Universiti Putra Malaysia

a world leader in new tropical agriculture

Proceedings of the International Conference on ANIMAL & HUMAN HEALTH SAFETY

PROCEEDINGS

- 6 - 8 December 2009
- Palm Garden IOI Resort
  Putrajaya, Malaysia

Editors

M. Ariff Omar • Rasedee Abdullah
Gurmeet Kaur Dhaliwal
Chen Hui Cheng • M. Murugaiyah
Kalthum Hashim • Ooi Peck Toung

Universiti Putra Malaysia

Universitas Airlangga Indonesia

Department of Veterinary Services Malaysia

Proceedings of the International Conference on

ANIMAL HEALTH & HUMAN SAFETY

Editors

M. Ariff Omar • Rasedee Abdullah
Gurmeet Kaur Dhaliwal
Chen Hui Cheng • M. Murugaiyah
Kalthum Hashim • Ooi Peck Toung

Universiti Putra Malaysia
Serdang • 2009

Perpustakaan Negara Malaysia Cataloguing-in-Publication Data

International Conference on Animal Health and Human Safety (2009: Putrajaya).
Proceeding of the International Conference on Animal Health and Human Safety, 6-8 December 2009, Palm Garden IOI Resort, Putrajaya, Malaysia / editors M. Ariff Omar ...[et al.].
ISBN 978-967-344-122-8
1. Animal health--Malaysia--Congresses. 2. Livestock--Malaysia--Congresses. I. M. Ariff Omar. II. Title.
636.089

Type face: Times New Roman
Size : 11/13.2 point

Typeset and Printed by
Universiti Putra Malaysia Press
43400 UPM Serdang
Selangor Darul Ehsan
Tel:03-89468851/89468854
Fax: 03-89416172
Emel: penerbit@putra.upm.edu.my

Content

Loop-mediated Isothermal Amplification (Lamp) as a Diagnostic Tool for Jembrana Disease Virus

Asmarani Kusumawati[1,2], Narendra Yoga Hendarta[2], Sri Hartati[1], Puji Astuti[1] and Tri Untari[1]

[1]*Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta, Indonesia,*
[2]*Study Center of Biotechnology, University Gadjah Mada, Yogyakarta, Indonesia*
Email: kartapati_2008@yahoo.com

Abstract

Jembrana disease virus (JDV) is the agent of an acute infectious Jembrana disease of Bali cattle (*Bos javanicus*), reported for the first time in Jembrana village in Bali, Indonesia. Jembrana disease is not unique to *Bos javanicus*, other types of cattle are also susceptible although the resulting lesion is milder. The aim of the research was to develop molecular diagnostic tool using loop-mediated isothermal amplification (LAMP). A set of primer specific to env-tm gene composing of outer primers (F3, B3), inner primers (FIP, BIP) and loop primers (IF, IB) designed from sequence data (genebank env-tm). Finally, due to its ease of use, LAMP is an efficient and specific diagnostic tool of Jembrana disease and an ideal method in conditions where sophisticated equipment are not available.

Keywords: Jembrana disease virus, loop-mediated isothermal amplification, LAMP, env-tm gene.

Loop-Mediated Isothermal Amplification (LAMP) as a Diagnostic Tool of Jembrana Disease Virus

Asmarani Kusumawati[a, b*], Narendra Yoga Hendarta[b], Pudji Astuti[a], Tri Untari[a] and Sri Hartati[a]

[a]Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta 55281, Indonesia
[b]Study Center of Biotechnology, University Gadjah Mada, Yogyakarta, Indonesia

* Corresponding author at : Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta 55281, Indonesia; e-mail : kartapati_2008@yahoo.com

Abstract

Jembrana Disease Virus (JDV) is the agent of an acute infectious Jembrana disease of Bali cattle (*Bos javanicus*), reported for the first time in Jembrana village in Bali, Indonesia. Jembrana disease is not unique to *Bos javanicus*, other types of cattle are also susceptible although the resulting lesion are milder. The aim of this research is to developed molecular diagnostic tool uses loop-mediated isothermal amplification (LAMP). A set of primers specific to *env-tm* gene and compose of outer primers (F3,B3), inner primers (FIP,BIP) and loop primers (IF, IB) were designed from sequence GeneBank envelope subunit transmembrane gene jembrana virus. Finally, due to its ease of use, LAMP is an efficient and specific diagnostic tool of jembrana disease, it is an ideal method in conditions where sophisticated equipment are not available.

Keywords: LAMP, Jembrana Disease Virus, *env-tm* gene

This paper was presented
at the conference on
Animal Health and
Human safety
Putrajaya, Malaysia
6-8 December 2009

Prof. Dr. Rasedee Abdullah
Chairman
Scientific Committee

Introduction

Jembrana virus is the agent of Jembrana virus disease (JVD), an acute and severe infectious disease of Bali cattle (*Bos javanicus*), reported for the first time in Jembrana village in Bali, Indonesia (Wilcox *et al.*, 1992). Presently, JVD has also been detected in other area of Indonesia, *e.g.* Lampung (Sumatra), West Sumatra and East Java provinces (Hartaningsih *et al.*, 1993). JVD is not unique to *Bos javanicus*. It turns out that other types of cattle are also susceptible, namely Friesian (*Bos taurus*) and crossbred Bali (*Bos javanicus* x *Bos indicus*) cattle although the resulting lesions are milder when compared to a case fatality rate of about 20% in *Bos javanicus* (Soeharsono *et al.*, 1995a). During the acute phase viral particles can be detected in saliva and milk and the titer of infectious virus in blood is high. Direct transmission of the disease occurs by the conjunctival, intranasal or oral routes. Infection by haematophagous arthropods has been suspected (Soeharsono *et al.*, 1995b). In experimentally infected animals, many Jembrana Disease Virus (JDV)-infected tissues were demonstrated early in the disease course which was consistent with the extremely high circulating viraemia during the febrile phase. The most infected organ is spleen but other organs are also highly infected, i.e. lymp nodes, lungs, bone marrow, liver and kidney (Chadwick *et al.*, 1998).

Antigenic cross-reactivity between the capsid protein of JDV and the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV) suggests that JV is a lentivirus (Wilcox *et al.*, 1995). The JV genome has been entirely determined. The genome consists of a single-stranded RNA, 7,732 nucleotides in length (Chadwick *et al.*, 1995b). Sequence comparaison clearly establihed that JV is actually a lentivirus, exhibiting retrovirus characteristics (Wilcox *et al.*, 1995a, 1995b). Though closely related, significant genomic differences were found between JV and BIV which may be related to the differences in pathogenicity between these two viruses (Chadwick *et al.*, 1995b). JV has been reported to be also related to human immunodeficiency virus (HIV). Some of its regulatory elements can functionally substitute for those of HIV. JV Tat is so able to activate not only its own long terminal repeat (LTR) but also that of HIV (Chen *et al.*, 2000). For this reason JV-based vectors may constitute a safe vector-mediated gene transfer, more readily acceptable than those from HIV for human gene therapy (Metharom *et al.*, 2000).

Envelope proteins JDV are interesting antigens that can be used in diagnosis and vaccination. During the acute phase viral particles can be detected in saliva and milk and the titer of infectious virus in blood is high. This characteristic can be advantegeously used for virus detection.

Loop-mediated isothermal amplification (LAMP) is the most recently developed molecular detection method (Notomi *et al.*, 2000; Parida *et al.*, 2008). This new generation of innovative gene amplification technique is known to be a very sensitive and fast detection method. It relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase, and the reaction is carried out under isothermal conditions, usually at 63°C the optimum temperature of the polymerase. Amplification proceeds by displacing and releasing single-stranded DNA (ss-DNA). Positive response can also be visually observed due to the production of whitish precipitate of magnesium pyrophosphate. This makes the method particularly attractive for routine analyses in conditions where sophisticated equipements are hardly available. A set of LAMP primers is composed of six primers (outer primers F3, B3; inner primers FIP; BIP; loop primers IF, IB), specifically recognizing eight distinct regions of the DNA target. This makes the amplification method highly specific for pathogen detection. The inner primers FIP and BIP consist of two parts, F1 and F2 and B1 and B2, respectively, linked by four T residue. F1 and F2 in one hand and B1 and B2 on the other hand are of opposite orientation. This helps the formation of a stem-loop structure at the end of the newly synthetized strand. Primers IF and IB further improve amplification efficiency. Amplification is initiated by an inner primer (e.g. FIP), followed by strand displacement by an outer primer.

The ss-DNA produced form a stem-loop structure at its 5'-end and serves as template for the second inner primer (BIP), giving rise to DNA structure with stem-loop at each end. This non-cycling process is followed by a cycling and exponential amplification. The strand displacement by an inner primer results in the production of the original structure, gaining an additional stem-loop. The process continues giving rise to the final structure which is a stem-loop cauliflower-like DNA structure, with alternately inverted repeats of the target in the same strand.

This paper describes a LAMP technique, based on *env-tm* gene, developed as a rapid, sensitive and convenient detection tool of *Jembrana Virus*. We used RNA as genomic source of JV, so the reaction was established using *Reverse Transcriptase*-LAMP (RT-LAMP) reaction. Efficiency of amplification was compared to that of conventional PCR.

Materials and methods

Isolation of total RNAs

Total RNAs were isolated from lymph nodes of infected animal using TRIzol reagent (Life Technologies), according to the manufacturer recommandation. Briefly, approximately 70 mg of tissue were homogenized in 1 mL of TriZol then let for 10 min. at room temperature. After centrifugation at 12,000 rpm, for 10 min. in a bench minicentrifuge, the supernatant was treated with 0.2 mL chloroforme then precipitated with 0.5 mL isopropanol. The pellet was washed with 70% ethanol and dissolved in 100 µL of steril DEPC-treated water. The purity and concentration of the RNA preparation were spectrophotometrically established.

Purification of viral RNA

Viral particles, isolated by sucrose gradient, were incubated for some minutes at room temperature in 10 mM Tris-HCl pH 7.5 and 0.4% SDS. RNA was purified by a combination of phenol and chloroforme treatment. RNA was precipitated with isopropanol and washed with 70% ethanol, then dissolved in steril DEPC-treated water. RNA purity and concentration were spectrophotometrically determined.

Isolation and amplification of env-tm gene

ENV-TM gene were isolated and amplified from total RNAs or purified viral RNA using Titan One Tube RT-PCR kit (Roche), according to the instruction manual provided. This kit allows the reverse transcription of RNA and the PCR amplification in a one step reaction. The primers, derived from a published sequence (Chadwick *et al.*, 1995b), used in the RT-PCR reactions are as follows. Primers for *env-tm* gene: upstream primer ATAGGATCC **ATG**GCCGTG-GGGATGGTCATAT (initiation codon **ATG** in bold letters, introduced *Bam*HI site underlined), downstream primer CAGCGGATCCTCCAAGCTACGTGTC (introduced *Bam*HI site underlined). RT-PCR reactions were performed in 50 µL of buffer, containing 0.4 µM of each primer, 1 µL enzyme mix (AMV reverse transcriptase, Taq DNA polymerase, and a proofreading polymerase) and variable amounts of RNA, with the following conditions: 50°C for 30 min. (reverse transcription step); 94°C for 2 min.; 35 cycles of 94°C for 40 sec., 55°C for 40 sec, 68°C for 1 min. 30 sec; 68°C for 10 min. then 4°C. After RT-PCR completion, 1 µL of Taq polymerase was added and the reaction continued for 10 min. at 72°C.

Cloning in pCR2.1-TOPO and transfection in E. coli

Cloning in pCR2.1-TOPO plasmid was carried out using TOPO TA Cloning system from Invitrogen, according to the instruction manual. Topoisomerase reaction was done in 6 µL, containing 1 to 2 µL of the RT-PCR/PCR reaction products (see above) and 1 µL TOPO

vector, for 5 min. at 22°C. Transformation was done using TSS method (*E. coli* DH 5α). TSS competent *E. coli* DH 5α bacteria were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD_{600nm} around 0.6) in LB containing 20% PEG 6,000 (w/v), 10% DMSO (v/v) and 70 mM $MgCl_2$. Different amounts of the transformation were spread on LB agar plate containing 50 µg/mL ampicillin, 40 µL of 40 mg/mL X-Gal and 40 µL of 100 mM IPTG (transformation of *E. coli* DH 5α) or 40 µL of 40 mg/mL X-Gal (transformation of *E. coli* TOP 10) and incubated at 37°C, overnight (OVN). Positive transformants, containing plasmid with an insert, are white or light blue colonies. The resulting constructs will be named pCR-TM (containing *env-tm* gene). A mixture of pCR-TM-positive clones were then cultured OVN and plasmids prepared using a miniprep plasmid preparation kit (Roche), according to the instruction manual.

Preparation of env-tm genes

env-tmgenes were excized from 5 µg of a mixture of pCR-TM constructs, by respectively single digestion with *Bam*HI, using the conditions as above, except for the phosphatase treatment which was omitted. The DNA fragments obtained were purified by electrophoresis on agarose gel and then eluted and quantified.

Preparation of pGEX-2T. vector pGEX-2T based constructs

Insertion of *env-tm* genes was carried out by ligation of the purified inserts to respectively single-digested (*Bam*HI) pGEX-2T. Ligation was done in 20 µL of buffer with 200 units of ligase (BioLabs), at 16°C, OVN. The amount of the vector (5.4 kb) was 50 ng while the inserts added were about 40 ng. Considering the length of the inserts, about 1.1 kb for *env-tm*, the ratio of insert/vector was about 5/1 and 4/1 respectively. The ligation products were transfected in *E. coli* DH 5α, using the TSS methodology. Different amounts of transformation were spread on ampicilline-agar plate and cultured for OVN, at 37°C. Transformants were cultured in 5 ml LB at 37°C for 7 H or OVN. Colonies were cultured in LB at 37°C for 7 H or OVN. Plasmids were prepared by alkaline lysis method and dissolved in 50 µL 10 mM Tris-HCl pH 7.5 and 0.5 mg/mL RNAses and incubated at 37°C for 30 min. Analyses of clones were carried out by single digestions with *Bam*HI, *Eco*RI and *Eco*RV (for *env-tm* construct, named pGEX-TM).

LAMP Reaction

A set of LAMP primers of *env-tm* [localization within *env-tm* gene and orientation in sense (S) or antisense (AS) given in brackets], consisting of outer primers F3 (S,731-748), B3 (AS,941-924) inner primers FIP (-47F1,SA,142-120,tttt,F2,S,76-94), BIP (–52 tttt...ttt..tttt/aaaa ggg/cccc) and loop primers IF (AS, 798-777), IB (S, 870-890), were "manually" designed, based on a sequence data. They were those previously described (Chadwick *et al.*,1995). The amplified zone of the target is 211 bp length (between F3 and B3). Primers F3 and B3 were also used in conventional RT-PCR.

LAMP reactions were carried out at 63°C for 60 min. The reaction mixture contained 2.0 µM each of FIP and BIP, 1.0 µM each of IF and IB, 0.2 µM each of F3 and B3, 1400 µM of dNTP mix (Promega, Madison, WI, USA), 0.6 M betaine monohydrate (Sigma-Aldrich), 6 mM MgSO4, 8 U of Bst DNA polymerase (large fragment; New England Biolabs Inc.), along with 1 x of the supplied buffer and specific amounts of template, in a final volume of 25 µL. Minimal time for amplification was determined by incubation for 20, 40 and 60 min. Amplification was also tested at 65°C. The degree of sensitivity was established by performing reaction at 63°C for 60 min, using serial 10-fold dilutions of template.

Amplification products were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel. LAMP-positive results are characterized by the appearance of ladder-like pattern on agarose gel, with many bands of different sizes up to the loading well. Positive responses were also visually detected due to the whitish colouration or by adding SYBR Safe and observation under a UV lamp. Cloned *env-tm* gene (pGEX-TM) was used as positive control and pGEX-2T without insert as negative control. Cloned pGEX-TM was previously isolated using DNAzol as described previously.

Results and Discussion

Isolation and amplification of env-tm genes

ENV proteins are important antigens of Jembrana virus, the agent of acute infectious disease of Bali cattle (*Bos javanicus*). env protein comprises 2 subunits i.e. SU and TM. In this work, we isolated and amplified the entire coding sequences *env-tm* (about 1.1 kb) subunits by RT-PCR as the genome of Jembrana virus is constituted of a single-stranded RNA molecule. The primers used were derived from the published sequence data (Chadwick *et al.*, 1995b). An initiation codon ATG was added and new restriction enzyme sites were introduced for cloning in pGEX-2T vector. Primers for the isolation of *env-tm* gene were conceived in a such manner that the gene insertion in pGEX-2T vector, i.e. in *Bam*HI sites, will be obligatorily in Sens orientation. RT-PCR reactions were performed, using a single step RT-PCR kit, as described in Materials and Methods, using total RNAs or isolated viral RNA as matrix. A Taq DNA polymerase treatment was added to complete the amplification process. As seen, the length of the DNA fragments corresponded to the expected one about 1.1 kb. for *env-tm* genes respectively. The additional Taq polymerase treatment was aimed to increase the extents of 3' adenine overhang thus to increase so the cloning efficiency in pCR2.1 vector, using TOPO TA cloning system.

Obtaining of pCR2.1-based constructs

Cloning in pCR2.1 vector was done using TOPO TA cloning kit. This system enables direct cloning of the RT-PCR products Thanks to topoisomerase and the 3' adenine overhang of the RT-PCR/PCR products, insertion of exogeneous DNA fragments is highly enhanced when compared to the classical insertion by DNA ligase. The colony was obtained and ampicilline resistant white or light blue colonies are bacterial transformants, having harvested plasmid containing an insert. A mixture white colonies were cultured and plasmids prepared using a miniprep plasmid preparation from Roche. Inserts corresponding to *env-tm* coding sequences were excized by respectively a single digestion with *Bam*HI. Inserts were purified by electrophoresis on agarose gel then eluted and quantified.

Construction and analyses of pGEX-TM vectors

As described in Materials and Methods while *env-tm* coding sequences were inserted in *Bam*HI site. The corresponding DNA fragments, consequently cut as earlier described, were inserted by ligation as described in Materials and Methods. For efficient insertion, we used a molar ratio insert/vector of 4/1 to 5/1. pGEX-2T constructs containing *env-tm* subunit gene will be called pGEX-TM .

To determine and analyse positive clones, bacterial colonies were cultured and plasmids were prepared by alkaline lysis method. Clones were analyzed by digestion as follows: double single digestion with *Bam*HI for pGEX-TM constructs. that the insertion is 1,1 bases in length. According to the expected DNA fragments obtained, 2 fragments of 4.9 and 1.1 kb.

Cloning in pCR2.1 plasmid, carried out using the TOPO TA Cloning system from Invitrogen, takes advantage of the topoisomerase reaction for easy, rapid and efficient insertion of DNA fragments with single 3' adenine overhang in the specially engineered vector. Positive transformants, containing plasmid with an insert, are white or light blue colonies, allowing thus easy identification of positive clones. Although the RT-PCR, used for the isolation and amplification of *env-tm* genes, already gives rise to 3' adenine overhang, by adding a Taq DNA polymerase treatment, obtaining positive clones was enhanced.

Cloning efficiency in pGEX-2T was increased by ligation of the insert in one site but de-phosphorylated (the case of *env-tm* construct).

RT- PCR reaction result

RT-PCR was carried out in a final volume of 25 µL, using the LAMP outer primers F3 and B3 with the following conditions : First cDNA synthesis at 45^0C for 45 min, AMV inactivation 94^0C for 2 min 50C, DNA amplification 40 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec, elongation at 68°C for 2 min; additional elongation at 68°C for 7 min. PCR was also performed under the same conditions for cDNA amplification using isolated pGEX-TM.

LAMP reaction result

Test of LAMP was done using cloned *env- tm* gene (pGEX- TM) or pGEX-2T as template or in absence of any template. Reaction was performed at 63°C or 65°C for 20, 40 and 60 min. Amplification worked well at both temperatures and was already fully efficient at 40 min incubation. A ladder-like pattern of bands of sizes up to the loading well was observed on agarose gel. Results were also visually established. Primers were specific to *env-tm* gene as negative response was obtained with pGEX-2T. The experiment also showed that the loop primers did not induce self-amplification of primers, in absence of any template. It has heen shown that this occurs with certain sequences of loop primers (Pillai et al., 2006). For standardization, LAMP reactions were henceforth carried out at 63°C, the optimum temperature of *Bst* polymerase, for 60 min. Optimum temperature (63^0C) and time (60 min) for RT-LAMP condition here was obtained from several experiment that we have done.

Efficiency of LAMP and comparison with conventional RT-PCR as diagnostic tool

The sensitivity of the reaction was established using serial 10-fold dilutions of templates. Amplification efficiency was compared to that of RT-PCR using the outer primers F3 and B3 specific to *env-tm*. Using pGEX-TM as template, LAMP positive response was still observed with 1 fg of plasmid (150 molecules of target). *env-tm*-based RT-PCR was still positively responding with 1 pg of plasmid though faintly. Detection threshold using Avogadro constant, established using RNA extracted from infected bovine spleen tissue indicated that the upper limit of detection was higher than 100 molecules viral particles.

We demonstrated in this experiment that the *env-tm*-based RT-LAMP was found to be a very sensitive diagnostic tool of Jembrana Disease Virus, contrarily to conventional RT-PCR using the same target. The lower sensitivity of RT-PCR for detecting the viral particles in organs or tissues may arise from the intricate population of genes and sequences within the total RNA extracted from infected organs or tissues which comprises virus as well as host-cell RNA. It has been suggested that contrarily to conventional RT-PCR, RT-LAMP is not adversely affected by irrelevant DNA targets (Notomi *et al.*, 2000). With the increasing complexity of RNA population, RT-PCR efficiency decreases and the difference between RT-LAMP and RT-PCR is consequently more pronounced.

Owing to its rapidity, sensitivity and ease of use, RT-LAMP methodology is suited for routine health controls, particularly in conditions where sophistical and expensive equipments are not available. It is also appropriate for field application in Veterinary Medicine.

Acknowledgment

This work was partly financed by the Fundamental Research Grant from Gadjah Mada University, Indonesian Government Competency Grant DIKTI and partly supported French Embassy in Indonesia .

References

Chadwick, B.J., Coelen, R.J., Sammels, L.M., Kertayadnya, G. and Wilcox, G.E. (1995a). Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. *J. Gen. Virol.*, **76**: 189-192.

Chadwick, B.J., Coelen, R.J., Wilcox, G.E., Sammels, L.M., and Kertayadnya, G. (1995b). Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. *J. Gen. Virol.*, **76**: 1637-1650.

Chadwick, B.J., Desport, M., Brownlie, J., Wilcox, G.E., and Dharma, D.M. (1998). Detection of Jembrana disease virus in spleen, lymp nodes, bone marrow and other tissues by in situ hybridization of paraffin-embedded sections. *J. Gen., Virol.*, **79**: 101-106.

Chen, H., He, J., Fong, S., Wilcox, G., and Wood, C. (2000). Jembrana disease virus Tat can regulate human immunodeficiency virus (HIV) long terminal repeat-directed gene expression and can substitute for HIV Tat in viral replication. *J. Virol.*, **74**: 2703-2713.

Hartaningsih, N., Wilcox, G.E., Dharma, D.M., and Soetrisno, M. (1993). Distribution of Jembrana disease in cattle in Indonesia. *Vet. Microbiol.*, **38**: 23-29.

Metharom, P., Takyar, S., Xia, H.H., Ellem, K.A., Macmillan, J., Shepherd, R.W., Wilcox, G.E., and Wei, M.Q. (2000). *J. Gene Med.*, **2**: 176-185.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification reaction of DNA. Nucl. Ac. Res., 28, e63.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L. & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev. Med. Virol., 18, 407-421.

Soeharsono, S., Wilcox, G.E., Dharma, D.M., Hartaningsih, N., Kertayadnya, G., and Budiantono, A. (1995a). Species differences in the reaction of cattle to Jembrana disease virus infection. *J. Comp. Pathol.*, **112**: 391-402.

Soeharsono, S., Wilcox, G.E., Putra, A.A., Hartaningsih, N., Sulistyana, K., and Tenaya, M. (1995b). The transmission of Jembrana disease, a lentivirus disease of Bos javanicus cattle. *Epidemiol. Infect.*, **115**: 367-374.

Wilcox, G.E., Chadwick, B.J., and Kertayadnya, G. (1995). Recent advances in the understanding of Jembrana disease. *Vet. Microbiol.*, **46**: 249-255.

Wilcox, G.E., Kertayadnya, G., Hartaningsih, N., Dharma, D.M., Soeharsono, S. and Robertson, T. (1992). Evidence for a viral aetiology of Jembrana disease in Bali cattle. *Vet. Microbiol.*, **33**: 367-374.