ISOLATION AND CLONING OF ENV-TM SUBUNIT GENE OF JEMBRANA DISEASE VIRUS IN THE PROKARYOTIC EXPRESSION VECTOR pGEX-2T

ISOLASI DAN KLONING GEN *ENV* SUBUNIT *TM* VIRUS PENYAKIT JEMBRANA PADA VEKTOR EKSPRESI PROKARIOTIK pGEX-2T

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ABSTRACT

Jembrana disease virus (JDV) is agent of a severe infectious disease of Bali cattle. *Env protein* is one of JDV structural proteins. This work was aimed to construct a prokaryotic expression vector based on pGEX-2T for recombinant protein production in *E. coli*. *Env-tm* gene was excised from our previous construct pCR-TM with *Bam*HI and inserted in this site in pGEX-2T. Due to the primer conception for the gene isolation and the procedures used for cloning, insertion efficiency in pGEX-2T and transfection in *E. coli* DH 5α were very high. 1,500-2,000 recombinant bacterial colonies were obtained. Six plasmids out of seven analyzed were positive recombinant plasmids. The inserted gene was either in sense (2 out of 6) or anti-sense (4 out of 6) orientation. Sense-oriented clones will produce correct recombinant ENV-TM protein.

Key words: Jembrana virus, pGEX-2T, Recombinant protein, env-tm gene.

ABSTRAK

Penyakit virus jembrana (PVJ) adalah *agent* penyebab penyakit infeksius akut pada sapi Bali. Protein ENV adalah salah satu protein yang sebagian besar menyusun struktur protein immunogenik. Pekerjaan bertujuan untuk membuat konstruksi vector ekspresi prokariotik yang didasarkan pada pGEX-2T untuk nantinya dapat memproduksi protein rekombinan pada E.coli. Gen env-tm dipotong dari kontruksi pCR-CA dengan BamHI dan insersi tersebut dimasukkan pada *site* yang sama pada pGEX-2T. Berkat konsepsi primer untuk isolasi gen dan prosedur yang digunakan untuk kloning, hasil efisiensi kloning pada pGEX-2T dan hasi transformasi pada E. coli $DH_5\alpha$ sangat tinggi. Dalam penelitian ini diperoleh koloni bakteri rekombinan 1.500-2000 koloni. Dari 7 koloni yang dianalisa, 6 diantaranya menunjukkan positif plasmid rekombinan. Dari 6 klon yang positif plasmid rekombinan 2 diantaranya berorientasi sens dan 4 yang lainnya berorientasi anti-sens. Klon yang berorientasi positif akan menghasilkan protein rekombinan ENV-TM.

Kata kunci : Jembrana virus, pGEX-2T, protein rekombinan, env-tm gene.

INTRODUCTION

Jembrana disease virus (JDV) is the agent of Jembrana disease (JD), a severe infectious disease of Bali cattle (*Bos javanicus*), reported for the first time in Jembrana village in Bali, Indonesia (Wilcox *et al.*, 1992). JD has also been detected in other area of Indonesia, namely Lampung (Sumatra), West Sumatra and East Java provinces (Hartaningsih *et al.*, 1993). JD is not unique to *Bos javanicus*. JD turns out to be able to infect other types of cattle, namely Friesian (*Bos taurus*) and crossbred Bali (*Bos javanicus* x *Bos indicus*) cattle although the resulting lesions are milder (Soeharsono *et al.*, 1995a). Viral particles can be detected in saliva and milk during the acute phase and the titter 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 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 occurs between JDV and the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV). This suggests that JDV is a lentivirus (Wilcox *et al.*, 1995). Its genome, entirely determined, is composed of a single-stranded RNA, 7,732 nucleotides in length (Chadwick *et al.*, 1995b). Sequence comparison 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 in pathogenicity between these two viruses (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. JDV 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).

Env protein is one of the structural proteins of JDV. It is composed of 2 subunit, i.e. su and tm. We describe in this paper the isolation and the cloning of the gene encoding for *env-tm* transmembrane subunit in the prokaryotic expression vector pGEX-2T.

MATERIALS AND METHODS

Isolation of env-tm gene

Env-tm gene was excised from our previous construct pCR-TM (Asmarani Kusumawati *et al.*, submitted) by a single digestion. 5 μg pCR-TM were digested with *Bam*HI (Roche) (15 units) in 50 μL buffer 2, for 1 h 30 min at 37°C. The resulting *env-tm* fragment, about 1.1 kb in length, was purified by electrophoresis on a 0.8% agarose gel and eluted using Gene Clean II kit (BIO101) (glassmilk-based procedure).

Preparation of pGEX-2T for cloning

The multi-cloning sites of pGEX-2T consist of 5'-*Bam*HI-*Sma*I-*Eco*RI-3'. Insertion of *env-tm* gene was done in *Bam*HI site. Digestion was performed as for *env-tm* gene isolation. As singly digested vector will self-ligate during the ligation step, *Bam*HI-digested pGEX-2T was further treated with alkaline phosphates (Roche), by adding 20 μl of phosphates buffer containing 2 units of enzyme and incubation at 37°C for 1 h. Linearzed pGEX-2T was purified by electrophoresis on a 0.8% agarose gel and eluted with Gene Clean II kit (BIO101) as above.

pGEX-2T-based env-tm construction

Insertion of *env-tm* gene was carried out by ligation of the purified gene and the *Bam*HI-cut pGEX-2T. Ligation was done in 20 μL of buffer with 200 units of DNA ligase (BioLabs), at 16°C, for 16 h. The amount of the vector was 50 ng while the DNA fragment added were 50 ng. Considering the length of the *env-tm* gene (about 1.1 kb) and that of pGEX (4.9 kb), the ratio of insert/vector was about 5/1.

Transfection in E. coli DH 5α

Half of the ligation products were transfected in *E. coli* DH 5α, using the TSS methodology. Briefly, *E. coli* DH 5α bacteria at log phase (OD_{600nm} = 0.6) were pelleted by centrifugation (Sorvall, 4,000 rpm, 4°C, 10 min). Bacterial pellet was then dissolved in one tenth of the initial culture volume in ice-cold TSS (10% PEG 6,000 (w/v), 5% DMSO (v/v), 35 mM $MgCl_2$, in LB medium). 0.2 ml of bacterial solution was used for transfection by adding the half of the ligation products. The mixture was incubated on ice for ≥30 min, heat-shocked at 42°C for 90 sec and put on ice again for some minutes. It

was then incubated at 37°C for ≥30 min. Different amounts of transformation were spread on LB/ampicilline-agar plate and cultured for 16 h, at 37°C.

Preparation of plasmid

Ampicilline-resistant bacterial colonies obtained following transfection were cultured in 5 ml LB-ampicilline at 37°C for 7 h or 16 h. Plasmids were prepared by alkaline lysis method. Briefly, pellet bacteria was 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 was 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 maximum speed (13,000 rpm) at room temperature. The supernatant 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 and slightly dried.

Analysis of clones

Plasmid pellet was 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. Analysis was carried out by digestion of 2.5-5,0 μL plasmid solution with 5 units *Bam*HI, at 37°C for 1 h 30 min. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel. For orientation determination, plasmids were digested with 5 units *Eco*RI or 5 units *Eco*RV, in digestion conditions as above and analysis was done in the same manner.

RESULTS AND DISCUSSION

Construction of pGEX-TM and obtention of recombinant bacterial clones

Jembrana disease virus (JDV) is agent of a severe infectious disease of Bali cattle (*Bos javanicus*). *Env* is one of the structural proteins of JDV. It is composed of two subunits su and tm. We have isolated the gene encoding for the transmembrane subunit *env-tm* by RT-PCR reaction using a one step reverse transcription-amplification procedure from the RNA genome (Asmarani Kusumawati *et al.*, submitted for publication). The gene we isolated comprises the entire coding sequence of *env-tm*, including the hydrophobic C-terminal domain, an additional upstream sequence containing an initiation codon ATG, new site for *Bam*HI and a sequence encoding for 3 amino acids and an additional downstream sequence allowing amplification downstream of the stop codon and containing a new site for *Bam*HI. The added restriction sites were intended for efficient cloning in pGEX-2T and protein expression in *E. coli* without modification of the reading frame.

Insertion of *env-tm* gene was realized by ligation in alkaline phosphates treated-*Bam*HI site of pGEX-2T, as described in Materials and Methods. Transfection was done in *E. coli* DH 5α by TSS method. Figure 1 shows that the transfection resulted in a high number of bacterial transformants. About 1,500-2,000 bacterial colonies were obtained, resulting from transfection of half of the ligation products i.e. corresponding to only 25 ng of plasmid. It appeared therefore that ligation and transfection efficiency was very high. For an easy description, the resulting positive construct of *env-tm* in pGEX-2T will be referred as pGEX-TM.

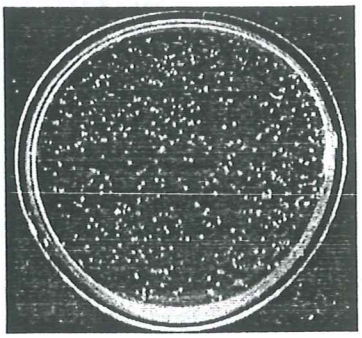

Figure 1. Recombinant bacterial colonies obtained following transfection. Ligation products were transfected in *E. coli* DH 5. Transformation was cultured on LB-ampicilline-agar plates for 16 h at 37°C.

Clone analysis and determination of positive recombinant pGEX-TM

Plasmids were prepared from bacterial transformants by the alkaline lysis method as described in Materials and Methods. They were then digested with *Bam*HI and the digestion products analyzed by electrophoresis on agarose gel. Digestion results are shown in Figure 2. pGEX-2T is 4.9 kb in length while *env-tm* gene is about 1.1 kb long. Insertion in pGEX-2T will result in recombinant plasmid of about 6.0 kb. *Bam*HI digestion of positive clones will result in 2 fragments of 4.9 kb (vector) and of about 1.1 kb (*env-tm* gene). pGEX-2T without insert, resulting from self-ligation, will give rise to a unique fragment of 4.9 kb.

As clearly seen in figure 2, 6 clones out of 7 analyzed gave rise to 2 fragments of 4.9 kb and 1.1 kb respectively. 6 clones were thus positive recombinant plasmids with *env-tm* insertion. This result shows that cloning efficiency was very high, resulted from correctly digested vector and insert and vector treatment with alkaline phosphates prior to the ligation step. Alkaline phosphates treatment eliminates the 5'-phosphate group of DNA, preventing thus self-ligation of *Bam*HI-cut pGEX-2T to occur. Cloning efficiency was also increased by excess of insert with regard to vector during the ligation step. Efficient cloning also arose from the procedure used for insert preparation. Insert excision of *env-tm* gene from pCR-TM construct enabled digestion control as the resulting digestion product, exhibing a length of 1.1 kb, can be easily observed in analysis. This is not the case with RT-PCR or PCR product digestion since enzyme cutting of amplification products is not very effective and the digested and not digested fragments exhibit similar length.

Discrimination of pGEX-TM in sense from anti-sense orientation

*Bam*HI enzyme allowed us determine

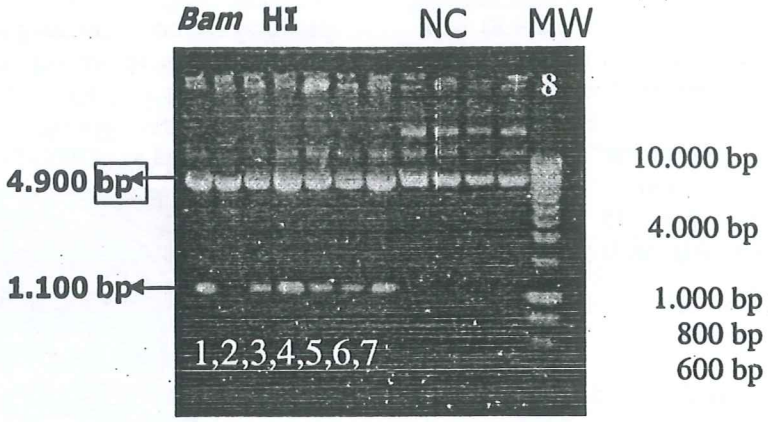

Figure 2. Plasmid analysis by *Bam*HI digestion. Digestion was performed as described in Materials and Methods. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel. 7 clones were analyzed (1-7). Nc: non digested clones; MW: molecular weight marker.

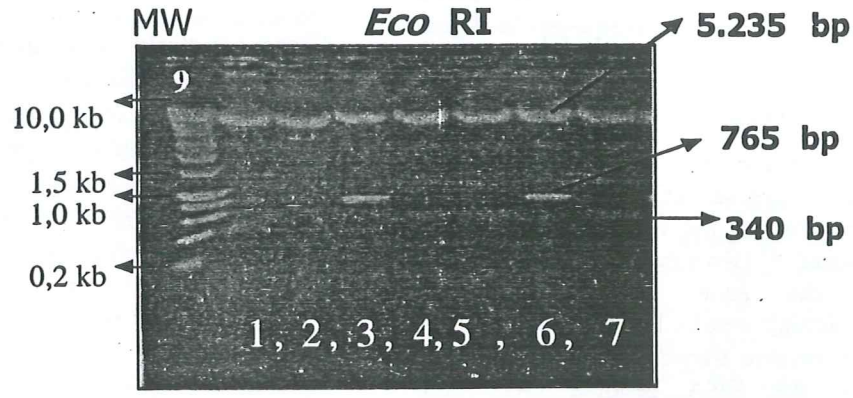

Figure 3. Analysis by *Eco*RI digestion. Digestion with *Eco*RI was carried out as described in Materials and Methods. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel. 7 clones were analyzed (1-7).; MW: molecular weight marker

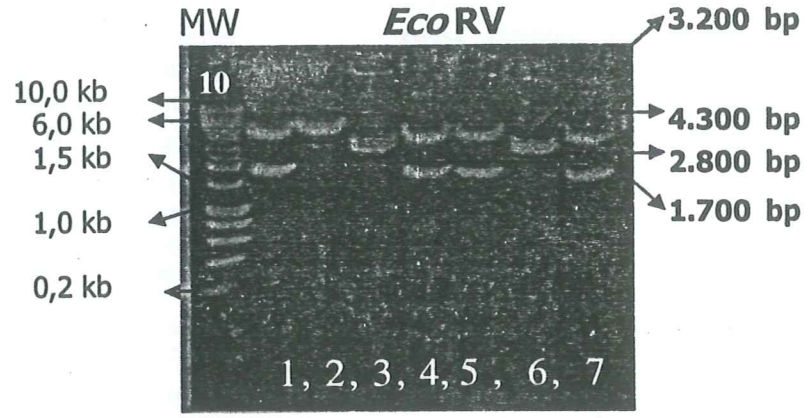

Figure 4. Clone analysis by *Eco*RV. Digestion with *Eco*RV was done as described in Materials and Methods. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel. 7 clones were analyzed (1-7).; MW: molecular weight marker

positive and negative clones. However single site insertion would give rise to recombinant plasmids in sense or anti-sense orientation and *Bam*HI digestion did not allow to discriminate sense from anti-sense orientation. Only genes in sense orientation will produce correct recombinant proteins. The orientation of *env-tm* gene in pGEX-2T construction was established by digestion with *Eco*RI or *Eco*RV. Table I shows prediction of fragments (number and length) obtained after restriction digestions.

Table 1. Prediction of DNA fragments obtained by restriction digestions

	pGEX-2T	pGEX-TM/Sens	pGEX-TM/ AntiSens
*Bam*HI:	4,900	4,900, 1,100	4,900, 1,100
*Eco*RI:	4,900	5,235, 765	5,665, 340
*Eco*RV:	4,900	≈3,200, ≈2,800	≈4,300, ≈1,700

Figure 3 shows results obtained by *Eco*RI digestion of the 7 clones already analyzed by *Bam*HI (Fig. 2). Clone 6, previously established as negative clone, only gave rise to one fragment of 4.9 kb (empty vector). This analysis showed that clones 2 and 5 were positive recombinant pGEX-TM in sense orientation (abstention of fragments 5.2 and 0.7 kb) and clones 1, 3, 4, 7 are positive recombinant pGEX-TM in anti-sense orientation (abstention of fragments 4.9 and 1.1 kb).

Results obtained by *Eco*RV digestion, illustrated in figure 4, actually confirmed results obtained by *Eco*RI digestion (Fig. 3). Therefore clones 2 and 5 are actually positive recombinant clones in sense orientation and clones 1, 3, 4 and 7 positive recombinant clones in anti-sense orientation. Only *env-tm* gene in clones 2 and 5 will be transcribed to mRNA and correct recombinant *env-tm* produced in *E. coli*.

The work described in this paper constitutes the first step in the production of *env-tm* recombinant protein in *E. coli*. Cloning in this vector enables the expression of proteins fused to GST (glutathione S-transferase from *E. coli*). As no method is available for the purification of *env-tm* protein, this fusion allows a simple protein purification procedure by GST-specific affinity chromatography with glutathione sepharose column. Isolated and purified proteins can be obtained, either fused or not to GST, thanks to the peptide link that constitutes a digestion site for thrombin. By the concept of the construction, once cut from GST, the recombinant *gag-ca* protein will be of full length and contain additional peptide G-S-A at the N-terminal end so that there will be no sterical hindrance resulting from fusion. Finally, cloning in pGEX-2T also allows direct gene transfer in a pGEX-based baculovirus transfer vector or in the eukaryotic expression vector pcDNA3.1.

CONCLUSION

The final aim of this research works is the concept and production of recombinant protein, DNA vaccines and transgene vaccines against Jembrana disease. The coding sequences of *env-tm* have been isolated and amplified by one step RT-PCR reaction from the viral genome, followed by a Tag DNA polymerase treatment. RT-PCR product was directly cloned in pCR2.1, using topoisomerase reaction. A great number of positive clone have been obtained which were further analyzed by restriction digestions. The excised *env-tm* and was then purified for the insertion in prokaryotic expression vector pGEX-2T. These vector have been cut by the corresponding enzymes and purified.

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