteria were grown overnight at 37°C at 225 rpm in a 50 ml culture of standard 2X YT medium with ampicillin (50 µg/ml, Sigma). Forty ml of overnight culture was added to 1 L of 2X YT medium plus ampicillin and grown at 37°C for 4 h at 225 rpm. Protein production was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (Sigma) for 4 h. The protein was purified as recommended in the QIAExpressionist Handbook (Qiagen) and an ELISA protocol was optimised.

One hundred ng of protein was coated onto each well of a Maxisorp 96-well plate (Nunc) and incubated overnight at 4°C. Plates were then washed 3 times with PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.05% Tween 20). One hundred µl of serum diluted 1:100 in PBS-T with 5% w/v skim milk (Fonterra) was added to each well for 1 h at 37°C. Plates were then washed 3 times with PBS-T and 100 µl of a 1:2 000 dilution of anti-bovine IgG-HRP (ICN) in PBS-T-skim milk was added to each well for 1 h at 37°C after which they were washed 3 times with
PBS-T and once with PBS. Colour development was induced by the addition of peroxidase substrate (Bio-Rad) and the reaction stopped by the addition of 2% w/v oxalic acid and absorbance read at 405 nm. BIV hyperimmune serum was included in every plate as a positive control and serum from uninfected cattle was used as a negative control.

Results

Clinical observations

The 13 Bali cattle infected with BIV-R29 and the 6 BFL-only controls did not develop any clinical signs of disease during the observation period. Rectal temperatures and total leukocyte counts remained normal throughout the experiment and no gross lesions were observed during post-mortem examination of the 7 cattle killed 65 days after infection.

Quantification of BIV proviral DNA load

Proviral DNA was detected in PBMC of all cattle inoculated with virus during the 65 days after infection. Proviral DNA was first detected 8 dpi in 4 of the 13 animals (Table 1), it was detected in all 13 cattle 20 dpi but in only 2/13 at 40 dpi and 3/6 at 60 dpi. Maximum proviral DNA titres were 6.2 x10³ proviral genome copies/100 000 CE.

Quantification of BIV RNA load

Plasma viral RNA was detected in 8 of the 13 cattle from 4 to 14 dpi (Table 4.2). Viral titres ranged from 4.2 x 10¹ to 1.0 x 10⁴ genome copies/ml plasma.
Table 4.1. BIV proviral DNA detection and quantification by conventional and qPCR in cattle inoculated with BIV-R29.

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<td>84.6</td>
<td>100</td>
<td>15.3</td>
<td>60.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

aNT, not tested.
b+, positive result by conventional PCR.
cNumber of BIV proviral genome copies/100 000 peripheral blood mononuclear cell-equivalents.
dNA, not available
Table 4.2. BIV viral RNA genome copies/ml of plasma determined by qPCR in cattle inoculated with BIV-R29.

<table>
<thead>
<tr>
<th>Cattle</th>
<th>0-2(^a)</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>14</th>
<th>15-34(^b)</th>
<th>15-62(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB169</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>CB170</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>CB171</td>
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<td>CB174</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>5.9 x10(^3)</td>
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<tr>
<td>CB176</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>CB178</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.2 x10(^2)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>6.1 x10(^1)</td>
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<td>-</td>
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<td>1.3 x10(^3)</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>5.6 x10(^3)</td>
<td>6.0 x10(^2)</td>
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</tbody>
</table>

\(^a\)represents results from days 0,1 and 2 p.i.
\(^b\)represents results from days 15, 16, 18, 20, 27 and 34 p.i.
\(^c\)represents results from days 15, 16, 18, 20, 27, 34, 40, 41, 43, 45, 47 to 53, 55 to 58, 60 and 62 p.i.
Viruses RNA and proviral DNA in tissues 65 days after infection

Proviral DNA and/or viral RNA were detected in all 6 cattle killed 65 dpi (Table 4.3). Proviral DNA was detected in at least 1 tissue type of each animal and most commonly in lymphoid tissues. Viral RNA was detected less frequently than proviral DNA and only in the spleen and prescapular lymph nodes. The levels of viral RNA detected by qRT-PCR were very low, bordering on undetectable.

Serological response to BIV-R29 infection

Antibody to the BIV TM\(^c\) and CA antigens were detected by ELISA (Figures 4.1 and 4.2). Increased ELISA absorbance readings with the BIV TM\(^c\) peptide were detected 12 dpi and were detected in most cattle by 20 dpi. The antibody response to the CA antigen was markedly less than to the TM\(^c\) peptide and a strong antibody response to this protein was detected in one animal only between 34 and 56 dpi (Figure. 4.2).

None of the control group developed detectable TM or CA antibody.
Table 4.3. Viral RNA and proviral DNA quantification in tissues after necropsy of experimentally infected Bali cattle.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cattle</th>
<th>CB177</th>
<th>CB178</th>
<th>CB179</th>
<th>CB180</th>
<th>CB181</th>
<th>CB182</th>
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<tbody>
<tr>
<td>Spleen</td>
<td>Provirus</td>
<td>+ (&lt;20)</td>
<td>+ (&lt;20)</td>
<td>+ (&lt;20)</td>
<td>-</td>
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<td>+ (&lt;20)</td>
<td>204.0</td>
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<tr>
<td>Lung</td>
<td>Provirus</td>
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<td>+ (&lt;20)</td>
<td>+ (&lt;20)</td>
<td>+ (&lt;20)</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Bone marrow</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>+ (&lt;20)</td>
<td>+ (&lt;20)</td>
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<td>Provirus</td>
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<td>29.28</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Proviral genome in tissues quantified using qPCR.

*b* Viral RNA genome copies in tissues quantified using qRT-PCR.

*c* Samples with values less than 20 but consistently above the negative control.

*d* Numbers refer to BIV proviral genome copies/100 000 cell equivalents.

*e* Numbers refer to BIV viral RNA genome copies/µg total RNA.
Figure 4.1. TM IgG in serum of cattle after infection with BIV-R29. Antibody was detected by ELISA with a BIV TM peptide antigen. Absorbances were normalised to day 0 readings. ELISA absorbances from mock infected cattle (CB183 – CB187) are also shown. Top: individual results in 17 cattle. Bottom: box and whisker plot of the values in the 13 cattle infected with BIV.
**Figure 4.2.** CA IgG in serum of cattle after infection with BIV-R29. Antibody was detected by ELISA with a BIV CA antigen. Absorbances were normalised to day 0 readings. ELISA absorbances from mock infected cattle (CB183 – CB187) are also shown. Top: individual results in 17 cattle. Bottom: box and whisker plot of the values in the 13 cattle infected with BIV.
Discussion

The detection of BIV provirus in PBMC in all 13 cattle over the course of the experiment and within tissues at the end of the experiment 65 dpi, the transient detection of viral RNA in plasma from 8 of 13 infected cattle and an antibody response to BIV in inoculated cattle, confirm that the Bali cattle were productively infected with BIV.

No clinical signs of infection were observed in any of the infected Bali cattle. The absence of significant clinical effects is similar to other experiments where BIV had been inoculated into B. taurus (Carpenter et al., 1992; Heaton et al., 1998; Isaacson et al., 1995; Zhang et al., 1997a). The greater susceptibility to JDV of Bali cattle than B. taurus is not reflected in their susceptibility to BIV. This absence of clinical signs in Bali cattle in response to BIV would be consistent with the presence of a BIV-like virus in Bali cattle on the island of Sulawesi in Indonesia where antibody to JDV has been detected but there is no evidence of Jembrana disease (Hartaningsih, personal communication).

BIV proviral DNA was detected in PBMC from 8 dpi until the conclusion of the experiment at 65 dpi but was not detected in all animals at all sampling occasions. The highest proportion of BIV proviral DNA positive animals was at 14 and 20 dpi when 84.4 and 100% of animals were positive, suggesting this was a peak period of virus replication and indicating an acute phase for BIV infection. After this period the virus appeared to persist in PBMC, at a level that was often undetectable. Modifications to the PCR assays may have assisted in the detection of very low proviral DNA copy numbers.

Provirus was detected in a wide variety of tissues at 65 dpi when the experiment was concluded, similar to a previous report of infection in B. taurus (Zhang et al., 1997a).
The levels of proviral DNA were low but highest in the lymphoid tissues examined, spleen and lymph nodes. The low titres were similar to those reported in other lentivirus infections, including African green monkeys persistently infected with SIV<sub>agm</sub> (Gueye et al., 2004) and in horses asymptomatically infected with a cell-adapted pathogenic EIAV (Harrold et al., 2000).

Although previous studies involving experimental infection of <i>B. taurus</i> with BIV have detected viral RNA within PBMC subpopulations, either by RT-PCR (Baron et al., 1995; Wu et al., 2003) or by in situ hybridisation (Carpenter et al., 1992), this study appears to be the first report documenting the transient nature of the plasma viraemia in the period soon after infection, similar to that reported in many other lentivirus infections (Langemeier et al., 1996; Miyake et al., 2006; Ryan et al., 2003; Stewart et al., 2005). While provirus was detected in all 13 infected cattle, viral RNA was detected in 8 of 13 cattle and only during the period from 4 to 14 dpi. The levels of virus RNA detected never exceeded $1 \times 10^4$ genome copies/ml plasma, much less than those detected during infection of Bali cattle with the genetically related JDV where titres of up to $1.6 \times 10^{12}$ genome copies/ml plasma have been detected during the acute disease (Stewart et al., 2005). The transient nature of the plasma viraemia could be viewed as escape of the virus from host control. While provirus was widespread in the tissues that were tested, viral RNA was detected in the spleen and prescapular lymph node only of some animals and was at low levels. Perhaps the level of replication of BIV relative to JDV may be associated with the relative lack of pathogenicity of BIV compared to JDV.

There was a rapid and strong response against TM but a poor antibody response to the CA protein in most infected cattle. The TM response to BIV infection was similar to that reported by Scobie et al. (1999) in cattle infected with the BIV FL112
isolate and an antibody response was detected as early as 2 weeks pi. The weak CA response detected in all but one of the 13 infected Bali cattle was surprising as in *B. taurus* there is normally a strong response to CA between 2 and 4 weeks pi (Isaacson et al., 1995; Whetstone et al., 1991). Seroconversion to a *gag* precursor also occurred between 2 and 4 weeks pi in rabbits infected with BIV (Pifat et al., 1992). The reason for the poor antibody response to CA in Bali cattle is unknown. In response to infection with the genetically and antigenically related JDV, Bali cattle normally mount a strong albeit delayed immune response against CA (Hartaningsih et al., 1994) but as in the Bali cattle infected with BIV, a subset of cattle infected with JDV mount a poor antibody response to the JDV CA and a strong antibody response to JDV TM (Ditcham et al., 2009). A 10 to 100 times greater antibody response to envelope proteins compared to CA proteins was also observed in horses in response to EIAV (O'Rourke et al., 1988).

Results from this study indicate not only that BIV is non-pathogenic in Bali cattle but that maximum virus replication occurred soon after infection and prior to the onset of a significant antibody response, certainly prior to the onset of a significant antibody response to the CA protein. It is likely that future attempts to detect BIV infection in Bali cattle and possibly other cattle species using PCR based assays, would have greatest chance of success soon after infection and before the onset of a significant antibody response.
Chapter 5: Bovine immunodeficiency virus infection alters the dynamics of subsequent Jembrana disease virus infection

Summary

To determine whether BIV infection is capable of protecting against superinfection with JDV, 15 animals were infected with BIV-R29 and 42 days after BIV infection, 9 of the BIV infected and 4 mock BIV infected animals were superinfected with JDV-Tabor7. All cattle were successfully infected with BIV, shown by the presence of proviral DNA and, in a subset of cattle, a transient viraemia. Strong antibody responses against the TM glycoprotein and poor antibody responses against the CA protein were also detected. Despite the development of immune responses against TM, a region known to contain cross-reactive epitopes, all cattle became infected with JDV, as indicated by the development of typical clinical signs of Jembrana disease and an acute phase viraemia.
Introduction

The 2 bovine lentiviruses, BIV (Gonda et al., 1987) and JDV (Chadwick et al., 1995a; Kertayadnya et al., 1993), are genetically and antigenically related but differ markedly in pathogenicity. The incidence of clinical Jembrana disease and serological surveys indicate JDV is common in the Bali cattle population in parts of Indonesia (Hartaningsih et al., 1993). A BIV-like non-pathogenic bovine lentivirus is also suspected to occur in the cattle population on Bali island (Barboni et al., 2001) and antibody to JDV has been detected in Bali cattle on the island of Sulawesi where there is no clinical evidence of Jembrana disease in the Bali cattle population (Desport et al., 2005). Antibody to JDV and BIV cannot be differentiated due to the presence of numerous cross-reactive epitopes on the CA (Desport et al., 2005; Kertayadnya et al., 1993) and TM proteins (Burkala et al., 1998).

A protective immunity against JDV infection has been induced by vaccination with inactivated whole virus antigens (Ditcham et al., 2009). It was considered possible that infection of Bali cattle with a non-pathogenic BIV-like lentivirus might also induce a protective immune response against Jembrana disease. Non-pathogenic strains of other lentiviruses have been shown to induce protective immunity against pathogenic strains of the same virus. Infection of domestic cats with non-pathogenic lion lentivirus or puma lentivirus ameliorated the effects of subsequent wild-type FIV infection (Terwee et al., 2008; VandeWoude et al., 2002). Similar results were obtained in domestic cats pre-infected with chimeric FIV (generated by substituting part of env of clade A FIV\textsubscript{PET} with a corresponding region of clade B FIV\textsubscript{M2}, or vice-versa) and subsequently inoculated with FIV\textsubscript{PET} or FIV\textsubscript{M2} (Giannecchini et al., 2007). Infection of macaques infected with attenuated SIV\textsubscript{mac} also ameliorated the effects of challenge with pathogenic SIV\textsubscript{mac} (Cranage et al., 1998; Sharpe et al., 2004) and
SIV<sub>sm</sub> (Nilsson et al., 1998) although attenuated SIV<sub>mac</sub> did not provide protection against the more divergent HIV-2 (Nilsson et al., 1998).

This Chapter describes an experiment to determine whether prior infection of Bali cattle with BIV would provide protection against superinfection with pathogenic JDV infection 42 days after the initial BIV infection. This experiment was expected to provide information that would increase our understanding of the effect JDV would have on the Bali cattle population if it were introduced onto the island of Sulawesi where BIV is suspected to occur in the cattle population of that island.

**Materials and methods**

*Animals*

Nineteen cattle approximately 18 months of age were obtained from Nusa Penida, an island adjacent to Bali where Jembrana disease has never been detected and the cattle have been consistently negative to antibody to JDV and BIV (Hartaningsih et al., 1993). The cattle were housed indoors as previously described (Soeharsono et al., 1990). Cattle were screened by PCR and ELISA to ensure they were not infected with bovine lentiviruses prior to challenge (Chapter 3). Six weeks prior to infection with BIV the cattle were vaccinated against BVDV with Pestigard® (Pfizer), as previously described (Chapter 4).

*Viruses*

The BIV-R29 for infection of cattle was obtained from J Brownlie and M Collins, Royal Veterinary College, UK. The virus was cultured in primary BFL cells and titrated in BFL cells as previously described (Chapter 4). An estimated 1.82 x 10<sup>4</sup> TCID<sub>50</sub> was inoculated into each animal, half subcutaneously and half intravenously.
The JDV <sub>Tab87</sub> (Chadwick et al., 1995b) used for infection of cattle was prepared by infection of antibody-negative cattle with a suspension of frozen spleen harvested from infected cattle as described previously (Soeharsono et al., 1990). Plasma from the infected cattle was obtained 2 days after the development of a febrile response typical of Jembrana disease, and the approximate ID<sub>50</sub> in the plasma determined using an antigen-capture ELISA as described previously (Stewart et al., 2005). An estimated 1 000 ID<sub>50</sub> of the virus was inoculated intravenously into each animal.

**Experimental infection and sampling of cattle**

Nine cattle were inoculated with BIV at day 0 and with JDV 42 days later (CB190–CB197 and CB205). Six cattle were inoculated with BIV at day 0 and were not subsequently inoculated with JDV (CB198–CB202 and CB204). Four cattle were inoculated with uninfected BFL cells in RPMI medium at day 0 and subsequently with JDV 42 days later (CB203, CB206, CB208 and CB210).

Animals were observed daily for clinical signs of disease. Rectal temperatures were measured daily for the duration of the study. Heparinised blood samples were obtained as required by venipuncture of the jugular vein and used for extraction of DNA from PBMC, extraction of RNA from plasma and serum for serological tests.

**Extraction of DNA and RNA from peripheral blood**

PBMC were purified from 10 ml of heparinised blood using a Ficoll-Paque Plus<sup>TM</sup> (GE Healthcare) gradient as recommended by the manufacturer. DNA was extracted from the PBMC using a QIAamp DNA Mini Kit (Qiagen) as recommended by the manufacturer. Viral RNA was extracted from plasma using the QIAamp Viral RNA Extraction Kit (Qiagen) as recommended by the manufacturer.
Detection of BIV proviral DNA

The integrity of genomic DNA was confirmed using gene-specific GAPDH primers as previously described (Chapter 4). BIV proviral DNA loads were determined using a conventional PCR assay as described previously (Chapter 4).

Quantification of BIV and JDV RNA in plasma

BIV RNA was quantified as described previously (Chapter 4) and JDV RNA as described by Stewart et al. (2005), with the following exceptions: 1X RT-PCR Reaction Mix for Probes (Bio-Rad), 0.2 µl iScript Reverse Transcriptase for One-Step RT-PCR (Bio-Rad) and 2 µl RNA extracted from plasma was added to each reaction. The reaction was made up to 10 µl using nuclease free water (Bio-Rad).

ELISA

The BIV TM antibody response was determined by ELISA using a cyclic BIV TM (BIV TM<sub>c</sub>) peptide as described previously (Chapter 4). The JDV TM antibody response was determined by ELISA using a cyclic JDV TM (JDV TM<sub>c</sub>) peptide as previously described (Ditcham et al., 2009) as while there was extensive cross-reactivity between JDV and BIV TM antigens, our experience was that the JDV TM<sub>c</sub> peptide provided greater sensitivity in the detection of JDV TM antibody than did the BIV TM<sub>c</sub> antigen. The BIV CA antibody response was determined using an ELISA with a recombinant BIV CA antigen as previously described (Chapter 4) except that 100 ng of protein was coated onto each well and serum was tested at a dilution of 1:100.
Analysis of data

To determine whether prior BIV infection would induce protection against subsequent JDV infection, differences between the viral load (VL) on the first and second day of the febrile period when the rectal temperature exceeded 39.3°C, the peak VL, the duration and magnitude of the VL where the area under the curve (AUC) was $>10^6$ genomes/ml, and the duration of the febrile period, were compared in JDV-infected cattle that had been previously infected with BIV and in cattle not previously infected with BIV, as described previously (Desport et al., 2009a; Ditcham et al., 2009). The AUC where the VL was $>10^6$ for each animal was estimated and linear interpolation between consecutive observations, in combination with the previously described piecewise-linear model was used to supply missing data caused by variations in sampling intervals (Ditcham et al., 2009). The duration of the infectious period was defined as the period where VL was $>10^6$ genomes/ml. A baseline of $10^6$ was chosen (Ditcham et al., 2009) as bloodmeal residues of between 4–10 nl have been reported on the mouthparts of tabanid flies and EIAV at $10^6$ ID/ml in blood can be transmitted by a single fly (Foil et al., 1987). For analysis of the febrile response, rectal temperatures were divided into ranges adapted from a previously described method (Muraguri et al., 1999) as low fever ($>39.3 \, ^\circ\text{C} – 40.2 \, ^\circ\text{C}$), moderate fever ($40.3 \, ^\circ\text{C} – 41.2 \, ^\circ\text{C}$) and high fever ($> 41.2 \, ^\circ\text{C}$). Student’s t-test was used for statistical comparison of the magnitude and duration of plasma VL and febrile responses and $P$-values $< 0.05$ were considered statistically significant.
Results

Clinical and virological observations in cattle after BIV inoculation

Fifteen BIV inoculated Bali cattle and 4 mock-infected control cattle were monitored for 42 days and then superinfected with JDV. During the initial 42 day period after BIV infection, the BIV-infected cattle did not exhibit any change in rectal temperature or other clinical signs of disease.

BIV proviral DNA was detected in PBMC initially 7 dpi in one animal and subsequently in PBMC of all 15 cattle inoculated with BIV, confirming that all animals were infected (Table 5.1). BIV proviral DNA was detected in most cattle after 17 dpi but at no single time point was BIV proviral DNA detected concurrently in all 15 cattle. BIV proviral DNA was not detected in the mock-infected cattle.

Plasma viral RNA was detected in 5 of the 15 cattle in the period from 8 to 13 dpi (Table 5.2) and plasma viral titres ranged from 1.04 to 4.25 log_{10} genome copies/ml plasma.

An antibody response to the BIV TM^c and CA antigens was detected by ELISA (Figure 5.1). Antibody to the BIV TM^c peptide was detected from 10 dpi and in the majority of cattle by 41 dpi (Figure 5.1A). Antibody to the BIV CA antigen was detected from 19 dpi (Figure 5.1C) but the CA response was markedly less than the response to the BIV TM^c peptide (Figure 5.1A). Only some of the BIV-infected cattle developed CA antibody and only one seroconverted strongly in the 42 day period after infection. None of the mock-infected cattle developed BIV TM^c or CA antibody (Figure 5.1B and 5.1D).
Figure 5.1. TM\(^c\) and CA IgG response in serum of cattle after inoculation with BIV, shown as box and whisker plots of the ELISA absorbance values. (A) Response detected by BIV TM\(^c\) peptide in all 15 cattle inoculated with BIV at day 0. (B) Response detected by His-BIV CA antigen in all 15 cattle inoculated with BIV at day 0. (C) Response detected by BIV TM\(^c\) peptide antigen in all 8 cattle mock inoculated at day 0. (D) Response detected by His-BIV CA antigen in all 8 cattle mock inoculated at day 0.
**Table 5.1.** BIV proviral DNA detected by PCR in cattle inoculated with BIV and prior to infection with JDV.

<table>
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</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td><strong>Cattle inoculated with BIV at day 0 and then JDV at day 42</strong></td>
<td></td>
</tr>
<tr>
<td>CB190</td>
<td></td>
</tr>
<tr>
<td>CB191</td>
<td>-</td>
</tr>
<tr>
<td>CB192</td>
<td>-</td>
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<td>CB196</td>
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<td>CB197</td>
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<td>CB198</td>
<td>-</td>
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<tr>
<td>CB199</td>
<td>-</td>
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<tr>
<td>CB200</td>
<td>-</td>
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<tr>
<td>CB201</td>
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<tr>
<td>CB202</td>
<td>-</td>
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<td>CB203</td>
<td>-</td>
</tr>
<tr>
<td>CB204</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cattle inoculated with BIV only at day 0</strong></td>
<td></td>
</tr>
<tr>
<td>CB205</td>
<td>-</td>
</tr>
<tr>
<td>CB206</td>
<td>-</td>
</tr>
<tr>
<td>CB207</td>
<td>-</td>
</tr>
<tr>
<td>CB208</td>
<td>-</td>
</tr>
<tr>
<td>CB209</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cattle inoculated with JDV only at day 42</strong></td>
<td></td>
</tr>
<tr>
<td>CB210</td>
<td>-</td>
</tr>
</tbody>
</table>

**Percent BIV-inoculated cattle positive**

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>15</th>
<th>85</th>
<th>53</th>
<th>47</th>
<th>60</th>
</tr>
</thead>
</table>

**Cumulative % positive**

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>15</th>
<th>93</th>
<th>93</th>
<th>93</th>
<th>100</th>
</tr>
</thead>
</table>

**Percent mock-inoculated cattle positive**

|         | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

\(^a\) and + denote negative and positive PCR results, respectively, for BIV proviral DNA.

\(^b\) not tested.
Table 5.2. BIV viral RNA genome copies/ml (log_{10}) of plasma determined by qPCR in cattle inoculated with BIV at day 0 (all cattle) and JDV at day 42 (CB190 – CB205 only).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Days after BIV inoculation</th>
<th>Days after JDV inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 - 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>CB190</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB191</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB192</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB193</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB194</td>
<td>-</td>
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<tr>
<td>CB195</td>
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<td>-</td>
</tr>
<tr>
<td>CB196</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB197</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CB205</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*<sup>a</sup> represent results from 0 and 1 dpi.  
*<sup>b</sup> negative.  
*<sup>c</sup> represents results from 14 - 17, 19, 21, 28, 35 and 41 - 42 dpi.  
*<sup>d</sup> represents results from 44, 46 and 48 - 50 dpi.  
*<sup>e</sup> represents results from 56 - 59, 61 and 63 dpi
Clinical and virological observations after JDV inoculation

Forty two days after BIV infection, 9 of the 15 BIV-infected cattle and 4 of the mock-infected cattle were inoculated with JDV and monitored for a further 15 to 19 days. All the JDV-inoculated cattle developed a febrile response but there were differences in the responses in the previously BIV-infected and non-BIV-infected groups (Figure 5.2). Excluding 2 animals CB190 and CB205 that responded uncharacteristically to JDV infection and were considered atypical responders (see below), the febrile response in the BIV-infected group started 2 days earlier (a mean of 7 dpi compared to 9 dpi, \( P = 0.058 \)), the peak of the febrile response occurred earlier (a mean of 10 dpi versus 12 dpi, \( P = 0.067 \)), the peak VL also occurred significantly sooner (a mean of 10 dpi versus 12 dpi, \( P = 0.008 \)) and there was a significantly earlier conclusion of the febrile response (a mean of 13 dpi compared to 15 dpi, \( P = 0.033 \), Table 5.3). There were no significant differences between groups in regards to the duration and severity of the febrile response (Table 5.3). The 6 BIV-only controls did not develop a febrile response or other clinical signs during the observation period (Figure 5.2).

JDV plasma RNA was detected in all cattle inoculated with JDV from 3 to 19 dpi (Figure 5.3). The maximum VL was 2.18 x 10^{11} genome copies/ml plasma. Excluding the 2 atypical responders, the cattle infected with JDV, regardless of whether they had been challenged previously with BIV, developed a viraemia typical of that reported previously (Desport et al., 2009a; Stewart et al., 2005). As shown in Figure 5.3, the pattern and magnitude of the viraemia was also similar between groups, but the viraemia started and finished earlier in the cattle infected previously with BIV, although these differences were not statistically significant; accordingly, in cattle previously infected with BIV there was a reduction in the mean duration of
the JDV viraemia that was >10^6 genomes/ml plasma (P = 0.068). There were no significant differences between previously BIV-infected and non-BIV-infected groups in the VL on the first or second day of the febrile period, the peak VL or the total AUC when the viraemia was ≥10^6 genomes/ml plasma (Table 5.4).

Two animals, CB190 and CB205, responded atypically to JDV infection. The cattle had a late onset fever (Figures 5.2 and Table 5.3) and the dynamics of their viraemia were different to those in the other cattle (Figure 5.3), similar to atypical responders previously reported (Desport et al., 2009a). These animals had an erratic viraemia increasing to a maximum titre of approximately 10^{10} genome copies/ml which continued through to the conclusion of the experiment. As the viraemia in these animals did not decrease before the experiment concluded, the data provided for the duration of the viraemia in Table 5.4 is an underestimation of the actual values.

After inoculation with JDV, plasma BIV RNA was again detected in 4 of the 9 superinfected cattle (Table 5.2). The titre of BIV RNA during this period ranged from 1.40 to 2.57 log_{10} genome copies/ml plasma.

Antibody to the JDV TM c antigen was detected (Figure 5.4) in the 15 day period after JDV infection in a majority of cattle previously infected with BIV (Figure 5.4A), but was not detected in any of the JDV-infected cattle that had not been infected previously with BIV (Figure 5.4B). ELISA absorbances to the CA protein increased at 4 days after JDV infection in the cattle that had been previously infected with BIV (Figure 5.4D). BIV CA ELISA absorbances remained low in the cattle infected with JDV only (Figure 5.4F).
Figure 5.2. Mean rectal temperatures in cattle inoculated with JDV. Y-error bars represent the standard deviations of rectal temperatures. Shown are the rectal temperatures in cattle which had been previously inoculated with BIV and responded to JDV in a typical fashion (○; CB191 – CB197), in cattle which had been previously infected with BIV and responded to JDV in an atypical fashion (▼; CB190 and CB205), JDV-only control cattle (●; CB203, CB206, CB208 and CB210) and BIV-only control cattle JDV (■).
Table 5.3. Effect of previous BIV infection of cattle on the febrile response following JDV infection.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Days after infection</th>
<th>Duration of febrile response (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak VL</td>
<td>Onset of Peak febrile response</td>
</tr>
<tr>
<td>Cattle infected with BIV 42 days previously</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB190</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>CB191</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>CB192</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>CB193</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>CB194</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>CB195</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>CB196</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>CB197</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>CB205</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Mean</td>
<td>11</td>
<td>9</td>
</tr>
</tbody>
</table>

(10)<sup>a</sup> (7)<sup>a</sup> (10)<sup>a</sup> (13)<sup>a</sup>

Variance 15.28 22.75 17.75 13.50 5.50 1.36 0.53 2.75

Cattle not previously infected with BIV

<table>
<thead>
<tr>
<th>Animal</th>
<th>Days after infection</th>
<th>Duration of febrile response (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak VL</td>
<td>Onset of Peak febrile response</td>
</tr>
<tr>
<td>CB203</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>CB206</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>CB208</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>CB210</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Mean</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>

Variance 0.25 0.92 1.67 1.58 2.25 3.33 0.25 3.33

\(P\)-value<sup>b</sup> 0.441 0.487 0.412 0.449 0.425 0.153 0.233 0.376

(0.008)<sup>c</sup> (0.058)<sup>c</sup> (0.067)<sup>c</sup> (0.033)<sup>c</sup>

<sup>a</sup> mean calculated excluding atypical responders CB190 and CB205.

<sup>b</sup> \(P\)-values represent statistical differences between animals infected with BIV and then JDV and those infected with JDV only.

<sup>c</sup> \(P\)-value calculated excluding atypical responders CB190 and CB205.
Figure 5.3. Mean plasma viral loads (JDV RNA genome copies/ml plasma) in animals inoculated with JDV at day 0. Y-error bars represent the standard deviations of viral loads. Shown are viral loads in cattle which had been previously infected with BIV and responded to JDV in a typical fashion (○; CB191 – CB197), JDV-only control cattle (●; CB203, CB206, CB208 and CB210), and cattle which had been previously infected with BIV and responded to JDV in an atypical fashion (▼; CB190 and CB205).
Table 5.4. Effect of previous BIV infection on the dynamics of the JDV viral load (VL) in plasma after JDV inoculation.

<table>
<thead>
<tr>
<th>Animal</th>
<th>VL 1\textsuperscript{st} day of febrile period (log\textsubscript{10})</th>
<th>VL 2\textsuperscript{nd} day of febrile period (log\textsubscript{10})</th>
<th>Peak VL (log\textsubscript{10})</th>
<th>AUC $\geq 10^6$ genome copies/ml (log\textsubscript{10})</th>
<th>Total days VL$\geq 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle infected with BIV 42 days previously</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB190</td>
<td>6.61</td>
<td>9.77</td>
<td>11.34</td>
<td>10.15</td>
<td>5.60</td>
</tr>
<tr>
<td>CB191</td>
<td>7.35</td>
<td>10.63</td>
<td>10.12</td>
<td>11.76</td>
<td>11.20</td>
</tr>
<tr>
<td>CB193</td>
<td>8.89</td>
<td>9.26</td>
<td>9.26</td>
<td>9.68</td>
<td>8.60</td>
</tr>
<tr>
<td>CB194</td>
<td>1.91</td>
<td>4.64</td>
<td>9.98</td>
<td>10.25</td>
<td>7.60</td>
</tr>
<tr>
<td>CB195</td>
<td>9.23</td>
<td>10.12</td>
<td>10.57</td>
<td>10.98</td>
<td>10.10</td>
</tr>
<tr>
<td>CB196</td>
<td>9.22</td>
<td>9.25</td>
<td>10.31</td>
<td>10.44</td>
<td>9.60</td>
</tr>
<tr>
<td>CB197</td>
<td>9.36</td>
<td>9.93</td>
<td>10.72</td>
<td>11.00</td>
<td>8.50</td>
</tr>
<tr>
<td>CB205</td>
<td>11.25</td>
<td>10.68</td>
<td>10.68</td>
<td>10.70</td>
<td>9.20</td>
</tr>
<tr>
<td>Mean</td>
<td>8.04</td>
<td>9.06</td>
<td>10.08</td>
<td>10.55</td>
<td>9.00</td>
</tr>
<tr>
<td>Variance</td>
<td>7.00</td>
<td>4.04</td>
<td>0.27</td>
<td>0.57</td>
<td>1.91</td>
</tr>
<tr>
<td>Cattle not previously infected with BIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB203</td>
<td>9.98</td>
<td>10.28</td>
<td>10.73</td>
<td>11.18</td>
<td>16.40</td>
</tr>
<tr>
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<td>9.40</td>
<td>10.01</td>
<td>10.18</td>
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<td>10.02</td>
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<td>9.16</td>
<td>10.13</td>
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<td>13.40</td>
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<td>9.60</td>
<td>10.26</td>
<td>10.64</td>
<td>12.53</td>
</tr>
<tr>
<td>Variance</td>
<td>1.65</td>
<td>0.41</td>
<td>0.10</td>
<td>0.15</td>
<td>7.77</td>
</tr>
</tbody>
</table>

\[P\text{-value}\]^a = 0.624 0.692 0.931 0.404 0.068

\[^a\] P-values represent statistical differences between groups of animals infected with BIV 42 days prior to infection with JDV and those infected with JDV only.
Figure 5.4. IgG response detected by JDV TMc peptide and His-BIV CA after inoculation with JDV, shown as box and whisker plots of the ELISA absorbance values. (A) Response detected by JDV TMc peptide in cattle infected with JDV at day 0 and infected with BIV 42 days earlier. (B) Response detected by JDV TMc peptide in cattle infected with JDV at day 0 but not infected previously with BIV. (C) Response detected by JDV TMc peptide in cattle not infected with JDV at day 0 but infected with BIV 42 days earlier. (D) Response detected by His-BIV CA in cattle infected with JDV at day 0 and infected with BIV 42 days earlier. (E) Response detected by His-BIV CA in cattle infected with JDV at day 0 only. (F) Response detected by His-BIV CA in cattle not infected with JDV at day 0 but infected with BIV 42 days earlier.
**Discussion**

Under the conditions with which the experiment was conducted, prior BIV infection did not prevent subsequent JDV infection or result in significant amelioration of the normal response of Bali cattle to JDV infection. Although it did not alter the normal clinical response to JDV infection, prior infection of cattle with BIV altered the dynamics of their response to JDV and resulted in an earlier onset of Jembrana disease. Although many of the effects were not significantly different, cattle previously infected with BIV developed an earlier onset of fever, an earlier peak febrile response, a significantly earlier peak VL and a significantly earlier resolution of fever following superinfection with JDV. Although prior BIV infection did not cause a difference in the total AUC, there was (in cattle previously infected with BIV) a reduction in the duration of the viraemia that exceeded $10^6$ genome copies/ml of plasma. The differences were only evident when 2 atypical responders, identified in a small percentage of all cattle infected with the JDV$_{Tab87}$ strain of JDV (Desport et al., 2009a), were removed from the analysis. The lack of statistical significance in those animals that responded typically was probably at least partly due to the wide variance in the response to JDV of the cattle previously infected with BIV. A case fatality rate of about 21% is normally evident in experimentally JDV-infected Bali cattle (Desport et al., 2009a) but no fatalities occurred in any of the JDV-infected cattle during the 14 day observation period after infection so no effect on the JDV-associated case fatality rate of prior BIV infection was determined.

The enhanced early replication of a superinfecting virus seen here has not been reported previously in superinfections with other related lentiviruses. An earlier onset of disease has, however, been reported with CAEV, JDV, EIAV and FIV following vaccination. Goats vaccinated with inactivated CAEV developed arthritis.
more rapidly after CAEV infection than control animals (McGuire et al., 1986). Cattle vaccinated with a tissue-derived JDV vaccine had earlier peak VL than control animals (Ditcham et al., 2009). Horses vaccinated with a recombinant EIAV subunit vaccine and then challenged with EIAV displayed severe enhancement of viral infection and exacerbation of disease (Montelaro et al., 1996). Goats vaccinated with a T-cell priming Gag peptide from CAEV had transiently enhanced virus replication after CAEV infection compared with control animals, potentially via T-cell enhancement of virus replication (Nenci et al., 2007) and similar findings have been reported in FIV vaccine studies (Richardson et al., 1997). The presence of an active, cross-reactive T-helper cell immune response may explain the earlier start of viraemia and accelerated febrile response. JDV has recently been identified as replicating in IgG-containing cells (Desport et al., 2009b) and alternatively, the earlier replication of JDV in BIV-infected cattle may be related to the B-cell stimulatory activity of BIV (Whetstone et al., 1997).

The lack of amelioration of the febrile response and the replication of JDV in the cattle previously infected with BIV occurred despite a strong antibody response to the BIV TM and, in a proportion of the cattle, an antibody response to the CA at the time of JDV inoculation. Due to the close antigenic relationship between JDV and BIV (Desport et al., 2005; Kertayadnya et al., 1993) the result was not expected as other lentiviruses have been shown to offer protection against infection with closely related heterologous viruses. Domestic cats infected with non-pathogenic puma or lion lentiviruses developed humoral and cell-mediated immune responses against both homologous and heterologous FIV isolated from domestic cats (VandeWoude et al., 2003), suppressed FIV-induced CD4+ T-cell depletion (Terwee et al., 2008; VandeWoude et al., 2002) and FIV-induced plasma and PBMC viral loads.
(VandeWoude et al., 2002). Domestic cats pre-infected with a chimeric FIV and subsequently infected with fully virulent FIV ameliorated the clinical effects of the virulent challenge virus in some challenged cats, and reduced viral RNA and proviral DNA loads in others (Giannecchini et al., 2007). A lack of neutralising antibody response against the TM glycoprotein could explain why JDV infection occurred despite a strong anti-TM antibody response.

It is possible that the R29 strain of BIV may have become attenuated since its isolation in 1969 (Whetstone et al., 1997) and that this may have affected the result obtained. It is also possible that the 42 day period between BIV infection and subsequent JDV infection was too short for an effective protective immunity to develop. Although there was a strong antibody response to the TM protein of BIV at the time of JDV infection, and a rapid antibody response to TM and CA after JDV infection, which is normally delayed until at least 6 weeks after infection (Hartaningsih et al., 1994), this response may be unrelated to the development of a protective immune response which may require a longer period to develop with the bovine lentiviruses. However, in a similar experiment, protection against SIV in macaques could be achieved within 21 days of infection with a live attenuated SIV, with partial protection against wild-type SIV provided within 10 days of inoculation (Stebbings et al., 2004). In contrast, after wild-type FIV infection there was no protection against the effects of heterologous virus challenge until 2 to 3 years after infection when animals exhibited a reduced virus load and sometimes a reduced decline of CD4+ T-cells (Pistello et al., 1999). The possibility that longer term infections with BIV may induce a protective response to JDV infection needs further investigation as it would perhaps offer a low cost means of immunizing cattle against Jembrana disease.
The dose of BIV used for infection and the challenge dose of JDV may also be factors responsible for lack of resistance to JDV infection following BIV infection. Studies of SIV\textsubscript{mac} in macaques have shown that the dose of primary virus affects the level of resistance to infection with a pathogenic virus (Cranage et al., 1998). Macaques given higher doses, 2 000 - 20 000 TCID\textsubscript{50}, of SIV\textsubscript{macC8} were shown to completely resist infection with SIV\textsubscript{mac}, shown by a lack of virus detection by PCR and a lack of virus isolation, whereas animals given lower doses of primary virus, 2 – 200 TCID\textsubscript{50}, were protected against a loss of CD4 cells only.

While there was no evidence that prior BIV infection provided protection against the pathological effects of subsequent JDV infection, there was also no evidence that the prior BIV infection exacerbated subsequent JDV infection, as reported in cases of HIV superinfection. Epidemiological observations suggest that infection with a second heterologous strain of HIV-1 has in the majority of cases, accelerated disease progression after infection, reviewed previously (Smith et al., 2005). It was observed in the current experiments that JDV infection in cattle previously infected with BIV reactivated replication of the BIV, and this appears to be an observation seen during heterologous lentivirus infections. Reactivation of primary infection virus has also been seen in HIV-2 infected macaques superinfected with SIV\textsubscript{mac} (Petry et al., 1995) and in HIV-2 infected baboons superinfected with heterologous HIV-2 (Locher et al., 1997). Reactivation might be associated with the potent transactivation function of the JDV Tat protein, which was shown to be a potent transactivator not only of its own LTR but also the BIV and HIV LTR \textit{in vitro} (Chen et al., 2000; Chen et al., 1999). JDV Tat may function similarly \textit{in vivo} in Bali cattle.

There is no evidence of the outcome of mixed infections with JDV and non-pathogenic BIV-like viruses under field situations in the Bali cattle population of
Indonesia. Although mixed infection with JDV and a second BIV-like virus has been reported to occur in Bali cattle on Bali island (Barboni et al., 2001), the difficulty of detecting BIV-like virus in cattle and the close antigenic relationship of the 2 viruses has so far precluded the epidemiological study of mixed infection by JDV and possible non-pathogenic BIV-like viruses. BIV proviral DNA is only transiently present within PBMC of experimentally infected Bali cattle (Chapter 4), making the virus difficult to detect even when the animals are known to be infected.
Chapter 6: Humoral immune responses to Jembrana disease virus detected using overlapping synthetic peptides spanning the MA, CA and SU regions of JDV

Summary

The mapping of linear B-cell epitopes on the MA, CA and SU regions of Jembrana disease virus is described. One hundred and fifty five overlapping peptides that spanned these regions were synthesised and used in an ELISA format to screen a panel of bovine sera from animals experimentally infected with JDV$_{\text{Tab87}}$, JDV$_{\text{Pul01}}$ or BIV-R29. Six immunoreactive (IR) peptides, representing 6 potential epitopes, were identified when the set of peptides was screened with sera taken following JDV$_{\text{Tab87}}$ infections; 1 in MA, 1 in CA and 5 in SU. Numerous IR peptides were identified when the set of peptides was screened with JDV$_{\text{Pul01}}$ sera. BIV-R29 sera also reacted with many peptides, including the IR peptides identified with the JDV$_{\text{Tab87}}$ sera. However, BIV-R29 sera did not react with some peptides and a combination of these peptides would enable detection of JDV-only seropositive cattle. These peptides include MA18, MA19, SU93, SU95, SU103, SU119 and SU135.
**Introduction**

The occurrence of clinical Jembrana disease and serological surveys for antibody reactive to JDV indicate that JDV infection is widespread in some islands of Indonesia including Bali, Java, Sumatra and Kalimantan (Indonesian Borneo) but the absence of clinical Jembrana disease and the occurrence of antibody to JDV suggests the occurrence of a non-pathogenic bovine lentivirus, possibly related to BIV, on the island of Sulawesi (Hartaningsih, personal communication). A BIV-like, non-pathogenic bovine lentivirus was also reported to occur in the cattle population on Bali island (Barboni et al., 2001) but this has not been confirmed and efforts to detect BIV proviral DNA in PBMC of cattle on Bali island were unsuccessful (Chapter 3).

While JDV and BIV are sufficiently different genetically that they can be differentiated utilising a number of PCR assays (Lew et al., 2004; Lewis et al., 2009) low proviral DNA loads in PBMC after infection precludes their use as a screening tool. They are very similar antigenically and attempts to differentiate antibody to the 2 viruses using ELISA and WIB procedures have been unsuccessful due to the presence of numerous cross-reactive epitopes on the CA, MA and TM proteins (Desport et al., 2005). Using recombinant, overlapping JDV Gag proteins, previous attempts have been made to define specific epitopes that differentiate between the 2 bovine lentiviruses antigenically, however these were unsuccessful (Desport et al., 2005). A number of recombinant proteins were produced and reacted with JDV and BIV hyperimmune sera as well as monoclonal antibodies in a WIB. These attempts identified at least three epitopic domains within MA and CA, including the Major Homology Region (MHR) but could not identify an epitope(s) that differentiated between the two viruses (Desport et al., 2005). A monoclonal antibody directed against an epitope which spans the cleavage site between BIV MA and the p2L
protein was reported to differentiate between BIV and JDV antibody using a WIB assay (Lu et al., 2002; Zheng et al., 2001) but the epitope involved does not appear to be immunogenic in cattle and linear forms of this epitope can not be used to differentiate between bovine lentivirus infections (Desport et al., 2005). Reliable serological surveillance for JDV infection in cattle in Indonesia requires the use of serological techniques that can differentiate the 2 infections.

Various epitope mapping studies have employed the use of expression libraries and recombinant proteins to map epitopes and then fine mapping using synthetic peptide strategies (Bertoni et al., 1994; Chong et al., 1991a; Chong et al., 1991b; Rosati et al., 1999). These approaches were used to map the antigenicity of the SU glycoproteins of EIAV (Ball et al., 1992) and CAEV (Valas et al., 2000) and the antigenicity of CAEV TM (Bertoni et al., 1994). In this Chapter, synthetic peptides were used to map the epitopes of JDV MA, CA and SU proteins to find epitopes which can be used for differentiating the 2 viruses in serological assays.

**Materials and methods**

*Source of animal sera*

A panel of bovine sera from experimentally infected Bali cattle was used in this study. The cattle were infected with either JDV_{Tab87}, JDV_{Pul01} or BIV-R29 during various studies of the response of cattle to these viruses (Desport et al., 2009; Ditcham et al., 2009; Chapters 4 and 5) and serum samples were acquired at various dpi. Pre-infection serum samples from the same cattle were used to test the background level of reactivity to each peptide.

Hyperimmune JDV and BIV sera, a gift from N. Hartaningsih (Indonesia) were also tested. JDV hyperimmune serum was created by inoculating a bovine lentivirus-free
animal with plasma taken from an animal during the febrile phase after infection with 
JDV_{Tab87}. The animal then received 7 booster inoculations intramuscularly at 2 week 
internals with tissue-derived JDV vaccine. Serum was taken 2 weeks after the final 
inoculation. BIV hyperimmune serum was produced in a bovine lentivirus-free 
animal with cell-culture preparations of BIV-R29 prepared as described in Chapter 4. 
This cell culture material was emulsified in Freund’s incomplete adjuvant and 
inoculated 3 times at 2-week intervals and serum was collected 2 weeks after the 
final inoculation. The first inoculation was from freshly harvested cell culture 
material while the final 2 inoculations were from frozen cell culture material. The 
reactivity of the JDV hyperimmune serum has previously been reported (Desport et 
al., 2005). The BIV hyperimmune serum reacted with JDV CA and BIV CA and SU 
proteins on a WIB.
Overlapping synthetic peptides

One hundred and fifty five overlapping synthetic peptides were constructed to cover the entire amino acid sequence of MA, CA and SU of JDV\textsubscript{Tab87} (accession number U21603). The peptides were designed to be 16 amino acids long and to overlap each other by 11 amino acids. This length allows for coverage of the three regions while the minimal overlap enables mapping to a fine specificity and takes into account the significant expense of peptide production. The peptides were synthesised by automated 9-fluorenylmethyloxycarbonyl chemistry (Mimotopes, Australia) as previously described (Valas et al., 2000). An amino-terminal biotinylated tetrapeptide (Ser-Gly-Ser-Gly) was added to all peptides to facilitate epitope accessibility and absorption to streptavidin-coated wells. All peptides were dissolved in 200 µl of cell culture grade, 100% DMSO (Sigma-Aldrich). For use in the ELISA, peptides were further diluted 1:200 in PBS-T (PBS containing 0.1% Tween-20 [Sigma] and 0.1% sodium azide [Sigma]).

ELISA

Serum samples were tested at a dilution of 1:50 against each peptide in a standard ELISA according to the peptide manufacturer’s instructions. Plates were coated with 100 µl of 5 µg/ml stock streptavidin (Sigma-Aldrich) and left to evaporate overnight at 37°C. Plates were blocked for 2 h with 200 µl of blocking solution (0.01 M PBS containing 2.5% gelatin [Bio-Rad], 5% rabbit serum [Invitrogen], 1% sodium caseinate [MP Biomedicals], 0.1% Tween 20 [Sigma]). All incubation steps were conducted at room temperature (~25°C) on a rocker platform. Plates were then washed 4 times with PBS-T. Peptides were further diluted 1:4 in PBS-T and 100 µl of peptide solution were added to each ELISA well, in duplicate, and incubated for 1 h. Plates were again washed 4 times. Serum was diluted 1:50 using PBS-T and
100 µl added to each well. Plates were incubated for 1 h then washed a further 4 times. Goat anti-bovine IgG HRP-conjugated (MP Biomedicals) was diluted 1:2 000 in 0.01 M PBS with additional 1% sheep serum (Invitrogen), 0.1% Tween 20 and 0.1% sodium caseinate, 100 µl was added to each well. Plates were incubated for 1 h then washed 4 times with PBS-T and 2 times with PBS only. Substrate (SigmaFast OPD; Sigma) was prepared as per the manufacturer’s instructions and 100 µl was added to each well. After colour development, the reactions were stopped with 3 M H₂SO₄ and read at 450 nm.

**Statistical analysis**

A model previously developed and described to map linear epitopes in HIV-1 was used to analyse the data produced in the ELISA (Loomis-Price et al., 1997). The cut-off for reactivity of individual peptides was determined as follows: the median and first quartile values (of the optical densities) were determined separately for each serum sample and for each block of peptides. The data were normalised by subtracting the median reactivity of the set from each value in the set and the standard deviation was calculated:

\[
\text{Standard deviation (}\sigma\text{)} = \frac{\text{median} – \text{first quartile}}{0.675}
\]

The data were then divided by the calculated standard deviation and expressed as normalised reactivity (\(\sigma\)) compared to the median. Measurements above a cut-off of 5 \(\sigma\) were considered positive.

The overlapping peptide set was screened with pre-infection sera to determine the level of background binding present for each serum sample. Unless the level of reactivity increased over time, the peptide was scored as non-reactive.

Immunoreactive (IR) peptides were defined as those which reacted with 75% or
greater of the serum samples tested and immunodominant (ID) peptides defined as those which reacted with 100% of the serum samples tested, as previously reported (Ball et al., 1992).

Results

When the overlapping peptides were tested against sera from cattle after infection with JDV\textsubscript{Tab87}, a number of peptides were not reactive against any of the sera tested, including peptide MA18 in MA (Figure 6.1A), peptides CA34, CA35, CA42 and CA62 in CA (Figure 6.2A) and peptides SU78, SU85, SU92, SU121, SU138, SU145, SU148 and SU149 in SU (Figure 6.3A). One peptide (MA24) was identified as IR in MA (Figure 6.1A), one peptide (CA70) was identified as IR in CA (Figure 6.2A) and 4 peptides (SU112, SU152, SU154 and SU155) were as IR in SU (Figure 6.3A). The locations of these peptides on the linear amino acid sequence of JDV\textsubscript{Tab87} are shown in Figures 6.5 and 6.6. No peptides were identified as ID in MA, CA or SU using the Ball et al. (1992) definitions. Using the less stringent definitions reported in a separate study (Valas et al., 2000), whereby IR peptides were defined by greater than 20% reactivity with bovine sera and ID peptides defined by greater than 58% reactivity, 22 MA peptides were IR and 1 was ID, 27 CA peptides were IR and 4 were ID, and 44 SU peptides were IR and 8 were ID.

When the overlapping peptides were screened with serum taken from cattle experimentally infected with JDV\textsubscript{Pul01}, a number of peptides were not reactive against the JDV\textsubscript{Pul01} sera, including peptides MA6, MA10, MA13, MA14 and MA22 in MA (Figure 6.1B), peptides CA26, CA27, CA38, CA39, CA43, CA44, CA55, CA62, CA63, CA67, CA68 and CA70 in CA (Figure 6.2B) and peptides SU78-80, SU83, SU84, SU86-88, SU90, SU94, SU95, SU111, SU112, SU114, SU116, SU131, SU135, SU136, SU147 and SU154 in SU (Figure 6.3B). Numerous peptides
were IR against the sera from JDV\textsubscript{Pul01} infected animals including peptides MA4, MA15, MA21 and MA25 in MA (Figure 6.1B), peptides CA41, CA49, CA52 and CA69 in CA (Figure 6.2B) and peptides SU100, SU106, SU108, SU110, SU115, SU118, SU120, SU121, SU126, SU145 and SU150 in SU (Figure 6.3B). Peptides CA53, SU96 and SU102 were ID.

When the overlapping peptides were screened with serum taken from cattle experimentally infected with BIV, peptides not reactive with the BIV sera tested included peptide MA10, MA12 and MA18 in MA (Figure 6.1C), CA33, CA42 and CA63 in CA (Figure 6.2C) and peptides SU74, SU83, SU85, SU86, SU88, SU92, SU93, SU95, SU103, SU105, SU111, SU114, SU119, SU135 and SU137 in SU (Figure 6.3C). Peptide 25 in MA (Figure 6.1C), peptides CA29, CA53 and CA55 in CA (Figure 6.2C) and peptides SU126, SU129, SU100, SU118 and SU123 in SU (Figure 6.3C) were all IR and no peptides were ID against these sera.

The 2 hyperimmune serum samples tested reacted against different peptides. JDV\textsubscript{Tab87} hyperimmune serum reacted particularly strongly against peptide SU83 as well as peptide MA5 (Figure 6.4). In contrast, BIV hyperimmune serum reacted strongly to peptides in 3 regions of CA: CA57 and 58, peptides CA44 and CA45, and CA65 (Figure 6.4).
Figure 6.1. Reactivity to peptides derived from the MA sequence of JDV_{Tab87} to serum samples taken (A) 56-159 days after infection of cattle with JDV_{Tab87}, (B) 71-102 days after infection of cattle with JDV_{Pul01} and (C) 35-101 days after infection of cattle with BIV.
Figure 6.2. Reactivity to peptides derived from the CA sequence of JDV$_{\text{Tab}87}$ to serum samples taken (A) 56-159 days after infection of cattle with JDV$_{\text{Tab}87}$, (B) 56-159 days after infection of cattle with JDV$_{\text{Tab}87}$ and (C) 35-101 days after infection of cattle with BIV.
Figure 6.3. Reactivity to peptides derived from the SU sequence of JDV~Tab87~ to serum samples taken (A) 56-159 days after infection of cattle with JDV~Tab87~, (B) 71-102 days after infection of cattle with JDV~Pul01~ and (C) 35-101 days after infection of cattle with BIV.
Figure 6.4. Reactivity of hyperimmune serum to overlapping peptides spanning the MA (peptides MA1-25), CA (peptides CA26-71) and SU (peptides SU72-155) of JDV. A: reactivity with JDV Tab87 hyperimmune sera. B: reactivity with BIV-R29 hyperimmune sera. Connecting lines are shown for clarity and are not meant to imply continuous data. Horizontal line (-----) indicates the cut-off of 5 $\sigma$. 
Figure 6.5. Linear representation of JDV MA and CA epitopes. A panel of sera taken from animals experimentally infected with JDV_{Tab87} were reacted in ELISA against the panel of JDV MA and CA synthetic peptides. Bovine humoral epitopes are portrayed along the linear amino acid sequence of the JDV_{Tab87} gag precursor protein. Grey highlighted sequences delineate immunoreactive peptides which are recognized by 75% or more of the bovine sera. The BIV gag precursor protein sequence is also shown below the JDV sequence for comparison. The sequences were aligned using ClustalW2 (Larkin, 2007). “*” indicates identical residues, “:” indicates conserved substitutions and “.” indicates semi-conserved substitutions.
Figure 6.6. Linear representation of JDV SU epitopes. A panel of sera taken from animals experimentally infected with JDV Tab87 were reacted in ELISA against the panel of JDV SU synthetic peptides. Bovine humoral epitopes are portrayed along the linear amino acid sequence of the JDV Env precursor protein. Grey highlighted sequences delineate immunoreactive peptides which are recognized by 75% or more of the bovine sera. Three immunoreactive peptides are shown at the carboxyl end of the protein; these are distinguished by the grey highlighted text (1), the underlined text (2) and the bolded text (3). The BIV Env precursor protein sequence is also shown below the JDV sequence for comparison. The sequences were aligned using
ClustalW2 (Larkin, 2007). “*” indicates identical residues, “:” indicates conserved substitutions and “.” indicates semi-conserved substitutions.

**Discussion**

The investigations conducted were designed to use synthetic peptides to identify antigenic sites on the MA, CA and SU that would react differentially to antibody to JDV and BIV. Synthetic peptides have been used previously to map antigenic sites in a number of lentiviruses, including EIAV (Ball et al., 1992; Grund et al., 1996; Soutullo et al., 2007), the SRLV CAEV and VMV (Mordasini et al., 2006; Rosati et al., 1999; Valas et al., 2000) and HIV-1 (Loomis-Price et al., 1997; Neurath et al., 1990). They have also been used with other virus systems including human cytomegalovirus (Greijer et al., 1999), *Foot and mouth disease virus* (Geysen et al., 1987) and *Epstein-Barr virus* (Middeldorp et al., 1988). The peptides were able to identify specific linear sites which were immunogenic in the native protein antigens. It is recognised, however, that a limitation of these studies as well as the current study is that the use of synthetic peptides cannot identify discontinuous conformation-dependent epitopes which may represent important antigenic determinants of viral proteins.

Another limitation of this current study was that it was necessary to use serum from BIV-R29 infected animals to screen the peptides and examine for differences in the reactions comparative to those in JDV-infected cattle. This BIV strain may not necessarily have a close antigenic relationship to the putative non-pathogenic bovine lentivirus in Indonesia. However, this was the only BIV serum available and sera from antibody-positive cattle in Sulawesi, which would have been a suitable alternative, were unavailable for this study.
Sera from cattle experimentally infected with JDV\textsubscript{Tab87} or JDV\textsubscript{Pul01} were used to screen the peptides for JDV-reactive epitopes as these are 2 reasonably well defined strains that have been detected in Indonesia and the samples had a well documented history. Of a number of JDV strains sequenced, JDV\textsubscript{Pul01} is the most divergent from JDV\textsubscript{Tab87} in \textit{env} and \textit{gag} regions (Desport et al., 2007) and we sought to determine whether the differences extended to the humoral response. The amino acid sequence homology between the two strains in the entire \textit{env} region and part of the \textit{gag} regions is 97% (Desport et al., 2007). Differences also existed between the reaction of cattle infected with these 2 JDV strains in regards to peak viral loads and duration of viraemia (Desport et al., 2009a). Differences were detected in the reactivity of the peptides between the JDV\textsubscript{Tab87} and JDV\textsubscript{Pul01} sera. The JDV\textsubscript{Pul01} sera had a larger number of IR peptides, with 3 ID peptides CA53, SU96 and SU102. These differences could be attributed to the fewer number of cattle analysed and it would be of interest to screen the peptides with more JDV\textsubscript{Pul01} sera to confirm these results.

The synthetic peptides used in this study encompassed the complete JDV\textsubscript{Tab87} MA, CA and SU regions. The MA, CA and SU proteins were chosen for investigation for a number of reasons. Firstly, the strongest and earliest immune responses against JDV are directed at CA (Hartaningsih et al., 1994; Kertayadnya et al., 1993) although a subset of cattle do not mount an immune response against CA (Desport et al., 2009a; Ditcham et al., 2009). Matrix was chosen as BIV was reported to contain at least one unique epitope in the CA that is absent in JDV, at the 6.4-kDa N terminus of the 29-kDa CA adjacent to MA (Zheng et al., 2001) and strong antibody responses against MA have been previously identified in experimentally infected animals (Desport et al., 2005; Kertayadnya et al., 1993). SU was included as anti-Env responses have been reported to persist beyond 190 weeks after BIV infection whilst
the Gag response wanes 40 weeks after infection (Isaacson et al., 1995), *env* sequences between JDV strains are reasonable well conserved (Desport et al., 2007) and there are a significant number of differences between the sequence of the SU regions of JDV and BIV (Chadwick et al., 1995b, Figure 6.6, Table 2.2). Little information is currently available detailing the antigenicity of JDV SU due to problems expressing recombinant SU.

In the ELISA used to examine differential reactivity of the peptides, a cut-off of 75% and greater positive reactivity to represent a significant B-cell epitope was used, as reported by others (Ball et al., 1992). Other studies have utilised lower cut-offs of 33% (Kusk et al., 1992) and 50% (Valas et al., 2000) but a higher cut-off was used in the current study to identify highly reactive B-cell epitopes in the JDV proteins.

There is potential for the use of IR peptides, those reacting with a high percentage of serum samples tested, and ID peptides reacting with all serum samples, to facilitate the development of a peptide-based ELISA able to identify most animals infected with JDV or both JDV and BIV depending on the peptides used. Several potentially useful IR peptides were detected.

One IR MA peptide was identified which spanned amino acids 116 to 131 of the CA and MA proteins. This peptide spanned the MA-CA border and indicates that the antibodies which recognise this peptide are produced in response to the uncleaved Gag precursor protein.
Sera from long-term JDV infections (>12 months after infection) were reported to recognise a protein of the same size as MA (Kertayadnya et al., 1993) and these findings identify an epitope within MA. Some JDV-infected cattle (CB83-86) have been reported not to respond to MA within 175 dpi when tested in an ELISA using recombinant MA (Ditcham et al., 2009) but MA24 reacted with sera from all of these cattle (Table 6.1). The difference between the responses reported by Ditcham et al. (2009) and those in the current study may be due to differences associated with the sequences covered by the antigens. The responses reported by Ditcham et al. (2009) were those generated when using a full length recombinant MA while MA24 spans the MA-CA border, encompassing additional sequence. Peptide ELISA has been shown to be more sensitive than an ELISA using recombinant proteins (Rosati et al., 1999). Coincidentally, most variation in JDV Gag occurs just before the predicted cleavage point between the MA and CA proteins (Desport et al., 2007) and this, combined with the identification of an IR peptide in this region, suggests this may be a region under pressure from the immune system.

The IR CA peptide reported here is within amino acids 346 – 360 of the Gag precursor protein and spans the CA-NC border. Like the MA response, it also suggests that the antibodies are produced in response to the uncleaved Gag precursor polyprotein. Hyperimmune sera against whole JDV and BIV were reported to not recognise recombinant proteins encompassing this region, presumable because of the differences in the way hyperimmune sera is raised compared to sera from natural or experimental infections (Desport et al., 2005). As shown in Figure 6.1, JDV hyperimmune serum did react to this peptide while BIV hyperimmune sera did not. A highly immunogenic domain has been reported in a similar location for EIAV (Chong et al., 1991b).
There were no IR peptides found within the MHR in CA when the peptides were screened with JDV\textsubscript{Tab87} sera. The lack of an JDV\textsubscript{Tab87} IR peptide in this region is surprising given that previous studies have shown that it is likely to be an epitopic domain within JDV (Desport et al., 2005). The MHR is conserved among retroviruses and is essential for virus assembly, maturation and infectivity (Mammano et al., 1994) and is assumed to account for some of the cross-reactivity observed between the Gag proteins of HIV, EIAV and BIV. Previous studies have shown cross-reactivity between BIV hyperimmune sera and the JDV MHR (Desport et al., 2005), although confirmation is required. Peptides CA52-59 spanned the MHR and although they were not identified as IR in the current study, a number of peptides had high reactivities against JDV\textsubscript{Tab87} sera, including CA53–55, 57 and CA58. Peptide CA57 had the second highest reactivity of all the CA peptides with a reactivity of 62.5%. The CA53 peptide was a JDV\textsubscript{Pul01} ID peptide and when screened with BIV sera, peptides CA53 and CA55 were found to be IR, confirming that this is one region of cross-reactivity. The BIV hyperimmune serum used in this study also strongly reacted to this region (Figure 6.4). JDV hyperimmune serum also reacted to the MHR, albeit more weakly than the BIV hyperimmune serum.

Surprisingly, the major homology region identified as an immunodominant domain in the CA proteins of many of the lentiviruses was not identified as IR or ID using this method of analysis. This may indicate that the level of stringency was too high or may be due to the characteristics of the sera chosen or the conformation of the peptides. Previous studies have shown that conformational changes to peptides, such as converting the peptides to a cyclical form, can help to increase reactivity (Scobie et al., 1999). Further studies are required to clarify this discrepancy.
Five IR peptides were found within SU. Peptide SU112 spanned amino acids 201–216 in the central portion of the protein. The most IR peptide, reactive with 87.5% of the sera tested, was SU134 and this spanned amino acids 311–326. The remaining 3 peptides, SU152, SU154 and SU155, clustered around the carboxyl end of SU and spanned amino acids 401–430. The reduction in reactivity for peptide SU153 compared with the reactivity for peptides SU152 and SU154 indicated that there may be 2 different epitopes at this location, as reported in CAEV SU (Valas et al., 2000). The clustering of IR peptides around the carboxyl end of SU is similar to CAEV (Bertoni et al., 2000; Valas et al., 2000), HIV-1 (Palker et al., 1987) and EIAV (Ball et al., 1992; Grund et al., 1996), although these viruses also have IR regions at the amino end of SU which were not identified in this current study of JDV. FIV also has an epitope at the carboxyl end of SU, progressing into the start of TM (Pancino et al., 1993), similar to the region encompassed by peptides SU154 and SU155 in the current study. It was suggested that the antigenicity of terminal segments of proteins, including lentiviral SU glycoproteins, is because these regions are frequently surface orientated and thus exposed and immunogenic in their native state (Ball et al., 1992; Valas et al., 2000; Van Regenmortel, 1999b).

No ID peptides were identified with the JDV_{Tab87} serum. This might be associated with the outbred nature of the Bali cattle population, as has been suggested in the T-cell epitope mapping in FIV (Dean et al., 2004). A number of ID peptides were identified with the JDV_{Pul01} serum including 1 in CA and 2 in SU (Figures 6.2 and 6.3). Reactivities with the JDV_{Tab°/87} sera to these peptides ranged from 25 to 50% (Figures 6.2 and 6.3). Further investigations are required to determine whether the differences are attributed to strain variation or to the number of samples analysed.
The differential reaction of some peptides to JDV sera compared to BIV sera suggests some of these peptides could be used to form antigens for potential JDV-specific and broadly reactive bovine lentivirus serological assays. Peptides which were reactive against 50% of sera taken from JDV\textsubscript{Tab87}, JDV\textsubscript{Pul01} and BIV-R29 would be useful to include in a bovine lentivirus serological assay as these should detect a majority of cattle infected with these bovine lentiviruses. The areas encompassed by these peptides are significant regions of cross-reactivity since a majority of both JDV and BIV sera reacted to these peptides.

Peptides that were not recognised by BIV sera are potential candidates for inclusion in a JDV-specific ELISA antigen, an approach previously suggested in CAEV (Valas et al., 2000). Unfortunately, none of these peptides were IR or ID against the JDV sera only. However, a combination of 5 of these peptides, SU93, SU95, SU103, SU119 and SU135 from SU, would have reacted with 87.5% of the JDV-only sera, although this specific combination would not have detected animals infected with JDV\textsubscript{Pul01}. The addition of 2 extra peptides, MA18 and MA19 would provide reactivity with the JDV\textsubscript{Pul01} sera. It is important to note, however, that this approach would potentially provide a JDV-specific serological assay and not a BIV-specific assay. Previous diagnostic assays have combined multiple antigens in an ELISA format (Khan et al., 2006) and it would be practical to combine these peptides in this format.

This study needs to be extended by testing sera from naturally infected cattle as well as by testing the longitudinal responses to the IR peptides in experimentally infected cattle, and applying the tests in a clinical setting in Indonesia. Consideration could also be given to using selected peptides in the formulation of vaccines capable of protecting against JDV, as has been reported in a number of lentivirus systems.
including SIV (Belyakov et al., 2001; Nehete et al., 2008) and HIV-1 (Hovanessian et al., 2004). Due to the persistent nature of the anti-TM antibody response in BIV infections (Isaacson et al., 1995, Scobie et al., 1999), the TM glycoprotein may also be a promising linear antigenic target and therefore extending this study to the TM glycoprotein may yield a potential antigen for inclusion in a differential serological assay. It would also be of interest to test the response of cattle superinfected with both BIV and JDV and their pattern of reactivity over time.
Chapter 7: General discussion

The close genetic and antigenic relationship between BIV and JDV raised 2 issues that were investigated and are reported in this thesis. First, in animals that are infected with both viruses, it was hypothesised that there might be cross-protective immunity or other interaction between the 2 viruses that could modify their pathogenesis. Second, there is a need for serological tests that will differentiate antibody to the pathogenic JDV and other non-pathogenic bovine lentiviruses. The presence of a BIV-like virus in cattle on the island of Sulawesi where Jembrana disease does not occur could have a marked effect on the events that might occur if JDV spreads to that island. In an endemic area, previous infection with a non-pathogenic bovine lentivirus like BIV might ameliorate the effect of subsequent infection with JDV, resulting in subclinical JDV infections. Immunosurveillance for JDV infection would be affected by the presence of a second antigenically cross-reactive but non-pathogenic bovine lentivirus in the cattle population of Indonesia.

The investigations reported in this thesis have provided information that clarifies these issues.

Evidence was provided previously for the occurrence of a non-pathogenic bovine lentivirus in Bali cattle in Indonesia, although this was based on serological evidence only and the virus has not been detected. This serological evidence, particularly that presented by Barboni et al. (2001), is difficult to evaluate because other investigations have demonstrated a very close antigenic relationship between JDV and BIV and there are no reagents or tests available that allow differentiation of antibody to the 2 types of virus in cattle sera (Desport et al, 2005). Antibody to JDV was detected in \textit{B. javanicus} in Sulawesi where there is no evidence of Jembrana disease (Hartaningsih, personal communication) suggesting the presence of a non-
pathogenic bovine lentivirus antigenically related to JDV. Blood from a seropositive animal in Sulawesi was inoculated into Bali cattle in an attempt to transmit the non-pathogenic bovine lentivirus but these attempts were unsuccessful (Hartaningsih, personal communication). For further investigation of the effects of the Indonesian strain of this non-pathogenic bovine lentivirus in Bali cattle, an attempt was made to detect the virus in cattle on the island of Bali (Chapter 3). A large number of cattle were screened using PCR and serological assays which detect both BIV and JDV. While a number of cattle were identified that contained proviral JDV DNA, BIV proviral DNA was not detected and the investigations reported in Chapter 3 therefore provide no evidence for the occurrence of a second non-pathogenic bovine lentivirus in these cattle. Isolation of virus in cell culture was not attempted in this study due to a lack of suitable facilities.

The lack of evidence of a BIV-like virus reported in Chapter 3, however, was insufficient to eliminate the possibility that there is a second non-pathogenic bovine lentivirus in the cattle population of Bali as only a limited number of samples from one area were screened and the seroprevalence was lower than expected. Previous investigators have concluded that BIV is difficult to detect in cattle. While numerous studies have reported serological evidence for BIV infection (Barboni et al., 2001; Bhatia et al., 2008; Horzinek et al., 1991; Meas et al., 1998; Meas et al., 2000a; Whetstone et al., 1990) there have been only 3 reports of isolation of the virus from cattle (Meas et al., 1998; Suarez et al., 1993; Van der Maaten et al., 1972). Suarez et al. (1993) were only successful in the isolation of virus on 2 occasions from many samples and only after 4 blind passages of samples in cell culture. A very exceptional result was reported from Japan, where an unusually high isolation rate of BIV in cell culture was reported from PBMC and milk-derived leukocytes from BIV-antibody-
positive dairy cattle. These isolates had 99.0 to 99.7% nucleotide sequence identity with the R29 strain of BIV within a 258 nucleotide amplicon from the pol region. These authors were also able to identify BIV proviral DNA by nested PCR and Southern blot in cattle and buffalo samples from Japan, Cambodia and Pakistan (Meas et al., 1998; Meas et al., 2000a; Meas et al., 2000b). The reports by Meas and colleagues are interesting as they suggest that infection with BIV is widespread and reasonably easy to detect in buffalo and cattle in Asian countries, and that virus isolation was possible from antibody-positive cattle.

The study of BIV infection in Bali cattle reported in Chapter 4 is the first report of the pathogenesis of BIV infection in this species. Because of the unusual susceptibility of Bali cattle to JDV and Malignant catarrhal fever virus (Soesanto et al., 1990) it was hypothesised that these cattle might also show a greater susceptibility to BIV than do B. taurus but their susceptibility appeared generally similar to the effects reported in B. taurus (Scobie et al., 1999; Suarez et al., 1993; Whetstone et al., 1990). We also considered that BIV in Bali cattle might have very different effects to those observed in B. taurus, as some lentiviruses do not cause disease in their natural host (VandeWoude et al., 2006) but do so in heterologous hosts, a phenomenon best described with SIV strains (Apetrei et al., 2004) but also recognised with other lentiviruses such as puma and lion lentivirus infection in domestic cats (VandeWoude et al., 1997). It has been reported that infection of B. taurus with JDV resulted in a mild disease only, of much less severity that observed in B. javanicus (Soeharsono et al., 1995a), and the reciprocal event, greater susceptibility of Bali cattle to BIV was a potential outcome.

Bali cattle inoculated with BIV did not develop clinical signs within 62 days of infection and this suggested that if the putative bovine lentivirus present in Sulawesi...
is indeed related to BIV-R29, then it should have no significant effect on animal health in the Bali cattle population. A result that is significant in terms of the optimal method for detection of natural BIV infection in Bali cattle, and possibly *B. taurus*, was that there was a transient period of viraemia detected after infection and it is during this period that the virus would be most easily detected. However, in natural infections, the timing of infection is unknown and therefore these results show that the choice of assay is an important one. If a recent infection is suspected, then a genome based assay would be most likely to detect infection but if a longer term infection is suspected then a ELISA using a TM peptide would be more appropriate. This transient period of viraemia was analogous to that which occurs in other animal lentivirus infections such as JDV infection of Bali cattle (Stewart et al., 2005), EIAV infection of horses (Harrold et al., 2000) and SIV<sub>smm-PBj14</sub> infection of pig-tailed macaques (Dewhurst et al., 1990; Fultz et al., 1989), although BIV was found to have significantly lower titres than these viruses. It is suggested that BIV infection of *B. taurus* should be re-examined using sensitive techniques such as qRT-PCR to see whether a similar acute phase viraemia is produced. Sensitive techniques need to be used in such an investigation as the level of viraemia in Bali cattle infected with BIV was low and virus was not detected consistently in any animal, reflecting the difficulty of detecting BIV infection even in experimentally infected cattle. The majority of infected cattle had a significant TM antibody response but a poor CA response but this occurred after the transient viraemic period, suggesting that detection of BIV proviral DNA would be most successful in antibody-negative cattle, similar to the result obtained for JDV in field cattle (Chapter 3). The ability to detect BIV prior to the onset of an antibody response but not afterwards is in contrast to the
report of the high frequency of isolation of BIV from antibody-positive cattle in Asia (Meas et al., 1998).

A limitation of this current study of BIV-R29 in Bali cattle is that an Indonesian BIV isolate was not discovered and could not be used for experimental studies, and the BIV-R29 isolate used might not reflect what would occur with the putative BIV-like virus present in Indonesia. However, other Asian isolates have been shown to be closely related to BIV-R29 (Meas et al., 1998). The R29 strain of BIV may have a different pathogenesis to the BIV-like virus present in Indonesian cattle. The results obtained may also not be typical of BIV infection under field conditions as it is considered by some that the BIV-R29 strain may have, since its isolation in 1969 (Van der Maaten et al., 1972), become attenuated during its prolonged storage and passage in cell culture (Whetstone et al., 1997). However, an unsuccessful attempt was made to increase the virulence of BIV-R29 by its serial passage through cattle (Whetstone et al., 1990), a technique known to increase the virulence of EIAV (Orrego et al., 1982), and perhaps this inability to increase its virulence reflects a natural low pathogenicity. Other BIV strains isolated from Florida were thought to be more pathogenic than BIV-R29, causing a B-cell lymphocytosis in experimentally infected cattle, but the effects of the Florida isolates in cattle were still very mild (Suarez et al., 1993; Whetstone et al., 1997). It is possible, however, that the inoculation of the Florida isolates into Bali cattle may produce results different to that observed with the R29 strain.

The lower antibody response to the CA versus the TM antigen was an unexpected finding in BIV-infected Bali cattle. Animals infected with lentiviruses normally develop a strong antibody response to CA and this response is the first to be detected (Rosati et al., 2004; Soutullo et al., 2007). A strong response to CA has also been
documented in Bali cattle infected with JDV (Hartaningsih et al., 1994) although there is a subset of JDV infected Bali cattle that do not develop a detectable CA antibody response (Desport et al., 2009a; Ditcham et al., 2009). A loss of Gag-reactivity in the presence of a sustained Env response has previously been reported in *B. taurus* infected with BIV (Isaacson et al., 1995).

In several lentivirus systems, infection with a non-pathogenic lentivirus is able to protect against subsequent superinfection with a closely related pathogenic lentivirus (Cranage et al., 1998; VandeWoude et al., 2002). An investigation of whether the same was true for BIV and JDV in Bali cattle showed that BIV-R29 infection did not induce a protective immune response against subsequent infection 42 days later with JDV. All BIV-infected animals inoculated with JDV became infected and had a viraemic phase and a febrile response typical of JDV infection. However, while previous BIV infection did not protect against subsequent JDV infection, there was no enhancement of JDV infection even though it did cause an earlier onset and resolution of fever that was associated with JDV infection. The result suggests that if BIV-R29 is related to the BIV-like lentivirus circulating within the cattle population of Sulawesi, if JDV were introduced into the cattle population of Sulawesi it would not lead to disease enhancement.

The lack of any evidence of a protective immunity against JDV as a consequence of previous BIV infection was unexpected, considering the extensive antigenic cross-reactivity of JDV and BIV proteins and evidence of an antibody response to BIV at the time of superinfection with JDV. It will be necessary to determine if protection against JDV can be achieved by altering the parameters of the superinfection. Perhaps if the dose of BIV used to infect the cattle was increased this would affect the result: higher doses of *SIV*<sub>macC8</sub> provided greater protection against challenge
with SIV\textsubscript{mac} (Cranage et al., 1998). Alternatively, the time interval between BIV and JDV infection could be important: greater protection has been provided by a decreased time of 21 days between superinfection with closely related simian lentiviruses (Stebbings et al., 2004) but it is also possible that a longer interval may be required for the development of a cell-mediated immune response. Although desirable, analysis of cell-mediated immune responses was not possible and so the type of immune response required to control infection could not be adequately assessed.

An important technical requirement for immunosurveillance of JDV infection in Indonesia is the development of reliable and specific serological tests. JDV and BIV contain an extensive array of cross-reactive epitopes on several proteins (Desport et al., 2005; Kertayadnya et al., 1993) and using serological assays, differentiation of infection by the 2 viruses using serological assays is currently not possible. The investigation reported in Chapter 6 where a series of overlapping peptides spanning the MA, CA and SU regions of JDV was used to identify epitopes that would react specifically to JDV and not BIV has identified potential peptides that could be used for this purpose. Seven peptides were identified within the MA-CA and the CA-NC junctions, while 5 were identified in SU, that reacted with >75% of sera from JDV-infected cattle. Unfortunately, these peptides also reacted with some sera from BIV-infected cattle and could not therefore be used for the development of a JDV-specific serological assay. A combination of these peptides could, however, be used as an alternative antigen for the development of a broadly-reactive bovine lentivirus serological test. A number of peptides were identified which reacted with sera from JDV-infected cattle only and hence have potential for development of a JDV-specific serological assay. These peptide combinations must now be tested using an extensive
array of sera from naturally infected cattle from areas of Indonesia where infection with both BIV and JDV have been suspected, such as Bali, and in regions where infection with a non-pathogenic BIV is suspected and where Jembrana disease has not been detected, such as Sulawesi. A longitudinal series of serum samples from individual cattle also needs to be tested to determine how the antibody response to the peptides changes over time.

In conclusion, the results reported in this thesis have made a significant original contribution to our understanding of BIV infection in Bali cattle that indicate BIV is unlikely to be a significant pathogen in these cattle. The results obtained have also provided insights into the interaction of BIV and JDV in Bali cattle which suggest that previous BIV infection will not provide significant cross-protective immunity against subsequent JDV infection, and that BIV infection is unlikely to provide a simple method of vaccinating cattle against JDV infection. The results have also provided information about epitopes of the Gag and SU proteins of JDV that indicate there are potential epitopes on these proteins that could be used for the development of JDV-specific serological tests needed in Indonesia.
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