CONSTRUCTION OF AN EXPRESSION VECTOR BASED ON pCDNA3.1(+) EXPRESSING JEMBRANA DISEASE VIRUS ENV-TM SUBUNIT GENE IN EUKARYOTIC SYSTEM

Asmarani Kusumawati[1,2], Basuki Hidayat[2], Soesanto Mangkoewidjojo[1], Joannes Sri Widada[3,4,*]

Faculty of Veterinary Medicine[1], Centre of Biotechnological Study[2], Faculty of Biology[3], Gadjah Mada University, Yogyakarta 55281, Indonesia; UMR 5098, University Montpellier II, 34095 Montpellier Cedex 5, France[4]

ABSTRACT

Jembrana disease virus (JDV) is agent of a highly infectious disease in *Bos javanicus*, discovered for the first time in Jembrana village, Bali, Indonesia. Its genome, composed of a single-stranded RNA has been entirely sequenced. ENV protein is an interesting antigen of JDV. We have previously isolated *env-tm* subunit gene by a single-step RT-PCR reaction. We report in this paper the construction of a pcDNA3.1(+)-based vector for ENV expression in eukaryotic cells. *Env-tm* gene was excized from the previous construct pCR2.1-TM by a digestion with *Bam*HI and inserted in *Bam*HI site of pcDNA3.1(+) plasmid. The inserted gene comprises the entire coding sequence, including the C-terminal hydrophobic domain and an additional ATG for correct expression in eukaryotes. 6 out of 7 clones analyzed were positive. This high cloning efficiency was due to correct *Bam*HI cut of pcDNA3.1(+) which was then followed by alkaline phosphatase treatment. Gene orientation was further determined by *Eco*RI and *Eco*RV digestions. 2 out of 6 positive clones were in sens orientation while the other 4 were in anti-sens orientation. Clones in sens orientation will be used for protein expression in eukaryotic system.

Keywords : Jembrana Disease Virus (JDV), *env-tm* gene, pcDNA 3.1 (+)

ABSTRAK

Virus penyakit Jembrana (Jembrana Disease Virus/JDV) adalah agen sangat infeksius penyebab penyakit Jembrana. Penyakit tersebut menyerang sapi, terutama sapi Bali (*Bos Javanicus*). Genom JDV berupa RNA untai tunggal (ssRNA) dengan ukuran 7.732 basa nukleotida. Genom JDV telah disekuen dengan lengkap. Protein Env, terutama protein subunit transmembran merupakan antigen penting dari JDV karena immunogenik. Telah berhasil diisolasi gen subunit TM *env-tm* dengan reaksi "single step RT-PCR". Penelitian ini bertujuan mengkonstruksi pcDNA 3.1 (+) untuk mengekspresikan protein Env subunit TM dalam sel-sel eukariotik. Gen *env-tm* dipotong dari pCR-TM dengan pemotongan menggunakan *Bam*HI dan disisipkan dalam site *Bam*HI dari pcDNA 3.1 (+). Gen yang disisipkan tersusun atas seluruh sekuen termasuk "C-terminal hydrophobic domain" dan ditambah ATG untuk ketepatan ekspresi dalam jasad eukariot. Hasilnya, 7 dari 8 klon yang dianalisis positif plasmidnya tersisipi gen *env-tm*. Tingginya efisiensi kloning karena ketepatan pemotongan pcDNA 3.1 (+) dengan *Bam*HI yang kemudian diikuti perlakuan dengan alkaline phosphatase. Orientasi gen selanjutnya ditentukan dengan pemotongan menggunakan *Eco*RI dan *EcoRV*. Hasilnya, 1 dari 7 klon positif membawa gen *env-tm* dengan orientasi sens, sementara 6 klon lain membawa gen *env-tm* dengan orientasi anti-sens. Plasmid dengan sisipan gen *env-tm* berorientasi sens digunakan untuk ekspresi protein dalam system eukariotik.

Kata kunci : Virus Jembrana, gen *env-tm*, pcDNA 3.1 (+).

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, Jembrana disease has also been detected in other area of Indonesia, like Lampung (Sumatra), West Sumatra and East Java provinces (Hartaningsih *et al.*, 1993). JVD is not unique to *Bos javanicus*. It has been reported since that other types of cattle can be infected, namely *Bos taurus* and crossbred Bali (*Bos javanicus* x *Bos indicus*) cattle. The lesions resulted are nevertheless milder when compared to a case fatality rate of about 20% in *Bos javanicus* (Soeharsono *et al.*, 1995a). Viral particles can be detected in saliva and milk during the acute phase and the titer of infectious virus in blood is high. Known direct transmission of the disease occurs by the conjunctival, intranasal or oral routes (Soeharsono *et al.*, 1995b). Extremely high circulating viraemia was observed during the febrile phase in experimentally infected animals and many JDV-infected tissues were demonstrated early in the disease course. 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 elements of JDV and the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV) suggests that JDV is a lentivirus (Wilcox *et al.*, 1995). The JDV genome has now been entirely sequenced. It is composed of a single-stranded RNA, 7,732 nucleotides in length (Chadwick *et al.*, 1995b). Sequence comparaison clearly established that JDV is actually a lentivirus, exhibiting retrovirus characteristics (Wilcox *et al.*, 1995a, 1995b). Though closely related, significant genomic differences were found between JDV and BIV which may be related to the differences observed in their respective pathogenicity (Chadwick *et al.*, 1995b).

JDV has been reported to be also related to human immunodeficiency virus (HIV). Some of its regulatory elements can functionally substitute for those of HIV (Chen *et al.*, 2000). For this reason JDV-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).

ENV protein of JDV is an interesting antigen. *Env* gene is expressed as a precursor protein that is further matured into two subunits, i.e. SU (surface) and TM (transmembrane) proteins. In this paper, we describe the cloning of the entire coding sequence of *env-tm* subunit gene, including the sequence encoding for the C-terminal hydrophobic domain, in the eukaryotic expression vector pcDNA3.1(+).

MATERIALS AND METHODS

Isolation of env-tm gene

The isolation of *env-tm* gene has reported in our previous work (Asmarani Kusumawati *et al.*, 2002). Briefly *env-tm* gene was isolated using a single-step RT-PCR reaction and cloned in pCR2.1-TOPO using topoisomerase reaction methodology. *Env-tm* gene was excized from the resulting positive clone, called pCR-TM, by *Bam*HI digestion as follows. pCR-TM plasmid was prepared from recombinat *E. coli* TOP10. A mixture of pCR-TM positive recombinant bacteria were cultured in 20 ml of LB-ampicilline, at 37°C, for one night. Bacteria were harvested by centrifugation (Sorvall, 3,000 rpm, 4°C, 15 min). Plasmid was purifed using a miniprep plasmid kit (Roche), according to the instruction manual. 5 µg of plasmid was digested with 15 units of *Bam*HI in 40 µl of adequat buffer, for 1 h 30 min, at 37°C. 2 µL of the digestion products were analysed by electrophoresis on 0.8% agarose gel. The *env-tm* gene, about 1.1 kb in length, was purified by electrophoresis on a 0.8% agarose gel and eluted using Gene Clean II kit (BIO101) (glass milk-based procedure) then quantified.

Preparation of pcDNA3.1 vector

Insertion of *env-tm* gene was done in *Bam*HI site of pcDNA3.1(+). 5 µg of pcDNA3.1(+) (Invitrogen) was digested with 15 units of *Bam*HI (Roche) in 40 µl of adequate buffer at 37°C for 2h 30 min, followed by a treatment with alkaline phosphatase, by adding 20 µL phosphatase buffer, 1 unit alkaline phosphatase and incubation at 37°C for 1 h 30 min. Linearized and dephosphorylated pcDNA3.1(+) was purified by electrophoresis on a 0.8% agarose gel and eluted with Gene Clean II kit (BIO101) (glass milk-based procedure) then quantified.

Construction of pcDNA3.1-env-tm

Ligation:

Insertion of *env-tm* gene was carried out by ligation of the purified gene to *Bam*HI-digested and dephosphorylated pcDNA3.1(+). Ligation was done in 20 µL of buffer with 200 units of ligase (BioLabs), at 16°C, OVN. The amount of vector (5.4 kb in length) used was 50 ng while the *env-tm* gene added was about 50 ng. Considering the length of the gene, i.e. about 1.1 kb, the ratio of insert:vector was so about 5:1.

Bacterial transformation:

Ligation products were transfected in *E. coli* DH 5, using the TSS methodology (Asmarani Kusumawati *et al.*, 2002). Briefly, TSS-competent bacteria were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD_{600nm} around 0.6) in LB containing 10% PEG 6,000 (w/v), 5% DMSO (v/v) and 35 mM $MgCl_2$. 0.2 ml competent bacteria were mixed to half of the ligation products, let for 30 min in ice then heat-shocked at 42°C for 90 sec and let in ice again for

some minutes. 0.5 ml of SOC were added and bacteria were incubated with agitation at 37°C, for 30 min. Different amounts of transformation were spread on ampicilline-agar plate and cultured for OVN, at 37°C. Individual bacterial colonies were cultured in 5 ml LB at 37°C for one night. Bacteria were then harvested by centrifugation at 4,000 rpm (in Sorvall centrifuge), for 15 min, at 4°C.

Plasmid preparation

Plasmids were extracted using alkaline lysis method. Briefly, bacterial pellets were dissolved in 0.3 ml buffer 1 (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and incubated for 5 min at room temperature. 0.3 ml of buffer 2 (0.2 M NaOH, 1% SDS) was then added and the solution mixed without vortexing. Finally 0.3 ml of buffer 3 (2.55 M potassium acetate, pH 4.8) was added and the solution mixed without vortexing then centrifuged in a minicentrifuge for 15 min at maximal speed (13,000 rpm) at room temperature. The supernatant (0.8 ml) was then precipitated by addition of 0,7 ml isopropanol and centrifugation (minicentrifuge, 13,000 rpm, 15 min, room temperature). The pellet was washed with 70% ethanol then slightly dried. It was then 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.

Clone analysis

Clones were analyzed by *Bam*HI digestion. Digestion was carried out in 20 µL of buffer, containing 2.5-5.0 µL of plasmid solution, 5 units enzyme, at 37°C, for 1 h 30 min. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel.

RESULTS AND DISCUSSION

Isolation of env-tm gene and preparation of cloning vector

ENV protein is an important antigen of Jembrana disease virus (JDV), the agent of acute infectious disease of Bali cattle (*Bos javanicus*). It is composed of 2 subunits i.e. SU (surface) and TM (transmembrane) proteins. *Env-tm* subunit gene, about 1.1 kb in length, was excized from our previous construct pCR-TM by *Bam*HI digestion, as described in Materials and Methods. *Env-tm* gene we previously isolated by a one step RT-PCR reaction and cloned in pCR2.1-TOPO plasmid, comprises the entire coding sequence, including the sequence encoding for the C-terminal hydrophobic domain, an additional initiation codon ATG upstream the gene and a new site for *Bam*HI upstream and downstream the gene, allowing further cloning in expression vectors. Figure 1 shows DNA fragments resulting from *Bam*HI digestion of pCR-TM. As seen, a fragment of about 1.1 kb corresponding to *env-tm* gene length was obtained, in addition to a fragment of 3.9 kb (pCR2.1-TOPO). The 1.1 kb fragment was excized from the gel and purified.

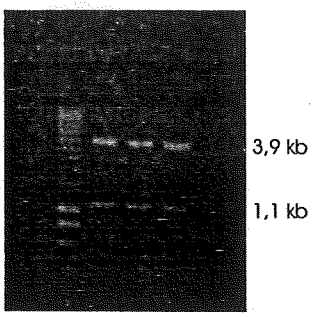

Fig 1. Insert corresponding to *env-tm* coding sequences were excised by enzyme *Bam*HI-*Eco*RI

Figure 2 shows pcDNA3.1(+) preparation for *env-tm* gene cloning. Shown in this figure analysis of *Bam*HI digestion and analysis of linearized vector after purification. Electrophoretic analysis showed that only a single DNA fragment was obtained. It corresponded to the linearized pcDNA3.1(+), i.e. 5.4 kb in length. Obtaining correct linearized vector was very important as cloning efficiency depends on complete vector digestion. Isolation of linearized cloning vector also prevented contamination of non digested vector which will give rise to negative clones. *Bam*HI digestion of pcDNA3.1(+) was followed by alkaline phosphatase treatment which dephosphorylates the 5'-end so that plasmid self-ligation will not occur. This will enhance the obtention of positive clones as only bacterial transformants harvesting a plasmid with an insert will grow in ampicilline-containing medium.

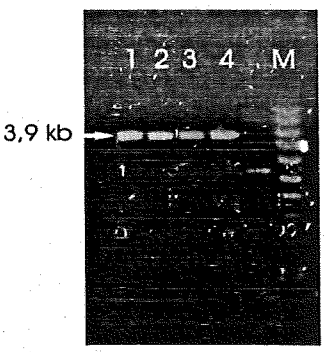

Fig 2. analysis of digested vector pcDNA 3.1 (+) were excised by enzym BamHI.

Construction of pcDNA-TM plasmid for env-tm expression in eukaryotic system

Previous cloning of *env-tm* gene in pCR2.1-TOPO plasmid using topoisomerase methodology (Asmarani Kusumawati *et al.*, 2002) allowed us to easily and rapidly isolate *env-tm* gene. This system enables clone determination solely by the coloration of bacterial colonies and our conception of primers used in RT-PCR reaction made easy gene preparation for cloning in pcDNA3.1(+). Cloning in pcDNA3.1(+) plasmid was carried out in *Bam*HI site, as described in Materials and Methods. Figure 3 shows bacterial

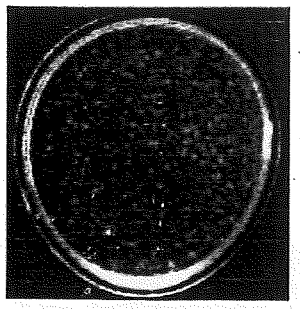

fig 3. Show the cloning pcDNA-TM transformation in *E.coli* DH5?

transformation with half of the ligation products which represented 25 ng of plasmid. As seen, more than 1,500-2,000 ampicilline-resistant bacterial colonies were obtained. They represented recombinant bacteria harvesting pcDNA3.1(+) plasmid either with or without *env-tm* gene insertion. 7 bacterial colonies were cultured and plasmids prepared by alkaline lysis method. Positive clones were determined by *Bam*HI digestion. 6 out of 7 clones analysed were positive, giving rise to 2 DNA fragments of 5.4 kb (pcDNA3.1(+)) and of about 1.1 kb (*env-tm* gene) respectively (Fig. 4). This high cloning efficiency was due to correct insert preparation on one hand and on the other hand to correct digestion of pcDNA3.1(+) followed by alkaline phosphatase treatment. It is possible to by-pass direct cloning of RT-PCR products in pCR2.1-TOPO and to use the RT-PCR products for cloning in pcDNA3.1(+). This procedure seems to be more rapid. In fact, it is not the case because correct digestion of amplification products is difficult to realize and digestion can not be easily controled. Therefore cloning will be much less efficient and more time is required. Excision of *env-tm* gene from pCR-TM construct can be controled therefore correct digestion can be easily established. The high cloning efficiency was also attained because the cloning vector was treated

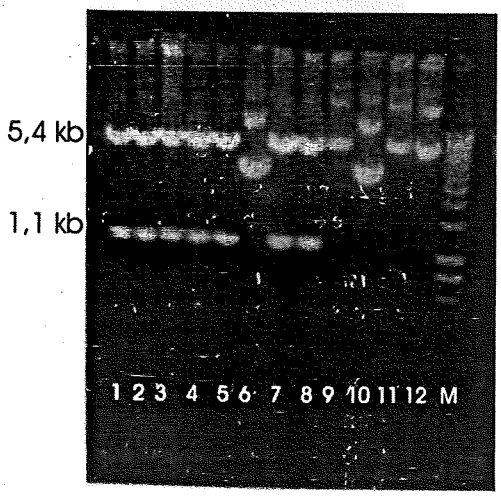

fig 4. Analysis of constructs base on pcDNA-TM were excised by enzym BamHI by electrophoresis on agarose gel.

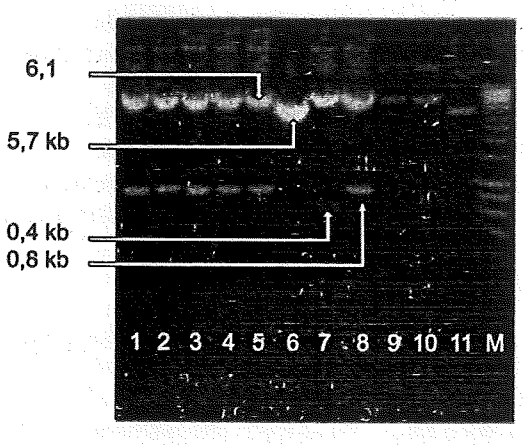

Fig. 5 Analysis of constructs base on pcDNA-TM were excised by enzym EcoRI by electrophoresis on agarose gel.

with alkaline phosphatase following *Bam*HI digestion. Singly-cut vector will be re-circularized during the ligation step. Dephosphorylation of the 5'-phosphate group prevented this process hence increased the efficiency of insert ligation in plasmid.

As *env-tm* gene was inserted in a single site, insertion occurs in sens as well as in anti-sens orientation with regard to the CMV promoter of pcDNA3.1(+). Orientation was determined by *Eco*RI and *Eco*RV digestion. Table I gives the expected number and length of DNA fragments obtained by these digestions.

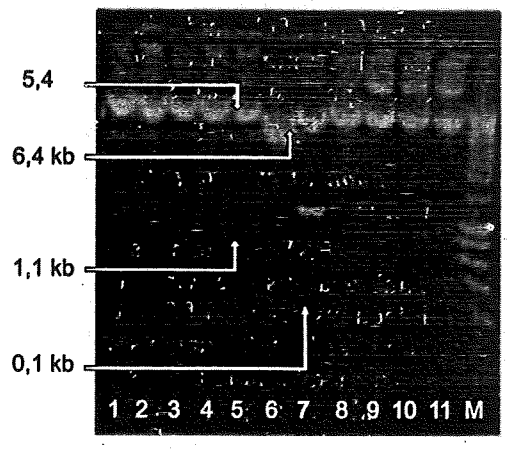

Fig. 6 Analysis of constructs base on pcDNA-TM were excised by enzym EcoRV by electrophoresis on agarose gel

All the 7 clones already analysed with *Bam*HI, were digested with *Eco*RI and *Eco*RV, as described in Materials and Methods. Figure 5 shows *Eco*RI digestion analysis. It confirmed that clone 2 was actually a negative clone, giving rise to a single fragment corresponding to pcDNA3.1(+) length. As seen in this figure, 2 out of 6 positive clones gave rise to two fragments of around 5.7 kb and around 0.75 kb.

Table I : Orientation determination of pcDNA-TM constructs
Expected sizes resulting from *Eco*RI and *Eco*RV digestions (in bp)

	pcDN3.1(+)	pcDNA-TM/Sens	pcDNA-TM/AntiSens
EcoRI :	5,400	5,705; 755	6,118; 345
EcoRV :	5,400	6,403; 60	5,400; 1,063
BamHI :	5,400	5,400; 1,063	5,400; 1,063

They are positive clones in sense orientation. The other 4 clones, giving rise to fragments around 6.0 kb and less than 0.4 kb, were positive clones in anti-sense orientation. Analysis by *Eco*RV confirmed that clones 2 and 5 are in sense orientation and that clones 1, 3, 4 and 6 are in anti-sens orientation (Fig. 6). Figure 7 represents the map of *env-tm* gene construct in pcDNA3.1(+) we called pcDNA-TM.

The work described in this paper constitutes the first step towards *env-tm* subunit gene expression in eukaryotic system. The gene we inserted in the eukaryotic expression vector pcDNA3.1(+) comprises the entire coding sequence, including the sequence encoding for the highly hydrophobic C-terminal domain. The gene also contains an additional initiation codon ATG upstream of the first codon GCC (coding for Ala) of *env-tm* gene. Due to eukaryotic translation mechanism, the ATG we introduced was intended for correct protein expression in eukaryotic cells.

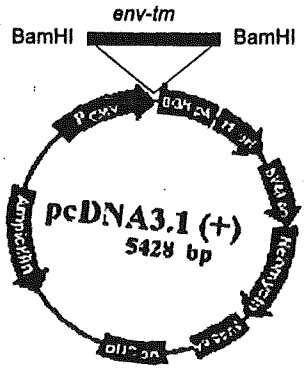

Figure 7 The map of pcDNA-TM

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). Novel bovine lentiviral vector based on Jembrana disease virus. *J. Gene Med.*, **2**: 176-185.

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.