Vaccination reduces the viral load and the risk of transmission of Jembrana disease virus in Bali cattle

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Abstract

The efficacy of a tissue-derived vaccine, which is currently used in Indonesia to control the spread of Jembrana disease in Bali cattle, was determined by quantifying the viral load in plasma following experimental infection with Jembrana disease virus. Virus transmission is most likely to occur during the acute phase of infection when viral titers are greater than 106 genomes/ml. Vaccinated cattle were found to have a 96% reduction in viral load above this threshold compared to control cattle. This would reduce the chance of virus transmission as the number of days above the threshold in the vaccinated cattle was reduced by 33%. Viral loads at the onset and resolution of fever were significantly lower in the vaccinated cattle and immune function was maintained with the development of antibody responses to Env proteins within 10-24 days post challenge. There was, however, no significant reduction in the duration of the febrile period in vaccinated animals. The duration and severity of clinical parameters were found to be variable within each group of cattle but the quantification of viral load revealed the benefits of vaccinating to reduce the risk of virus transmission as well as to ameliorate disease.

Keywords

Jembrana disease virus
Bovine lentivirus
Vaccine

Introduction

Jembrana disease (JD) is an acute, sometimes fatal infection of the immune system of Bali cattle in Indonesia, which first occurred in Bali in 1964. The causative agent, Jembrana disease virus (JDV), is a bovine lentivirus (Kertayadnya et al., 1993) which is now endemic in Bali and has spread to Bali cattle on the islands of Sumatra, East Java and Kalimantan (Hartaningsih et al., 1993; Wilcox, 1997). Experimental infection with JDV typically results in the development of a febrile response and leukopenia within 5–12 days of challenge accompanied by high viral titers of 10^8 ID_{50}/ml equating to between 10^{10} and 10^{11} RNA genome copies/ml plasma (Soeharsono et al.,1995; Stewart et al., 2005). The viremia peaks between 8–12 days post-challenge and then declines as the febrile response and leukopenia resolve. Recovered cattle remain persistently infected for at least 25 months but do not appear to suffer any disease progression (Soeharsono et al., 1990). Recovered animals develop a delayed humoral response several weeks after infection prompting the assumption that antibody is not a major factor in natural resolution of the acute disease process (Hartaningsih et al., 1994). Recovered cattle are resistant to reinfection and neutralizing antibody responses have been detected after 4–6 months in some cattle (Soeharsono et al., 1990). Transmission of JDV has been reported to be similar to equine infectious anaemia virus (EIAV) and is likely to occur via hematophagous arthropods during the acute stage of the disease when viral titers in blood are high (Issel and Foil, 1984; Soeharsono et al., 1995). There is also evidence of transmission of JDV during the acute stage of JD to susceptible cattle in close contact in the absence of hematophagous arthropods, probably by virus in secretions infecting cattle by the conjunctival, intranasal or oral routes, which has been experimentally reproduced (Soeharsono et al., 1995). 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; Hawkins et al., 1976).

A vaccine that can effect a reduction in the duration and magnitude of viremia would be expected to reduce the risk of transmission in an outbreak of JD. The most successful vaccines against animal lentiviruses are those which are based on whole or inactivated virus preparations. Some degree of protection has been induced with inactivated viral preparations in cats against feline immunodeficiency virus (Hosie et al., 2000; Yamamoto et al., 1993), in horses against EIAV (Issel et al., 1992) and against intravaginal challenge with $SHIV_{89.6}$ in macaques mucosally immunised with inactivated $SHIV_{89.6}$ (Ambrose et al., 2003). However, potentiation of lentiviral disease following vaccination is occasionally seen in goats vaccinated against caprine arthritis encephalitis virus (Russo et al., 1993).

To control JD in Indonesia, an inactivated tissue derived viral vaccine, JDVacc, has been developed that contains detergent inactivated virus in homogenised spleen taken from infected donor animals at the peak of viremia (Hartaningsih et al., 2001). The efficacy of this vaccine has been determined by analysis of clinical signs, in particular the development and duration of the febrile response that develops after experimental challenge with JDV (Hartaningsih et al., 2001). Two doses of JDVacc (given 1 month apart) is the currently recommended vaccination regime for Bali cattle in areas where JD outbreaks are still occurring in Indonesia. Whilst the development of a febrile response is a classical indicator of JD, we have recently found that 15% of animals experimentally challenged with JDV did not develop a febrile response, despite high levels of viral replication in some cases (Desport et al., 2009). The development of a qRT-PCR assay in our laboratory for the detection of JDV RNA in plasma (Stewart et al., 2005) has enabled the quantification of the plasma viral loads during the acute stage of infection. In this study, we sought to determine the efficacy of the 2 dose vaccination regime in protecting cattle in a JD outbreak situation by analysing viral loads (VL) and immune responses

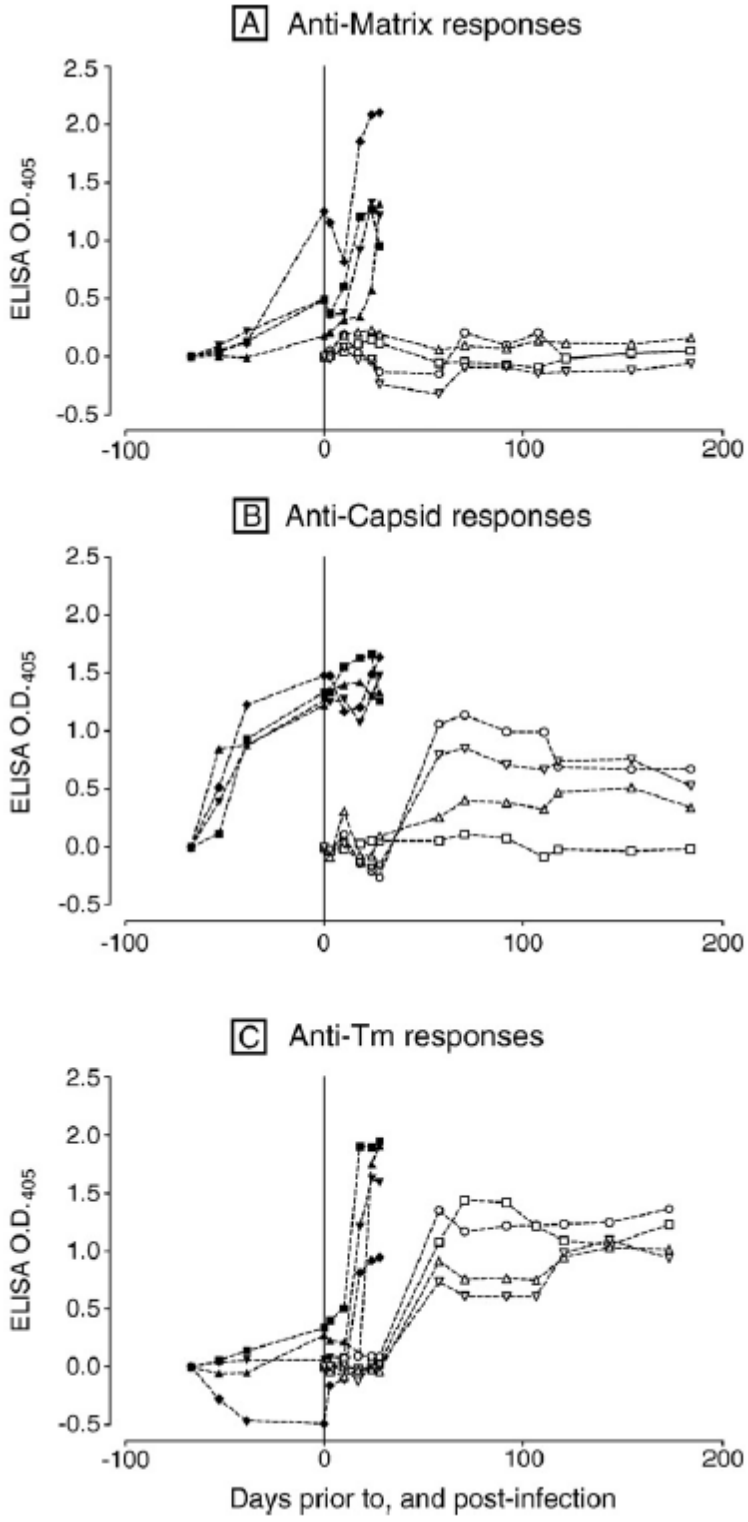

Fig. 1. ELISA absorbances indicating the development of IgG responses detected using immobilized recombinant JDV p16 (A), recombinant JDV p26 (B), and circularized JDV Tm peptide (C) after vaccination with JDVacc (days -67 and -39) and infection with 1000 ID_{50} $JDV_{TAB/87}$ (day 0). Sequential serum samples were obtained from vaccinated cattle CB75 (■), CB76 (▲), CB77 (▼), CB78 (◆) and control cattle CB83 (□), CB84 (Δ), CB85 (▽) and CB86 (◇).

after vaccination and challenge. A plasma VL of 10^6 JDV genome copies/ml, approximately equivalent to 10^4 ID_{50}/ml (Stewart et al., 2005) or < 1 ID_{50} of EIAV per tabanid fly (Hawkins et al., 1976), was chosen as a threshold above which the risk of transmission of virus is increased. A piecewise linear regression model (Brandin et al., 2006) was fitted to the data and used to estimate missing data at the end of the plasma viraemia so that area under the curve (AUC)≥10^6 genome copies/ml could be calculated and compared in vaccinated and control cattle.

Results

Development of IgG responses after vaccination and challenge

Bali cattle were vaccinated twice with JDVacc and IgG antibody responses against five JDV recombinant proteins determined using a combination of ELISA and Western blot. Responses to the JDV matrix protein (p16) were detectable in only one of the vaccinates prior to challenge but all of the vaccinates seroconverted to this antigen 10–24 days post-infection (Fig. 1A). All vaccinated cattle were found to have seroconverted to the recombinant JDV p26 after the first vaccination with increasing ELISA absorbance values after the second vaccination that were maintained after experimental infection (Fig. 1B). Antibodies specific to JDV Tat were not detected in any of the vaccinated or control cattle at any of the available sampling times (data not shown). None of the vaccinated cattle had detectable antibody response against JDV ΔSu (Fig. 2) or JDV Tm peptide (Fig. 1C) prior to challenge but all cattle seroconverted to both antigens between 10 and 24 days post challenge indicating an anamnestic immune response. In contrast, the control group showed the characteristic delay in seroconversion to JDV antigens with responses detectable against JDV p16, p26 and Tm peptide beginning at 56 days post-infection (Fig. 1). CB84 appeared to seroconvert

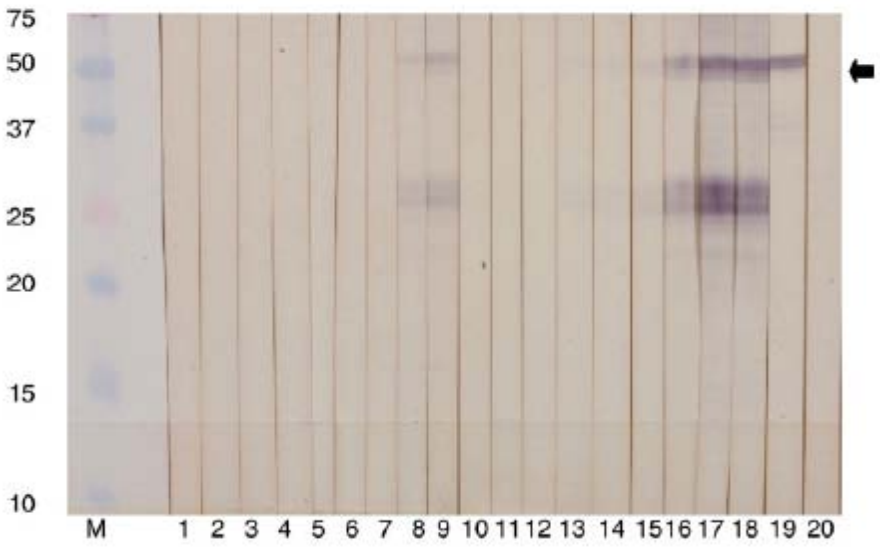

Fig. 2. Western immunoblot indicating the development of IgG responses detected using immobilized JDV ΔSU-GST after vaccination with JDVacc (days -67 and -39) and infection with 1000 ID_{50} $JDV_{TAB/87}$ (day 0). Sequential serum samples were obtained from vaccinated cattle CB76 on days -67 (lane 1), -53 (lane 2), -39 (lane 3), 0 (lane 4), 3 (lane 5), 10 (lane 6), 17 (lane 7), 24 (lane 8), 28 (lane 9) and CB77 on days -67 (lane 10), -53 (lane 11), -39 (lane 12), 0 (lane 13), 3 (lane 14), 10 (lane 15), 17 (lane 16), 24 (lane 17), 28 (lane 18). Positive (lane 19) and negative (lane 20) control sera were also included.

strongly to the JDV Tm peptide (Fig. 1C) but only weakly to JDV p26 compared to the other control animals which had increasing ELISA absorbances from day 60 (Fig. 1B). None of the control animals developed antibodies that recognized the JDV p16 (Fig. 1A) or JDV ΔSu antigens (data not shown).

Comparison of clinical responses after challenge with JDV

The efficacy of this vaccine has been previously determined by using its effect on the duration and magnitude of the febrile response and the severity of the leukopenia as correlates of protection (Hartaningsih et al., 2001). Three of the 4 animals in the control group developed typical febrile responses (a rectal temperature of ≥39.3 °C) within 7–9 days of infection (Fig. 3). The fourth control animal, CB84, developed a transient mild fever on day 12 post-infection. In the vaccinated group, 2 of the 4 cattle developed febrile responses after a similar period whilst CB76 developed fever 17 days post-infection and CB78 did not develop elevated temperatures at any stage (Fig. 4). The duration in days for the low, moderate and total febrile periods is shown in Table 1. There were no significant differences in the observed data collected for the clinical responses that developed following challenge with $JDV_{TAB/87}$ between the vaccinated and the control cattle. However, if the atypical responders (CB78 and CB84) were excluded from the statistical analysis, the duration of the febrile response in the vaccinated cattle was reduced (P=0.067) with the period of moderate fever being significantly shorter (P=0.051) in the vaccinated cattle (Table 1).

Viral loads after vaccination and challenge with JDV

Although 2 of the 8 cattle failed to develop typical febrile responses, all animals had detectable levels of circulating plasma viral RNA over the course of the experiment (Figs. 3 and 4). The atypical control animal, CB84, exhibited an undulating low level plasma VL, peaking at $\log_{10}$=7.93 genomes/ml (Fig. 3 and Table 2). The vaccinated animal that did not develop a febrile response (CB78) was found to have a plasma VL similar to the other vaccinates with a peak of $\log_{10}$=10.17 genomes/ml (Fig. 4 and Table 2). The VL on the second day of the febrile response and on the first day after the febrile response were significantly lower in the vaccinated animals compared to the controls (Table 2). Peak plasma VL occurred earlier in the febrile period and declined more rapidly than in the control group, whereas higher levels of plasma virus persisted in the febrile controls for several days and in CB83 and CB86, did not peak until 6 days after onset of fever (Fig. 3). Significant differences (P=0.017) in peak VL were only observed when the atypical responders were not included in the analysis (Table 2).

Comparison of observed and fitted data

The fitted piecewise linear regression was used to supply missing points in the VL as they were not sampled for long enough to determine when their VL dropped to $<10^6$ genomes/ml. The fitted and observed data points for both vaccinated and control cattle are shown in Figs. 3 and 4. There were no significant differences in the mean model parameters estimated for the two groups. The mean duration of the plateau phase (R–M), corresponding to the period in the disease where viral replication was matched by viral clearance, was reduced in vaccinated animals (this approached significance P=0.077). The Chi-squared goodness-of-fit test indicated there was no difference between the observed data and the piecewise linear regression model for all animals (P=0.66–0.99 for controls, and P=0.32–0.99 for vaccinates). The lower P values seen in 2 animals in the vaccinated group were due to the fitted line deviating from the measured results in the pre-febrile period of viral replication. The proportion of the sums-of-squares from these points was 73% and 86% of the total, respectively.

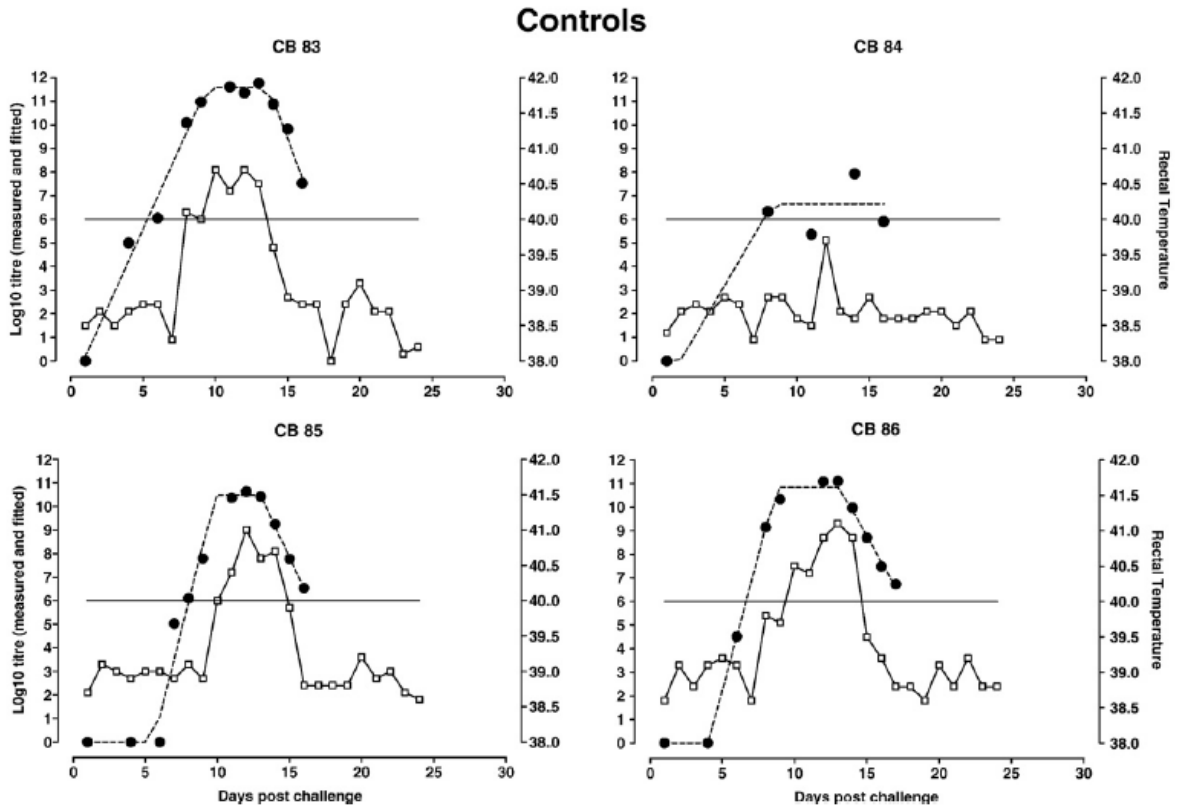

Fig. 3. Dynamics of $JDV_{TAB/87}$ replication in control GST vaccinated animals (CB83–86) after infection with 1000 ID_{50}/ml $JDV_{TAB/87}$ (day 0). Rectal temperatures (□) were collected for 24 days post-infection. Plasma VL (JDV genome copies/ml plasma) was determined using qRT-PCR (●) and transformed to $\log_{10}$ scale. Differences in VL were calculated using a threshold of 10^6 genome copies/ml plasma (solid line) and AUC was determined from measured plasma VL (●) and fitted VL, calculated using the piecewise linear regression model (- -), where measured data was unavailable.

This deviation from the fitted line was due to a reduction in virus replication rates between 4–8 days post-challenge in the vaccinated animals, particularly in CB 75 and CB 78. This was not observed to the same extent in the control animals.

Viral load in plasma of vaccinates and controls

The estimated mean total AUC was 5.4544×10^{11} for control cattle and 2.2780×10^{10} genomes/ml for the vaccinated animals. The estimated mean AUC$\geq10^6$ genomes/ml was 5.4541×10^{11} and 2.2772×10^{10} equating to 99.995% and 99.961% of the total VL for control and vaccinated animals respectively. In vaccinated cattle the AUC$\geq10^6$ genomes/ml was reduced by 96% compared to the control cattle with a lower 95% confidence limit (LCL) of 80%. When these values were transformed to the $\log_{10}$ scale for statistical analysis (Table 2), vaccinated typical responders were found to have a significantly lower AUC$\geq10_6$ genomes/ml compared to normal control cattle (P=0.018). There was a 33% reduction in the mean duration of time that the plasma VL of vaccinated animals exceeded 10^6 genomes/ml which was significantly different (P=0.031) from the control cattle (Table 2).

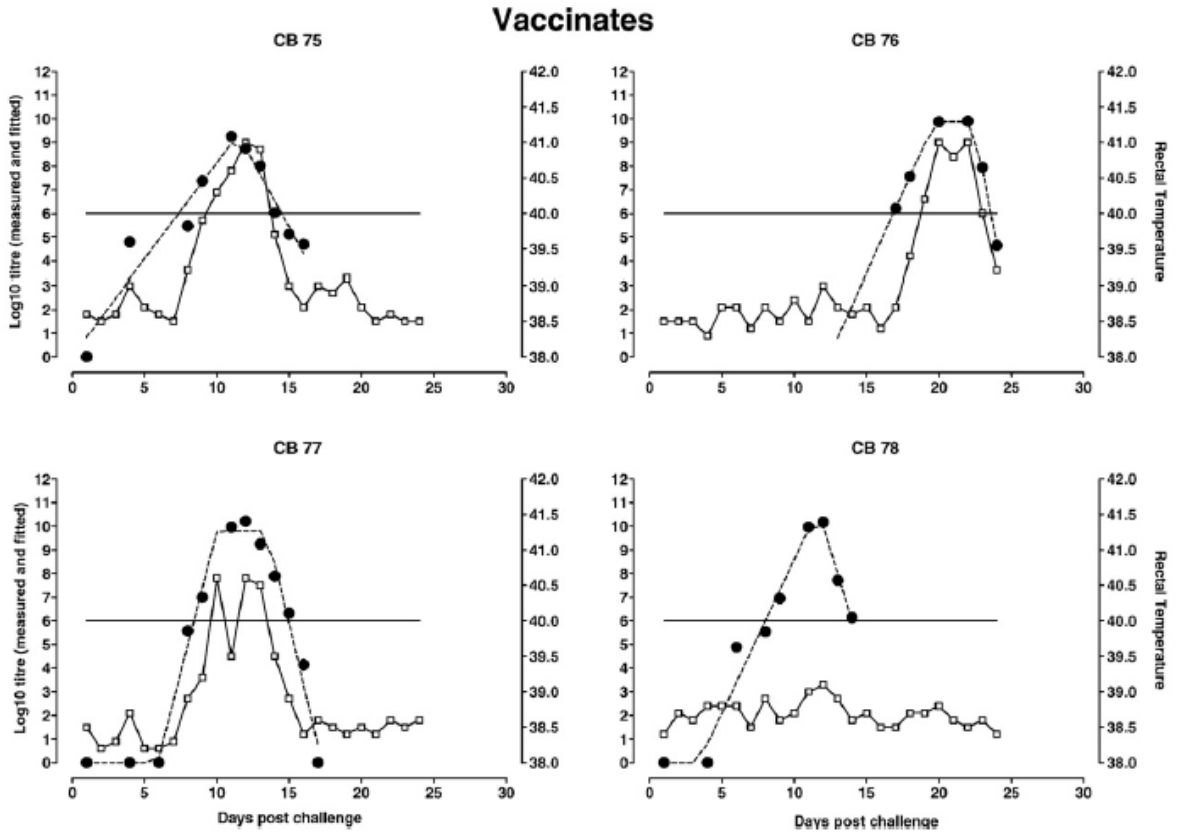

Fig. 4. Dynamics of $JDV_{TAB/87}$ replication in individual JDVacc vaccinated animals (CB75–78) after infection with 1000 ID_{50}/ml $JDV_{TAB/87}$ (day 0). Rectal temperatures (□) were collected for 24 days post-infection. Plasma VL (JDV genome copies/ml plasma) was determined using qRT-PCR (●) and transformed to $\log_{10}$ scale. Differences in VL were calculated using a threshold of 10^6 genome copies/ml plasma (solid line) and AUC was determined from measured plasma VL (●) and fitted VL, calculated using the piecewise linear regression model (- -), where measured data was unavailable.

Discussion

The protection provided after vaccination with a tissue-derived inactivated virus vaccine that is currently used for the control of Jembrana disease in Indonesia has not previously been determined. JDVacc has been shown to reduce the duration and severity of JD and it is likely that this amelioration of the acute phase of disease is sufficient to achieve a reduction in the mortality rates and reduce the transmission of virus to other susceptible cattle (Hartaningsih et al., 2001). The use of VL, a piecewise linear regression model and a transmission threshold value has provided a method to determine vaccine efficacy in the context in which the vaccine is used. The benefit of a significant reduction in VL in the vaccinated cattle during the early febrile response and recovery period is revealed when expressed as a 33% reduction in the number of days when the plasma VL is above the transmission threshold. Although this is an estimated threshold value for the transmission of JDV, this encompasses >99.9% of the total VL in both groups of animals. Further studies are required to determine the efficacy of JDVacc when used to control the spread of JDV in outbreak situations.

Table 1
Comparison of observed data (days post infection) for the onset, peak and end of the febrile response and peak viral load together with duration (days) of low (39.3–40.2 °C), moderate (40.3–41.2 °C), high (>41.2 °C) and total febrile periods following challenge with 1000 ID_{50} $JDV_{TAB/87}$ in vaccinated (CB75–78) and control (CB83–86) Bali cattle

Cattle no.	Peak VL	Number of days post infection			Duration of febrile response (days)		
		≥39.3 °C	Peak °C	<39.3 °C	Low	Moderate	Total
CB 75	10	8	11	14	2	4	6
CB 76	21	17	21	23	3	3	6
CB 77	11	9	12	14	2	3	5
CB 78	11	N/A[a]	11 (39.1 °C)	N/A	0	0	0
Mean	13.25	11.33	13.75	17	1.75	2.5	4.25
Var	26.92	24.33	23.58	27	1.58	3	8.25
CB 83	12	7	11	14	3	4	7
CB 84	13	12	12	13	1	0	1
CB 85	11	9	11	15	2	4	6
CB 86	12	7	12	15	3	5	8
Mean	12	8.75	11.5	14.25	2.33	3.25	5.5
Var	0.66	5.58	0.33	0.92	1.33	4.92	9.67
P-value	0.331	0.235	0.212	0.228	0.277	0.307 (0.051)[b]	0.288 (0.067)[b]

[a] N/A = not applicable.
[b] *P*-value calculated excluding atypical responders CB78 and CB84.

The absence of detectable neutralizing antibodies after vaccination with JDVacc in the original study (Hartaningsih et al., 2001), together with poor antibody responses to Tm prior to challenge in this study indicates that this vaccination regime does not engender the development of strong humoral responses to Env antigens which would be required to achieve sterilizing immunity. The use of JDVacc has been demonstrated to induce antibody responses to several viral proteins (Hartaningsih et al., 2001) but it was not possible to correlate specific responses with protection from severe JD in such small groups of cattle. Although antibodies are not believed to play an important role in the clearance of JDV during natural infections, neutralizing antibodies have been reported to develop 4–6 months post-infection (Hartaningsih et al., 2001) and passive antibody treatment of macaques prior to challenge with SHIV has induced sterilizing immunity (Mascola et al., 1999).

The responses to the Gag proteins appeared to develop early after vaccination but this reflects their relative abundance compared to Env proteins in the vaccine preparation. The variation seen in the JDV ΔSU responses of the vaccinated cattle compared to the controls could have been due to the use of detergent in the preparation of JDVacc as this would have denatured the native SU protein. Alternatively, the production of a truncated recombinant SU antigen in an Escherichia coli expression system may not represent the immunodominant epitopes that are present on the native full length protein. Indeed, only a clone representing the extracellular domain of BIV Env was able to detect SU-

Table 2
Comparison of plasma VL on the first and second days of febrile response, first day after the febrile response, peak VL, area under curve (AUC) for plasma VL≥10^6 genome copies/ml and duration (days) of AUCN10^6 genome copies/ml following challenge with 1000 ID_{50} $JDV_{TAB/87}$ in vaccinated (CB75–78) and control (CB83–86) Bali cattle

Cattle no.	Log_{10} VL 1st day ≥ 39.3 °C	Log_{10} VL 2nd day ≥ 39.3 °C	Log_{10} VL 1st day < 39.3 °C	Log_{10} peak VL	Log_{10} AUC ≥ 10^6 genome copies/ml	Days AUC ≥ 10^6 genome copies/ml
CB75	7.37	8.31	5.11	9.25	9.53	6
CB76	7.56	8.71	4.65	9.90	10.43	7.4
CB77	8.48	9.98	6.32	10.21	10.51	7.5
CB78	N/A [a]	N/A	N/A	10.17	10.50	6.2
Mean	7.80	9.00	5.36	9.88	10.23	6.8
Var	0.35	0.76	0.75	0.20	0.22	0.6
CB 83	10.08	10.97	9.82	11.76	12.22	12.2
CB 84	7.08	N/A	7.93	7.93	8.33	7.3
CB 85	10.06	10.36	6.53	10.63	11.02	9
CB 86	9.15	10.33	7.48	11.10	11.62	11.9
Mean	9.09	10.55	7.94	10.36	10.80	10.1
Var	1.99	0.13	1.91	2.83	2.95	5.6
P-value	0.086 (0.007) [b]	0.037	0.015	0.309 (0.017) [b]	0.282 (0.018) [b]	0.031

[a] N/A = not applicable.
[b] *P*-value calculated excluding atypical responders CB78 and CB84.

specific antibodies after experimental infection with BIV (Chen et al.,1994). The absence of a detectable response to JDV Tat was not unexpected, as JDV Tat is only reported to be highly expressed early in infection (Setiyaningsih et al., 2008) and is unlikely to be present at high levels in JDVacc. The development of antibody responses to both Su and Tm in all of the vaccinated cattle at 3 weeks post-infection suggested that a normal memory B cell response developed following vaccination and challenge. The immunosuppression that is characteristically observed with JD (Wareing et al., 1999) did not appear to inhibit the development of virus specific antibodies following infection. This would be an additional benefit of vaccination as infected cattle frequently succumb to secondary infections as a result of the immunosuppressive effect of JDV (Dharma et al., 1991). Circulating antibodies to JDV in the control animals were not detectable until at least 8 weeks post-infection, confirming earlier studies (Hartaningsih et al., 1994). Unfortunately, analysis of the cellular immune responses to vaccination and long-term analysis of the persistence of the virus after vaccination were not possible with the resources available in Bali.

Vaccinated animals exhibited lower peak VLs compared to the controls, indicating that a low level of virus neutralization or reduction in early spread of virus may be due to the humoral immune responses generated after vaccination. The role of neutralizing antibodies in providing protection in other lentivirus challenge systems is uncertain and controversial (Issel et al., 1992; Sattentau, 1996) and in some cases antibody-dependent enhancement of virus replication or absence of any correlation between protection and humoral parameters have been reported (Mazzetti et al., 1999; Raabe et al., 1999). The most significant effects of JDVacc appear after the peak VL when the vaccinates cleared circulating virus much faster than the control animals. The elevated rectal temperatures at lower viral titers in the vaccinated group suggest that the effective viral clearance was due to a primed CMI response.

We have recently observed that 15% of cattle experimentally infected with JDV, including CB84 in this study, fail to develop the typical clinical signs associated with JD (Desport et al., 2009). These cattle typically have much lower peak VL and AUC ≥10^6 genomes/ml compared to normal responders. This suggests that there may be a threshold of disease induction where a particular virus burden is required to trigger the release of pro-inflammatory cytokines and other factors which can

cause pyrexia. This phenomenon has been observed for EIAV and is proposed to be between 5×10^7 to 1×10^8 RNA copies/ml for infection in horses (Cook et al., 2003). The consistent reduction in VL and febrile responses in the vaccinates compared to the typical controls and the lower peak VL in CB84 correlates well with the virus threshold theory that reduction of viral titers can ameliorate disease as well as reducing the risk of virus transmission. Cats with rapidly progressive FIV infection have been shown to have viral RNA loads 1–2 $\log_{10}$ higher than long term survivors and this difference in VL is present early in infection (Diehl et al., 1996). It was not possible to attribute the absence of a febrile response after vaccination and challenge in CB78 to efficacy of JDVacc, particularly as this animal had a peak VL which was similar to the other vaccinates. Comparable results have been seen in SIV-infected macaques in vaccine trials, with varying patterns of virus replication apparently unrelated to the vaccines being tested (Silvera et al., 2002; Waisman et al., 1996). A decrease in the magnitude and duration of the febrile response has been the most commonly employed correlate of protection for JDVacc (Hartaningsih et al., 2001) but the trend for a febrile response to be induced at lower levels of virus in the vaccinated animals in this study highlights the balance between effective immune activation and potentiation of viral replication. Immune activation has been shown to facilitate viral replication in several different lentiviral infections (Fauci et al., 1991; Poli and Fauci, 1993) and in some cases disease potentiation has occurred post-vaccination (Issel et al., 1992; Richardson et al., 2002).

When the JDVacc preparation was originally tested, 4 of the 10 vaccinated animals did not develop a febrile response or leukopenia after challenge (Hartaningsih et al., 2001). Differences between the two challenge experiments could have arisen due to the method of preparation of virus by passage through a donor animal or could be related to the additional vaccination and lower challenge dose used in the earlier study. The serological responses to viral antigens other than JDV p26 were only apparent after the third vaccination in the previous study indicating that a more mature immune response had developed prior to virus challenge. Protection against homologous EIAV challenge in 14 out of 15 ponies was achieved after 5–8 vaccinations with an inactivated whole virus vaccine although it was not sufficient to prevent infection when a heterologous EIAV strain was used as the challenge virus (Issel et al., 1992). JDV strains in Bali appear to be highly genetically conserved (3% amino acid heterogeneity in *env*) and it is expected that JDVacc should offer good protection to cattle on this island (Desport et al., 2007). However, a JDV strain detected in Indonesian Borneo (Kalimantan) was divergent from strains detected in Bali (13% amino acid heterogeneity in *env* compared to $JDV_{TAB/87}$) and it will be important to determine whether using $JDV_{TAB/87}$ as the vaccine strain will offer sufficient protection against virus strains in Kalimantan.

In conclusion, despite the problems inherent in attempting immunological control of lentivirus infections and atypical responses to challenge within small groups of animals, it was noted that vaccination of Bali cattle with inactivated virus only marginally reduced the duration of the acute clinical signs of infection yet significantly reduced the magnitude and duration of VL after challenge with JDV. These factors would be expected to reduce the window of opportunity for transmission of JDV via close contact or hematophagous arthropods such as tabanid flies to naïve cattle thus helping to contain the spread of virus. By determining the duration and magnitude of the $VL\geq10^6$ genomes/ml, we have developed a much improved method for assessing the efficacy of JDVacc after challenge, which is directly relevant to its use in JD outbreak situations.

Materials and Methods

Animals

Eight female Bali cattle between 6 and 12 months of age were purchased and housed as previously described (Soeharsono et al., 1990). Animals were housed for 1 week prior to vaccination and were tested by ELISA (Hartaningsih et al., 1994) to ensure that they were JDV seronegative.

Vaccination of cattle

The vaccine, JDVacc, was prepared as previously described (Hartaningsih et al., 2001). Briefly, the capsule was removed from the spleen of an animal experimentally infected with the $JDV_{TAB/87}$, and was homogenised at a ratio of 10% w/v in Medium 199 at 4 °C. Virus was inactivated by the addition of Triton-X 100 to a final concentration of 1% v/v and subsequent stirring for 30 min at room temperature. The antigen preparation was mixed and homogenised with a mineral oil adjuvant (MOA) at a ratio of 2:1 MOA:antigen.

Two vaccinations, each of 1 ml, were administered intramuscularly into the neck of four cattle at 67 and 39 days before infection with $JDV_{TAB/87}$. The four control animals were given 3×1 mg/ml doses of glutathione-s-transferase (GST) homogenised in an equal volume of incomplete Freund's adjuvant (IFA) at 67, 53 and 32 days before experimental infection. Cattle were monitored daily for any signs of clinical disease and the absence of any febrile response after vaccination indicated that the vaccine preparation did not contain infectious virus.

Experimental infection with $JDV_{TAB/87}$

All animals in this experiment were challenged intravenously with an estimated 1000 ID_{50} of $JDV_{TAB/87}$, quantified using capture ELISA (Stewart et al., 2005). Clinical signs and rectal temperatures were monitored daily up to 23 days post-infection. Blood samples were collected into EDTA for plasma collection on days 0, 3, 5, 7 and any days when the rectal temperature was ≥39.5 °C. Serum samples were collected at 0, 4, 5, 10 and 13 weeks post-vaccination for all cattle and samples from weeks 15, 17 and 26 were taken from the control GST vaccinated group only.

Viral RNA quantification

Viral RNA was prepared from 280 µl of plasma from each timepoint using a QIAmp Viral RNA mini kit (QIAGEN) following the manufacturer's instructions, with a final elution volume of 60 µl in buffer EB. qRT-PCR was performed as previously described using Access RT-PCR kit (Promega) (Stewart et al., 2005). Each sample was assayed in duplicate using 2 µl RNA in a reaction volume of 25 µl with fluorescence excited at a wavelength of 470 nm and detected at a wavelength of 510 nm. The one-step RT-PCR protocol consisted of an RT step at 48 °C for 45 min, a 3 min inactivation step at 95 °C, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 60 °C for 30 s. The fluorogenic probe and primers were designed to amplify and detect a 121 bp fragment of the JDV gag gene as previously described (Stewart et al., 2005). The limit of detection of the assay was 9.8×10^2 JDV viral RNA copies over 35 cycles, equivalent to 4.2×10^4 JDV genome copies/ml plasma.

Immunoblot and ELISA analysis of anti-JDV IgG responses

Serum samples were tested using a combination of ELISA and Western immunoblot for the presence of antibodies to recombinant JDV proteins using viral antigens expressed in E. coli strain JM109. All recombinant viral proteins were derived from $JDV_{TAB/87}$sequences (Chadwick et al.,1995) and cloned into the PinPoint expression system (Promega) as previously described (Desport et al., 2005). The JDV recombinant constructs included p16 (matrix protein encoded by nucleotides 229–604), p26 (capsid protein encoded by nucleotides 604–1287), ΔSU (N-terminal truncated surface unit protein encoded by nucleotides 5556–6463) and Tat1 (encoded by nucleotides 5010–5303). Western immunoblots were prepared using 2000 ng of the recombinant proteins and after overnight transfer, membranes were blocked using 5% skimmed milk in PBST for 30 min at room temperature. Test sera were diluted 1:50 in blocking solution and incubated for 1 h at room temperature. A 1:2000 dilution of rabbit anti-bovine IgG-HRP (BioRad) in PBS/T was used, followed by detection using the Bio-Rad HRP detection reagents. Serological responses were further investigated by ELISA using Maxisorb plates (Nunc). Coating antigens were either recombinant JDV p26 (12.5 ng/well) or JDV p16 (50 ng/well) or a cyclic JDV Tm peptide KVQTGLGCVPRGRYGHFD (1 μg/well) equivalent to the BIV peptide TMA2 which has previously been identified as encompassing the principle immunodominant domain (Scobie et al., 1999). All dilutions were prepared in 5% skimmed milk powder in PBS/T and plates were incubated at each step for 1 h at 37 °C and then washed 3 times with PBS/T. A 1:2000 dilution of rabbit antibovine IgG-HRP in PBS/T (BioRad)was used as the secondary antibody in each assay and the BioRad HRP substrate reagent was added to the plates and allowed to develop for 15 min. The reaction was stopped with 2% oxalic acid and the absorbance was measured at 405 nm (A_{405}).

Piecewise linear regression and statistical analysis

In order to supply missing data caused by variations in disease onset or sampling intervals, a piecewise linear model was used which was fitted separately for the available data from each animal. The model had 5 parameters; 3 inflection points (D, M and R), a growth rate (a) and clearance rate (b). D, M and R were defined by time in days to initial appearance of virus in plasma (day D) observed after infection, when maximum plasma VL was achieved (day M) and when net viral loss began (day R). In the model, t is time in days post-infection and *v* is virus per ml. The four stages are defined in the model as:

Delay stage	$\mathrm{Ln}(v) = 0$	for $t \leq D$,
Growth stage	$\mathrm{Ln}(v) = a(t - D)$	for $D < t < M$,
Plateau stage	$\mathrm{Ln}(v) = a(M - D)$	for $M \leq t \leq R$,
Clearance stage	$\mathrm{Ln}(v) = a(M - D) - b(t - R)$	for $R < t$.

R–M represented the duration of the plateau period where viral growth and loss were approximately equal. Because the “slopes” *a* and *b* were determined on the log (Ln) viral and linear time scales they represented growth and clearance rates respectively on the untransformed viral scale. The model parameters were estimated by minimizing the total residual sum of squares between the observed and fitted values and parameter estimates for the two groups were compared with a two-tailed Students 2-sample t test. The sums-of squares for the differences between the measured data points and the fitted

points were calculated, and the Chi distribution used to assess the goodness of fit of the piecewise regression.

Analysis of vaccine efficacy

Vaccine efficacy was determined by testing for differences in: (i) VL on first and second days of fever and on first day after the end of fever; (ii) duration of time and magnitude of VL where AUC$\geq 10^6$ genomes/ml; (iii) duration of the febrile period. The total VL (AUC) for each animal was estimated by summing the areas under each pair of observations that form sets of trapezoids. The trapezoidarea was determined as h*([v+w]/2), where h was the time in days between consecutive viral determinations (denoted v and w) in genomes/ml. Where critical observations were missing (i.e. the start and finish of viral excretion) the piecewise model was used to estimate the time of these events so that a total AUC could be estimated for each animal. The duration of the infectious period was defined as the period where VL$\geq 10^6$ genomes/ml. This was determined as described above but with a baseline set at 10^6 rather than zero. Linear interpolation between consecutive observations was used to more accurately determine the time when VL exceeded or fell below 10^6 genomes/ml. In three of the control animals (CB83, 85 and 86) and one vaccinated animal (CB78) the sampling finished before the viremia fell below 10^6 genomes/ml so the piecewise model was used to generate this data. This required extrapolation beyond the last data point for 1 day for three animals and for 2 days for CB83 (Figs. 3 and 4). For data analysis in this study, rectal temperatures were divided into ranges adapted from the method of Muraguri (Muraguri, Kiara, and McHardy, 1999) taking 39.3 °C as the lowest significant pyrexia. The ranges were defined as low fever (39.3 °C–40.2 °C), moderate fever (40.3 °C–41.2 °C) and high fever (N41.2 °C).

Statistical analysis

The mean and 95% confidence limits for percentage viral load and infectious period reductions were estimated using the method of Coles et al. (1992). The VL on the first and second days of elevated rectal temperatures (≥39.3 °C), the first day after the febrile period had resolved and the peak and area under the curve$\geq 10^6$ genome copies/ml values were $\log_{10}$ transformed to stabilise the variance between groups. Student's t test was used for statistical comparison of observed data and magnitude and duration of plasma VL. P-values<0.05 were considered statistically significant.

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