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Analysis of Jembrana disease virus replication dynamics *in vivo* reveals strain variation and atypical responses to infection

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ABSTRACT

Jembrana disease virus (JDV) is an acute lentiviral infection of Bali cattle in Indonesia. Data generated during a series of cattle infection experiments was examined and significant differences were identified in the mean plasma viral load on the first and second days of the febrile response in cattle infected with $JDV_{TAB/87}$ compared to those infected with $JDV_{PUL/01}$. The peak and total viral loads $\geq 10^6$ genome copies/ml during the acute stage of the disease were significantly higher in $JDV_{TAB/87}$ infected cattle. $JDV_{PUL/01}$ infected cattle developed peak rectal temperatures earlier than the $JDV_{TAB/87}$ cattle but there were no differences in the duration of the febrile responses observed for the 2 groups of animals. The plasma viremia was above 10^6 genome copies/ml for almost 3 days longer in $JDV_{TAB/87}$ compared to $JDV_{PUL/01}$ infected cattle. Atypical responses to infection occurred in approximately 15% of experimentally infected animals, characterized by reduced viral loads, lower or absent febrile responses and absence of p26-specific antibody responses. Most of these cattle developed normal Tm-specific antibody responses between 4–12 weeks post-infection.

Introduction

Jembrana disease virus (JDV) is a lentivirus which was first reported in Indonesia in 1964, causing high mortalities in Bali cattle (*Bos javanicus*) on the island of Bali. The disease is now endemic in Bali and has subsequently occurred in Bali cattle in Java, Sumatra and Kalimantan (Indonesian Borneo). Infection of Bali cattle with JDV causes an acute febrile response lasting 5–12 days after a short incubation period, and animals characteristically develop anorexia, leukopenia mainly due to a lymphopenia, oral erosions and enlargement of superficial lymph nodes during the febrile period (Soesanto et al., 1990). High levels of infectious virus (10^8 ID_{50}/ml plasma) were detected during the febrile response by titration in susceptible animals, reducing to 10^5 ID_{50}/ml on the first day after the febrile period and further declining to about 10 ID_{50}/ml at 42 and 72 days after infection (Soeharsono et al., 1995). The case fatality rate was estimated to be 17% (Soeharsono et al., 1990) and surviving animals were found to be resistant to re-infection, did not appear to suffer relapses and remained viremic for at least 2 years (Soeharsono et al., 1990). There is evidence of immunosuppression during the acute stage of Jembrana disease resulting in a delayed seroconversion to viral proteins (Hartaningsih et al., 1994). Attempts to cultivate JDV *in vitro* have so far been unsuccessful (Wilcox et al., 1992). An inactivated tissue-derived whole virus vaccine has been produced which has been found to ameliorate the febrile response and the leukopenia that occur post-infection (Hartaningsih et al., 2001).

The early responses to JDV infection in cattle were determined by monitoring the development of clinical signs, without quantification of the virus load (VL) during infection. Methods for quantification of lentiviruses without using traditional cell culture techniques are now well established, with surrogate assays such as measurement of HIV-1 p24 capsid protein, reverse transcriptase (RT) activity and amount of viral genomic RNA all currently being used (Marozsan et al., 2004; Schutten et al., 2000; Sutthent et al., 2003). The recent development of a JDV specific quantitative real-time reverse transcription PCR assay (qRT-PCR) has enabled the quantification of viral RNA genomes in plasma samples taken during the course of infection, providing data that can be used to assess vaccine efficacy and strain differences (Stewart et al., 2005). A piecewise linear regression model has recently been used to facilitate calculation of area under the curve (AUC) to demonstrate a reduction in the duration and magnitude of the plasma VL after vaccination with an inactivated whole virus vaccine and homologous challenge with $JDV_{TAB/87}$ (Ditcham et al., 2009).

Most lentiviruses have relatively labile genomes which are prone to variation and the development of quasispecies is common (Delwart et al., 1994; Leroux et al., 2001). A recent phylogenetic analysis of JDV *env* sequences has revealed that the $JDV_{PUL/01}$ strain clustered

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separately from other Bali strains and that there is a 7 nucleotide insertion in the U3 region of the LTR relative to $JDV_{TAB/87}$ (Desport et al., 2007). Differences in viral tropism and replication rates have been attributed to variations in this region of the LTR in Maedi-visna virus and Equine infectious anemia virus (EIAV) (Barros, Andresdottir, and Fevereiro, 2005; Lichtenstein et al., 1999). In addition, only 1 of 20 cattle was successfully infected after plasma from a $JDV_{PUL/01}$ donor animal on the second day of fever (rectal temperature ≥39.5 °C) was diluted 10^{-5} to give an estimated challenge dose of 1000 ID_{50}. The plasma from $JDV_{TAB/87}$ infected animals has been shown to consistently contain $\geq 10^8$ ID_{50}/ml at this stage of the febrile response (Soeharsono et al., 1990). We sought to determine whether there was any evidence of variation in replication dynamics and viral loads between $JDV_{TAB/87}$ and $JDV_{PUL/01}$ strains *in vivo* by comparing the viral loads during the acute stage of the disease in groups of cattle that had been experimentally infected with JDV.

Results

Clinical responses to infection with $JDV_{TAB/87}$ and $JDV_{PUL/01}$

The rectal temperatures of 26 Bali cattle experimentally infected with JDV were compared to determine the range of values that are normally observed during the acute phase of Jembrana disease (Table 1). In 19 of the 26 experimentally infected animals, the clinical disease followed a typical course; an incubation period of 5–12 days, followed by a febrile response with a range of clinical signs, resolving as the rectal temperature returned to normal 13–17 days post-infection (Table 1). The groups that were infected with $JDV_{PUL/01}$ developed peak rectal temperatures on average 1.5 days before those infected with $JDV_{TAB/87}$ but there were no significant differences in the duration or magnitude of the febrile responses or the number of days to recovery (Table 1). This is illustrated in Fig. 1 by the rectal temperature of CB137 ($JDV_{PUL/01}$) which peaked at 11 days post-infection compared to the temperature of CB210 ($JDV_{TAB/87}$) which peaked at 12 days post-infection. However, peak VL developed 8–14 days post-infection with no difference in the mean time to peak VL in either group (Table 1). The observed and fitted data generated using the piecewise linear regression were closely aligned for the typical responders in both groups of animals (CB210 and CB137 shown in Fig. 1).

Four animals CB64, CB84, CB134 and CB136 did not develop a typical febrile response but instead a transient mild fever for 1–2 days but they were all found to have detectable levels of circulating virus (Fig. 2). Two patterns of viral replication were seen in these animals. In CB64, CB84 and CB134 there was an undulating viremia increasing to a maximum titer of approximately 10^8 genomes/ml which resulted in differences between the observed and fitted VL data (Fig. 2). In CB136 there was a more typical pattern of viral increase and decrease but with lower viral titers compared to the typical responders. In addition, 3 cattle CB109, CB111 and CB156 died after infection with $JDV_{PUL/01}$. CB111 exhibited a severe leukopenia (data not shown) and detectable viremia within 3 days of infection resulting in a poor fit between observed and fitted data (Fig. 3). After a prolonged period of elevated rectal temperatures (13 days) this animal subsequently died. CB109 also developed an early transient fever and died as the febrile response was resolving. CB156 appeared to be following the typical course of infection with the febrile response resolving at 13 days post-infection but was unable to clear the virus and subsequently died (Fig. 3). This failure to clear virus was a common feature of these animals as they were all found to have high plasma viral loads at death but Fischers exact test did not indicate any significant differences ($P = .140$) in fatalities between the 2 groups of cattle. These were the only fatalities giving a mortality rate of 21% for cattle experimentally infected with $JDV_{PUL/01}$.

Table 1
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 for 26 cattle experimentally infected with 1000 ID_{50}/ml $JDV_{TAB/87}$ (CB61–CB210) or $JDV_{PUL/01}$ (CB108–CB158)

Cattle no.	Day post-infection				Duration of febrile response (days)			
	Peak VL	≥39.3 °C	Peak °C	<39.3 °C	Low	Moderate	High	Total
CB 61	10	5	11	14	5	3	0	8
CB 62	11	8	11	15	3	3	1	7
CB 63	8	7	11	14	0	6	1	7
CB 64	**14**	**16**	**17**	**19**	**2**	**0**	**0**	**2**
CB 83	12	7	11	14	3	4	0	7
CB 84	**13**	**12**	**12**	**13**	**1**	**0**	**0**	**1**
CB 85	11	9	11	15	2	4	0	6
CB 86	12	7	12	15	3	5	0	8
CB 203	12	8	13	15	4	5	0	9
CB 206	12	3	13	15	6	1	0	7
CB 208	11	8	11	14	2	2	1	5
CB 210	12	9	12	17	3	5	0	8
Mean	**11.5**	**8.3**	**12.1**	**15**	**2.8**	**3.2**	**0.3**	**6.3**
Var	**2.1**	**9.9**	**2.7**	**2.33**	**2.5**	**3.8**	**0.2**	**5.5**
CB 108	10	5	12	14	3	4	2	9
CB 109	10	(4) 9	(4) 11	(5) 13 died 14	4	1	0	5
CB 110	11	10	12	13	2	1	0	3
CB 111	14	(3) 12	(7) 13	(10) 18 died	9	4	0	13
CB 134	**14**	**4**	**4**	**5**	**1**	**0**	**0**	**1**
CB 135	13	9	11	16	4	2	2	8
CB 136	**12**	**12**	**12**	**13**	**1**	**0**	**0**	**1**
CB 137	12	9	11	15	2	5	0	7
CB 138	10	9	12	15	1	4	0	5
CB 154	11	8	10	14	1	5	0	6
CB 155	11	7	10	14	4	2	1	7
CB 156	11	8	10	13 died 14	4	2	0	6
CB 157	11	10	10	14	3	1	0	4
CB 158	10	9	10	14	2	2	1	5
Mean	**11.4**	**8.6**	**10.6**	**13.6**	**2.9**	**2.4**	**0.4**	**5.7**
Var	**1.8**	**4.7**	**4.2**	**7.5**	**4.2**	**2.8**	**0.5**	**9.2**
***P*-value**[a]	**0.901**	**0.721**	**0.062**	**0.156**	**0.901**	**0.285**	**0.482**	**0.637**

Atypical responders are highlighted in bold.
[a] *P*-values represent statistical differences between animals infected with $JDV_{TAB/87}$ and $JDV_{PUL/01}$.

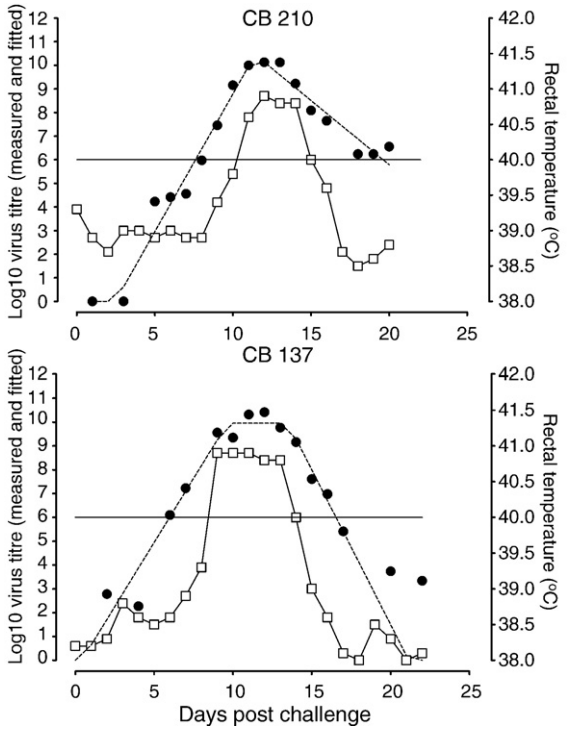

Fig. 1. Dynamics of viral replication in CB210 after infection with 1000 ID_{50}/ml $JDV_{TAB/87}$ and CB137 after infection with 1000 ID_{50}/ml $JDV_{PUL/01}$. Rectal temperatures (□) were collected for up to 22 days post-infection. Plasma VL (JDV genome copies/ml plasma) was determined using qRT-PCR (●) 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.

Comparison of $JDV_{TAB/87}$ and $JDV_{PUL/01}$ replication dynamics

The mean plasma VL on the first and second days of the febrile response in the cattle infected with $JDV_{PUL/01}$ were significantly lower ($P=.046$ and $P=.033$ respectively) than in cattle infected with $JDV_{TAB/87}$ (Table 2). Similarly, the mean peak VL was significantly lower in $JDV_{PUL/01}$ infected animals ($P=.049$). $JDV_{TAB/87}$ infected cattle were found to have a mean log_{10} plasma VL $\geq 10^6$ genomes/ml of 11.2 compared to 10.26 for $JDV_{PUL/01}$ infected cattle during the acute stage of Jembrana disease. This is approaching significance ($P=.056$) and is reflected in the significant difference of almost 3 days in the mean number of days that cattle were found to have plasma VL above this threshold in the 2 groups of animals ($P=.018$) (Table 2).

Analysis of variance within strains

When the variation in clinical responses to infection with either strain of JDV was analysed within groups infected with the same strain there were no significant differences. However cattle infected with $JDV_{PUL/01}$ in group IV were found to have a significantly higher peak VL ($P=.033$) and AUC $\geq 10^6$ genomes/ml ($P=.034$) than the cattle in groups V and VI. In cattle infected with $JDV_{TAB/87}$ significant differences in VL were observed in the cattle in group II on the first ($P=.056$) and second ($P=.033$) days of elevated rectal temperatures (>39.3 °C) when compared to groups III and VII.

Development of antibody responses post-infection

The 8 typical responders that were sampled beyond the recovery period of Jembrana disease developed detectable anti-capsid antibody responses 5–15 weeks post-infection (Fig. 4). A small transient increase in antibody levels was seen in some animals at 2 weeks

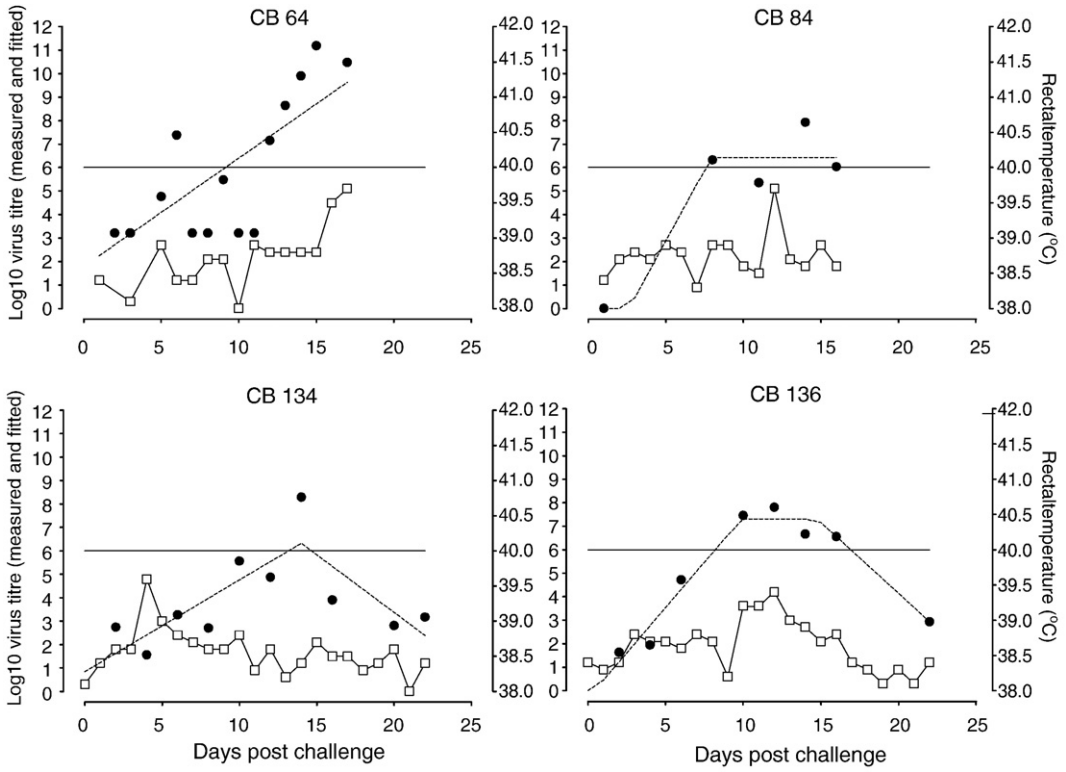

Fig. 2. Dynamics of viral replication in atypical responders after infection with 1000 ID_{50}/ml $JDV_{TAB/87}$ (CB64 and CB84) or 1000 ID_{50}/ml $JDV_{PUL/01}$ (CB134 and CB136). Rectal temperatures (□) were collected for up to 22 days post-infection. Plasma VL (JDV genome copies/ml plasma) was determined 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.

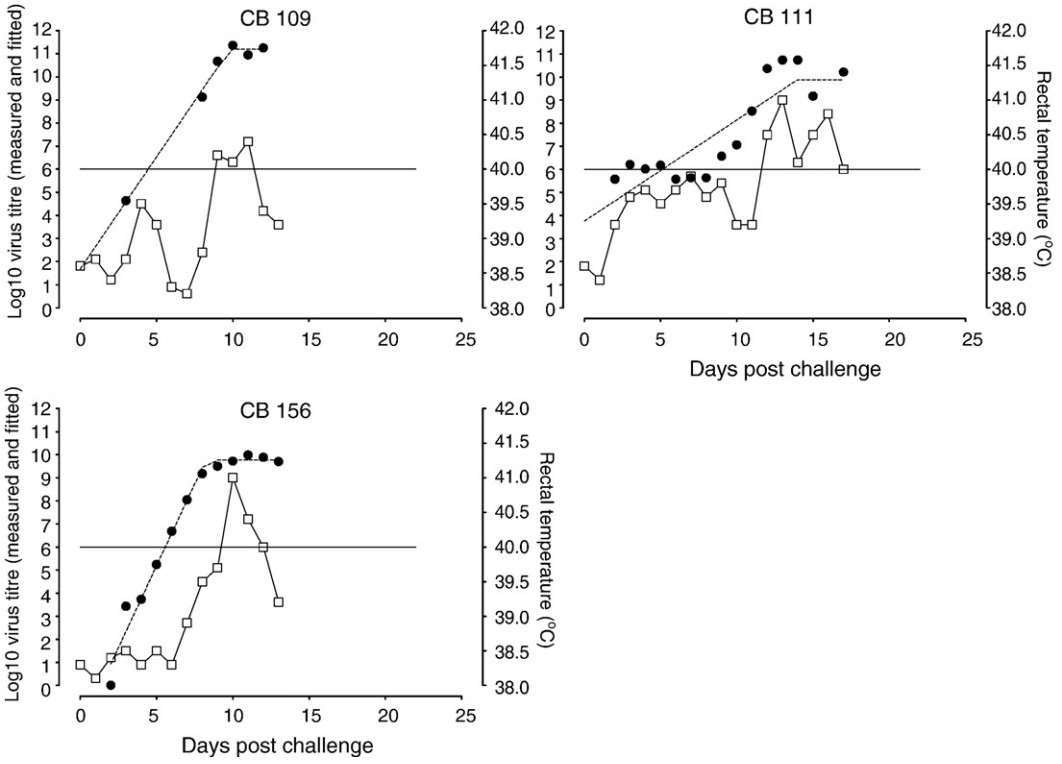

Fig. 3. Dynamics of viral replication in fatal cases of Jembrana disease after infection with 1000 ID_{50}/ml $JDV_{PUL/01}$. Plasma VL (JDV genome copies/ml plasma) was determined using qRT-PCR (●) and transformed to $\log_{10}$ scale. Differences in VL were calculated using a threshold of 10^6 genome copies/ml plasma (solid line) and AUC was determined from measured plasma VL (●). Rectal temperatures (□) were collected until the day of death.

post-infection and all animals appeared to reach a peak or plateau by 26 weeks. The 4 atypical responders did not develop responses that were detectable using the JDV p26-his ELISA within the period of sampling (Fig. 5) although very weak responses were observed by Western immunoblotting (data not shown). Antibody responses detected using the JDV Tm^c peptide were observed in all cattle, including the atypical responders with the exception of CB134.

Discussion

Experimental infection with different strains of JDV resulted in a broad spectrum of responses, ranging from an absence of any clinical signs of disease in 15% of cattle to fatalities in 11.5% of cattle. This high level of animal to animal variation can obscure the effects of vaccination or strain variation when using small numbers of animals per group. Quantification of the magnitude and duration of VL $\geq 10^6$ genome copies/ml has revealed the benefits of using JDVacc, a whole virus inactivated vaccine that is currently used in Indonesia (Ditcham et al., 2009) and highlighted the problems associated with correlating febrile responses with vaccine efficacy. The data presented in this study reveals differences in the magnitude and duration of plasma VL generated after infection with $JDV_{PUL/01}$ compared to $JDV_{TAB/87}$ which could be associated with the phylogenetic separation of $JDV_{PUL/01}$ from other Bali strains based on *env* sequences (Desport et al., 2007). Animals infected with $JDV_{TAB/87}$ exhibited higher and longer plasma VL compared to those infected with $JDV_{PUL/01}$ which could be due to both host and virus factors. Co-infection with other pathogens, particularly those such as helminths which cause immune activation, are host-specific contributing factors which have been identified in the spread and progression of HIV in developing countries (Bentwich et al., 2000). Small changes in lentivirus gene sequences can confer significant phenotypic changes in the virus. Simultaneous mutations in the *capsid* and *vif* genes have been shown to confer a highly pathogenic phenotype on a non-pathogenic infectious clone of Maedi-visna virus (Gudmundsson et al., 2005). Differences in LTR sequences have been correlated with an increased replication capacity of EIAV *in vitro* (Madden and Shih, 1996) and it is possible that the 7 nucleotide insertion in the U3 region of the LTR of $JDV_{PUL/01}$ (Desport et al., 2007) may be contributing towards at least some of the differences in the viral replication dynamics. Expression and regulation of mRNA transcripts can influence both lentiviral replication and disease pathogenesis (Schiltz et al., 1992; Unger, Stout, and Luciw, 1991). We have recently reported differences in the splicing patterns of mRNA transcripts encoding the Tat proteins of these two virus strains (Setiyaningsih et al., 2008). Two small non-coding exons were identified in transcripts obtained from $JDV_{PUL/01}$ whereas only one non-coding exon was identified in the $JDV_{TAB/87}$ transcripts. Small non-coding exons have been identified in HIV-1 mRNA leader sequences and are associated with changes in gene expression and the cell type from which the transcripts were derived (Krummheuer et al., 2001).

Atypical responses were seen in 4 of the cattle infected with either $JDV_{TAB/87}$ or $JDV_{PUL/01}$ and their lower peak VL was associated with reduced or absent clinical signs. Atypical responses to infection have been reported in a small proportion of mock-vaccinated cats in feline immunodeficiency virus (FIV) vaccine trials where they were found to be naturally resistant to FIV, even after more than one challenge (Verschoor et al., 1996). Two EIAV strains appear to have different disease induction capabilities in horses which correlate with nucleotide differences in the 3′ region of the genome. Based on these observations, it has been postulated that a threshold level of EIAV is required for induction of acute disease in horses (Cook et al., 2003). Similarly, high levels of $SIV_{smmPBj14}$ in the plasma of pig-tailed macaques was associated with a virus threshold beyond which a synergistic cycle of lymphoid activation and viral replication accelerates uncontrollably resulting in the acutely lethal syndrome (Schwiebert and Fultz, 1994).

Table 2
Comparison of plasma VL on the first and second days of febrile response, peak VL, area under curve (AUC) for plasma VL $\geq 10^6$ genome copies/ml and duration of AUC $\geq 10^6$ genome copies/ml for 26 cattle experimentally infected with $JDV_{TAB/87}$ (CB61–CB210) or $JDV_{PUL/01}$ (CB108–CB158)

Animal	Log_{10} VL 1st day ≥39.3 °C	Log_{10} VL 2nd day ≥39.3 °C	Log_{10} peak VL	Log_{10} AUC $\geq 10^6$ genome copies/ml	Days AUC $\geq 10^6$ genome copies/ml
CB 61	11.34	11.81	12.22	12.74	13.8
CB 62	11.09	11.47	11.97	12.57	15.7
CB 63	10.56	11.29	11.29	11.89	12.9
CB 64	**10.48**	**10.12**	**11.19**	**11.41**	**8.9**
CB 83	10.08	10.97	11.76	12.22	12.2
CB 84	**7.08**	**N/A**	**7.93**	**8.33**	**7.3**
CB 85	10.06	10.36	10.63	11.02	9
CB 86	9.15	10.33	11.10	11.62	11.9
CB 203	9.98	10.28	10.73	11.18	16.39
CB 206	9.40	10.01	10.18	10.55	10.30
CB 208	7.54	8.95	10.02	10.22	10.70
CB 210	9.16	10.00	10.13	10.60	13.40
Mean	**9.66**	**10.55**	**10.81**	**11.20**	**11.88**
Var	**1.68**	**0.66**	**1.48**	**1.46**	**7.65**
CB 108	6.29	6.37	10.64	11.19	11.7
CB 109	10.67	11.36	11.36	11.87	10
CB 110	9.95	11.19	11.19	11.65	10
CB 111	6.21	6.01	10.74	11.18	11.8
CB 134	**1.57**	**N/A**	**8.29**	**8.29**	**3**
CB 135	9.74	10.46	10.58	11.23	13.9
CB 136	**7.81**	**N/A**	**7.81**	**8.22**	**9.2**
CB 137	9.56	9.35	10.42	10.78	11.1
CB 138	8.08	10.35	10.35	10.46	8.7
CB 154	8.43	8.79	9.19	9.42	6.9
CB 155	8.83	9.54	9.69	10.14	9.6
CB 156	9.18	9.50	9.99	10.48	7.8
CB 157	8.83	8.94	8.94	9.35	8
CB 158	8.29	9.06	9.06	9.32	6.5
Mean	**8.10**	**9.24**	**9.87**	**10.26**	**9.16**
Var	**5.14**	**2.74**	**1.16**	**1.39**	**7.34**
***P*-value**[a]	**.046**	**.033**	**.049**	**.056**	**.018**

Atypical responders are highlighted in bold.
[a] *P*-values represent statistical differences between animals infected with $JDV_{TAB/87}$ and $JDV_{PUL/01}$.

The variation observed between groups of cattle infected with the same strain of virus indicated that there were some group specific factors contributing towards these differences. The significantly higher VL in cattle in group II at the onset of the febrile response could have occurred as a result of a higher titer of infectious virus in the challenge dose prepared for this group. There were no differences between the peak VL for these groups confirming earlier studies which showed that there was no detectable relationship between the dose and the severity and duration of the disease induced (Soeharsono et al., 1990). The differences between groups infected with $JDV_{PUL/01}$ were due to higher peak VL and AUC $\geq 10_6$ genome copies/ml in cattle in group IV compared to groups V and VI. This suggests that group specific factors such as poor nutrition (unpublished observation) or the introduction of additional infectious agents in the challenge dose may have contributed to the higher VL and subsequent fatalities of 50% of the cattle in this group.

During the recovery phase of Jembrana disease the earliest detectable immune response is against the JDV capsid protein (Hartaningsih et al., 1994). Capsid antibody responses are used as diagnostic indicators of bovine lentiviral infections (Barboni et al., 2001; Burkala et al., 1999; Meas et al., 2000) but the absence of detectable responses in 4 of the 12 animals in this study suggests that these diagnostic assays may be underestimating the prevalence of infection. Failure to mount a detectable antibody response has also been observed in other lentivirus infections. Immune dysfunction in persons who are HIV-1-infected but who remain persistently seronegative has been suggested to account for the lack of detectable antibody response, although in this case the absence of antibodies appears to correlate with quick onset or rapid progression of disease in 50% of these cases (Ellenberger et al., 1999). FIV genome-positive seronegative cats have been reported after being housed with seropositive FIV-infected cats and it is thought that this could be due to delayed seroconversion, infection with defective virus or atypical routes of infection (Dandekar et al., 1992). Robust antibody responses to envelope glycoproteins have been reported in the absence of core antigen p28 specific antibodies in African green monkeys naturally infected with SIV_{AGM} (Norley et al., 1990). This was associated with a lack of virus trapping in lymph nodes and non-progression to AIDS and it is suggested to occur as a result of T-cell tolerance to gag resulting in protection from the catastrophic demise of the immune system (Norley and Kurth, 2004). The significance of these different humoral responses in JDV infected cattle remains to be determined.

A linear relationship between the infectious doses used in JDV experimental infections and the incubation period before onset of disease has been identified in previous studies and provides some additional evidence of differences in replication rates between JDV strains (Soeharsono et al., 1990). Tenfold dilutions of whole blood from donor animals infected with either Singaraja/86 strain ($JDV_{SING/86}$) or $JDV_{TAB/87}$ were inoculated into naïve cattle and a consistently longer incubation period was seen in the cattle challenged with $JDV_{SING/86}$ compared to those challenged with the same dilution of $JDV_{TAB/87}$.

The comparison of plasma VL on the first 2 days of fever in cattle infected with different strains of JDV clearly showed that $JDV_{PUL/01}$ infected cattle developed fever at a lower plasma VL compared to cattle infected with $JDV_{TAB/87}$. Thus the infection of only 1/20 cattle with a 10^{-5} dilution of plasma from a $JDV_{PUL/01}$ donor animal taken on the second day of fever was almost certainly due to a lower titer of virus rather than an atypical response to infection. The development of the JDV p26 capture ELISA has provided an indirect method to quantify VL in donor animals and was used for preparation of

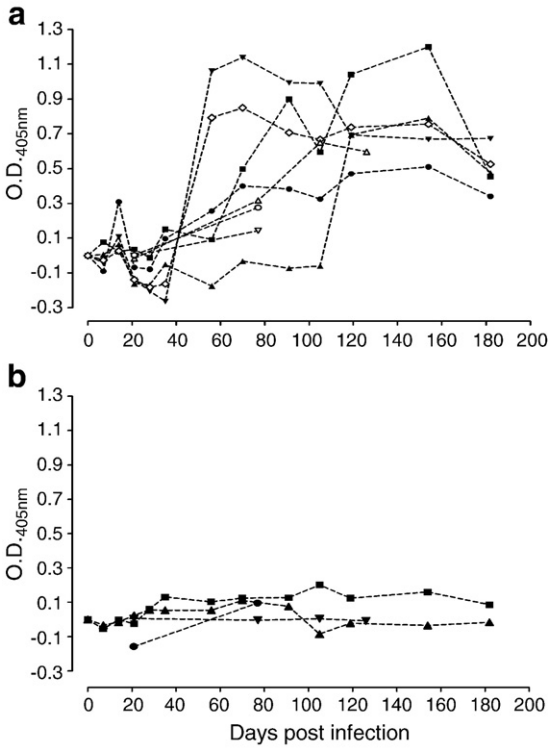

Fig. 4. Development of IgG responses to recombinant JDV p26 in (a) typical CB61 (■), CB62 (▲), CB83 (▼), CB85 (♦), CB86 (●), CB135 (♦), CB137 (x) and CB138 (I) and (b) atypical CB64 (■), CB84 (▲), CB134 (▼) and CB136 (♦) responders after experimental infection with $JDV_{TAB/87}$ (CB61–64, CB83–86) or $JDV_{PUL/01}$ (CB134–138).

challenge doses for each of the groups examined in this study (Stewart et al., 2005). Unfortunately the lower plasma VL in $JDV_{PUL/01}$ infected animals did not correlate with any attenuation of the virus as all of the mortalities were observed in cattle infected with this strain of virus. The correlation between lower or absent febrile responses in the atypical responders and reduced peak viral loads suggests that a threshold of disease induction may exist for JDV and that a vaccine which is able to effect a 10–100 fold reduction in viral load may be sufficient to control the spread of Jembrana disease in Indonesia.

Materials and methods

Experimental infection with $JDV_{TAB/87}$ or $JDV_{PUL/01}$

Female Bali cattle 6–12 months of age were purchased from Nusa Penida, an island off the coast of Bali that is free of Jembrana disease. The animals were housed indoors and tested by ELISA for JDV capsid serum antibody (Hartaningsih et al., 1994) to ensure that they had not been infected previously. Live viral challenge doses were prepared by inoculation with virus strains $JDV_{TAB/87}$ or $JDV_{PUL/01}$ into seronegative donor animals as described previously (Soeharsono et al., 1995). The plasma viral load in the donor animals was determined using antigen capture ELISA as described previously (Stewart et al., 2005) and fresh plasma samples were diluted to give approximate challenge doses of 1000 ID_{50}/ml. The cattle used in this study were derived from a series of experimental infection trials and therefore different donor animals were used to prepare challenge doses for each group (Table 3). Sampling intervals varied between the groups and only cattle from groups II, III and V were sampled beyond the acute stage of the disease for between 11 and 26 weeks post-infection. Rectal temperatures were taken daily for 21 days post-infection. For data analysis in this study, rectal temperatures were divided into ranges adapted from the method of Muraguri et al. (1999) taking 39.3 °C as the lowest

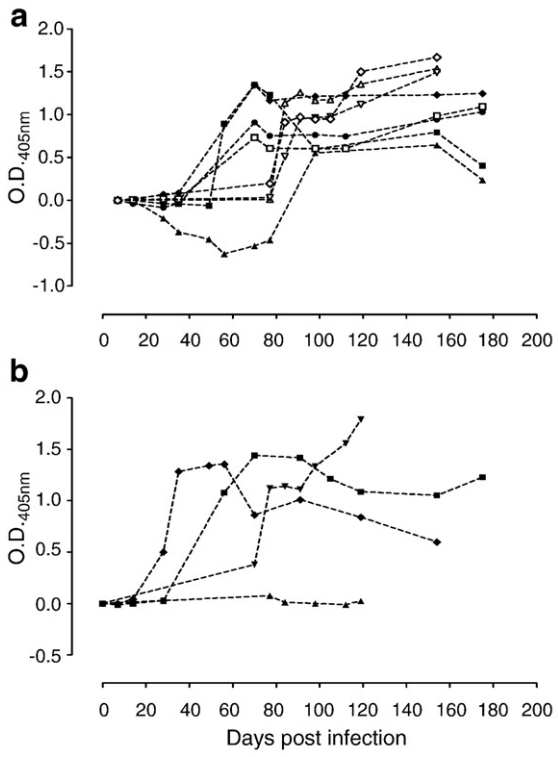

Fig. 5. Development of IgG responses to recombinant JDV Tm^c peptide in (a) typical CB61 (■), CB62 (▲), CB83 (▼), CB85 (♦), CB86 (●), CB135 (♦), CB137 (**x**) and CB138 (**I**) and (b) atypical CB64 (■), CB84 (▲), CB134 (▼) and CB136 (♦) responders after experimental infection with $JDV_{TAB/87}$ (CB61–64, CB83–86) or $JDV_{PUL/01}$ (CB134–138).

Table 3
Cattle numbers, groups and strain of JDV used for infection

	Cattle no.					JDV strain
Group II	CB61	CB62	CB63	CB64		$JDV_{TAB/87}$
Group III	CB83	CB84	CB85	CB86		$JDV_{TAB/87}$
Group IV	CB108	CB109	CB110	CB111		$JDV_{PUL/01}$
Group V	CB134	CB135	CB136	CB137	CB138	$JDV_{PUL/01}$
Group VI	CB154	CB155	CB156	CB157	CB158	$JDV_{PUL/01}$
Group VII	CB203	CB206	CB208	CB210		$JDV_{TAB/87}$

significant temperature indicating fever. The ranges were defined as low fever (39.3 °C–40.2 °C), moderate fever (40.3 °C–41.2 °C) and high fever (>41.2 °C). Whole blood samples were taken into EDTA for preparation of plasma and PBMC and serum samples were prepared by centrifugation from clotted blood taken into tubes without EDTA. All samples were stored at −80 °C until analysis. Clinical signs were monitored until recovery.

Viral RNA quantification

Plasma samples were centrifuged (8000 g, 5 min) prior to extraction of RNA. Viral RNA from 140 µl (280 µl for CB83–86) of recently thawed plasma from each sample was extracted using a QIAmp Viral RNA mini kit (Qiagen) following the manufacturer's instructions, with a final elution volume of 60 µl in buffer EB. Extracted RNA was stored at −80 °C until assayed. The primer set utilized was JDV *gag*1f (TGGGAAGCATGGACAATCA) and JDV *gag*1r (GGAGACCCGTCAGATGTGGA) which specifically amplified a 118 bp fragment of the JDV *gag* gene. The fluorogenic probe (CCCACAACTTAGAAAGAACTTCCCCGCTG) was labeled at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher dye BHQ-1. The qRT-PCR reactions were performed in a Rotor-Gene 3000 (Corbett Research). RNA samples from groups II–IV were quantified using an Access RT-PCR kit (Promega) as previously described (Stewart et al., 2005) using 2 µl of extracted viral RNA per reaction. 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. RNA samples obtained from the animals in group V, VI and VII were analyzed using a premixed 2× mastermix (Iscript, BioRad), with the addition of both primers and fluorogenic probe to a final concentration of 100 nM as above, and ultrapure water added to give a final 1× concentration. MMLV reverse transcriptase was used in this assay following the manufacturers' instructions. One microliter of RNA sample was added to 9 µl of mastermix and the one-step RT-PCR protocol consisted of an RT step of 50 °C for 10 min, a 5 min inactivation step at 95 °C with a 2 s pause at 92 °C to prevent thermal overshoot, followed by 40 cycles of 92 °C for 2 s, 95 °C for 15 s and 58 °C for 30 s and 60 °C for 30 s. A standard curve was generated in each run by amplification of duplicate serial 10-fold dilutions of a plasmid containing nucleotides 19–2881 of $JDV_{TAB/87}$ strain, encompassing the target amplicon. Copy numbers of the plasmid added to each standard reaction were calculated, with one copy of plasmid equivalent to one viral genome.

Development of anti-JDV IgG responses post-infection

Serum samples were tested by ELISA using JDV capsid protein with a hexa-histidine tag (JDV p26-his) produced from a plasmid containing JDV capsid in pTrcHisB (a kind gift from Dr. M. Collins). A JDV TM peptide (KVQTGLGCVPRGRYCHFD) reported to encompass the principal immunodominant domain of JDV Tm (Barboni et al., 2001) was synthesized 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). Sera were tested at dilutions of 1:100 (JDV p26-his) or 1:16 (JDV Tm^c peptide) using Maxisorb plates (NUNC) coated with 12.5 to 50 ng of antigen per

well or with 1 μg JDV Tm^c peptide per well diluted in 0.1 M $NaHCO_3$ buffer (pH 9.5). Blocking was performed at room temperature with 5% skimmed milk in PBS-0.05% Tween 20 (PBST) for 1 h. Serum dilutions in blocking solution were incubated for 1 h at 37 °C followed by a 1:2000 dilution of horseradish peroxidase labeled rabbit anti-bovine IgG (ICN) in PBS/T for 1 h at 37 °C. HRP substrate reagent (BioRad) was added to the wells and allowed to develop for 15 min. The reaction was stopped with 2% oxalic acid and the absorbance read at 405 nm.

Statistical analysis of viral load and temperature differences

Differences between $JDV_{TAB/87}$ or $JDV_{PUL/01}$ were assessed by analyzing changes in rectal temperature and plasma VL in experimentally infected cattle. Rectal temperatures were divided into ranges adapted from the method of Muraguri et al. (1999) using 39.3 °C as the lowest significant fever. The VL on the first and second days of elevated rectal temperatures (>39.3 °C) and the peak and area under the curve $\geq 10^6$ genome copies/ml values for VL were $\log_{10}$ transformed to stabilize the variance between groups. Variation between animals infected with different strains and between groups infected with the same strain was tested using ANOVA (Minitab). The area under curve (AUC) of the plasma VL $\geq 10^6$ genomes/ml was used to assess differences in duration and magnitude of the VL. This threshold was chosen as it has been shown to represent 99.995% of the total VL for the control group in a recent vaccine trial (Ditcham et al., 2009) and requires minimal estimation of data points. Three aspects of plasma VL were compared: (i) peak VL; (ii) AUC plasma VL $\geq 10^6$ genomes/ml; and (iii) number of days where plasma VL $\geq 10^6$ genomes/ml. The VL (AUC) for each animal was estimated by summing the areas under each pair of observations that form sets of trapezoids. The trapezoid-area 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. The VL $\geq 10^6$ genomes/ml was determined with a baseline set at 10^6. Linear interpolation between consecutive observations was used to more accurately determine the time when VL exceeded or fell below 10^6 genomes/ml. Sampling did not always continue for long enough for the VL to reduce to 10^6 genomes/ml so the piecewise linear model described previously (Ditcham et al., 2009) was used to extrapolate beyond the last data point.

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