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Final report

project

Large-scale production of a vaccine and diagnostic reagents for Jembrana disease in Indonesia

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1 Acknowledgments

The significant contribution and cooperation of PT Vaksindo Satwa Nusantara in the development of a commercial Jembrana disease vaccine is gratefully acknowledged.

The significant cooperation of the staff of Pusat Veterenaria Farma (PUSVETMA) toward the commercial development of serological kits for immunosurveillance of Jembrana disease is gratefully acknowledged.

2 Executive summary

Jembrana disease, an acute disease with a 20% case fatality rate, is a peculiarly Indonesian problem as it produces a severe disease in Bali (Bos javanicus) cattle and has been detected in Bali, Java, Sumatra and Kalimantan only. Bali cattle are farmed and used in significant numbers in Indonesia where they are owned almost exclusively by smallholder farmers and contribute to poverty reduction of smallholder farmers, with a consequent value-adding effect in rural (usually poorer) areas of Eastern Indonesia.

The disease is caused by a bovine lentivirus, Jembrana disease virus (JDV). Future control of the virus requires a combination of commercially available diagnostic and surveillance methods, and a vaccine. This project involved the development of sustainable methods for the production of diagnostic reagents and a vaccine.

A recombinant polyprotein containing two virus proteins of JDV, the capsid (CA) and Tat proteins, was produced. Vaccination of small groups of experimental cattle with this vaccine elicited very high titres of CA and Tat antibody prior to challenge with JDV four weeks after the second vaccine dose. The vaccine ameliorated the hyperpyrexia and severe lymphopenia typically seen in cattle infected with JDV but as expected, did not provide complete protection.

The technology required for the production of the recombinant CA/Tat protein for Jembrana disease was transferred to the private veterinary vaccine company in Indonesia. The technical capability of the company to produce the vaccine was established and success in producing a batch of the vaccine was achieved. Further progress was delayed by change of ownership of the company. A commitment by DGLS to use the vaccine was made and the process of licensing the vaccine is well advanced. Preliminary approval for the vaccine was obtained from Balai Besar Pengujian Mutu dan Sertifikasi Obat Hewan (BBPPMSOH).

A medium scale field trial of the vaccine indicated that under field conditions the vaccine had no significant adverse effects in vaccinated animals, and elicited a strong and persistent antibody response.

The capacity to sustainably produce a recombinant protein antigen as a basis for commercial serological (ELISA and Western immunoblotting) kit for surveillance of Jembrana disease virus infection has been established at Pusat Veterenaria Farma (PUSVETMA) with guidance from project staff at the Balai Besar Veteriner Denpasar.

The potential for the development of an alternative potentially low cost heterologous live virus vaccine against Jembrana disease by using the non-pathogenic bovine immunodeficiency virus (BIV) was investigated and the hypothesis that prior infection of cattle with BIV would protect against subsequent infection with JDV was tested. Infection of BIV-infected cattle with pathogenic JDV, 42 days after infection with BIV, did not affect JDV replication and it also did not result in amelioration of the clinical signs of Jembrana disease in the JDV-infected cattle. This result was unexpected considering the close genetic and antigenic relationship of the two viruses.

A major problem with serological surveillance of Jembrana disease in Indonesia is that both JDV and the antigenically and genetically related BIV are present in cattle and it is not possible to differentiate the two infections serologically. Significant progress in developing a type-specific serological test that would differentiate antibody to the two viruses was made during the project and this could result in a type-specific test.

To ensure that diagnostic reagents and the vaccine would work with all strains of JDV, further analysis of the genome of JDV was undertaken that revealed that there is genetic diversity in JDV. Strains from Bali and Kalimantan differed phylogenetically and it is possible they are the result of separate incursions of JDV from the natural host.

3 Background

Jembrana disease, an acute disease associated with a 20% case fatality rate, is a peculiarly Indonesian problem as it produces a severe disease only in Bali (Bos javanicus) cattle which are farmed and used in significant numbers only in Indonesia, and the causative virus Jembrana disease virus occurs only in Indonesia although it is most closely related to a non-pathogenic virus detected in other countries and including Australia.

Initial outbreaks of the disease in naïve areas are always associated with a high mortality rate of about 20% in the affected areas, followed by an endemic situation with a lower mortality rate. In endemic areas there are periodic occurrences of smaller outbreaks in localised areas. It first occurred in Bali cattle in 1964 in Bali, when within 12 months it killed 26,000-70,000 (estimates vary) of a total population of about 300,000 Bali cattle on the island. It was detected in Lampung province in Sumatra in 1976 (where it was initially called Rama Dewa disease) and then later spread to West Sumatra. It was detected in East Java in 1978 (where it was initially called Banuwangi disease) and in Kalimantan in the early 1990s. The source of infection in Java and Sumatra was most likely the illegal movement of persistently infected cattle from Bali. The source of infection in Kalimantan is unknown. The disease is now endemic in Bali, Java, Sumatra and Kalimantan. There is a potential threat of the transmission of the disease to Sulawesi and other areas of Indonesia where there is a significant Bali cattle population.

Jembrana disease is a continuing threat to the cattle industry in Indonesia, Cattle in Indonesia are owned almost exclusively by smallholder farmers and contribute to poverty reduction of smallholder farmers, with a consequent value-adding effect in rural (usually poorer) areas of Eastern Indonesia. The utilisation of the cattle as draft power enables smallholder farmers to double food production from their available land; they are therefore critical to the security of food production within Indonesia. The occurrence of Jembrana disease in Bali also prevents access to the national and international cattle market by Balinese smallholder farmers. It is policy of the Directorate General of Livestock Services (DGLS) that the development of the cattle industry in Indonesia will be based on Bali cattle. Precise figures on the cattle industry in Indonesia are difficult to obtain but 1995 figures (Buku Statistik Peternakan, 1995) indicate that there were then in excess of 11 million cattle, of which about 27% were Bali cattle. The majority of these cattle are in Nusa Tenggara Barat (NTB), Nusa Tenggara Timur (NTT), Maluku and Sulawesi, where Jembrana disease does not now occur, the cattle have no immunity to the disease, and outbreaks of Jembrana disease would have devastating effects.

The future control of Jembrana disease requires a combination of excellent diagnostic and surveillance methods, and an appropriate vaccine. During project AS1/2000/029 conducted from 2001 to 2004, suitable methods for the control of Jembrana disease were developed. The major developments were the use of modern molecular technologies to produce methods to control the disease: (a) a vaccine, and (b) a serological antigen for immunosurveillance. The technology developed was appropriate for the commercial production of the products.

Potential vaccines were based on recombinant protein technology, and it was demonstrate in laboratory trials that these vaccines induced a protective immunity in cattle, as measured by a reduction in the duration of the febrile response, a reduction in virus load after challenge with virulent virus, and absence of fatalities. These recombinant protein vaccines had significant advantages over the tissue vaccine that has been used previously in Indonesia, in terms of safety, standardisation and quality control procedures, and ability to produce them on a sustainable basis.

The recombinant protein vaccines that were trialled have been produced only on a small scale at Murdoch University, from where they were transferred to Indonesia for the

laboratory trials. The next step and the major objective of this project is the development within Indonesia of methods for production of vaccines on a medium scale and a sustainable basis. This will involve transfer of the technology to a commercial vaccine company, although there is a problem with this aim: the anticipated number of doses of vaccine required per year is too small for a private commercial company to invest significantly in research and development. A strategy to produce the vaccine on a larger and sustainable basis within Indonesia has, however, been developed. Discussions were held with a small vaccine company (Vaksindo) for the production and quality control of the vaccine. The intention was for the vaccine to be distributed by the Directorate General of Livestock Services (DGLS) and the provincial veterinary services; the distribution arrangement would mirror that which has operated within Indonesia for the distribution of the cattle Haemorrhagic Septicaemia (Pasteurella) vaccine used widely throughout Indonesia for many years.

Methods for the diagnosis of bovine lentivirus infections including Jembrana disease which have been developed are now being utilised by the Veterinary Disease Investigation Centre in Denpasar (Balai Besar Veteriner Denpasar). These are important in the early detection and confirmation of disease outbreaks enabling more rapid disease control measures to be instigated. Training in methodology has been provided to other regional Disease Investigation Centres throughout Indonesia, primarily in areas where the disease is endemic. The key test developed utilises a recombinant JDV protein antigen for an ELISA and provides a method that can be used for routine immunosurveillance, an essential biosecurity technique, and is also useful as a diagnostic tool. The Balai Besar Veteriner Denpasar will be responsible for the production of the recombinant protein antigen, the assembly of kits incorporating all essential reagents and including control sera, and distribution of the kits to other Disease Investigation Centres throughout Indonesia. It was anticipated that production of these kits will be performed on a commercial basis by Pusat Veterenaria Farma (PUSVETMA).

4 Objectives

The aims of this project were

1. To progress the technology for Jembrana disease diagnosis and vaccination that were developed during a previous project AS1/2000/029 so that the vaccine and diagnostic kit could be produced in a sustainable manner within Indonesia,
2. To examine the interaction of Jembrana disease virus with another non-pathogenic bovine lentivirus detected in Indonesia as this will be important in determining what will happen when Jembrana disease occurs in new previously uninfected areas.
3. To improve the specificity of the current Jembrana disease serological test.

The specific objectives were:

1. Develop the capacity within Indonesia for the production of a recombinant protein vaccine for Jembrana disease
2. Confirm the safety and efficacy of the recombinant protein vaccine under field conditions
3. Develop a serological test kit for distribution to provincial veterinary laboratories throughout Indonesia
4. Determine if the non-pathogenic BIV will provide protection against the pathogenic JDV.
5. Develop a serological test that will specifically detect antibody to Jembrana disease virus and will not cross-react with the non-pathogenic BIV that is present concurrently with JDV in Indonesia. This will be attempted by three approaches.

5 Methodology

Objective 1. Develop the capacity within Indonesia for the production of a recombinant protein vaccine for Jembrana disease.

This required the following

- Provision of assistance to Vaksindo in the production of a recombinant protein vaccine, using already established expression systems.

  A GST-tagged recombinant polyprotein containing both JDV CA and Tat proteins was selected for use based on previous results that indicated a combination of the capsid (CA) and Tat proteins of JDV gave a protective immune response. Methods were devised to express both proteins as a single polyprotein in a single expression using a recombinant plasmid containing the capsid and tat genes of JDV and a bacterial expression system. This system was considered relatively simple and amenable to production methods that could be applied in Indonesia. The use of a single expression, rather than two expressions, was expected to reduce costs and simplify the production.

  The objective was approached in several phases

  - to transfer to Vaksindo the technology for small scale production of the proteins, This will involve replication of the plasmid-containing BL21 strain of E. coli, induction of expression of the Capsid-Tat protein with IPTG, purification and solubilisation of the protein from the bacterial cell mass (while monitoring by Western immunoblotting), adjusting protein to a standard concentration. Once this is achieved, to then
  - produce the proteins on a medium scale in current Vaksindo fermenters, and if satisfactory yields cannot be achieved with these facilities, to
  - produce the proteins in fermenters at LIPI Biotechnology where greater control of bacterial growth conditions will be possible.
- cooperate with BBPMSOH (Balai Besar Pengujian Mutu dan Sertifikasi Obat Hewan), the Indonesian vaccine and drug licensing authority, to ensure registration of the vaccine for use in cattle in Indonesia. This is a two-stage process, initially involving an application detailing all available information on the vaccine, and then an animal efficacy trial conducted at the Disease Investigation Centre Denpasar by BBPMSOH staff.
- involve a QA consultant in the processes to be adopted at Vaksindo to provide advice on the preparation of a standard operating procedure.

Objective 2. Confirm the safety and efficacy of the recombinant protein vaccine under field conditions.

This was achieved by the following activities.

- production at Murdoch University of sufficient vaccine for vaccination of 100 cattle.
- selection by Balai Besar Veteriner Denpasar of an appropriate area in the Tabanan district of Bali for testing, in collaboration with provincial veterinary service, then vaccination (twice at monthly intervals) of 100 cattle and placebo vaccination of an additional 100 cattle.
- monitor the vaccinated cattle at monthly intervals for evidence of disease by clinical observations and farmer interviews.
- collect sera at start and at 3 month intervals for serological testing.

- monitor sera for development and persistence of antibodies to JDV using ELISA and Western immunoblotting procedures. This last activity was undertaken jointly by the Australian and Indonesian partners.

Objective 3. Develop a serological test kit for distribution to provincial veterinary laboratories throughout Indonesia.

This required project staff at the Balai Besar Veteriner Denpasar to

- produce antigens and assemble serological kit for detection of Jembrana disease virus antibody in cattle.
- small scale production of the recombinant protein antigen at Disease Investigation Centre Denpasar.
- develop standard quality control procedures for assessment of the antigen including testing on a range of standard reference sera.
- assemble a test kit with antigen (probably antigen-coated ELISA trays), Western blot strips, other essential reagents, and positive and negative serum controls.
- involve PUSVETMA (Pusat Veterenaria Farma) in anticipation of eventual transfer of technology for the production of serological test kit.
- hold a workshop for provincial Disease Investigation Centre laboratory staff to demonstrate and publicise kit.

Objective 4. Determine if the non-pathogenic BIV will provide protection against the pathogenic JDV.

This involved:

- infection of cattle with the infectious clone of the American R-29 strain of BIV and super-infection with Jembrana disease virus. This experiment will also provide valuable reagents for the detection of BIV and development of type-specific serological tests.
- analysis of result to determine if prior BIV infection will ameliorate the effects of infection with Jembrana disease virus.

Objective 5. Develop a serological test that will specifically detect antibody to Jembrana disease virus and will not cross-react with the non-pathogenic BIV that is present concurrently with JDV in Indonesia.

Three methods were attempted to achieve this objective.

1. Develop an epitope map of Jembrana disease virus proteins to identify regions that do not react with sera from the BIV-infected cattle and cattle from Sulawesi, and then utilise these epitopes to develop a type-specific serological test. This work was conducted by the Australian partner.

First, a series of truncated proteins based on the capsid (CA), matrix (MA) and envelope (Env) proteins were to be produced and used as antigens for the detection of antibody in sera of cattle infected with JDV and cattle infected with BIV. Antigens were sought that would react with sera from either JDV or BIV but not both.

It became evident during the course of these investigations that the truncated proteins produced lacked the specificity required. Therefore additional studies were undertaken in which short synthetic peptides were designed based on the CA, MA and Env proteins, and each of these peptides were used as an antigen. This study formed a component of a PhD thesis by Ms Tegan McNab, expected to be completed in 2009.

2. Undertake sequence analysis of the genome of 3 additional strains of Jembrana disease to identify unique regions of the JDV genome that are present consistently in all strains and not present in BIV. If regions are identified that are consistently present in JDV but not in BIV then attempts will be made to express proteins from these sequences and to test them as potential type-specific antigens. The study will add to the data already available: currently complete sequence data available for one strain from Bali, with partial sequence of a number of other strains from Bali, Sumatra and Kalimantan. This study was the basis of a PhD project by Mr Shane Peterson which is expected to be completed in 2009...
3. Identify and characterise the non-pathogenic lentivirus present in cattle in Sulawesi, involving both the Balai Besar Veteriner Denpasar and the Australian partner and requiring
   - Collection of tissues from antibody-positive cattle in Sulawesi
   - PCR amplification of the genome of virus from antibody-positive cattle in Sulawesi and comparison of this genome with that of Jembrana disease virus
   - identification of regions that are specific to Jembrana disease virus
   - use of these regions to produce recombinant proteins suitable as type-specific antigens for serological tests.

6 Achievements against activities and outputs/milestones

Objective 1: To develop the capacity within Indonesia for the production of a recombinant protein vaccine for Jembrana disease

no.	activity	outputs/ milestones	completion date	comments
1.1	Vaksindo produce a recombinant protein vaccine, using established expression systems	Transfer to Vaksindo the technology for laboratory scale production of the proteins.	Completed in Year 2	A fermenter was purchased jointly by Vaksindo and the project and initially shipped to Murdoch University where conditions to operate the fermenter and produce proteins on a medium scale were established.
		Produce the proteins on a medium scale in Vaksindo fermenters	Progressed but not completed	Fermenter and other essential small equipment shipped to Vaksindo. Essential constructs required for production of recombinant CA and Tat proteins for vaccine transferred to Vaksindo. Small scale production and protocol for production of CA/Tat vaccine established at Vaksindo by project staff from Murdoch University. Initial problems by Vaksindo staff in operation of fermenter and production of proteins overcome. Technical capability of Vaksindo to produce vaccine established. Vaksindo changed ownership in 2008 (Year 3 of project) and they ceased work on project [although there has been continuing expression of interest in vaccine by new management].
		Vaccine tested for efficacy in cattle at Balai Besar Veteriner Denpasar	Not completed	Unable to conduct efficacy testing in cattle with Vaksindo product.
1.2	Cooperate with BBPMSOH to register vaccine	Submit initial application detailing all available information on the vaccine.	Year 2	Documentation required by BBPMSOH prepared by Vaksindo and submitted. Provisional approval for vaccine obtained.
		Work with BBPMSOH to conduct an animal efficacy trial at the Disease Investigation Centre Denpasar	Not completed	Final efficacy testing by BBPMSOH not conducted as commercial product not produced.

PC = partner country, A = Australia

Objective 2: To confirm the safety and efficacy of the recombinant protein vaccine under field conditions by achieving the following activities…

no.	activity	outputs/ milestones	completion date	comments
2.1	Determine safety and efficacy of vaccine under field conditions.	Production at Murdoch University of sufficient vaccine for vaccination of 100 cattle.	Year 1	Production of batch of vaccine successful.
		Selection of appropriate area in the Tabanan district of Bali for testing, in collaboration with provincial veterinary service.		Vaccine area selected in Tabanan district as proposed.
		Vaccination (twice at monthly intervals) of 100 cattle (twice) and placebo vaccination of an additional 100 cattle.		Vaccination of 100 cattle and placebo vaccination of additional 100 cattle successful.
		Monitor cattle for history of disease at monthly intervals by clinical observations and farmer interview. Collect sera at start and at 3 month intervals and conduct serological tests		Cattle were monitored for 12 months for adverse clinical signs that could be attributed to vaccine and for antibody response to vaccine components.
		Analyse data and final conclusion of safety from field trial.		Vaccine associated with minimal side effects and farmer acceptance of vaccine very favourable. A strong and persistent antibody response to vaccine components was detected in vaccinated cattle.

PC = partner country, A = Australia

Objective 3: To develop a serological test kit for distribution to provincial veterinary laboratories throughout Indonesia

no.	activity	outputs/ milestones	completion date	comments
3.1	Develop serological kit at Balai Besar Veteriner Denpasar and then investigate commercial development of the kit at PUSVETMA	Small scale production of the recombinant protein antigen at Balai Besar Veteriner Denpasar Development of standard quality control procedures for assessment of the antigen including testing on a range of standard reference sera	Year 1	Technology to produce recombinant protein CA protein of Jembrana disease virus for use in serological tests (ELISA and Western immunoblotting) well established at Balai Besar Veteriner Denpasar.
		Assembly of test kit with antigen (probably antigen-coated ELISA trays), Western blot strips, other essential reagents, and positive and negative serum controls. Involvement of PUSVETMA in anticipation of eventual transfer of technology to them.	Completed in Year 3 by collaboration between DIC and PUSVETMA	The capacity of PUSVETMA to produce a serological test kit for distribution to other provincial veterinary centres on a commercial basis has been achieved by collaboration between Balai Besar Veteriner Denpasar and PUSVETMA.

3.2	Workshop to demonstrate serological kit		Completed	Training of PUSVETMA staff and staff from various provincial veterinary centres in ELISA methodology for JDV has been conducted.

PC = partner country, A = Australia

Objective 4: To determine if the non-pathogenic BIV will provide protection against the pathogenic JDV

no.	activity	outputs/ milestones	completion date	comments
4.1	Infection of cattle with the infectious clone of the American R-29 strain of BIV and super-infection with Jembrana disease virus.	R-29 strain of BIV transferred to Balai Besar Veteriner Denpasar Cattle purchased and infected with BIV and some super-infected with JDV. Analysis of results to determine if prior infection with BIV will ameliorate the effects of infection with Jembrana disease virus.	Completed successfully Year 3	The R-29 strain of BIV was shown to replicate in Bali cattle but without the production of clinical signs. It produced a transient plasma viraemia soon after infection but to persist in a proviral form for at least 2 months after infection. There was a rapid antibody response to the TM protein and a slightly more delayed response to the CA protein but the response to both proteins was much earlier than expected in response to JDV. Subsequent infection with pathogenic JDV 42 days after infection with BIV caused a transient reactivation of the BIV viraemia. It did not result in amelioration of the clinical signs of Jembrana disease in the JDV-infected cattle. This result, although clear, needs to be interpreted with caution as a longer interval between BIV infection and superinfection with JDV might have a different effect.

PC = partner country, A = Australia

Objective 5: To develop a serological test that will specifically detect antibody to Jembrana disease virus and will not cross-react with the non-pathogenic BIV that is present concurrently with JDV in Indonesia…

no.	activity	outputs/ milestones	completion date	comments
5.1	Develop an epitope map of Jembrana disease virus proteins to identify regions that do not react with sera from the BIV-infected cattle and cattle from Sulawesi, and then utilise these epitopes	Prepare truncated JDV capsid and envelope proteins by recombinant technology and purify for use as antigens in ELISA and Western immunoblots.	Completed in Year 2	Recombinant truncated proteins of JDV prepared and none specifically differentially detected antibody to JDV or BIV. An alternative approach investigated: the use of synthetic peptides.
		Conduct serological tests with JDV control sera and sera from Sulawesi	Continuing study to be completed mid 2009	Peptides produced to regions of CA, MA and SU of JDV. These were tested by ELISA against cattle antisera to JDV and BIV. A small number of peptides have shown

	to develop a type-specific serological test.	Recombinant protein antigens identified that react with either but not both control sera and sera from Sulawesi.	Continuing study to be complete mid 2009	indications of specificity (the ability to differentiate antibody to BIV and JDV) and one peptide is to an area of the genome of JDV that has not been reported as present in BIV and are being retested against additional antisera. These studies form a component of a PhD study by Tegan McNab and will be incorporated into the thesis [expected submission February 2010]. The initial results are promising in terms of the possible development of a type-specific serological test and this study will continue.
5.2	5.2. Sequence analysis of the genome of 3 additional strains of Jembrana disease	Sequence analysis of 3 strains JDV-infected cattle in Kalimantan and Bali and analysis of sequence data	Year 3	Genome analysis of two strains of JDV from Bali in the period from 1987 to 2001 and two strains from Kalimantan in 2001 and 2007, revealed that the Balinese and Kalimantan strains form two phylogenetic clusters which are 10% divergent and differed in the putative vpw and vpy genes. These may represent two separate incursions of JDV from the natural host. Although the natural host is unknown, there was evidence that buffalo (Bubalis bubalis) can be infected by JDV and they may be the reservoir host. This genetic diversity between virus strains on Bali and Kalimantan has potential implications for the transmission of the disease and potential differences in antigenicity of strains in the two islands, and hence future vaccine efficacy.
5.3	Characterisatio n of non-pathogenic bovine lentivirus in cattle in Sulawesi		Not completed successfully	All attempts to genetically characterise the non-pathogenic bovine lentivirus present in Sulawesi have been unsuccessful.

PC = partner country, A = Australia

7 Key results and discussion

Objective 1. To develop the capacity within Indonesia for the production of a recombinant protein vaccine for Jembrana disease

Methods were determined for the small scale production of a GST-tagged recombinant polyprotein containing both JDV CA and Tat proteins, with potential for use as a vaccine for the control of Jembrana disease. In small scale experiments, the CA/Tat-GST polyprotein was produced with yields of up to 1.2 g/L of 95% pure recombinant protein (sufficient for 600 doses/L at a rate of 2 mg per dose). Small experimental groups of cattle were vaccinated twice with 2 mg of this protein emulsified in an oil adjuvant and they were then challenged with a pathogenic JDV strain (Pul01) 4 weeks after the second dose. Vaccination elicited very high titres of CA and Tat antibody of IgG, IgM and IgA subclasses prior to challenge with JDV. The vaccine ameliorated the hyperpyrexia and severe lymphopenia typically seen in cattle infected with JDV but as expected, did not provide complete protection. These small scale experiments were conducted within a limited time frame to reduce costs and it is possible that greater efficacy of the vaccine would be obtained if a longer interval between vaccine doses and a longer interval between vaccination and challenge with JDV had been possible.

The technology required for the production of the recombinant CA/Tat protein for Jembrana disease was transferred to the private veterinary vaccine company (Vaksindo) in Indonesia. Assistance with the purchase of a suitable fermenter was provided and training in its operation and downstream processing of the product was provided. The technical capability of the company to produce the vaccine was established and success in producing a batch of the vaccine was achieved.

The production of a recombinant protein vaccine was new technology for the company but a challenge they accepted with enthusiasm. The company need continuing assistance with technical problems and while local Indonesian scientists (trained in Australia as part of their involvement with Jembrana disease research projects) are available to technically assist them, additional continuing support may be required.

Vaksindo changed ownership in 2008 (during Year 3 of project). This, and the sad loss of their laboratory manager, seriously affected the development of this project and resulted in temporary cessation of work on the production of the vaccine. There has been a continuing expression of interest in production of the vaccine by the new management.

A commitment by the DGLS to Vaksindo to purchase the recombinant Jembrana disease vaccine was made prior to change of ownership of the company.

Documentation required for licensing of the vaccine in Indonesia was prepared by Vaksindo and submitted to BBPMSOH and provisional approval for the vaccine was obtained. Final efficacy testing by BBPMSOH is still required and has not been conducted as commercial product was not produced at Vaksindo.

Objective 2. To confirm the safety and efficacy of the recombinant protein vaccine under field conditions.

A bacterially expressed recombinant JDV CA/Tat-GST polyprotein emulsified in a mineral oil adjuvant was tested for safety and immunogenicity under field conditions in a field trial in the Tabanan district of Bali. For the field trial, 100 cattle were inoculated twice with 2 mg of the polyprotein vaccine emulsified in an oil adjuvant and a similar number of cattle were similarly inoculated with a control vaccine containing chicken ovalbumin and recombinant GST. Strong antibody responses to the CA and Tat proteins were detected for 12 months post-vaccination. A low prevalence (≤3%) of adverse reactions to CA/Tat-GST polyprotein vaccine occurred, including abortion and a transient shock-like syndrome, but similar signs were also observed in the control group vaccinated with GST plus chicken

ovalbumin. The reason for the adverse reactions in response to vaccination were not determined.

The two vaccine doses induced a strong immune response to the CA and Tat components of the vaccine that persisted for 12 months in all vaccinated cattle under field conditions.

The results suggest the vaccine would be suitable for use under field conditions and would have no significant adverse effects in vaccinated animals. Exacerbation of the clinical signs of Jembrana disease in response to vaccination, as has been observed with some other lentivirus vaccines, has never been observed with this and other recombinant JDV protein vaccines or with the earlier whole virus vaccines that were used.

During conduct of the safety trial in the Tabanan region of Bali, it was noted that the cattle used which were less than 4 years of age were antibody-negative (had not been infected with JDV) and would likely be susceptible to infection, whereas many of the cattle 4 years or more in age were antibody-positive (previously infected). It appeared that in this region, JDV had not been circulating in cattle in the previous 4 years and no new infections had occurred. This provides some insight into the current kinetics of Jembrana disease virus infection in the Bali cattle population on the island: the cyclic nature of the infection in cattle depending probably on herd immunity, and the persistent nature of the antibody response.

Objective 3. To develop a serological test kit for distribution to provincial veterinary laboratories throughout Indonesia

The capacity to sustainably produce a recombinant protein antigen for serological surveillance of Jembrana disease virus infection was initially established at the Balai Besar Veteriner Denpasar. The test is based on the use of a recombinant JDV CA protein antigen. The technology for commercial production of the reagents for distribution to other provincial veterinary centres was then established at PUSVETMA by collaboration between the Balai Besar Veteriner Denpasar, and PUSVETMA who developed a biomolecular laboratory for this purpose. Commercial production and distribution of the kit should commence in 2010.

Objective 4. To determine if previous infection with the non-pathogenic BIV will provide protection against subsequent infection with the pathogenic JDV

The potential for the development of a simple to administer and low cost heterologous live virus vaccine against Jembrana disease by using the non-pathogenic bovine immunodeficiency virus (BIV) has been a consideration and the hypothesis that prior infection with BIV would protect against subsequent infection with JDV was tested.

The American R-29 strain of BIV was used for the investigations which were conducted at the Balai Besar Veteriner Denpasar. Reasons for the selection of this R-29 strain were that it has been well studied, it was known to be of low pathogenicity (at least in Bos taurus), and it was available as an infectious clone that would replicate well in bovine lung cells.

While there was one previous report indicating that BIV did not replicate in two Bali cattle (Whetstone et al., Bovine lentivirus infection in Bali cattle following inoculation with BIV. In "Jembrana disease and the bovine lentiviruses", ACIAR Proceedings 75, 1996), an initial study of the kinetics of BIV in Bali cattle at the DIC in Denpasar in 2008 clearly demonstrated that the R-29 strain would replicate in these cattle. it produced a transient plasma viraemia soon after infection and persisted in a proviral form in peripheral blood mononuclear cells for at least 2 months after infection (when the experiments were terminated). The infection was subclinical and there was no febrile reaction or alteration in peripheral blood leucocyte populations in the infected cattle, two changes that occur consistently in cattle infected with JDV. There was a rapid antibody response to the TM protein and a slightly more delayed response to the CA protein of BIV but in both cases

the antibody response occurred much earlier than that which occurs in Bali cattle in response to JDV infection. These results are being prepared for publication.

Subsequent infection of the BIV-infected cattle with pathogenic JDV, 42 days after infection with BIV, resulted in a transient reactivation of the BIV viraemia but it did not affect JDV replication and it also did not result in amelioration of the clinical signs of Jembrana disease in the JDV-infected cattle. This result, although clear, needs to be interpreted with caution as a longer interval between BIV infection and superinfection with JDV might have a different effect. An effective cell-mediated immune response might take much longer than 42 days to develop and experiments to confirm this could provide interesting results. The results are being prepared for publication.

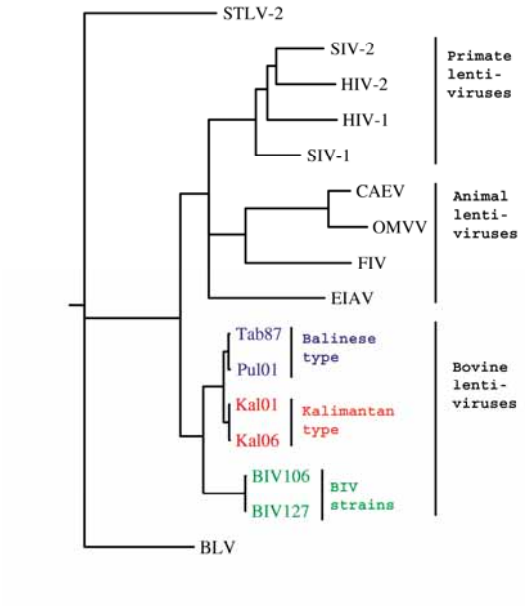
Objective 5. To develop a serological test that will specifically detect antibody to Jembrana disease virus and will not cross-react with the non-pathogenic BIV that is present concurrently with JDV in cattle in Indonesia

A major problem with serological surveillance of Jembrana disease in Indonesia is that there are two bovine lentiviruses in the cattle population of Indonesia: JDV and the antigenically and genetically related BIV or a BIV-like virus. While the two viruses have very different clinical effects in Bali cattle, they cannot be differentiated serologically and previous and current surveillance methods (based on either a whole JDV antigen or a recombinant JDV CA protein) are presumed to have detected antibody to both viruses. For example, in Sulawesi in which clinical Jembrana disease has not been detected, there is serological evidence for the presence of a virus that cross-reacts with JDV. Unfortunately, all attempts to genetically characterise the non-pathogenic bovine lentivirus present in Sulawesi have been unsuccessful so an assumption has been made that it is probably BIV.

To develop a more specific antigen that might differentiate antibody to the two viruses, recombinant truncated proteins of JDV were prepared but none specifically differentially detected antibody to JDV or BIV. These results have been published (Desport et al., 2005). However, more recent investigations using synthetic peptides have provided promising results in terms of the possible development of a type-specific serological test. The peptides tested were produced to regions of CA, MA and Env (including SU and TM) of JDV, areas that were considered most likely to produce type-specific differential results. These peptides were tested by using them as antigens in an ELISA against cattle antisera obtained from cattle infected with either JDV or BIV. Early results from these assays have indicated that a small number of peptides have specificity (the ability to differentiate antibody to BIV and JDV) and there was one very promising peptide that would be encoded by an area of the genome of JDV that is not present in BIV (there are multiple insertions and deletions in the genome of JDV relative to BIV). The promising peptides are being retested against additional antisera. These studies form a component of a PhD study by Ms Tegan McNab and the results will be incorporated into the thesis [expected submission February 2010] and will also be prepared for publication.

The assumption (hypothesis) has been made that there is a single strain of JDV in Indonesia that originated by cross-species transfer and led to the outbreak in 1964. This hypothesis was investigated to determine if it is correct and if there might be variation in the virus sufficient to affect its detection by currently available serological surveillance techniques and molecular diagnostic procedures. Variation has been detected. First, it was shown that not all strains of JDV have the same pathogenicity. During small scale vaccine trials it was noted that there is variation in pathogenicity of strains of JDV detected in Bali (Desport et al., 2009). Second, investigations into the genetics of JDV have demonstrated that there is genetic variation in JDV, more than was originally hypothesised. Our understanding of the genetics of JDV has previously been limited to the complete genome of one strain of virus only (the Tab87 strain), and partial sequence analysis of a number of other strains. Analysis of specific segments of the genome of viruses from Bali, Sumatra and Kalimantan (Desport et al., 2007) indicated that while most

strains of JDV in Bali and Sumatra were genetically conserved, differences occurred in a strain originating from Kalimantan. It was considered that the strains in Sumatra were very similar to strains from Bali (Desport et al., 2007) and probably originated by the illegal movement of cattle from Bali to these areas. The origin of strains in Kalimantan is unknown and as Jembrana disease is active in all three Kalimantan provinces, the genetics of strains in these areas was examined in more detail. Analysis of the complete genome of two strains of JDV from Bali detected in the period from 1987 to 2001 and two strains from Kalimantan in 2001 and 2007, revealed that the two Balinese strains isolated 14 years apart were very similar and supported earlier studies that indicated Balinese strains have been genetically stable over several years but that the Balinese and Kalimantan strains formed two phylogenetic clusters which were 10% divergent from each other (see figure below) and also differed in the putative vpw and vpy genes.



The genomic studies undertaken indicate that the Balinese and Kalimantan strains are genetically different and that the strains in Kalimantan did not originate from Bali. The occurrence of the two phylogenetic clusters suggests the possibility of two separate incursions of JDV from the natural host. Although the natural host is unknown, evidence was obtained that buffalo (Bubalis bubalis) can be infected by JDV and it is hypothesised that they may be the reservoir host. This has implications for the future control of Jembrana disease in Indonesia, including a possible need for restrictions on the movement of buffalo in which the virus might persist.

8 Impacts

8.1 Scientific impacts – now and in 5 years

A major scientific impact of the project is the improved capacity for diagnosis of Jembrana disease in Indonesia, and consequent potential for a faster response to the occurrence of disease during outbreaks. The specific benefits in this area have been:

- improved methodology for the diagnosis of Jembrana disease and the transfer of the diagnostic technology to provincial veterinary laboratories.
- the improved ability for immunosurveillance enabled by the ability to produce serological antigen with recombinant DNA technology, so eliminating the need to infect of Jembrana disease virus-free cattle to produce whole virus antigen, and ultracentrifugation of freshly collected plasma from infected cattle for purification of the previously used whole virus antigen. The production of a serological kit will facilitate this further.

The molecular biotechnology employed during the project has reinforced its potential application for disease investigation, and has resulted in its application to other diseases that have occurred in Indonesia, including but not limited to avian influenza, rabies and classical swine fever.

A molecular biotechnology capacity was established at PUSVETMA which will provide a mechanism for commercial production of other diagnostic kits and alternative vaccines by this company.

8.2 Capacity impacts – now and in 5 years

The development of a recombinant protein antigen for serological tests and a vaccine for Jembrana disease has involved new technology for Indonesia but it is likely to be an increasingly common method of vaccine production and antigen production in future. The skills learned and problems solved during the development of the Jembrana disease vaccine will have application and assist in the production of other future vaccines produced within Indonesia.

There has been an extensive training program associated with the Jembrana disease project that has resulted in the development of technical skills by the participants. These skills are now being applied to a variety of disease problems in Indonesia. The emphasis on hypothesis driven research has provided problem solving skills and improved research methodology to both Indonesian and Australian participants.

The following have received advanced postgraduate scientific and technical training in association with this project which have or are likely to lead to the award of a PhD: Dr Meredith Stewart (Australia), Dr Wayan Masa Tenaya (Indonesia) who is a holder of an Allwright Fellowship, Joshua Lewis (Australia), Tegan McNab (Australia), Dr William Ditcham (Australia) and Shane Peterson (Australia).

A project participant from Balai Besar Veteriner Denpasar, Dr Ni Luh Putu Agustini received a Master of Science degree from Udayana University for research undertaken in association with this project.

8.3 Community impacts – now and in 5 years

8.3.1 Economic impacts

Cattle in Indonesia play an important role in income generation for smallholder farmers. The utilisation of cattle as draft power enables smallholder farmers to double food production from their available land. The disposable income of these smallholders has an important add-on effect, and can be expected to make an important contribution to the economic conditions in rural (and usually poorer) communities.

In areas where the disease is endemic (Bali, Kalimantan, Java and Sumatra) the losses associated with Jembrana disease (mortalities, decreased ability to use affected animals as a source of draft power for rice farming) cause a reduction in the income of smallholder farmers. It has been estimated that the endemic situation is associated with a 2% mortality rate and a 4% loss in production. While the disease is absent in Sulawesi and Maluku, and in other areas in eastern Indonesia, the potential occurrence of the disease in these areas poses a major threat to the economy of the rural communities. Based on information from the islands where the disease has occurred (Bali, Java, Sumatra and Kalimantan), it is expected that an initial outbreak in the 'clean' areas will be associated with high mortality rates (around 20%), followed by an endemic situation. It is only by the development of an efficacious vaccination method that most of the current losses associated with the disease, and the possible future losses in the current disease-free areas, could be eliminated.

The development of Jembrana disease diagnostic methods requiring molecular biological methodology (PCR, RT-PCR, capture ELISA, ELISA and Western immunoblotting) in regional diagnostic labs at Medan, Bukittinggi, Lampung and Banjar Baru South Kalimantan has resulted in a rapid diagnostic capacity. This will assist the veterinary services to detect and prevent the spread of Jembrana disease.

8.3.2 Social impacts

The effects of the introduction of Jembrana disease into an area where it does not currently occur (eg. Sulawesi or Maluku) would be devastating socially, with an initial severe impact on small cattle-holders but with flow-on to the entire area. Early diagnosis and use of an effective vaccine will prevent much of this social hardship.

8.3.3 Environmental impacts

This project has provided the Government of Indonesia with the capacity to increase the population of Bali cattle. The fear of using Bali cattle by small farmers has been allayed by an improved understanding of the disease in Indonesia and the development of recombinant vaccines and reliable diagnostic methods.

8.4 Communication and dissemination activities

There have been multiple discussions between the Project Leaders and the DGLS regarding the vaccine and the involvement of Vaksindo in the manufacture of the vaccine and PUSVETMA in the commercial production of the serological test kit.

There have been multiple discussions between Vaksindo and the DGLS regarding the production of the vaccine.

A one day workshop on prospects of a recombinant protein vaccine for Jembrana disease was held in Denpasar at the commencement of the project, and attended by a wide section of DGLS staff.

A 5 day workshop on the development and use of recombinant protein antigen for serological tests for Jembrana disease was held at Surabaya by project staff from the

Balai Besar Veteriner Denpasar. This was attended by staff of PUSVETMA and including Dr Endang Pudjiastuti (Head of R & D Department), Dr.Rosmelati Situmeang M Kes (R & D), Dr SNR Anieka Rochmah (R & D), Dr. Rosmiati Wisindie (Head of Diagnostic Division), Dr. Nur Sjolikah (Head, Antisera Division), Dyah Estikoma (Analyst), Dr.Nana Rostita (Diagnostic Division), Budi Wiyatno (Diagnostic Division), Dr Jamilah Rohaniyati (Mammalian Vaccine Department) and Nidya Narulita (Viral Vaccine Department).

9 Conclusions and recommendations

This project has been the final project in a series of collaborations on Jembrana disease between Murdoch University and various Indonesian partners, particularly the Balai Besar Veteriner Denpasar (Veterinary Disease Investigation Centre, Denpasar). These projects have led to a markedly improved understanding of the disease, its diagnosis and methods for its control. They have also had a significant training component and have contributed to improved research and technical capacity for animal health research within Indonesia.

9.1 Conclusions

Methods were determined for the small scale production of a GST-tagged recombinant polyprotein containing both JDV CA and Tat proteins, with potential for use as a vaccine for the control of Jembrana disease.

The vaccine ameliorated the hyperpyrexia and severe lymphopenia typically seen in cattle infected with JDV but as expected, did not provide complete protection.

The technology required for the production of the recombinant CA/Tat protein for Jembrana disease was transferred to the private veterinary vaccine company (Vaksindo) in Indonesia.

A commitment by the DGLS to Vaksindo to purchase the recombinant Jembrana disease vaccine was made prior to change of ownership of the company.

Documentation required for licensing of the vaccine in Indonesia was prepared by Vaksindo and submitted to BBPMSOH and provisional approval for the vaccine was obtained.

A batch of the vaccine was tested for safety and immunogenicity under field conditions in Bali. Two vaccine doses induced a strong immune response to the CA and Tat components of the vaccine that persisted for 12 months. A low prevalence of adverse reactions occurred but as these also occurred in placebo vaccinated cattle they were unlikely to be directly associated with the vaccine.

The capacity to sustainably produce a recombinant protein antigen for serological surveillance of Jembrana disease virus infection was established by project staff at the Balai Besar Veteriner Denpasar. The technology was transferred to PUSVETMA by project staff from the Balai Besar Veteriner Denpasar and commercial production of an ELISA and Western immunoblotting kit is expected in 2010.

The potential for the development of a simple to administer and low cost heterologous live virus vaccine against Jembrana disease by using the non-pathogenic bovine immunodeficiency virus (BIV) has been a consideration and the hypothesis that prior infection with BIV would protect against subsequent infection with JDV was tested. The R-29 strain of BIV replicated in these cattle. Subsequent infection of BIV-infected cattle with pathogenic JDV, 42 days after infection with BIV, did not affect JDV replication and it also did not result in amelioration of the clinical signs of Jembrana disease in the JDV-infected cattle.

A major problem with serological surveillance of Jembrana disease in Indonesia is that there are two bovine lentiviruses in the cattle population of Indonesia: JDV and the antigenically and genetically related BIV or a BIV-like virus. Investigations of synthetic peptides have provided promising results in terms of the possible development of a type-specific serological test.

Variation has been detected in JDV. Not all strains of JDV have the same pathogenicity. There is genetic variation in JDV and while Balinese strains were very similar and supported earlier studies that indicated Balinese strains have been genetically stable over

several years, strains from Kalimantan differ genetically from strains in Bali and suggests the possibility of two separate incursions of JDV from the natural host.

9.2 Recommendations

Jembrana disease remains a significant problem in Indonesia: in endemic areas the disease appears to be cyclic and occurs periodically probably as herd immunity wanes. Outbreaks are currently present in Kalimantan and it remains a very significant threat to the Bali cattle population of areas where the disease does not currently occur and in which there are significant Bali cattle populations, including Sulawesi and Maluku provinces. Control of the disease requires commercial development of the reagents required for future control of the disease, including diagnostic reagents and a vaccine. Methods for sustainable production of a vaccine and diagnostic reagents have been developed but the technology, particularly, for large scale production of the vaccine is difficult and further assistance needs to be provided to a commercial organisation in the final commercial development of this product.

10 References

10.1 References cited in report

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Desport, M., Stewart, M.E., Mikosza, A.S., Sheridan, C.A., Peterson, S.E., Chavand, O., Hartaningsih, N. and Wilcox, G.E. (2007) Sequence analysis of Jembrana disease virus strains reveals a genetically stable lentivirus. Virus Research 126: 233-244.

Desport, M., Ditcham, W. G., Lewis, J. R., McNab, T. J., Stewart, M. E., Hartaningsih, N. and Wilcox, G. E. (2009) Analysis of Jembrana disease virus replication dynamics in vivo reveals strain variation and atypical responses to infection. Virology 386: 320-316.

Ditcham, W. G., Lewis, J. R., Dobson, R. J., Hartaningsih, N., Wilcox, G. E. and Desport, M. (2009) Vaccination reduces the viral load and the risk of transmission of Jembrana disease virus in Bali cattle. Virology 386: 317-324

10.2 List of publications produced by project

Desport M., Stewart ME, Sheridan CA, Ditcham WG, Setiyaningsih S, Tenaya WM, Hartaningsih N, and Wilcox GE. (2005). Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and non-pathogenic bovine lentiviruses. Journal of Virological Methods 124:135-42.

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Setiyaningsih, S., Desport, M., Stewart, M. E., Hartaningsih, N. and Wilcox, G. E. (2008) Sequence analysis of mRNA transcripts encoding Jembrana disease virus Tat-1 in vivo. Virus Research 132: 220-225.

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11 Appendixes

11.1 Appendix 1

Readers are referred to the following PhD theses that are available on-line and which contain greater detail of methodology used and results obtained during this project

Stewart, M. (2005) An investigation into aspects of the replication of Jembrana disease virus. PhD thesis, Murdoch University.
http://wwwlib.murdoch.edu.au/adt/pubfiles/adt-MU20051222.104106/02Whole.pdf

Setiyaningsih, S. (2006) Molecular and immunogenic analysis of Jembrana disease virus Tat. PhD thesis, Murdoch University.
http://wwwlib.murdoch.edu.au/adt/pubfiles/adt-MU20061019.150917/02Whole.pdf

Ditcham, W. (2007) The development of recombinant vaccines against Jembrana disease. PhD thesis, Murdoch University.
http://wwwlib.murdoch.edu.au/adt/pubfiles/adt-MU20071119.94111/02Whole.pdf

Further details of methodology and results will be available in 2010 in PhD theses by Lewis, J. (Recombinant proteins as vaccines and diagnostic antigens for the control of Jembrana disease virus infection in Indonesia), McNab, T. (Detection and differentiation of Jembrana disease virus and Bovine immunodeficiency virus infections in cattle), Peterson, S. (Genomic evolution of Jembrana disease virus) and Tenaya, W.M. (Aspects of the pathogenesis of Jembrana disease virus) which will become available on-line through the Murdoch University website http://wwwlib.murdoch.edu.au/adt/