Construction and Studies of Recombinant DNA Delivery System for CAV

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Introduction

Vaccine in general can be derived from live or attenuated virus, killed whole-cell virus, sub-unit component (LPS, protein), recombinant vaccine and DNA or RNA vaccine. Recombinant and DNA based vaccination constitutes two of the most recent approaches to vaccine development. DNA vaccine is a simple and versatile method of inducing both humoral and cellular responses to the antigen encoded by the plasmid, as well as protection against a variety of infectious agents. VP1 is a capsid protein and it is studied to develop a new DNA vaccine against CAV.

In recombinant bacterial systems, most of the bacteria used as vectors are derived from the Gram negative microorganisms. The use of *Lactococcus* as an alternative vector system has been considered because it is the genetically-best characterized lactic acid bacteria and due to its GRAS (Generally Regarded As Safe) status. Currently, there are several recombinant Lactococcal strains which has shown the capability to express heterologous proteins with vaccine potential (Robinson *et al.*, 1997). However, the major problem faced in studies using *Lactococcus* system is the lack of commercially available plasmid vectors in the market. Thus, it is important to construct potential plasmid vectors to be used for expressions in *Lactococcus*.

Materials and Methods

Construction of plasmid DNA. The VP1 gene was amplified and digested with *Eco*RI and *Kpn*I. The 1.4 kb of the gene was then ligated to the *Eco*RI – *Kpn*I double digested pcDNA3.1 vector. Recombinant plasmid was transformed into *E. coli* TOP 10 cells using heat-shock method by Sambrook *et al.* (1989). The cells were grown in LB medium containing ampicillin (50µg/ml) and incubated at 37°C.

Vero cells. The plasmid DNA was transformed into Vero cells (Africa green monkey kidney cells) by LIPOFECTAMINE 200 reagent (Life technologies). The transfected Vero cells were cultred in L15 medium with 10% fetal calf serum and 1% antibiotics at 37°C. the cells were then sub-cultured at 2 to 3 days intervals.

Indirect immunofluorescence test. The transfected Vero cells and untransfected control vero cells were plated on 4 chamber slide (Lab-Tek) respectively a day before the test. The cells were fixed in chilled acetone : methanol (1:1) for 10 min. A 50 µl of CAV VP1 monoclonal antibody (TropBio) diluted 1:1000 in PBS was applied to the cells. After 1 h of incubation at 37°C in a humidified atmosphere, the slides were washed 3 times in PBS, each time for 15 min. A 50 µl of a 1:100 dilution of FITC rabbit anti-mouse IgG (Kirkegaard and Perry Laboratories) was then applied to the cells for 1 h at 37°C and rinsed 3 times with PBS, each time for 15 min. The slides were then mounted in buffered glycerol (pH 7.4) and observed for flourescence using a flourescence microscope.

Indirect immunoperoxidase test. The transfected Vero cells and the untransfected control Vero cells were plated on 4 chamber slide (Lab-Tek) respectively a day before the test. The cells were fixed in chilled acetone: methanol (1:1) for 10 min. Non-specific binding sites were blocked by incubating the cells with 5% BSA for 1 h at 37°C. The blocking solution was then drained off. A 50 µl of CAV VP1 monoclonal antibody (TropBio) diluted 1:1000 in PBS was placed to the cells. After 1 h of incubation at 37°C in a humidified atmosphere, the slides were washed 3 times in PBS, each time for 15 min. A 50 µl of a 1:1000 dilution of HRP goat anti-mouse IgG (Kirkegaard and Perry Laboratories) was then applied to the cells and incubated 1 h at 37°C. The cells were then rinsed 3 times with PBS. The slides were then mounted in buffer glycerol (pH 7.4) and observed under a light microscope.

Bacterial strains and growth conditions. Lactococcus strain were grown in M17 medium supplemented with 0.5% glucose and incubated at 30°C where as *E. coli* strains were grown in LB medium and incubated at 37°C. When required, a total concentration of 5 g/ml of erythromycin was used for *Lactococcus* while 50 g/ml of ampicillin were used for *E. coli* cultures.

Construction of pMG36enisA. The genomic DNA of *L. lactis* ATCC strain 11454 was isolated using the method from Engelke *et al.* (1992) with minor modification. The nisA gene was amplified by PCR using primers design based on the published sequence of nisA gene (Engelke *et al.*, 1992) with the forward primer carrying an *Eco*R1 site. The gene itself carries a unique *Sac*1 site downstream of the nisin promoter region. The amplified gene was then digested with *Eco*R1 and *Sac*1 to obtain the 344 bp DNA fragment containing the promoter region of the nisin gene. This fragment was then cloned into the *Eco*R1 – *Sac*1 region of the expression vector pMG36e (Van de Guchte *et al.*, 1989). The constructed plasmid was then transformed into plasmidless *E. coli* XL1-Blue (Stratagene) using heat-shock method by Sambrook *et al.* (1989) and later electro-transformed into a plasmidless *Lactococcus lactis* MG1363 (Gasson et al., 1983) using the protocol from (Holo et al.and Nes, 1989) and selection was made on media containing erythromycin. Verification of the construct was done by restriction digestion

analysis of the plasmid with *Eco*R1 and *Sac*1, and by PCR amplification of the cloned promoter region. The newly constructed plasmid vector was designated as pMG36eRnisA.

Construction of pAJ03. An erythromycin resistant plasmid (pAJ01) was previously ligated to the *E. coli* cloning vector pUC19 at the *Eco*R1 site resulting a new shuttle vector named pAJ02 (Raha *et al.*, 2001). The P32 constitutive promoter together with the VP3 gene of the CAV from the previously constructed pMG36e-VP3 recombinant plasmid was amplified by PCR with the forward primer designed to carry a *Kpn*1 site while the reverse primer was designed to carry an *Xba*1 site. The amplified PCR product was then digested and cloned into the *Kpn*1 – *Xba*1 region of the pAJ02. The recombinant plasmid designated as pAJ3 was then transformed into *E. coli* XL1-Blue (Stratagene) and electrotransformed into plasmidless *L. lactis* MG1363. The transformants were selected on medium containing ampicillin and erythromycin respectively. Verification of the transformant was done by restriction digestion analysis and PCR amplifycation of the cloned DNA fragment.

Results and Discussions

The CAV VP1 gene was expressed in the Vero cells. Using indirect immunofluorescent test, the expression of VP1 was observed as green-yellowish fluorescence and the staining was predominantly in the cytoplasm of the Vero cells. For the indirect immunoperoxidase test, immuno-peroxidase staining was detected as brownish colour also predominantly in the cytoplasm. However, both immunostaining tests indicated low levels of expression of the VP1 gene in transfected Vero cells.

The amplification of the nisA gene, including the promoter region, resulted in a PCR product of 415 bp in size. The forward primer used was designed to carry an *Eco*RI site, while the downstream of the promoter region contains a unique *Sac*I. These two sites were then used for a double restriction digestion to isolate the promoter region and clone it in the *Eco*RI – *Sac*I region of the expression vector pMG36e. This resulted in a replacement of the P32 promoter with the inducible nisin promoter. Nisin promoter has been documented to be a strong inducible promoter (de Ruyter *et al.*, 1996). The replacement of the P32 promoter with the nisin promoter theoretically will improve on the expression ability of the plasmid vector. The constructed plasmid vector (pMG36eRnisA) retained the ability of pMG36e to replicate *in E. coli* as well as *L. lactis* MG1363. Thus, similarly to pMG36e, the newly constructed vector has the ability to use *E. coli* as an intermediate host for molecular cloning purposes.

The amplification of the P32 promoter together with the VP3 gene from the previously constructed pMG36e-VP3 resulted in a PCR product of about 700 bp. The digestion and cloning of the PCR product into the *Kpn*I – *Xba*I region of the plasmid pAJ02 resulted in a construction of a plasmid vector with the potential to express the VP3 gene of the CAV. The recombinant plasmid also showed the ability to replicate in both *E. coli* and *L. lactis* thus allowing the use of *E. coli* as an intermediate host for molecular purposes.

Although these two plasmids have the potential to be used in *Lactococcus* expression system, they are currently still in construction level. The efficiency of protein expression in these two newly constructed plasmids will be studied in near future.

Conclusions

The VP1 gene from chicken anemia virus (CAV) was successfully cloned into a commercially available expression vector pcDNA3.1 and this recombinant DNA molecule showed the ability to express this VP1 protein when transfected into Vero cells. However, the expression level was found to be low. Subsequently, efforts have been made to locally construct two expression vectors for *Lactococcus* expression system. The expression abilities of these vectors are yet to be studied.

Benefits from the study

This study looks for an alternative vaccine delivery system as well as contributed in a deeper understanding of using *Lactococcus* expression system for vaccine delivery. Throughout the course of this project, a total of 6 graduate and 14 undergraduate students were trained in molecular techniques.

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Project Publications in Refereed Journals

None.

Project Publications in Conference Proceedings

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Graduate Research

None.

I·R·P·A INTENSIFICATION OF RESEARCH IN PRIORITY AREAS

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