Production of Recombinant Non-Structural Protein 3ABC of Foot and Mouth Disease Virus (FMDV) Serotype O using Baculovirus-Insect Cell Expression System

การผลิตรีคอมบิแนนท์โปรตีนที่ไม่ใช่โปรตีนโครงสร้างชนิด 3 เอบีซี ของไวรัสโรคปากและเท้าเปื่อยซีโรไทป์โอด้วยระบบการแสดงออกของแบคคูโรไวรัสในเซลล์แมลง

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Abstract

Foot-and-mouth disease is one of the most highly contagious diseases of cloven-hooved ruminants. FMD is on the A list of infectious diseases of animals of the Office International des Epizooties (OIE) and

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has been recognized as the most important constraint to international trade in animals and animal products. Vaccination is one of the strategies to control FMD. However, it is very difficult to differentiate vaccinated animals from naturally infected animals. Currently the detection of antibodies to non-structural proteins (NSPs) is the ideal diagnostic approach to distinguish virus infected from vaccinated animals. In this studied, the NSP 3ABC DNA was successfully cloned and expressed using a baculovirus (AcNPV) vector, pFastBac™HTb in insect cells. The PCR products of 3ABC gene were approximately 1300 bp in size. The SDS-PAGE analysis of recombinant protein found that the size of recombinant protein was around 53 kDa. The dot and Western blot analyses showed the recombinant protein was specific to mouse anti-histidine monoclonal antibody and porcine anti-FMDV serotype O hyperimmune serum. Thus, the recombinant 3ABC protein may be useful for the development of the test for differentiates infected from vaccinated animals.

Key words: 3ABC, FMD, gene expression, baculovirus system, recombinant protein

บทคัดย่อ

โรคปากและเท้าเปื่อยเป็นโรคหนึ่งที่สามารถระบาดได้อย่างรวดเร็วมากในตระกูลสัตว์กีบ โรคปากและเท้าเปื่อยเป็นโรคที่จัดอยู่ในกลุ่ม A ขององค์การโรคระบาดสัตว์ระหว่างประเทศ และเป็นข้อจำกัดให้ประเทศที่มีการระบาดของโรคปากและเท้าเปื่อยไม่สามารถส่งออกสัตว์ หรือผลิตภัณฑ์จากสัตว์นั้นได้ การใช้วัคซีนเป็นวิธีการหนึ่งในการควบคุมการระบาดของโรคปากและเท้าเปื่อย แต่ปัญหาสำคัญในการทำวัคซีนคือไม่สามารถที่จะแยกภูมิคุ้มกันของสัตว์ที่เกิดจากการทำวัคซีนออกจากการติดเชื้อตามธรรมชาติได้ ปัจจุบันการตรวจแอนติบอดีพบว่าภูมิคุ้มกันต่อโปรตีนที่ไม่ได้เป็นโครงสร้างของไวรัสสามารถที่จะใช้แยกสัตว์ที่เกิดการติดเชื้อตามธรรมชาติออกจากสัตว์ที่ทำวัคซีน การศึกษาในครั้งนี้ได้ทำการโคลนยีน 3ABC ของเชื้อไวรัสปากและเท้าเปื่อยซีโรไทป์โอ และทำการผลิตรีคอมบิแนนท์โปรตีน 3ABC โดยใช้เซลล์แมลง ผลผลิตของปฏิกิริยาลูกโซ่โพลีเมอร์เลสของยีน 3ABC มีขนาดประมาณ 1300 คู่เบส การวิเคราะห์ด้วย SDS-PAGE พบว่ารีคอมบิแนนท์โปรตีน 3ABC พบว่ามีขนาดประมาณ 53 kDa นอกจากนี้การวิเคราะห์ด้วยวิธี dot blotและ Western blot พบว่ารีคอมบิแนนท์โปรตีน 3ABC ที่ได้ทำปฏิกิริยาจับได้อย่างจำเพาะกับ mouse anti-histidine monoantibody และ porcine anti-FMDV serotype O hyperimmune serum ดังนั้นรีคอมบิแนนท์โปรตีน 3ABC ที่ได้จะเป็นประโยชน์ในการนำไปพัฒนาชุดตรวจสอบโรคปากและเท้าเปื่อยเพื่อแยกสัตว์ที่ติดเชื้อตามธรรมชาติออกจากสัตว์ที่ทำวัคซีน

คำสำคัญ: 3เอบีซี,โรคปากและเท้าเปื่อย,การแสดงออกของโปรตีน,แบคคูโลไวรัส, รีคอมบิแนนท์โปรตีน

Introduction

Foot & Mouth Disease (FMD) is a highly contagious and economically devastating disease of cattle, swine and other cloven-hoofed ruminants (Bachrach, 1968). Symptoms vary in importance between species, but in general, the animals develop a fever and may get blisters in their mouths and on their feet as well as on their teats. FMDV is a non-enveloped, single-stranded, plus-sense RNA genome of approximately 8,500 bases belonging to the genus *Aphtovirus* in the family *Picornaviridae* (Rueckert, 1996). Picornaviruses are icosahidral particles, 30 nm in diameter with 60 copies of each of four proteins VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A) (King *et al*., 2000). These proteins are derived from the proteolytic cleavage of a single large precursor molecule. Seven serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) have been identified serologically, and multiple subtypes occur within each serotype (Bachrach, 1968). There are three serotypes (O, A and Asia1) in Thailand. FMD is on the A list of infectious diseases of animals of the Office International des Epizooties (OIE) and has been recognized as the most important constraint to international trade in animals and animal products (Leforban, 1999).

Current serological tests for FMD detect antibody to the structural proteins of the virus. Antibodies to capsid proteins are induced by both vaccination and infection. In the past, the test to detect the antibodies against to virus infection associated antigen (VIAA) was used to differentiate the vaccinated animals from infected animals (Newman *et al*., 1979; Pinto and Garland, 1979). However, this assay still suffer from several limitations, including low specificity, variable sensitivity and the fact that antibodies to 3D can arise from vaccination, particularly repeated vaccination (O'Donnel *et al*., 1996; Villinger *et al*., 1989). However, virus replication during infection results in the production of a number of non-structural (NS) proteins, of which some are immunogenic (Tesar *et al*., 1989). Animals, vaccinated with highly purified, NSP-free vaccines, produce antibodies against the structural proteins (SP) but not against NSP. FMD virus infection induces antibodies against both SP as well as NSP. Differentiation of infection from vaccination by detecting antibodies to NSP in infected ruminants has been described (Clavijo *et al*., 2004). To date, the detection by ELISA of an antibody response to the non-structural polyprotein 3ABC seems to be the most reliable indicator of a previous infection. NSP ELISAs are simple to perform and are suited to large scale application by a routine serological laboratory. Furthermore, an additional benefit of tests using NS proteins is the fact that a single test can be used to detect previous exposure to the virus regardless of the serotype of virus involved. In this study, the 3ABC gene of FMDV serotype O was cloned, sequenced and expressed using baculovirus expression system. The recombinant 3 ABC proteins were tested with serum from FMDV-infected pig.

Materials and Methods

1. Virus and RNA extraction

Field strain of FMDV serotype O was propagated in baby hamster kidney BHK-21 cell line. In brief, BHK-21 cells were cultured in a Minimum Essential Medium (Gibco®). Cell culture was inoculated with field strain of FMDV serotype O and the virus was harvested after the inoculated cells showing 75% of the cytopathogenic effect. In order to obtain the virus RNA, the inoculated cells were lysed using acid-guanidium-thiocyanate-phenol-chloroform method as described by Sambrook and Russell (2001).

2. cDNA synthesis and PCR

The first-strand cDNAs were obtained by reverse transcription using 1 μl of sample RNA, 10 mM dNTPs (Fermentas®), 2.5 μM Oligo-dT primers, 1X reverse transcriptase buffer (25 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 20 mM DTT), 5 mM $MgCl_2$, 0.4 U AMV reverse transcriptase and 0.4 U RNase inhibitor (Finzyme®) at 42°C for 50 min. The open reading frame of 3ABC was amplified by PCR using a specific forward primer containing a *SpeI* restriction enzyme site 5′-GGGGACTA GTGCCACCATGATTTCAATTCCTTCCCAAAA-3× and a specific reverse primer containing a *HindIII* restriction enzyme site 5′-GGGGAAGCTTCTACTC GTGGTG TGGTTCGGGGTC-3′. PCR reaction was performed with 1.0 U of Taq DNA polymerase (Invitrogen®) per sample in a total volume of 100 μl in reaction buffer containing 10 μl of cDNA templates, 0.25 mM dNTPs mix (Invitrogen®), 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$ (Invitrogen®) and 1 pgmol of sense and anti-sense primers. The PCR condition included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 90 sec, primer annealing temperature at 67°C for 90 sec and primer extension step at 72° C for 90 sec, and the final extension step was performed at 72°C for 20 min. Amplified products were analysed by electrophoresis on agarose gels.

3. Construction of expression vector

The 3ABC gene fragments were ligated to the baculovirus transfer vector, pFastBac ™HTb (Invitrogen®) at *spe*I and *HindIII* restriction sites. The ligation reaction was used to transform DH5α™ competent cells (Invitrogen®). The LB agar plates containing 100 μg/ml ampicillin and 7 μg/ml gentamicin was used to select the positive clones. The presence of the plasmid containing 3ABC gene and the orientation of insert have confirmed by PCR and endonuclease assay. The correctness of the clone was verified by DNA sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (BSU, Thailand). The nucleotide sequences of recombinant plasmid were then translated to amino acid sequence by DNASIS program. For phylogenetic study, the 3ABC sequences of 5 reported pandemic strains (PanAsia), O/SAR/19/2000 (accession no. AJ539140), O/JPN/ 2000 (accession no. AB079061), O/TAW/2/99bov (accession no. AJ539137), O/TIBET/ CHA/99

(accession no. AJ 539138) and O/SKR/2000 (accession no. AJ539139), were used to align with recombinant 3 ABC protein in this study using CLUSTALW program. In order to obtain recombinant baculovirus DNA, the recombinant plasmids were used to transform DH10Bac™ competent cells (Invitrogen®). Colonies containing recombinant bacmids were identified by showing the disruption of the *lacZ*α gene and also confirmed by PCR. High molecular weight mini-prep DNA was obtained from the selected *E. coli* clones and was then used to transfect insect cells.

4. Baculoviruses and cells

Sf21 cell line *(Spodoptera frugiperda*; Invitrogen®) was grown at 27°C using SF900II medium (Invitrogen®) supplemented with 4% FBS and 1×antibiotics (GIBCO/BRL). The recombinant baculovirus DNA was used to transfect Sf21 cells line using CellFECTIN® (Invitrogen®). At 72 hour post transfection, the recombinant baculovirus particles were collected from supernatant and viral titer was determined using plaque assay. Subsequently, the high-titer seed stock of recombinant baculovirus was produced by Sf21 insect cells at a multiplicity of infection (MOI) of 0.01 to 0.1 using Sf900 II SFM® medium (Invitrogen®) containing 4% fetal bovine serum (GIBCO/BRL) and 1× antibiotic. High-Five™ cell line *(Trichoplusia ni*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 2mM L-glutamine and 100 unit/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml of amphotericin B (antibiotic/antimycotic solution; GIBCO/BRL) was inoculated with recombinant baculoviruses at a multiplicity of infection (MOI) of 2 for the production of recombinant 3ABC protein. After 72 h post-inoculation, the infected High-Five cells were collected by centrifugation at 200xg for 10 min. The infected cells were resuspended in lysis buffer under denaturing conditions and the recombinant protein was analysed using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The theoretical size was calculated according to the amino acid sequence. The immunological reactive FMD NSP was elucidated by dot blot and western blot analyses.

5. Dot blotting analysis

The crude protein extracted from both wild-type infected cells and recombinant baculovirus infected cells was dotted on nitrocellulose membrane (Biorad®). The membrane was first treated with blocking buffer (5% powder milk in PBS) and then incubated with either swine anti-FMD hyperimmune sera (1:100) or mouse anti-histidine IgG monoclonal antibody (1:3000; Invitrogen®) for an hour. Following 3 times wash in PBS-Tween (0.05%), the membrane was incubated with either goat anti-swine IgG conjugated with horseradish peroxidase (1:3,000; Sigma®) or goat anti-mouse IgG conjugated with peroxidase (1:300; Sigma®) for one hour. After 3 times washing in PBS-Tween (0.05%), the membrane was finally incubated with diaminobenzidine solution (Sigma®) containing 1%

H_2O_2 for 5-10 min.

6. SDS-PAGE and Western blotting

The crude protein of both wild-type infected cells and recombinant baculovirus infected cells was analyzed using 10 % SDS-PAGE stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membrane using 400 mAmp at 4°C for 5 h. The membrane was first treated with blocking buffer (5% powder milk in PBS) and then incubated with mouse anti-histidine IgG monoclonal antibody (1:3000; Invitrogen®) for one hour. After 3 times washing in PBS-Tween (0.05%), the membrane was incubated with goat anti-mouse IgG conjugated with peroxidase (1:300; Sigma®) for one hour. Following 3 times wash in PBS-Tween (0.05%), finally it was incubated with diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min.

Results

1. Cloning of the gene encoding of 3ABC

The RT-PCR products of 3ABC gene were approximately 1300 bp in size (Fig.1). Alignment of the 3ABC amino acid sequences of the five PanAsia isolates and the recombinant 3ABC (r3ABC) sequence obtain from the present study is shown in Figure 2. All the PanAsia viruses 3ABC amino

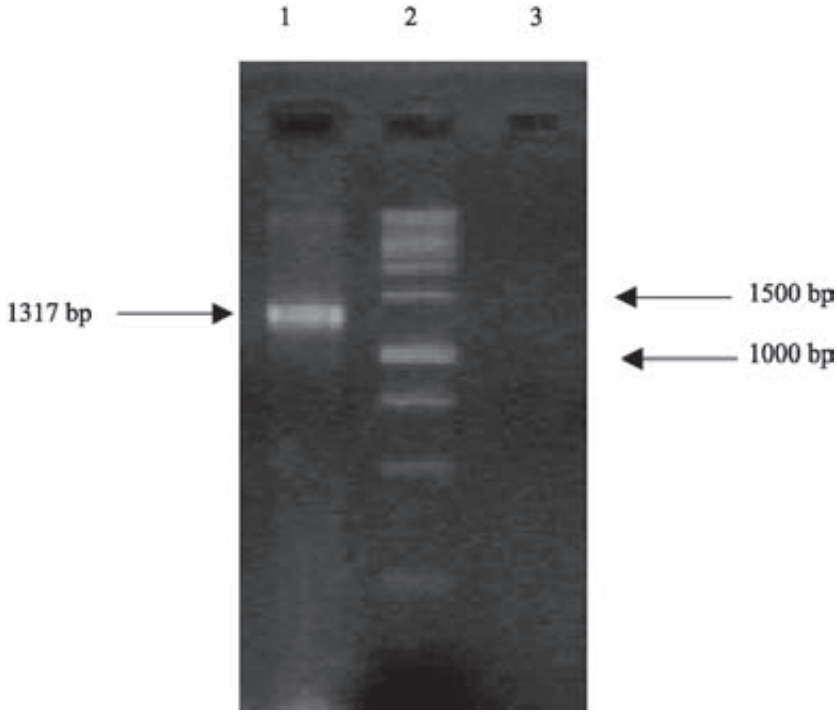

Figure 1 Agarose gel electrophoresis analysis of 3ABC gene product amplified by PCR. Lane 1: PCR products of 3ABC gene ; lain 2: 1 Kb DNA markers; lane 3: negative control.

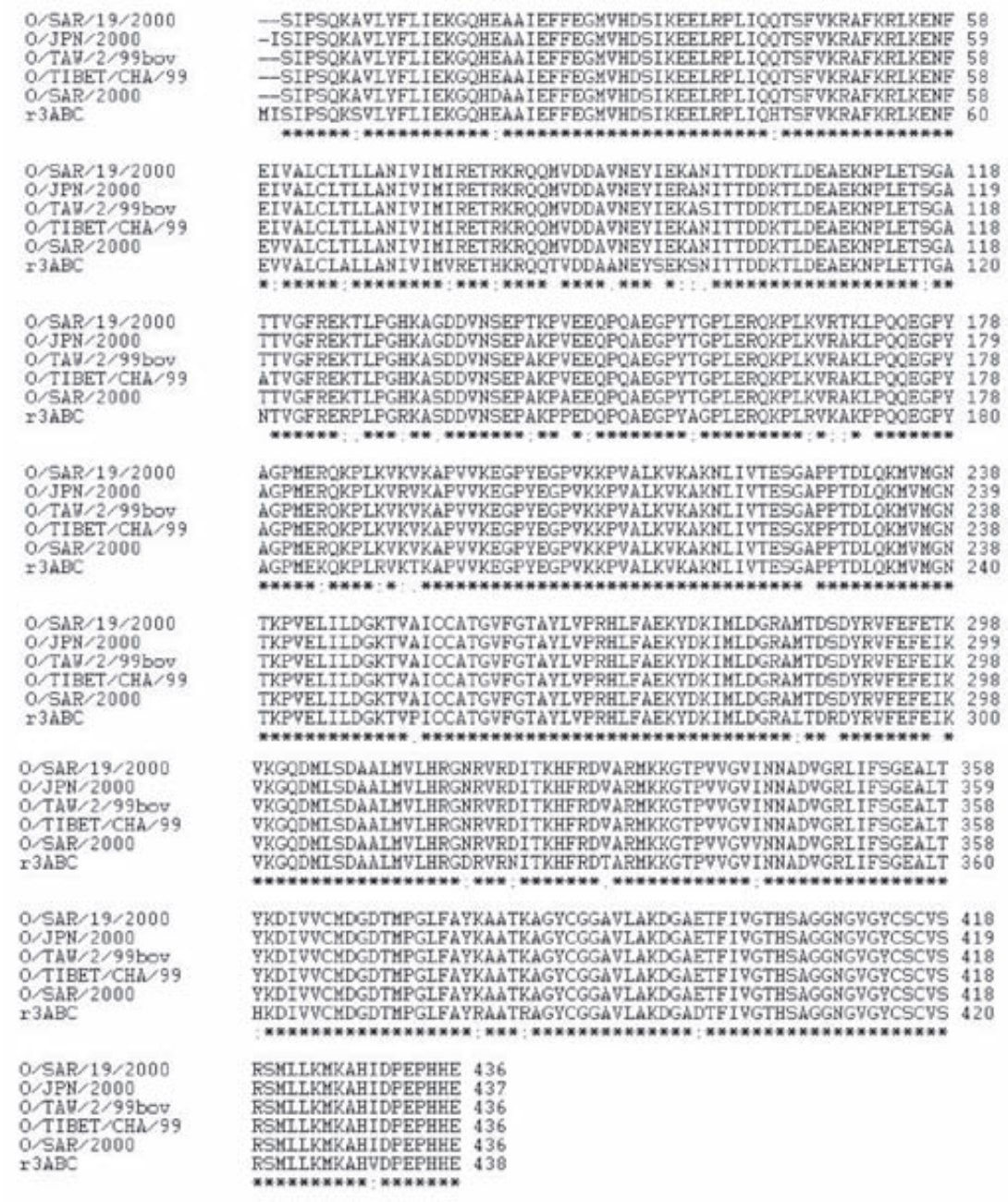

Figure 2 Alignment of deduced amino acid sequences for the recombinant 3ABC (r3ABC) with the known 3ABC sequences of the 5 PanAsia isolates of FMDV serotype O. Consensus line: asterisks (*) identical amino acid residues; colons (:) indicates conserved substitutions; and periods (.) indicate semi-conserved substitutions.

acid sequences are very closely related across the whole gene with 98-99 % homology. However, the r3ABC sequence had only 91 % homology with all PanAsia isolates. The relationships between the 5 PanAsia viruses and the r3ABC gene were determined using phylogenetic algolithms (Fig. 3).

2. Expression of the recombinant protein

The SDS-PAGE analysis of recombinant 3ABC protein revealed an extra band of protein which had the molecular mass (M_r) of 53 kDa when compared with wild-type infected cells (Fig. 4). The immunological reactive FMD NSP was determined by dot blot and western blot analyses. The dot blot analysis of crude protein from recombinant baculovirus infected cells using mouse anti-histidine monoclonal antibody and porcine anti-FMDV hyperimmune sera showed the positive results (Fig. 5). The western blotting using the anti-histidine antibody showed the positive band at the molecular mass approximately 53 kDa, but the protein from wild-type infected High-Five cells was negative (Fig.6).

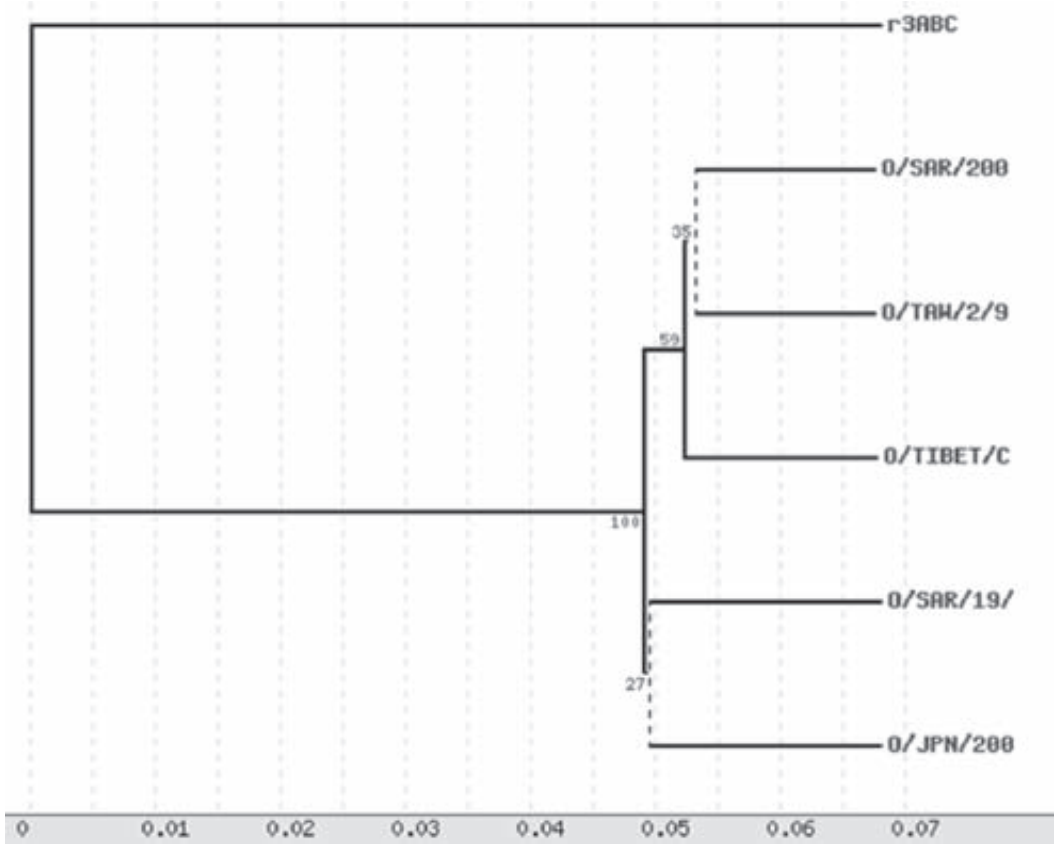

Figure 3 Phylogenetic tree of the new r3ABC and the known 3ABCs of the PanAsia virus strains. The tree was constructed using CLUSTALW and branch length values are indicated.

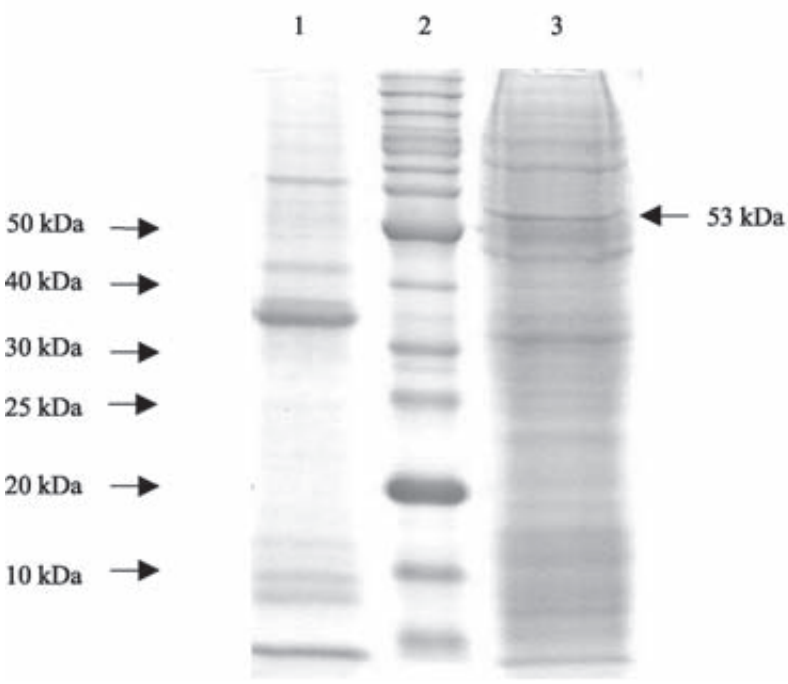

Figure 4 Analysis of expressed products of 3ABC fusion gene in baculovirus transfected High-Five cells by 10 % SDS-PAGE. High-Five cells were infected with wild-type virus (lane 1) or recombinant baculovirus containing an insert that encodes a 3ABC (lane 3). Molecular mass markers are shown (lane 2). The arrow indicates a protein band with a molecular weight of 53 kDa which obtained from the cell lysate extracted from High-Five cells infected with recombinant baculovirus.

Figure 5 Dot blot analysis of the r3ABC protein in cell lysates from High-Five cells infected with the recombinant baculovirus (1) or wild-type virus (2). The dot blots were stained with mouse anti-histidine monoclonal antibody (a) or porcine anti-FMDV hyperimmune serum (b).

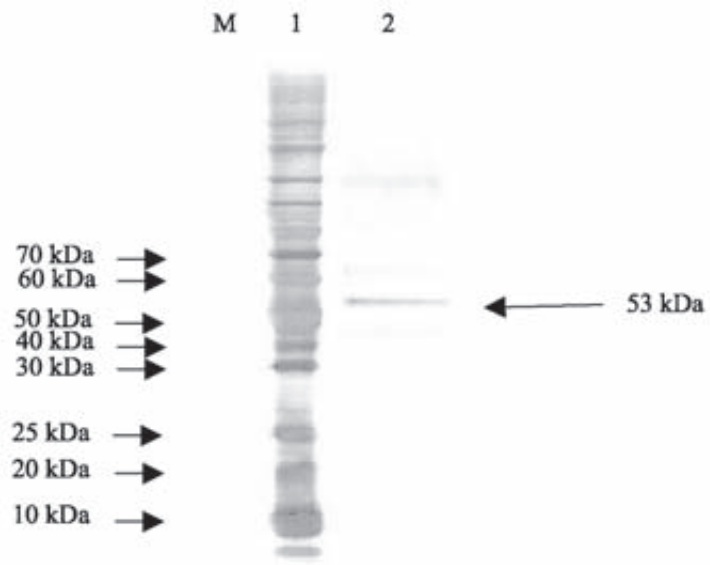

Figure 6 Western blot analysis of r3ABC protein cell lysates from High-Five cells infected with the recombinant baculovirus (Lane 1) or wild-type baculovirus (Lane 2). The Western blot was stained with mouse anti-histidine monoclonal antibody. Molecular mass (lane M) is indicated on the right. The arrow indicates a specific protein band with a molecular weight of 53 kDa that is recognized by mouse anti-histidine monoclonal antibody.

Discussion

In this study, the recombinant 3ABC was successfully cloned and expressed. The recombinant gene was sequenced and compared with previously reported polypeptide 3ABC sequences of the five PanAsia virus strains. The results showed that the r3ABC had 91 % homology with all the PanAsia isolates. The phylogenetic tree showed that the FMDV serotype O Thailand isolate was in the distinct branch from the 5 reported PanAsia virus isolates. The SDS-PAGE analysis of recombinant 3ABC produced by the insect cells had the size approximately 53 kDa. The recombinant 3ABC reacted positively with porcine anti-FMDV hyperimmune sera. The finding suggested that the recombinant 3ABC may display a conformation similar to the native viral protein. These results agreed with the previously reported (Bergman *et al*., 1993). The infected animals can be clearly differentiated from the vaccinated due to the presence of antibody to 2C 3A and 3ABC induced by viral replication following infection. Thus, recombinant 3ABC may be useful tools for the development of the diagnostic test kit for differentiates infected from vaccinated animals.

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