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Comparison of Immunoassay and Real-Time PCR Methods for the Detection of Jembrana disease virus Infection in Bali Cattle

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Abstract

A sensitive diagnostic assay for the detection of infections with the bovine lentivirus Jembrana disease virus (JDV) is required in Indonesia to control the spread of Jembrana disease. Immunoassays are used routinely but are compromised by cross-reactive epitopes in the capsid (CA) protein of JDV and the genetically related bovine immunodeficiency virus (BIV). JDV *gag*-specific primers were tested in a real-time PCR assay to detect proviral DNA in peripheral blood mononuclear cells from 165 cattle from the Tabanan district of Bali. JDV-specific amplicons were detected in 9% of the cattle and only 33% of the real-time PCR positive cattle were also seropositive. The delayed seroconversion that occurs after infection with JDV could explain the low concordance between these assays but other factors may be responsible. BIV proviral DNA was not detected in any of the PBMC DNA samples. A high concordance value of 98.6% was found between the JDV plasma derived antigen Western blot and the JDV p26-his recombinant protein ELISA. Only 21% of the seropositive cattle had detectable levels of proviral DNA suggesting that the proviral load in recovered cattle is low. A combination of real-time PCR and JDV p26-his ELISA is recommended for the detection of infection with JDV in Indonesia.

Keywords: Jembrana disease virus; bovine lentivirus; BIV; capsid; ELISA; immunoassay; transmembrane epitope

1. Introduction

Jembrana disease (JD) first occurred in the Jembrana district of the island of Bali in Indonesia in 1964 affecting Bali cattle (*Bos javanicus*). The disease spread quickly to cattle in surrounding districts in Bali and has since been reported in Sumatra, Java and Kalimantan (Desport et al., 2007; Hartaningsih et al., 1993; Soeharsono et al., 1995). Jembrana disease virus (JDV) is a lentivirus that is very similar genetically and related antigenically to the other bovine lentivirus, bovine immunodeficiency virus (Burkala et al., 1999; Chadwick et al., 1995; Desport et al., 2005; Kertayadnya et al., 1993). Unlike BIV, JDV causes an acute infection after a short incubation period of between 4 to 15 days (Kertayadnya et al., 1993; Soesanto et al., 1990). High plasma viral loads (>10^8 ID_{50}/ml) are detected as the cattle become pyrexic, coinciding with a marked leucopaenia which resolves as the temperature returns to normal and the plasma viraemia declines. Cattle infected with JDV are immunosuppressed transiently (Wareing et al., 1999) and do not develop detectable anti-capsid antibody responses until 5-15 weeks post-infection (Kertayadnya et al., 1993). The CA antibody response peaks between 23 and 33 weeks post-infection and persists beyond 59 weeks post-infection (Hartaningsih et al., 1994) whilst the virus has been shown to persist at low levels for at least 25 months after infection (Soeharsono et al., 1990).

Diagnosis of JD is achieved currently by monitoring clinical signs during the acute stage of the disease or by using immunoassays with purified plasma derived viral antigens to identify recovered cattle in field surveys (Hartaningsih et al., 1993; Hartaningsih et al., 1994). Recombinant protein antigens are

replacing viral antigens increasingly in diagnostic immunoassays for lentiviruses as they have been found to be more sensitive and easier to produce (Celer and Celer, 2001; de Andres et al., 2005). Capsid antigens are often used in these assays since antibodies to this protein develop first and are usually highly conserved within each lentiviral group (Burki et al., 1992; Houwers and Nauta, 1989; Zanoni et al., 1991). Recombinant CA and truncated transmembrane (TM) proteins have been used for the detection of BIV infections using Western blot assays (Abed et al., 1999). An immunodominant epitope was identified in the N-terminal portion of BIV TM (Chen et al., 1994) and a TM peptide ELISA was developed from peptide mapping this region (Scobie et al., 1999). The combination of recombinant CA and TM peptides has now been applied to diagnostic ELISAs for JDV and SRLV (Barboni et al., 2001; Saman et al., 1999). Unlike other lentiviral assays where serological diagnosis usually occurs well before the clinical stage of the disease due to the chronic nature of these infections, reliable detection of seroconversion to JDV using immunoassays cannot be achieved until 5-15 weeks after the onset of clinical disease (Desport et al., 2009). A recent review of diagnostic assays for small ruminant lentiviruses (SRLV) concluded that a combination of ELISA and PCR would be the most reliable way to ensure that both recently infected and animals in the post-seroconversion phase of infection would be detected (de Andres et al., 2005). JDV proviral DNA from recovered cattle has been amplified successfully using PCR (Desport et al., 2007) and a qRT-PCR assay is used currently to quantify plasma viral loads during the acute stage of the disease (Stewart et al., 2005).

The future control of JD in Indonesia will require a combination of reliable diagnostic assays and surveillance techniques. A comparison of immunoassays using a combination of JDV CA (p26) and transmembrane (TM) antigens and plasma derived JDV antigen was undertaken. In addition, an assessment of the utility of a Real-Time PCR assay for the early detection of JDV proviral DNA was also evaluated.

2. Materials & methods

2.1 Viral strain, plasmid & bacterial host cells

The JDV $_{TAB/87}$ CA plasmid construct in pTrcHisC (Invitrogen) was kindly supplied by Dr Margaret Collins (Barboni et al., 2001) and was transformed into BL21 (DE3) *E. coli* for protein expression and purification (JDVp26-his).

2.2 Primers and PCR cloning strategy

Plasmid DNA from JDV clone Jgag6 (Desport et al., 2005) containing the entire JDV *capsid* was used as template for production of a fused JDV p26/TM peptide construct similar to other studies (de Andres et al., 2005; Rosati et al., 2004). Amplification was performed using primers p26BamF and p26TMEcoR (Fig. 1) and the amplified product was digested with appropriate enzymes before ligation into pTrcHisA plasmid digested with BamHI and EcoRI. Plasmids were transformed into Top 10F' *E. coli* competent cells initially and the resulting construct containing the JDV *capsid* sequence from 604 – 1222 fused directly to the putative TM principal immunodominant domain epitope was confirmed by sequence analysis. The plasmid was also transformed into *E. coli* BL21 cells for protein expression studies (JDV

p26/TM-his). A JDV TM peptide (KVQTGLGCVPRGRYCHFD) which has been reported to encompass the principal immunodominant domain of JDV TM (Barboni et al., 2001) was synthesised in linear form (Proteomics International, Perth) and dissolved in 0.01 M ammonium acetate to form a cyclic peptide (JDV TM^c peptide) as previously described (Scobie et al., 1999).

2.3 Protein expression & purification

The JDV p26-his and JDV p26/TM-his positive colonies were grown in 2YT plus 1 mM ampicillin to early log phase culture and induced with 0.1 mM isopropyl-ß-D-thiogalactopyranoside with agitation. The bacterial cells were pelleted, washed once in PBS and resuspended in lysis buffer (50 mM Tris-HCL pH7.5, 50 mM NaCl, 10 mM imidazole and 5% [v/v] glycerol). The cells were lysed by sonication and the soluble lysate fraction was collected by centrifugation. Eight bed volumes of lysate were added to 1 bed volume of Ni-NTA agarose resin (QIAGEN) in chromatography columns (BioRad) and agitated for 2 h. The lysate was allowed to flow out and washing was performed using native wash buffer pH 8 (250 mM NaH_2PO_4, 2.5 M NaCl and 50 mM imidazole). Four bed volumes of elution buffer pH 8 (250 mM NaH_2PO_4, 2.5 M NaCl and 250 mM imidazole) were added and the eluate collected for further analysis. The purified fractions were analysed by SDS-PAGE and stained with Coomassie Brilliant blue to determine yield and purity. Densitometry was used to quantify the levels of recombinant protein.

2.4 Serum samples

A panel of 165 sera and DNA samples was collected from Bali cattle sourced from the Tabanan district of Bali where Jembrana disease seropositives have been reported previously (Hartaningsih et al., 1993). A panel of 10 positive sera from experimentally infected cattle and 30 negative cattle from the JDV-free island of Nusa Penida (Hartaningsih et al., 1993) were used as reference sera to determine cut-off values for the ELISA assays. All positive and negative reference sera were tested by plasma-derived antigen Western blot and JDV p26-his Western blot to confirm their immunological status.

2.5 ELISA

Checkerboard titration of antigens and serum dilutions were performed to determine the optimal signal-to-noise ratios for positive and negative sera. NUNC Maxisorb™ plates were coated with either 0.2 μg of JDV p26-his, 0.05 μg of JDV p26/TM-his or 1 μg JDV TM peptide (linear or cyclic) per well diluted in 0.1 M carbonate coating buffer pH 9.5 and incubated overnight. ELISA assays were conducted with 100 µL volumes of reagents, except the substrate where a 50 µL volume was added. Sera were tested at a dilution of 1:200 (JDV p26-his and JDV p26/TM-his) or 1:16 (JDV TM^c peptide). The plates were washed with PBS/T (PBS plus 0.05% Tween 20) twice before blocking in PBS/T plus 5% skimmed milk powder and incubated for 30 min. The blocking solution was removed and the plates rinsed twice with PBS/T. Diluted sera were incubated for 1 h at 37°C, washed 3 times with PBS/T and a 1:2,000 dilution of rabbit anti-bovine IgG conjugated to HRP secondary antibody (ICN) in PBS/T plus 5% skimmed milk powder was added for 1 h at 37°C. The plates were further washed twice with PBS/T and twice with PBS

before colour substrate reagent (BioRad) was added for 15 min. The reaction was stopped with 2% [w/v] oxalic acid and the absorbance readings were taken at OD_{405nm}.

2.6 Evaluation of ELISA results

User-defined two-graph receiver operating characteristic (TG-ROC) was used with a Microsoft EXCEL (version 11) spreadsheet to select the cut-off values for the different antigens using reference positive (n=10) and negative (n=30) sera (Greiner et al., 1995). This program plots the test sensitivity and specificity against a threshold (cut-off value), assuming the latter to be an independent variable. Two cut-off values were established representing the lower and upper limits of the intermediate range (IR) or 'borderline samples' with a pre-selected accuracy level (95% sensitivity and specificity).

2.7 Statistical analysis

The agreement between the ELISA, WB and PCR assays was assessed by concordance (percentage overall test agreement) and by kappa values (to test that agreement is beyond chance agreement) with the reference assay (JDV plasma derived antigen WB) plus a positive reaction in at least one other assay (Fleiss, 1981).

2.8 Western Blotting

Western blots were prepared using 2 μg of the recombinant proteins and after overnight transfer nitrocellulose membranes were blocked using 5% skimmed milk in PBS/T for 30 min at room temperature. Test sera were diluted 1:25 in

blocking solution and incubated for 1 h. A 1:2000 dilution of horseradish peroxidase labelled rabbit anti-bovine IgG (ICN) was used followed by detection using HRP detection reagents (BioRad).

2.9 Extraction of DNA from PBMC

PBMC were prepared using Ficoll density gradient centrifugation. Briefly, peripheral blood was collected into sterile 10 ml tubes (15% EDTA; Vacutainer). Tubes were then centrifuged at 900 *g* for 10 min. The buffy coat layer was collected and mixed directly with 2 ml of PBS, overlayed onto 6 ml Ficoll (Amersham) in a sterile 10 ml tube and centrifuged at 400 *g* for 20 min at 4°C. The interphase was collected, cells were washed 3 times in cold PBS and centrifuged at 300 *g* for 5 min and after the third wash, cells were resuspended with 1 ml PBS and stored at -20°C until required. Genomic PBMC DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer's instructions and stored at -20°C until use. Any extraction using the QIAamp® kit that yielded less than 0.05 μg/μl of DNA was concentrated by ethanol precipitation (Sambrook, 2001). DNA extracted from PBMC was assessed by PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described previously (Mohan et al., 2001) using primers GAPDHF (5' CCTTCATTGACCTTCACTACATGGTCTA 3') and GAPDHR (5' GCTGTAGCCAAATTCATTGTCGTTACCA 3'. Only samples which were amplified successfully with GAPDH primers were used in the study.

2.10 Amplification of JDV Proviral DNA

Genomic PBMC DNA samples were analysed for the presence of JDV proviral DNA by real-time PCR based on the method described previously (Stewart et al., 2005). All real-time PCR reactions were performed in 0.1 ml tubes and caps (Corbett Research) in a Corbett Rotor-Gene real-time PCR detection system. Each standard and sample was tested in duplicate. 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 $MgCl_2$, undefined stabilisers; Bio-Rad), 0.6 mM of each primer, 0.1 μM fluorogenic probe (Geneworks), 0.2 μg of extracted DNA and were made up to a final volume of 10 μl using ultra pure water. The one-step protocol consisted of a 5 min inactivation step at 95°C and 40 cycles of 92°C for 2 sec, 95°C for 15 sec and 58°C for 30 sec and a final step of 70°C for 10 min. Increases in reporter dye emission were examined in real-time by collecting data during the extension steps. Samples in which DNA copy numbers were above the limit of detection of the real-time PCR assay were defined as JDV proviral DNA positive. A number of samples which were amplified in the real-time PCR assay but were below the limit of detection, yet clearly distinguishable from the JDV-negative DNA control, were re-analysed using conventional PCR. The primer pair JDV1 (5' GCAGCGGAGGTGGCAATTTTGATAGGA 3') and JDV3 (5' CGGCGTGGTGGTCCACCCCATG 3') were used to amplify a 360 bp fragment within the *gag* open reading frame as described previously (Desport et al., 2007). Reaction conditions consisted of 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.88 mM of each primer (Invitrogen), 0.687 U *Taq* polymerase, 0.4 μg DNA and were made up to a final volume of 50 μl with ultra pure water. Unless stated otherwise, all reagents were from Fisher

Biotech. Reaction conditions for 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. Direct DNA sequencing was performed to confirm the presence of JDV proviral DNA using 0.01 µg of QIAquick kit (QIAGEN) purified amplicon.

2.11 Amplification of BIV proviral DNA

All seropositive animals were tested for the presence of BIV proviral DNA using 0.2 µg of extracted DNA in a real-time PCR assay as described previously (Lew et al., 2004).

3. Results

Samples from 165 Bali cattle from the Tabanan district of Bali were analysed by ELISA and Western blot for the presence of antibodies against JDV plasma derived antigen, JDV p26, JDV TM peptide or a fused protein construct encompassing both proteins. In addition, PBMC DNA from the same cattle was tested for the presence of JDV proviral DNA using PCR. Since there is no Gold Standard test for JDV, a sample was assigned “positive” status where a positive result was obtained in the reference JDV plasma derived antigen Western blot and at least one other assay. Only 24 of the 165 samples were found to be positive using this method giving a seroprevalence rate of just 14.54% (95% confidence interval, 9.2 to 19.9%). This is lower than the seroprevalence rate of 22.1% (95% confidence interval, 15.5 to 28.8%) which was reported in the Tabanan region in a previous study (Barboni et al., 2001; Hartaningsih et al., 1993) but is similar to another study performed in this region at the same time of year where a seroprevalence of 15.6% (95% confidence interval, 7.5 to 23.7%) was reported (Soeharsono et al., 1995).

3.1 Plasma derived antigen and JDV p26-his assays

Soluble JDV p26-his recombinant protein was purified successfully from the bacterial lysates and was tested initially against reference JDV positive and negative sera by ELISA to determine cut-off values for the field sera samples (Fig 3a). When the 165 field samples were tested, a 98.6% concordance between JDV positive and JDV p26-his seropositive samples was observed with 99.3% specificity (Table 1). A difference in sensitivity was observed when

the JDV p26-his protein was used in Western blot compared to ELISA with lower concordance (91.5%) and kappa values using Western blot (Table 1).

3.2 Comparison between JDV p26-his and JDV p26 /TM-his

The results obtained when reference positive and negative sera were tested in the JDV p26 /TM-his ELISA and the JDV p26-his ELISA were very similar with close median values for both assays (Fig. 3a). However, when the field samples were tested, the JDV p26/TM-his ELISA data gave a much lower median value for positive samples and significantly more false negatives ($p<0.0001$) by Fisher's exact test compared to those obtained with JDV p26-his ELISA (Fig 3b).

3.3 JDV TM peptide ELISA

Both the linear and cyclic versions of the JDV TM peptide were tested in ELISA with JDV positive and negative sera. Very low background OD values were observed with both peptides for the negative serum sample (Fig. 2). However, a much greater sensitivity was observed with the positive control serum sample and the JDV TM^c peptide compared to the linear peptide. When the field sera were tested only 8 of the positive serum samples were identified using the JDV TM^c peptide ELISA with a concordance value of 87.3% and a correspondingly low kappa value (Table 1). An additional 5 samples were positive in the JDV TM^c peptide ELISA but only 1 of these was positive in any other test (data not shown).

3.4 PCR and Sequencing

JDV specific PCR products were identified from a total of 15 PBMC DNA samples in this study of which only 5 were also seropositive (Table 1). All PCR positive samples were sequenced to confirm the presence of JDV specific products (data not shown). The sensitivity and concordance values were both low for this assay when compared to any of the serological tests (Table 1).

4. Discussion

In this study a comparison of diagnostic assays was undertaken to determine which is the most sensitive and reliable method for diagnosing infections with JDV. The plasma-derived whole virus antigen is increasingly difficult to produce in Indonesia as it requires the experimental infection of susceptible cattle with JDV (Hartaningsih et al., 1994; Kertayadnya et al., 1993). The JDV p26-his antigen was found to be more sensitive in ELISA than in Western blot confirming earlier studies (Barboni et al., 2001). Capsid specific antibodies are generally the first detectable response after the delayed seroconversion and therefore JDV p26 is an essential component for a JDV diagnostic assay. However, variable *gag* responses have been reported after experimental infection with BIV and JDV. BIV CA antibodies were detectable from 2 weeks until at least 2.5 years after infection (Whetstone et al., 1990) whilst Gag responses were found in one study to have declined by 40 weeks after infection and in a second study to have remained low or undetectable until 190 weeks after infection (Isaacson et al., 1995). We have observed weak to undetectable CA antibody responses in 15% of experimentally infected cattle which, despite having detectable plasma viraemia, also failed to develop the

classical febrile response to infection. Interestingly, all except one of these animals developed strong TM responses which were detected using the JDV TM^c peptide ELISA. This suggests that a diagnostic assay based on the detection of JDV p26 or TM peptide specific antibodies may offer greater sensitivity. Indeed, increased sensitivity was reported in an earlier study when JDV p26-his and JDV TM peptide were combined in a single ELISA assay although in this case the JDV TM peptide was linear and the assay was not compared to plasma derived antigen immunoassays (Barboni et al., 2001). In addition, serological detection of the genetically related SRLV was improved by using the combination of Maedi visna virus (MVV) CA and a TM peptide as coating antigens in an ELISA (Celer and Celer, 2001). This has been improved further by expressing the entire CA and TM epitope in a single fusion protein for serological detection of SRLV, Feline immunodeficiency virus and Equine infectious anaemia virus infections (Rosati et al., 2004). When a similar strategy was used for serological detection of JDV infections in this study, a large reduction in the sensitivity of the assay compared to JDV p26-his ELISA was observed when field samples were tested. It is possible that the addition of the JDV TM peptide sequence affected the folding of the CA protein resulting in a sub-optimal configuration in the ELISA plate well. This was supported further by the marked reduction in expression, purity and stability of the protein (data not shown). An increase in sensitivity to 100% was observed if the results from the JDV p26-his and TM^c peptide ELISAs were combined but this was accompanied by an increase in the number of false positives. Further investigations with a larger number of seropositive samples are required to determine whether the combination of these two antigens

improves the overall specificity and sensitivity of the diagnostic assay. A reduction in sensitivity was observed when MVV CA and whole TM protein were expressed as a single protein with sensitivity dropping from 88% using the indirect whole virus ELISA to 64% with the indirect fusion protein ELISA (DeMartini et al., 1999). However this was also accompanied by greatly reduced protein expression, stability and purity.

The detection of JDV proviral DNA positive cattle in this study was often not accompanied by a detectable antibody response. This could indicate that these cattle have become infected with JDV recently and consequently have not yet seroconverted. Many of the JDV proviral DNA positives were owned by farmers who also owned seropositive animals (data not shown). However, JDV proviral DNA could not be amplified from 79% of seropositives indicating that the proviral load in PBMC of cattle that have recovered from infection with JDV is very low to undetectable. A PCR assay for MVV was recently shown to have a diagnostic sensitivity of 56.7% and this has been attributed to the low number of infected monocytes in the blood (Karanikolaou et al., 2005). The estimated number of peripheral blood monocytes infected by MVV may be as few as 1 in 10^5 to 10^6 even in a diseased animal (Zhang et al., 2000). Greater sensitivity has been achieved with a nested PCR for diagnosis of SRLV infection and sequence diversity has been identified as a factor in amplification detection (Eltahir et al., 2006). Given the genetic stability of JDV strains within Bali this is an unlikely explanation for the failure to detect JDV proviral DNA and it is more likely to be due to the sensitivity of the assay (Desport et al., 2007).

The possibility that the seropositives were due to infection with BIV was addressed by testing samples with the sensitive real-time PCR assay (Lew et al., 2004) since serological differentiation between infections with JDV and BIV is not possible yet (Desport et al., 2005). BIV proviral DNA was not detected in any of the seropositive samples in this study. A recent Bayesian validation of the use of PCR and indirect fluorescent-antibody assay for the diagnosis of BIV infections concluded that a substantial misclassification of infection would be expected regardless of which assay was used (Orr et al., 2003). Difficulties in amplifying BIV successfully from PBMC DNA samples have been reported (Saman et al., 1999) and BIV proviral DNA was found to be undetectable in PBMC taken 12 months after experimental infection (Baron et al., 1998). It is therefore likely that the failure to detect any bovine lentivirus proviral DNA in 79% of the seropositive animals is due to low proviral loads. The tropism of JDV is currently under investigation to determine which subset of PBMC is likely to harbour proviral DNA.

In conclusion, the JDV p26-his ELISA was found to be a reliable assay for the detection of JDV seropositives that would have been detected previously using plasma derived antigen preparations in Indonesia. The addition of the TM peptide to make a fused p26/TM-his protein did not improve the sensitivity of the assay. A combination of JDV p26-his ELISA and real-time PCR is recommended as the most sensitive method for diagnosis of JDV infection in Indonesia. This is the first study to compare immunological and molecular detection methods for JDV and further studies are underway to determine the detectable proviral load in DNA extracted from PBMC of recovered cattle and

to identify peptides that could be used to diagnose infection with JDV specifically.

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References:

Abed, Y., St-Laurent, G., Zhang, H., Jacobs, R.M., Archambault, D., 1999. Development of a Western blot assay for detection of bovine immunodeficiency-like virus using capsid and transmembrane envelope proteins expressed from recombinant baculovirus. Clin Diagn Lab Immunol 6, 168-72.

Barboni, P., Thompson, I., Brownlie, J., Hartaningsih, N., Collins, M.E., 2001. Evidence for the presence of two bovine lentiviruses in the cattle population of Bali. Vet Microbiol 80, 313-27.

Baron, T., Betemps, D., Mallet, F., Cheynet, V., Levy, D., Belli, P., 1998. Detection of bovine immunodeficiency-like virus infection in experimentally infected calves. Arch Virol 143, 181-9.

Burkala, E.J., Ellis, T.M., Voigt, V., Wilcox, G.E., 1999. Serological evidence of an Australian bovine lentivirus. Vet Microbiol 68, 171-7.

Burki, F., Rossmanith, W., Rossmanith, E., 1992. Equine lentivirus, comparative studies on four serological tests for the diagnosis of equine infectious anaemia. Vet Microbiol 33, 353-60.

Celer, V., Jr., Celer, V., 2001. Detection of antibodies to ovine lentivirus using recombinant capsid and transmembrane proteins. J Vet Med B Infect Dis Vet Public Health 48, 89-95.

Chadwick, B.J., Coelen, R.J., Wilcox, G.E., Sammels, L.M., Kertayadnya, G., 1995. Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. J Gen Virol 76 (Pt 7), 1637-50.

Chen, P., Liu, Z.Q., Wood, C., 1994. Use of TrpE fusion protein to identify antigenic domains within the BIV envelope protein. J Virol Methods 47, 331-43.

de Andres, D., Klein, D., Watt, N.J., Berriatua, E., Torsteinsdottir, S., Blacklaws, B.A., Harkiss, G.D., 2005. Diagnostic tests for small ruminant lentiviruses. Vet Microbiol 107, 49-62.

DeMartini, J.C., Halsey, W., Boshoff, C., York, D., Howell, M.D., 1999. Comparison of a maedi-visna virus CA-TM fusion protein ELISA with other assays for

detecting sheep infected with North American ovine lentivirus strains. Vet Immunol Immunopathol 71, 29-40.

Desport, M., Ditcham, W.G., Lewis, J.R., McNab, T.J., Stewart, M.E., Hartaningsih, N., Wilcox, G.E., 2009. Analysis of Jembrana disease virus replication dynamics in vivo reveals strain variation and atypical responses to infection. Virology in press.

Desport, M., Stewart, M.E., Mikosza, A.S., Sheridan, C.A., Peterson, S.E., Chavand, O., Hartaningsih, N., Wilcox, G.E., 2007. Sequence analysis of Jembrana disease virus strains reveals a genetically stable lentivirus. Virus Res 126, 233-44.

Desport, M., Stewart, M.E., Sheridan, C.A., Ditcham, W.G., Setiyaningsih, S., Tenaya, W.M., Hartaningsih, N., Wilcox, G.E., 2005. Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and nonpathogenic bovine lentiviruses. J Virol Methods 124, 135-42.

Eltahir, Y.M., Dovas, C.I., Papanastassopoulou, M., Koumbati, M., Giadinis, N., Verghese-Nikolakaki, S., Koptopoulos, G., 2006. Development of a semi-nested PCR using degenerate primers for the generic detection of small ruminant lentivirus proviral DNA. J Virol Methods 135, 240-6.

Fleiss, J.L., 1981. Statistical methods for rates and proportions. Wiley, New York.

Greiner, M., Sohr, D., Gobel, P., 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J Immunol Methods 185, 123-32.

Hartaningsih, N., Wilcox, G.E., Dharma, D.M., Soetrisno, M., 1993. Distribution of Jembrana Disease in Cattle in Indonesia. Vet Microbiol 38, 23-9.

Hartaningsih, N., Wilcox, G.E., Kertayadnya, G., Astawa, M., 1994. Antibody response to Jembrana disease virus in Bali cattle. Vet Microbiol 39, 15-23.

Houwers, D.J., Nauta, I.M., 1989. Immunoblot analysis of the antibody response to ovine lentivirus infections. Vet Microbiol 19, 127-39.

Isaacson, J.A., Roth, J.A., Wood, C., Carpenter, S., 1995. Loss of Gag-specific antibody reactivity in cattle experimentally infected with bovine immunodeficiency-like virus. Viral Immunol 8, 27-36.

Karanikolaou, K., Angelopoulou, K., Papanastasopoulou, M., Koumpati-Artopiou, M., Papadopoulos, O., Koptopoulos, G., 2005. Detection of small ruminant lentiviruses by PCR and serology tests in field samples of animals from Greece. Small Rumin Res 58, 181-187.

Kertayadnya, G., Wilcox, G.E., Soeharsono, S., Hartaningsih, N., Coelen, R.J., Cook, R.D., Collins, M.E., Brownlie, J., 1993. Characteristics of a retrovirus associated with Jembrana disease in Bali cattle. J Gen Virol 74 (Pt 9), 1765-78.

Lew, A.E., Bock, R.E., Miles, J., Cuttell, L.B., Steer, P., Nadin-Davis, S.A., 2004. Sensitive and specific detection of bovine immunodeficiency virus and bovine syncytial virus by 5' Taq nuclease assays with fluorescent 3' minor groove binder-DNA probes. J Virol Methods 116, 1-9.

Mohan, M., Malayer, J.R., Geisert, R.D., Morgan, G.L., 2001. Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos. Mol Reprod Dev 60, 289-96.

Orr, K.A., O'Reilly, K.L., Scholl, D.T., 2003. Estimation of sensitivity and specificity of two diagnostics tests for bovine immunodeficiency virus using Bayesian techniques. Prev Vet Med 61, 79-89.

Rosati, S., Profiti, M., Lorenzetti, R., Bandecchi, P., Mannelli, A., Ortoffi, M., Tolari, F., Ciabatti, I.M., 2004. Development of recombinant capsid antigen/transmembrane epitope fusion proteins for serological diagnosis of animal lentivirus infections. J Virol Methods 121, 73-8.

Saman, E., Van Eynde, G., Lujan, L., Extramiana, B., Harkiss, G., Tolari, F., Gonzalez, L., Amorena, B., Watt, N., Badiola, J., 1999. A new sensitive serological assay for detection of lentivirus infections in small ruminants. Clin Diagn Lab Immunol 6, 734-40.

Sambrook, J., Russell, DW, 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scobie, L., Venables, C., Hughes, K., Dawson, M., Jarrett, O., 1999. The antibody response of cattle infected with bovine immunodeficiency virus to peptides of the viral transmembrane protein. J Gen Virol 80 (Pt 1), 237-43.

Soeharsono, S., Hartaningsih, N., Soetrisno, M., Kertayadnya, G., Wilcox, G.E., 1990. Studies of experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. J Comp Pathol 103, 49-59.

Soeharsono, S., Wilcox, G.E., Putra, A.A., Hartaningsih, N., Sulistyana, K., Tenaya, M., 1995. The transmission of Jembrana disease, a lentivirus disease of Bos javanicus cattle. Epidemiol Infect 115, 367-74.

Soesanto, M., Soeharsono, S., Budiantono, A., Sulistyana, K., Tenaya, M., Wilcox, G.E., 1990. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. J Comp Pathol 103, 61-71.

Stewart, M., Desport, M., Hartaningsih, N., Wilcox, G., 2005. TaqMan real-time reverse transcription-PCR and JDVp26 antigen capture enzyme-linked immunosorbent assay to quantify Jembrana disease virus load during the acute phase of in vivo infection. J Clin Microbiol 43, 5574-80.

Wareing, S., Hartaningsih, N., Wilcox, G.E., Penhale, W.J., 1999. Evidence for immunosuppression associated with Jembrana disease virus infection of cattle. Vet Microbiol 68, 179-85.

Whetstone, C.A., VanDerMaaten, M.J., Black, J.W., 1990. Humoral immune response to the bovine immunodeficiency-like virus in experimentally and naturally infected cattle. J Virol 64, 3557-61.

Zanoni, R.G., Nauta, I.M., Pauli, U., Peterhans, E., 1991. Expression in Escherichia coli and sequencing of the coding region for the capsid protein of Dutch maedi-visna virus strain ZZV 1050: application of recombinant protein in enzyme-linked immunosorbent assay for the detection of caprine and ovine lentiviruses. J Clin Microbiol 29, 1290-4.

Zhang, Z., Watt, N.J., Hopkins, J., Harkiss, G., Woodall, C.J., 2000. Quantitative analysis of maedi-visna virus DNA load in peripheral blood monocytes and alveolar macrophages. J Virol Methods 86, 13-20.

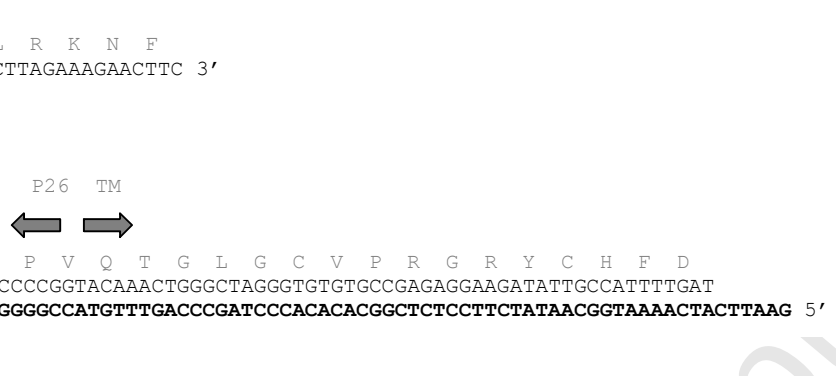


Fig. 1. Primer sequences used for the production of a construct encoding JDV p26/TM peptide. The reverse primer is shown in bold and the amino acid sequences are also indicated (grey). Grey arrows indicate the fusion between the C' terminus of p26 and the N' terminus of TM epitope.

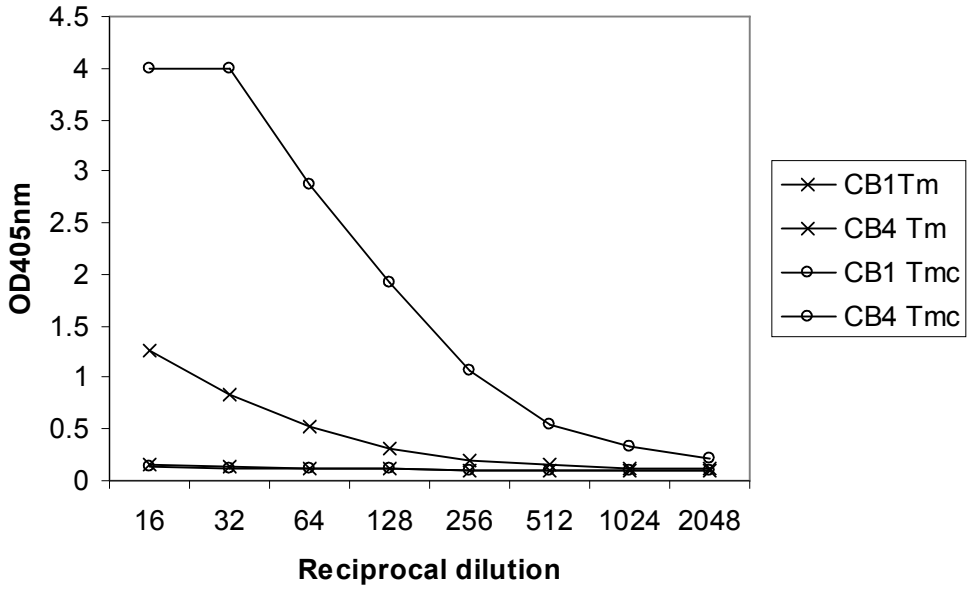
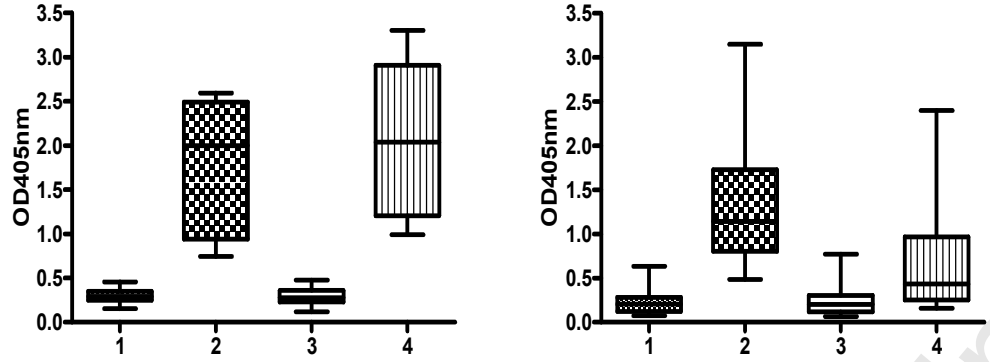


Fig. 2. Comparison of the ELISA absorbances obtained with reference negative (CB1) and JDV positive (CB4) serum samples with 1 µg JDV TM peptide or 1 µg JDV TM^c peptide.



(a) (b)

Fig. 3. Box plots of ELISA absorbances obtained using the JDV p26-his and JDV p26/TM-his purified proteins as coating antigens. The upper and lower edges of the boxes correspond to the 75th and 25th percentiles, respectively, with the central line representing the median and the vertical lines the range of values. (a) Reactivity of reference negative and positive bovine serum to JDV p26-his (lanes 1 and 2) and JDV p26/TM-his antigens by ELISA (lanes 3 and 4) respectively. (b) Reactivity of JDV native antigen negative and positive bovine field serum by Western blot in comparison to JDV p26-his (lanes 1 and 2) and JDV p26/TM-his antigens by ELISA (lanes 3 and 4).

Table. 1. Specificity, sensitivity, concordance and kappa values for ELISA, WB and PCR diagnostic assays compared to JDV infection status in 165 samples from a group of cattle. Cattle were assumed to have been infected with JDV if their samples were positive in the JDV plasma derived antigen WB and at least one other assay.

Diagnostic assay		JDV infection +	JDV infection -	Specificity	Sensitivity	Concordance	Kappa
p26-his ELISA	+	23	1	99.3%	95.8%	98.6%	0.95
	-	1	140				
p26/TM-his ELISA	+	9	5	96.5%	37.5%	87.9%	0.41
	-	15	136				
TM^{c} peptide ELISA	+	8	5	96.5%	33.3%	87.3%	0.37
	-	16	136				
p26-his + Tm^{c} ELISA	+	24	6	95.9%	100%	96.4%	0.87
	-	0	135				
p26-his WB	+	18	8	94.3%	75%	91.5%	0.64
	-	6	133				
PCR	+	5	10	92.9%	20.8%	82.4%	0.16
	-	19	131				