Development of Primary Cell Culture Assay for Evaluation of Gene Delivery into Shrimp by Chimeric Baculovirus

การพัฒนาวิธีใช้เซลล์เพาะเลี้ยงเพื่อตรวจสอบประสิทธิภาพของแบคคิวโลไวรัส ในการขนส่งยีนเข้าสู่กุ้ง

Suthiwat Khankaew (ศุทธิวัต ขันแก้ว)* Dr.Rapeepun Vanichviriyakit (ดร.รพีพรรณ วานิชวิริยกิจ)**
Dr. Pongsak Khunrae (ดร.พงศักดิ์ ขุนแร่)*** Dr.Triwit Rattanarojpong (ดร.ไตรวิทย์ รัตนโรจน์พงศ์)***
Dr.Kanokwan Poomputsa (ดร.กนกวรรณ พุ่มพุทรา)****

ABSTRACT

Baculovirus Vector System has been developed to deliver the gene of interest into various animal cells including shrimps. In this study, the chimeric baculovirus has been genetically engineered to deliver White Spot Syndrome Virus (WSSV) *vp28* gene into Pacific white shrimp, *Penaeus vannamei*. Moreover, the primary shrimp hemocyte was developed to assess the efficiency of baculovirus gene delivery as a rapid and more convenient approach. The chimeric baculovirus, expressing *vp28* was conveniently detected from the transfected hemocyte cells by Reverse Transcription PCR. The results showed that baculovirus was able to deliver the *vp28* since the *vp28* expression could be detected in hemocyte cells. Thus, this chimeric baculovirus is assumed to be able to deliver WSSV gene and express in shrimp. This assay can be conveniently used as preliminary test for various gene expression studies and vaccine development.

บทคัดย่อ

ระบบแบคคิวโลไวรัสได้ถูกพัฒนาให้ใช้ในการขนส่งยีนที่สนใจเข้าสู่เซลล์สัตว์หลายชนิดรวมถึงกุ้งด้วย การศึกษาครั้งนี้จึงได้พัฒนาแบคคิวโลไวรัสลูกผสมเพื่อขนส่งยีน *vp28* ของไวรัสตัวแดงดวงขาวเข้าสู่กุ้ง และยังได้พัฒนาวิธีตรวจสอบการขนส่งยีนเข้าสู่กุ้ง โดยใช้เซลล์เม็ดเลือดกุ้งที่เลี้ยงในอาหารเพาะเลี้ยงในหลอดทดลอง ซึ่งสามารถควบคุมปัจจัยต่างๆได้ดี ภายหลังการเติมแบคคิวโลไวรัสลูกผสมที่สร้างขึ้น สามารถตรวจสอบการเข้าสู่เซลล์กุ้งโดยตรวจสอบการแสดงออกของยีน *vp28* ด้วยเทคนิค Reverse Transcription PCR ซึ่งพบว่ามีการแสดงออกของยีนดังกล่าว ในเซลล์เม็ดเลือดกุ้ง ดังนั้นจึงเป็นการบ่งชี้ว่าแบคคิวโลไวรัสลูกผสมนี้จะเป็นพาหะที่ดีในการขนส่งยีนของไวรัสตัวแดงดวงขาวเข้าสู่เซลล์กุ้งได้ตามเป้าหมาย และน่าจะมีประสิทธิภาพเช่นเดียวกันเมื่อนำไปทดสอบกับกุ้ง จะเห็นได้ว่าการใช้ระบบเซลล์ยังเป็นระบบที่ดีสำหรับการทดสอบเบื้องต้น ในการศึกษาการแสดงออกของยีนต่าง เพื่อพัฒนาเป็นวัคซีนต่อไป

Key Words: Shrimp hemocyte, Baculovirus, Gene delivery

คำสำคัญ: เซลล์เม็ดเลือดของกุ้ง แบคคิวโลไวรัส การขนส่งยีน

* *Student, Master of Science Program in Applied Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi*

** *Lecturer, Department of Anatomy, Faculty of Science, Mahidol University*

*** *Lecturer, Department of Applied Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi*

**** *Assistant Professor, Department of Biotechnology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi*

Introduction

Nowadays, many shrimp farms encounter a problem with infectious diseases especially from viruses (Timothy, 2012). Although in shrimp farm have adopted a biosecurity system, but it remains a variety of viral disease still affecting shrimp. White spot syndrome virus (WSSV) is a highly mortal agent that leads to 100% mortality within 7-10 days. Therefore, the effective prevention of WSSV infection requires the knowledge of shrimp and virus interaction especially the role of WSSV genes and proteins involved in disease pathogenesis. Regarding to several unidentified function of WSSV genes and proteins, recent shrimp researches need the effective gene delivery system both *in vitro* and *in vivo* to discover their functions.

Several studies demonstrated that VP28 is one of the envelope protein mainly involving in the entry of virus into shrimp cell (Tsai et al., 2006) and is considered as the attractive target to produce the vaccine against WSSV infection in shrimp in various forms such as recombinant VP28 vaccine (Witteveldt et al., 2004), DNA vaccine encoding *vp28* (Rajesh et al., 2008) and *vp28* dsRNA (Kim et al., 2007).

Recently, Syed et al. (2009) developed the recombinant baculovirus able to express VP28 protein on its envelope by WSSV immediate early promoter 1 (ie1 promoter) and shown the capability to enter shrimp cell. The *vp28* was expected to be expressed in shrimp and to induce shrimp immunity. However, detection of the *vp28* gene expression delivered by baculovirus in shrimp tissues was affected by quality of shrimp tissues after collection and many factors during shrimp cultivation in the experiment.

Generally, the chimeric baculovirus will be injected into shrimp and then the target gene expression will be monitored in organs such as gills, pleopod or muscle. However, this method is time-consuming, inconvenient and has many interference factors. Thus, a rapid and more convenient assay was developed to assess the efficiency of baculovirus gene delivery. In this study, primary hemocyte culture of *Penaeus vannamei* were used as an alternative for assessment of *vp28* gene delivery and expression by chimeric baculovirus. The *vp28* gene expression can be easily detected in hemocytes by reverse transcription PCR (RT-PCR).

Objective of the study

The objective of this study was to develop a primary shrimp hemocyte culture system for assessment of chimeric baculovirus gene delivery into the shrimp.

Methodology

Experimental animals

Penaeus vannamei (10-14 g body weight) was obtained from Charoen Pokphand Foods PCL. The shrimps were screened for WSSV-free by real time PCR assays.

Preparation of WSSV viral inoculum

The WSSV stock was prepared by injection into healthy shrimps. The hemolymph was collected from moribund shrimp followed by centrifugation at 3000 × g for 20 min at 4 ºC. The supernatant was centrifuged at 8000 × g for 30 min at 4 ºC. The supernatant was passed through a 0.45 µm filter and then stored at -80 ºC before use. (Syed et al., 2009)

Generation of chimeric baculovirus

The pFASTBacHT A baculovirus transfer vector in which its polyhedrin promoter replaced by WSSV ie1 promoter was used in this study. Full

length *vp28* gene was amplified from WSSV genome using primers F-RsrII_VP28 (5'-CGC CGG TCC GTG TTG GTA AAG CCA AAC CAT GGA TCT TTC TTT CAC TCT TTC GG–3') and R-BamHI_VP28 (5'-CGC GGA TCC TTA CTC GGT CTC AGT GCC AGA G–3') and inserted into the pFASTBacHT A under the control of the WSSV ie1 promoter (pFastBacHT-ie1*vp28*). The PCR conditions used for *vp28* amplification was at 95 ºC for 2 min, followed by 35 cycles of 95 ºC for 30s, annealing for 30s at 55 ºC, extension at 72 ºC for 30s followed by final extension at 72 ºC for 5 min. The PCR product was analyzed by 1.5% agarose gel electrophoresis and DNA sequence analysis. Another plasmid without *vp28* insertion (pFastBacHT-ie1) was used as negative control.

The recombinant baculovirus was generated by integration of the *vp28* from pFastBacHT-ie1*vp28* into baculovirus genome within DH10Bac™ *Escherichia coli* (Life Technologies) according to the protocol of Bac-to-Bac system (Life Technologies). Recombinant baculovirus, rBV-VP28, were extracted from the DH10Bac™ and then transfected into insect cells to propagate the recombinant baculovirus. The insect cells, Sf9 cell lines isolated from *Spodoptera frugiperda*, were grown in Grace's insect medium supplemented with 10% fetal bovine serum (FBS), 2.5% lipid mixture (1000x) and the shear force protectant (0.25% pluronic acid) and incubated at 27°C. The virus particles were harvested on day-5 post transfection and the viral titer was determined by end point dilution assay according to Reed and Muench (1938). For the negative control, the pFastBacHT-ie1, a plasmid without *vp28* insertion, was also used to generate the recombinant, rBV-ie1, baculovirus using the same method.

Culture of primary shrimp hemocytes

Hemolymph from *Penaeus vannamei* was withdrawn from shrimp using appropriate syringe and needle containing sterile shrimp salt solution (450 mM NaCl, 10mM KCl, 10 mM EDTA and 10 mM HEPES pH 7.2) at a ratio of shrimp salt solution: hemolymph = 1:1 and gently mixed.. The 500 µl of 1.5×10^6 cells/ml hemocytes cell suspension were prepared in 2x Leibovitz's L-15 (Life Technologies) supplemented with 15% heat inactivated FCS, 2% Penicillin/ streptomycin, 0.5% NaCl at osmolarity of 720 mos/kg. The cells suspension were added into each well of 24 well plate and incubated at 27ºC for 1 h then the medium was removed and replaced with modified 2x Leibovitz's L-15.

Gene delivery assay in primary hemocyte culture

After 6 h incubation of the primary hemocyte culture at 27 ºC, the medium was removed and then washed with 2x Leibovitz's L-15. 300 µl of recombinant baculoviruses, rBV-ie1 and rBV-VP28, at 1×10^8 pfu/ml were added. WSSV suspension and wild-type baculovirus (AcMNPV), were also used as positive control and negative control, respectively. After 18 h, cells in each well were washed with 2x Leibovitz's L-15 for 3 times. The cells were collected by repeated pipetting and then RNA extracted according to the manufacturer's protocol (Ambion).

Detection of *vp28* expression

Total RNA extracted from cells obtained from 10 wells of each infections using 1 ml of Trizol LS reagent (Ambion). The RNA samples were treated with DNaseI, RNase-free (Thermo Scientific) to prepare the DNA-free RNA prior to cDNA synthesis for RT-PCR. One microgram of DNA-free RNA was used to cDNA synthesis according to the manufacturer's protocol (Thermo Scientific). The

vp28 was amplified from cDNA with the specific primers F-RsrII_VP28 (5'-CGC CGG TCC GTG TTG GTA AAG CCA AAC CAT GGA TCT TTC TTT CAC TCT TTC GG–3') and R-BamHI_VP28 (5'-CGC GGA TCC TTA CTC GGT CTC AGT GCC AGA G–3'). The PCR program was carried out as mentioned previously.

Results

The amplification and cloning of *vp28* into pFASTBac HT A

The *vp28* was successfully amplified by using WSSV genome as the template. The amplified *vp28* showed the molecular mass of 615 bp (Figure 1). The *vp28* was subsequently cloned into pFASTBacHT A to generate the recombinant vector (pFASTBacHT A /rBV-VP28). The result showed that the *vp28* was successfully cloned into pFASTBacHT A (data not shown) and the *vp28* was engineered into chimeric baculovirus via pFASTBacHTA, baculovirus transfer vector as demonstrated in Figure 2. The recombinant vectors were used to generate chimeric baculovirus in *Sf-9* cell according to the protocol of Bac-to-Bac system (Life Technologies).

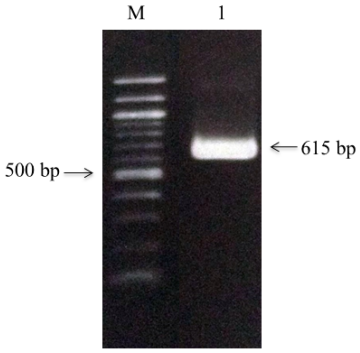

Figure 1 The PCR product of *vp28* showed the molecular mass of 615 bp (1) and M: 100bp DNA ladder

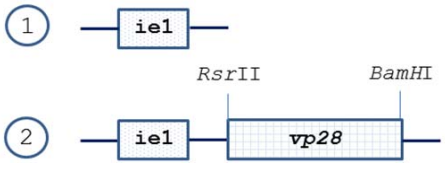

Figure 2 Diagram showing construction of *vp28* gene under the control of WSSV immediate early promoter 1 (ie-1) in pFASTBac HT A. (1) pFastBacHT-ie1 for negative control baculovirus, rBV-ie1 and (2) pFastBacHT-ie1*vp28* for recombinant rBV-VP28 baculovirus.

End point titration and expression of VP28 protein on chimeric baculovirus particle

After propagation of chimeric baculovirus rBV-ie1 and rBV-VP28 in *Sf-9* cell, the cell culture medium was collected and end point titration of the virus was determined. The result showed that the endpoint titration of rBV-ie1 and rBV-VP28 were 1.24×10^7 and 1.09×10^7 pfu/ml respectively. When analyzing the expression of VP28 on virus particles, it was shown that VP28 protein could be detected from chimeric baculovirus rBV-VP28 with the molecular mass of 28 kDa whereas no VP28 was observed from chimeric baculovirus rBV-ie1 which was the negative control sample (Figure 3).

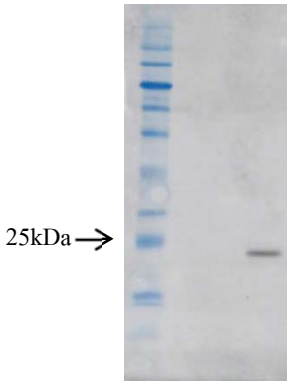

Figure 3 Western blot analysis of VP28 expression in recombinant baculovirus (rBV-ie1) (lane1) and rBV-VP28 (lane2). M: Protein Ladder (10-250 kDa).

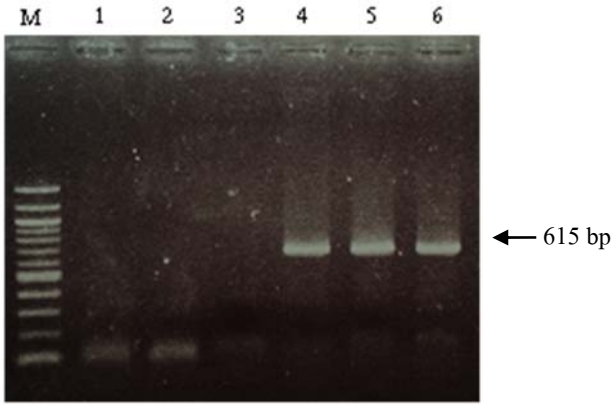

Figure 4 RT-PCR for detection of *vp28* expression in primary hemocyte culture at 18 h post infection. Lane 1: hemocytes without any virus infection, lane 2: wild-type baculovirus AcMNPV infection, lane 3: recombinant rBV-ie1 baculovirus infection, lane 4: recombinant rBV-VP28 baculovirus infection, lane 5: WSSV infection, lane 6: Positive control (615 bp) and M: 100 bp DNA ladder.

Gene delivery assay in primary hemocyte culture

When performing the entry ability of constructed chimeric baculovirus in shrimp hemocytes, the chimeric baculovirus was co-cultured with shrimp hemocytes and WSSV infected hemocytes were used as the positive control sample. The result found that *vp28* transcript could be examined from the hemocytes RNA cultured with rBV-VP28 and WSSV infected hemocytes at 18 h of culture (lane 4 and 5) (Figure 4). However, the *vp28* transcript could not be detected in wild type baculovirus, chimeric baculovirus rBV-ie1, respectively (lane 2 and 3) (Figure 4).

Discussion and Conclusions

Recent years, baculovirus system was adopted for gene delivery into insect, mammalian cells (Lu et al., 2007) and shrimp both *in vitro* and *in vivo* (Syed et al., 2009 and Syed, Kwang, 2011). Baculoviruses can be engineered to display the foreign peptides and proteins on the viral surface have proven particularly useful as immunogens or surface display that may provide further opportunities for enhancing and targeting baculovirus-mediated transduction of animal cells (Thomas et al., 2005). However, detection of gene expression in shrimp tissues and cells has been shown to be inconvenient because of many obstacles such as quality of shrimp samples that affect assessment of gene delivery efficiency. Furthermore, rearing of the shrimp is a time consuming process. Therefore, this system is possibly applied as the alternative gene delivery system in shrimp researches in order to study the WSSV genes and proteins functions *in vitro*. In this study, we showed that the *vp28* gene delivery by chimeric baculovirus carrying *vp28* can be easily detected in cultured shrimp hemocyte cell by RT-PCR. As the result, hemocytes is the important target cell to study WSSV genes and proteins functions because this shrimp cell type provides the advantages such as easy to culture as well as rapid and less time consuming when compares to other shrimp cells and tissues (Seena et al, 2010). Moreover, hemocyte is the major immune cell in shrimp (Sung et al., 1999), therefore, other genes of interest or immune-related genes of shrimp viruses can be delivered into shrimp cells using baculovirus system for shrimp protection against infectious disease and reasonable to study the mechanism of WSSV to alter shrimp defense system.

Our results showed that *vp28* can be easily detected in cultured shrimp hemocyte under the control of ie-1 promoter by RT-PCR. This demonstrated that the ie-1 promoter can function in shrimp hemocytes (Fang et al, 2009). As the result, suggested that the chimeric baculovirus rBV-VP28 can enter the shrimp cell. This modified and developed method has could be considered as one of protocols for the delivery of gene via the constructed chimeric baculvirus into the shrimp hemocytes. Hereafter, this system will be used as a tool to study the function of WSSV genes and proteins of interest that alter immune mechanism in shrimp hemocytes in order to gain the knowledge of shrimp and virus interactions in the molecular details.

Acknowledgements

This study was supported by Department of Applied Microbiology, Faculty of science, and Biotechnology Program, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi. We thank Charoen Pokphand Foods PCL for experimental animals and scholarships.

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